



Microbiota and Cow's Milk Tolerance

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Microbiota and Cow's Milk Tolerance



PhD Thesis

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National Food Institute

Technical University of Denmark

Front page pictures: Colourbox.com

Preface

This thesis represents the outcome of a PhD project conducted at the National Food Institute at The Technical University of Denmark in the period from December 2015 to March 2020. The project was conducted under the principal supervision of Senior Researcher Katrine Lindholm Bøgh, Head of the Research Group for Food Allergy and co-supervision from Senior Scientist Martin Iain Bahl and Professor Tine Rask Licht from the Research Group for Gut, Microbes and Health. The project was funded by the Danish Dairy Research Foundation.

Katrine Bækby Graversen
Kgs. Lyngby, March 2020

Summary

BACKGROUND

The default immunological response to dietary antigen exposure via the gastrointestinal tract is oral tolerance, while failure to develop or breakdown of tolerance results in food allergy, but the mechanism leading to this outcome in some individuals is not fully understood. It has been suggested that failure to develop tolerance is linked to an imbalance in intestinal microbiota caused by environmental or lifestyle factors. Cow's milk allergy is the most common food allergy in infants and young children. The best way to prevent cow's milk allergy in infants is by exclusive breastfeeding until introducing solid foods. However, in many cases breast milk must be supplemented with or replaced by infant formula, which is most often based on cow's milk proteins. The recommendation for allergy prevention in non-exclusively breastfed infants at high risk of developing allergy is highly debated. Infant formula based on hydrolysed cow's milk have been suggested as a good option due to their reduced allergenicity, but concerns have been raised about the possible reduced preventive capacity of these products. Alternative types of processing, such as heat-treatment, are being investigated with the aim of reducing allergenicity while maintain tolerogenicity, with the ultimate goal of producing safe and efficient products for prevention and treatment of cow's milk allergy. To accomplish this, more knowledge on how tolerance development is influenced by both protein and host-related factors is essential.

AIM AND METHODS

The aim of this project was to investigate whether and how (1) the physicochemical characteristics of whey products and (2) the gut microbiota composition of the host, independently and in combination, affect oral tolerance development, as well as the underlying mechanisms. This was investigated by inducing tolerance in Brown Norway rat models through oral administration of whey products with different physicochemical characteristics obtained by applying either heat-treatment (**Manuscript I**) or hydrolysis (**Manuscript III**).

The association between microbiota composition and acute immune regulation (**Manuscript II**) as well as tolerance development (**Manuscript III**) was assessed by manipulating the microbiota of rats by the antibiotic amoxicillin. The effect of amoxicillin on intestinal microbiota composition as well as on host intestinal permeability, morphology, humoral and cellular immune regulation was analysed. Finally, the effect of the whey products on the growth of gut bacteria derived from healthy infant donors was evaluated in an *in vitro* incubation study (**Manuscript III**).

RESULTS

Results presented in **Manuscript I**, revealed that the capacity of whey to prevent sensitisation of naïve rats and to desensitise already sensitised rats was not reduced by mild heat-treatment. Heat-treatment reduced the intraperitoneal sensitising capacity, but had no effect on oral sensitisation. However, oral provocation with heat-treated whey resulted in milder allergic symptoms compared to unmodified whey. Protein uptake studies showed that heat-treatment changed the uptake route of whey with less being absorbed through the epithelium but more into the Peyer's patches.

Results presented in **Manuscript III**, revealed that moderate hydrolysis did not reduce the primary preventive capacity of whey. A very diverse response was observed in the group administered with intact whey, and in that group sensitisation was not significantly different from the control group, which did not receive any product for prevention. To our knowledge, the present study is the first to show that moderately hydrolysed whey protein is superior to intact whey protein for preventing whey-specific IgE sensitisation.

Results presented in **Manuscript II**, revealed that daily intra-gastric administration of amoxicillin resulted in immediate and dramatic shifts in microbiota composition, characterised by reduced within sample (α) diversity, reduced variation between animals (β diversity), increased relative abundance of Bacteroidetes and Gammaproteobacteria, with concurrent reduction of Firmicutes, compared to the control group. After one week, the total fecal IgA level, relative abundance of small intestinal FoxP3⁺ regulatory T cells and goblet cell numbers were higher in the amoxicillin group compared to controls.

Results presented in **Manuscript III** revealed that amoxicillin-induced perturbation of the gut microbiota one week prior to and during tolerance induction did not affect the development of tolerance. In the group administered with the extensively hydrolysed whey (the product with the weakest tolerance inducing capacity) amoxicillin treated rats were actually better protected against allergic reactions than those with a conventional microbiota. In the light of epidemiological studies showing an association between early life antibiotic consumption and the development of food allergies, the observation that amoxicillin-induced perturbation of the gut microbiota promotes acute immune regulation (**Manuscript II**) and possibly tolerance development (**Manuscript III**) warrants further investigation.

Finally, *In vitro* incubation of infant faecal microbiota with whey products with different degree of hydrolysis included in **Manuscript III** indicated that moderately hydrolysed whey products promoted the expansion of the genus *Enterococcus*.

CONCLUSION AND PERSPECTIVES

Collectively, these results highlight both heat-treatment and moderate hydrolysis as potential methods for producing efficient and safe cow's milk-based products intended to prevent cow's milk allergy in infants at high risk of CMA, regardless of their gut microbiota composition. However, possible effects of hydrolysed products on infant microbiota composition indicated by the *in vitro* incubation study warrants further investigation.

Resumé (Danish)

BAGGRUND

Som udgangspunkt vil eksponering til fødevarer-allergener via mave-tarmkanalen resultere i dannelse af oral tolerance. Manglende udvikling eller nedbrud af denne tolerance resulterer i fødevarerallergi. De mekanismer, der fører til dette udfald hos enkelte individer, er ikke fuldt ud forstået. Det er blevet foreslået at manglende udvikling af tolerance kan være forbundet med en ubalance i bakteriesammensætning i tarmen forårsaget af miljø- eller livsstilsfaktorer. Komælksallergi er den mest almindelige fødevarerallergi blandt spædbørn og små børn. Den bedste måde at forebygge komælksallergi hos spædbørn er igennem fuldamping indtil introduktion af fast kost. I mange tilfælde må amning dog suppleres med eller erstattes af modernælkserstatning, som oftest er baseret på komælksproteiner. Det er omdiskuteret, hvilken type modernælkserstatning der bør anbefales til ikke-fuldammede spædbørn i højrisiko for at udvikle allergi. Modernælkserstatninger baseret på hydrolyseret komælk er blevet foreslået som en god mulighed på grund af den reducerede allergenicitet. Der er dog rejst bekymring for de reducerede forebyggende egenskaber af disse produkter. Alternative forarbejdningsmetoder såsom varmebehandling undersøges med henblik på at reducere allergenicitet og samtidig bevare tolerogenitet, med det mål for øje at fremstille sikre og effektive produkter til forebyggelse og behandling af komælksallergi. Mere viden om, hvordan toleranceudvikling påvirkes af både protein- og værtsrelaterede faktorer er afgørende for at opnå dette.

FORMÅL OG METODER

Formålet med dette projekt var at undersøge, hvorvidt og hvordan (1) de fysikokemiske egenskaber af valleprotein-produkter og (2) tarmenes mikrobiota uafhængigt og i kombination påvirker udviklingen af oral tolerance samt de underliggende mekanismer. Dette blev undersøgt ved at inducere tolerance i Brown Norway rotte-modeller igennem oral eksponering til valleprotein-produkter med forskellige fysikokemiske egenskaber opnået ved varmebehandling (**Manuskript I**) eller hydrolyse (**Manuskript III**).

Sammenhængen mellem mikrobiota-sammensætning og immunregulering (**Manuskript II**) samt toleranceudvikling (**Manuskript III**) blev undersøgt ved at manipulere rotters mikrobiota med antibiotikummet amoxicillin. Effekten af amoxicillin på tarmens mikrobiota-sammensætning såvel som på værtens tarmpermeabilitet, vævsmorfologi samt det humorale og cellulære immunsystem blev karakteriseret. Endelig blev effekten af valleprotein-produkterne på væksten af tarmbakterier fra raske spædbørn evalueret i et *in vitro* inkubationsforsøg (**Manuskript III**).

RESULTATER

Resultater præsenteret i **Manuskript I** viste, at kapaciteten til at forhindre sensibilisering af naïve rotter og til at desensibilisere allerede sensibiliserede rotter ikke blev reduceret ved mild varmebehandling af valle. Varmebehandling reducerede den intraperitoneale sensibiliseringsevne, men havde ingen effekt på orale sensibilisering. Dog gav oral provokation med varmebehandlet valle anledning til mildere allergiske reaktioner sammenlignet med ubehandlet valle. Måling af proteinoptag i tarmen viste at

varmebehandling ændrede optagelsesvejen således at en mindre andel blev absorberet gennem epitelcellelaget, hvorimod mere blev optaget igennem Peyerpletterne.

Resultater præsenteret i **Manuskript III** viste, at moderat hydrolyse ikke reducerede den forebyggende kapacitet af valle. Et meget forskelligartet respons blev observeret i gruppen, der fik intakt valle, og i denne gruppe var sensibiliseringen ikke signifikant forskellig fra kontrolgruppen, som ikke modtog noget produkt til forebyggelse. Så vidt vi ved, er dette studie det første der viser, at hydrolyseret valle-protein er bedre end intakt valle-protein til at forebygge valle-specifik IgE-sensibilisering.

Resultater præsenteret i **Manuskript II** viste, at daglig sondefodring med amoxicillin resulterede i øjeblikkelige og dramatiske ændringer i mikrobiota-sammensætningen, kendetegnet ved reduceret diversitet inden for hvert dyr, reduceret diversitet i mellem dyr, øget relativ mængde af Bacteroidetes og Gammaproteobacteria og samtidig reduktion af Firmicutes sammenlignet med kontrolgruppen. Efter en uge var det totale fækale IgA-niveau, relativ forekomst af FoxP3⁺ regulatoriske T-celler i tyndtarmen og antal bægerceller i tynd- og tyktarmen højere i amoxicillin-gruppen sammenlignet med kontrolgruppen.

Resultater præsenteret i **Manuskript III** viste, at amoxicillin-induceret ubalance af tarm-mikrobiotaen en uge før og under toleranceinduktion ikke reducerede udviklingen af tolerance i nogen af produktgrupperne. I den gruppe som fik højt hydrolyseret valle-protein (det produkt med de svageste tolerance-inducerende egenskaber) var de rotter der fik amoxicillin faktisk bedre beskyttede mod at udvikle allergiske reaktioner sammenlignet med de rotter der havde en konventionel mikrobiota. I lyset af epidemiologiske studier, der viser en sammenhæng mellem antibiotika forbrug i det tidlige liv og udviklingen af fødevarerallergi, er det overraskende, at amoxicillin-induceret ubalance af mikrobiotaen fremmede akut immunregulering (**Manuskript II**) og muligvis toleranceudvikling (**Manuskript III**), og dette bør eftervises inden der kan drages en håndfast konklusion.

Endelig indikerede *in vitro* inkubationsforsøg med tarmbakterier fra raske spædbørn og valleprotein-produkter med forskellig hydrolysegrad i **Manuskript III**, at produkterne med moderat hydrolyseret valleprotein fremmede væksten af bakterier af slægten *Enterococcus*.

KONKLUSION OG PERSPEKTIVER

Samlet set fremhæver disse resultater både varmebehandling og moderat hydrolyse som potentielle metoder til at producere effektive og sikre komælksbaserede modernælkserstatninger beregnet til at forebygge komælksallergi hos spædbørn i højrisiko for at udvikle allergi uanset deres mikrobiota-sammensætning. Eventuelle effekter af hydrolyserede produkter på spædbørns mikrobiota-sammensætning, som blev indikeret ved *in vitro* inkubationforsøget, bør dog undersøges nærmere.

List of manuscripts

Manuscript I

Graversen KB, Ballegaard AR, Kræmer LH, Hornslet SE, Sørensen LV, Christoffersen HF, Jacobsen LN, Untersmayr E, Smit JJ, Bøgh KL. Cow's milk allergy prevention and treatment by heat-treated whey – a study in Brown Norway rats. *Accepted for publication in Clinical and Experimental Allergy*.

Manuscript II

Graversen KB, Bahl MI, Larsen JM, Ballegaard AR, Licht TR, Bøgh KL. Short-term amoxicillin-induced perturbation of the gut microbiota promotes acute intestinal immune regulation in Brown Norway rats. *Accepted for publication in Frontiers in Microbiology*.

Manuscript III

Graversen KB, Larsen JM, Pedersen SS, Sørensen LV, Christoffersen HF, Jacobsen LN, Halcken S, Licht TR, Bahl MI, Bøgh KL. Moderately hydrolysed whey protein has a superior primary preventive capacity compared to intact whey independently of gut microbiota perturbation in Brown Norway rats. *In preparation*.

Manuscript not included in the thesis:

Michalovich D, Rodriguez-Perez N, Smolinska S, Pirozynski M, Mayhew D, Uddin S, Van Horn S, Sokolowska M, Altunbulakli C, Eljaszewicz A, Pugin B, Barcik W, Kurnik-Lucka M, Saunders KA, Simpson KD, Schmid-Grendelmeier P, Ferstl R, Frei R, Sievi N, Kohler M, Gajdanowicz P, **Graversen KB**, Bøgh KL, Jutel M, Brown JR, Akdis CA, Hessel EM, O'Mahony L. Obesity and disease severity magnify disturbed microbiome-immune interactions in asthma patients. *Nat Commun*. 2019 Dec 13;10(1):5711.

Abbreviations

AMX	Amoxicillin	ip	Intraperitoneal
ANCOM	ANalysis of COMposition of Microbiomes	iTreg	Induced Treg
ASV	Amplicon Sequence Variants	iW	Intact Whey Product
BEH	Ethylene Bridged Hybrid	LP	Lamina Propria
BLG	β -lactoglobulin	LPS	Lipopolysaccharides
BMDC	Bone Marrow-derived Dendritic Cells	M cell	Microfold cell
BN	Brown Norway	MFI	Mean Fluorescence Intensity
CMA	Cow's Milk Allergy	mHW	Mildly Hydrolysed Whey Product
CTR	Control	mLN	Mesenteric Lymph Node
CT	Cholera Toxin	NGS	Next-generation Sequencing
DADA	Divisive Amplicon Denoising Algorithm	nTreg	Natural Treg
DC	Dendritic Cells	PAGE	Polyacrylamide Gel Electrophoresis
DIG	Digoxigenin	PAS	Periodic Acid–Schiff
eHF	Extensively Hydrolysed Formula	PBS	Phosphate Buffered Saline
eHW	Extensively Hydrolysed Whey Product	PCoA	Principal Coordinate Analysis
FBS	Fetal Bovine Serum	pHF	Partially Hydrolysed Formula
FDR	False Discovery Rate	pHW	Partially Hydrolysed Whey Product
FITC	Fluorescein Isothiocyanate	PP	Peyer's Patches
FoxP3	Forkhead box P3	Qiime	Quantitative Insights Into Microbial Ecology
GF	Germ-free	RT	Room Temperature
GPC	Germeation Chromatography	SPF	Specific Pathogen Free
MHO	Human Milk Oligosaccharides	Th	T Helper Cell
HT	Heat-treated	TLR	Toll-like Receptor
Ig	Immunoglobulin	TGF- β	Transforming Growth Factor- β
IL	Interleukin	Treg	Regulatory T Cell
IBD	inflammatory bowel disease	WPI	Whey Protein Isolate

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1 Background

The default immunological response to dietary antigen exposure via the gastrointestinal tract is oral tolerance, while failure to develop or breakdown of tolerance results in food allergy, but the mechanism leading to this outcome in some individual is not fully understood [1].

Food allergy can be immunoglobulin E (IgE)-mediated or non-IgE mediated [2]. The focus of the current project is on IgE-mediated food allergy, which occurs in two phases: An initial sensitisation phase where allergen-specific IgE is produced by plasma cells via stimulation by T helper 2 (Th2)-derived cytokines, and a subsequent elicitation phase, where symptoms occur [3]. In an allergic individual, ingestion of foods containing the offending allergen will lead to cross-linking of allergens with IgE antibodies on the surface of tissue resident mast cells and circulating basophils, which causes degranulation and release of chemical mediators. This may give rise to symptoms in the gastrointestinal tract, the skin, the respiratory system and/or cardiovascular system [4,5]. In severe cases, allergic reactions can cause life-threatening anaphylactic shock associated with systemic vasodilation.

1.1 Cow's milk allergy

Cow's milk allergy (CMA) is the most common food allergy in infants and young children with a high national variation in prevalence of approximately 0.5-3% [6–8]. The prognosis for outgrowth of CMA with age is good and resolution rates above 50% within the first 1-5 years of life have been reported [8,9]. Despite the good prognosis for outgrowth of CMA, prevention should still be prioritised for important reasons: CMA is associated with the risk of severe reactions [10], CMA in infancy may negatively impact nutritional status and growth [11,12], and comorbidities are common thus many children suffering from CMA in infancy develop other allergic conditions later in life [13].

1.2 Cow's milk allergens

Cow's milk proteins are divided into two fractions: the curd and the whey, which constitute approximately 80 and 20% of the total proteins, respectively [14]. Multiple different cow's milk proteins can cause allergic reactions. The determination of what allergens an cow's milk allergic individual are sensitised to, called component resolved diagnosis, is currently not part of the standard clinical practice for CMA diagnosis [4], but is used for research purposes. Based on these studies, it appears that no single cow's milk allergen can be said to be the most allergenic, and most individuals suffering from CMA are sensitised to more than one cow's milk allergen [15,16]. The most frequently reported allergens that patients are sensitised to are caseins of the curd fraction, and β -lactoglobulin (BLG) and α -lactalbumin of the whey fraction, which are also the most abundant proteins in cow's milk [14].

1.2.1 β -lactoglobulin

The most abundant protein in the whey fraction is BLG, which constitutes approximately 50% of the total whey protein [17]. BLG is a relatively small globular protein with a molecular weight of 18.3 kDa [18]. BLG consists of 162 amino acids including five cysteine residues of which four form two disulphide bonds and one retains a free sulfhydryl group [19], which is buried inside the globular tertiary structure. At physiological pH, BLG occurs in a dimeric form, but dissociates into monomers at low pH. The allergenicity of BLG is believed to be related to the fact that it is relatively stable to digestion and has no human homologue [20]. However, BLG can be found in trace amounts in human breast milk due to transfer of dietary BLG to the milk [21].

1.3 Management of cow's milk allergy

Currently, no cure exists for any allergy including CMA, and strict avoidance of foods containing the offending allergen(s) is the only way to manage the disease [4]. For non-exclusively breastfed infants this implies the use of hypoallergenic infant formula, which is defined as one that is tolerated by 90% of cow's milk allergic infants (with a confidence interval of 95%) as determined by an appropriately designed clinical trial [22]. Hypoallergenic infant formula can be based on proteins from plants (e.g. soy or rice) or milk from other mammals than cow (e.g. camel, mare or donkey) or can be based on synthetic amino acids [4]. However, most hypoallergenic infant formula are based on processed cow's milk. Processing, such as hydrolysis and heat-treatment, affects the structure of the proteins and may thereby prevent their cross-linking to IgE thus avoiding the induction of an allergic reaction. Antibody-binding sites on an allergen are called epitopes. Epitopes can either be comprised of sequential residues in the primary structure (linear epitopes) or of residues that are brought together in the folded protein (conformation epitopes) [23]. Processing may affect linear and conformational epitopes differently [24,25].

1.3.1 Hydrolysis of cow's milk proteins

Enzymatic hydrolysis of proteins, also called proteolysis, breaks proteins into peptide fragments and free amino acids and may thereby destroy allergenic epitopes. Various enzymes can be applied for hydrolysis of cow's milk proteins including pancreatic enzymes (trypsin, chymotrypsin and pancreatin) [26] and bacteria-derived enzymes (such as Alcalase) [27]. Since different enzymes have different specificities for cleavage sites, the exact enzyme combination used is crucial for the resulting peptide profile.

Hydrolysis is often combined with other types of processing such as heat-treatment, application of high pressure and ultrafiltration [28]. Information about the processing of commercial ingredients for infant formula is in most cases not public available. Commercial products are (as a minimum) described according to the source material (whey or casein) and the classification as either extensively (eHF) or partially (pHF) hydrolysed formula based on the molecular weight of the peptides. However, there is currently no consensus for a strict definition of eHF and pHF. The American Academy of Pediatrics have suggested a definition of eHF as containing only peptides that have a molecular weight below 3 kDa, and

pHF as primarily containing oligopeptides with a molecular weight below 5 kDa [29]. High variation between size distribution [30] and peptide sequence profiles [31] has been reported among different commercial hydrolysed infant formulas based on the same protein source and within same classification. This implies that different infant formulas with same classification may have different immunological effects.

1.3.2 Heat-treatment of cow's milk proteins

Heat-treatment disrupts the secondary and tertiary protein structure by breaking hydrogen bonds, causing an unfolding of proteins also called denaturation. In BLG, unfolding may result in exposure of the hydrophobic sulfhydryl groups which were previously buried inside the folded protein [32]. Interactions of the sulfhydryl group with caseins or other unfolded whey proteins drive protein aggregation [33].

Conformational changes induced by heat-treatment may influence protein epitopes in various ways that either promote or prevent antibody binding: Unfolding may expose epitopes that were previously inaccessible inside the folded protein and/or dissociate conformational epitopes, while aggregation may cause formation of neo epitopes and/or masking of epitopes. The effect of heat-treatment on epitope accessibility depends on the specific heat-treatment applied and in particular the temperature used [34–36].

1.4 Intestinal degradation and uptake of cow's milk proteins

Dietary proteins are degraded in the stomach by acidic hydrolysis and pepsin, and further in the small intestine by pancreatic enzymes as well as by brush-boarder enzymes localized in the microvilli-covered surface of the intestinal epithelium [37]. Although that the majority of ingested proteins are degraded into amino acids and very short peptides in the intestinal lumen and during uptake by brush-boarder enzymes, it is evident that a small fraction do reach lamina propria and the circulation in an immune reactive form [38,39]. Many food allergens are characterised as being relatively resistant to pepsin and acid proteolysis [40], however this is not universal for all food allergens [41]. Most studies have found that BLG is resistant to pepsin and acid proteolysis [41], which is likely related to the compact structure containing two disulphide bonds [42].

The intestinal uptake of dietary antigens can occur through different pathways in the small intestine that are generally divided into paracellular and transcellular pathways (Fig. 1.1). Paracellular diffusion is regulated by tight junction protein complexes formed between adjacent epithelial cells, and should under normal conditions only allow the passage of very small molecules (less than 0.6 kDa) [43]. However, the passage of larger molecules can occur through the so-called "leak pathway" in the presence of inflammatory signals [44], thus paracellular transport may in some cases contribute to intestinal uptake of peptides.

Transcellular transport can either occur through epithelial cells, microfold (M) cells, goblet cells or phagocytes [45]. Epithelial cells cover the vast surface area of the intestinal cell wall. Transcellular uptake through epithelial cells favour small, soluble particles, and is generally associated with lysosomal antigen degradation; however, transcytosis without degradation do also occur [46]. M cells are found in the follicle associated epithelium covering lymphoid structures in the small intestine called Peyer's patches [47]. M cells are specialized in engulfing particulate luminal content and deliver it directly to dendritic cells (DCs)[48]. Additionally, antigens may be taken up via mucus producing goblet cells via the so-called goblet cell associated antigen passage [49] or sampled directly by phagocytes such as macrophages or DCs, that can extend their dendrites into the lumen without disrupting tight junction complexes [50,51].

Exact information about the relative contribution of the different pathways for proteins/peptides uptake remains elusive, is dependent on physicochemical characteristics of proteins/peptides such as size, polarity and solubility [45]. It has been suggested that the uptake route for a food antigens may determine what type of immune response that will be mounted [45,52,53].

1.4.1 Intestinal protein uptake in allergic individuals

In individuals suffering from existing food allergy, intestinal permeability of food allergens is increased [54–56]. This is a result of both IgE dependent CD23 receptor mediated transcellular transport (specific to the offending allergen) [57] and by increased non-specific paracellular permeability caused by release of tryptase from mast cells leading to redistribution of tight junction protein complexes [58,59].

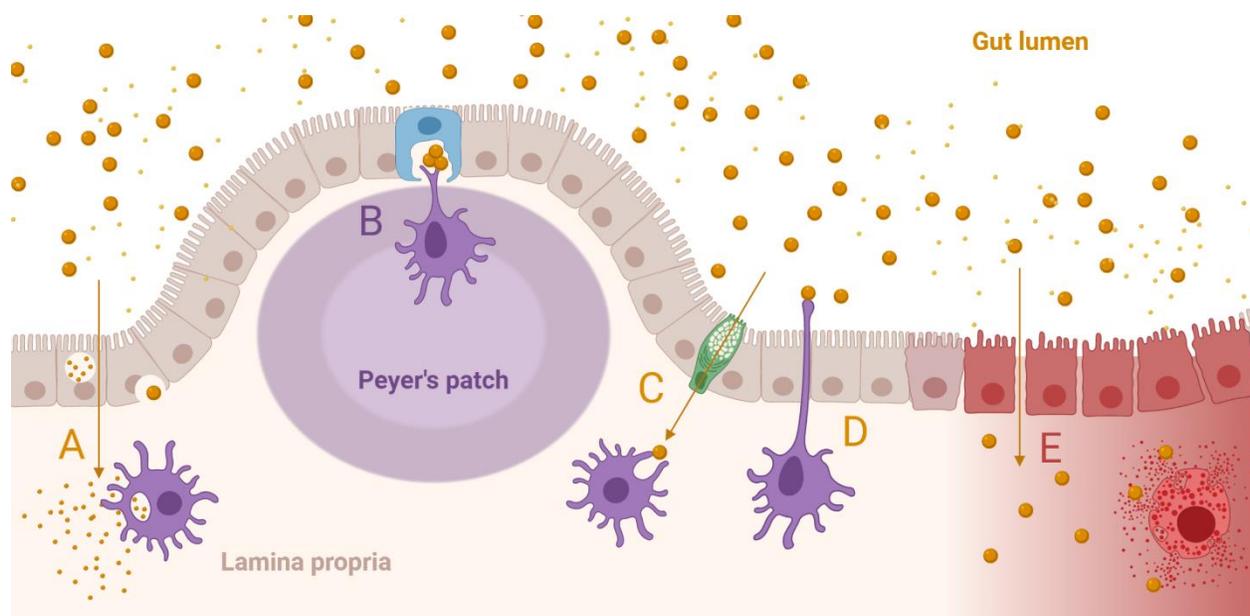


FIGURE 1.1: Dietary antigen uptake in the gut. Antigens may be taken up through epithelial cells, which are the most abundant cell type in the intestinal epithelial layer. The majority of antigens taken up by epithelial endocytosis are digested within lysosomes but some are left intact (A). Alternatively proteins may be taken up by microfold cells scattered in the follicle associated epithelium covering Peyer's patches (B), taken up via the goblet cell associated antigen passage (C), or sampled by phagocytes that extend their dendrites into the lumen (D). During overt inflammation or allergic reaction, antigens may enter lamina propria by paracellular diffusion (E). The figure was created with Biorender.com

1.5 Oral tolerance

In lamina propria or Peyer's patches of the small intestine, antigens are transferred to antigen presenting cells such as DCs which can degrade antigens by intracellular vesicles and present them on surface molecules, major histocompatibility complex class II, to activate naïve T cells [60]. A subset of regulatory DCs expressing CD103⁺ DCs are central for tolerance induction [51,61]. CD103⁺ DCs loaded with antigen in lamina propria, drain to mesenteric lymph nodes where they promote the differentiation of naïve T cells to become Forkhead box P3 (FoxP3⁺) regulatory T cells (Tregs) through retinoic acid and transforming growth factor- β (TGF- β) dependant mechanisms [62–64] (Fig. 1.2).

Tregs induced in the periphery are referred to as induced Tregs (iTregs), and distinguish from thymus derived natural Tregs (nTregs) by their low expression of the transcription factor Helios [65]. In contrast to nTregs, iTregs are essential for oral tolerance development [66,67]. Similarly, antigens delivered to DCs via M cells can activate Tregs locally in Peyer's patches; however, while mesenteric lymph nodes are found to be necessary for development of tolerance [64,68], Peyer's patches are not [69,70].

Upon activation, iTregs are imprinted the expression of gut homing factors that enables them to migrate to the lamina propria, which appears to be essential for oral tolerance [67,71]. In lamina propria, Tregs expand due to stimulation by tolerogenic cytokines secreted by tissue resident tolerogenic macrophage and epithelial cells [67].

It is well established that Tregs are essential for preventing Th2 immune responses, however the suppressive function is only partly understood [1,72]. It is known that Tregs can suppress the function of DCs [73], Th2 cells [74], B cells [75] and mast cells [76]. The suppression is mediated either by direct cell-cell contact e.g. by CTLA4, which is a negative co-stimulatory molecule, and/or by the secretion of anti-inflammatory cytokines including IL-10 and TGF- β [77].

It is well established that Tregs are essential for preventing Th2 immune responses, which likely involves Tregs suppressing the function of DCs [73], Th2 cells [74], B cells [75] and mast cells [76]. The suppression is mediated either by direct cell-cell contact e.g. by cytotoxic T lymphocyte antigen-4, which is a negative co-stimulatory molecule constitutively expressed on Tregs, and/or by the secretion of anti-inflammatory cytokines including IL-10 and TGF- β [77]. Additionally, Tregs promote IgA production by plasma cells via TGF- β dependant mechanisms [78–80].

In addition to Treg-mediated mechanisms, antigen-specific T cell anergy and depletion, which respectively refers to unresponsiveness and apoptosis, may also contribute to oral tolerance. It is the general perception that low-dose antigen primarily leads to induction of Tregs, while high-dose antigen leads to anergy and depletion [81,82].

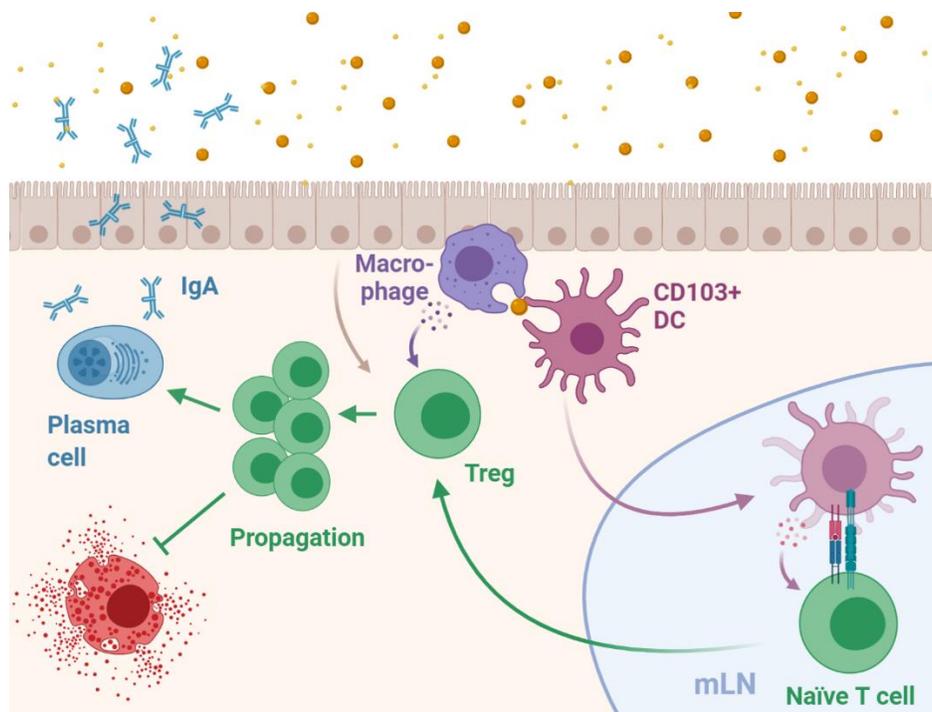


FIGURE 1.2: Regulatory T cell mediated oral tolerance. In lamina propria, antigens are taken up and degraded by CD103⁺ dendritic cells (DC) that migrate to the mesenteric lymph nodes (mLN) and activate antigen-specific naïve T cells to become regulatory T cells (Treg). Upon activation, Tregs are imprinted homing factors that enable them to migrate to lamina propria where they propagate due to activation by cytokine stimulation from tissue resident macrophages and epithelial cells. Tregs promote IgA secretion by plasma cells, but suppress the function of other cell types including suppression of mediator release from mast cells. The figure was created with Biorender.com

1.5.1 Immunoglobulin A and oral tolerance

At mucosal surfaces including that of the gastrointestinal tract, IgA is continuously secreted and contributes to the barrier function [83]. IgA is a neutralising antibody class, known to be important for the immunological defence against pathogenic bacteria and toxins, while the role of IgA in immune tolerance to dietary allergens remains elusive [1].

In breastfed infants, antigen-specific IgA transferred via the breast milk to the infant gut lumen may likely promote the development of tolerance upon first exposure to dietary antigens. IgA may affect the immune response by promoting the selective uptake of antigen-IgA complexes via M cells [84] and by promoting the interaction with DCs [85]. Two studies report that the breast milk from mothers of cow's milk allergenic infants have lower levels of total and cow's milk-specific IgA than those of healthy infants [86,87].

However, in the context of established tolerance or allergy, the impact of antigen-specific IgA remains largely unknown. Results from one study in ovalbumin-sensitised IgA deficient mice revealed that systemic transfer of ovalbumin-specific IgA is protective against anaphylaxis triggered by the oral exposure to ovalbumin, indicating that IgA may mediate antigen-specific immune exclusions [88].

1.6 Gut microbiota and food allergy

The observation of increasing prevalence of allergic diseases within the last decades [5] has been connected with concurrent changes in environmental and lifestyle factors [89]. Already in 1989, Strachan identified an association between family size and hay fever, and based on that suggested the so-called “hygiene hypothesis” [90]. In the following years, this concept has been extrapolated to other autoimmune and allergic diseases including food allergy. Furthermore, the contribution of the gut microbiota in explaining the correlation between lifestyle/environmental factors and these diseases is becoming increasingly appreciated [91].

1.6.1 Infant gut microbiota colonisation and composition

Until recently, it was believed that infants are born sterile, but studies now indicate that some colonisation takes place already *in utero* [92,93]. Colonisation increases dramatically upon birth, initially by facultative aerobes such as *Eschericia/Shigella*, *Enterococcus* and *Streptococcus* [94]. After few days, the gut becomes anaerobic and strict anaerobes such as *Bifodobacterium*, *Bacteroides*, *Lactobacillus* and *Veillonella* expand [94,95]. In early life, colonisation pattern is influenced by the mode of delivery: While vaginally delivered infants harbour many taxa from their mother’s own microbiota, the microbiota of infants born by C-section is more alike the general skin microbiota indicating that the vertical transfer of bacteria from the mother to the child may be obstructed by C-section [94,96]. Numerous studies have found associations between allergic diseases including food allergies and C-section [97,98].

During infancy, the gut microbiota composition is affected by breastfeeding status [94,99]. Human breast milk is a source of multiple components that shape the infant gut microbiota composition such as human milk oligosaccharides (HMOs) that are non-digestible carbohydrates [100–102], IgA [103] and cytokines [104], as well as live bacteria [105]. The HMOs have attracted much attention in recent years because human cohort studies have found associations between the composition of maternal milk oligosaccharides and the development of food allergy in the infant [106,107].

Another factor that influence gut microbiota composition is antibiotic treatment. While the gut microbiota in adults rapidly returns to the baseline with respect to the major taxa shortly (1-2 weeks) after cessation of antibiotic treatment [108–111], changes appear to persist for longer in infants especially after multiple courses of antibiotics [112]. Children exposed to antibiotics within their first year of life shows delayed microbiota maturation (reduced diversity, increased facultative aerobes) compared to those not exposed to antibiotics [95]. Most epidemiological studies suggest an association between antibiotic exposure in early life and the development of CMA [113–115].

1.6.2 Microbiota and cow’s milk allergy pathogenesis

Studies have found that the gut microbiota composition differs between healthy infants and those suffering from CMA [116] or food allergies in general [117]. From these studies it remains unknown whether the differences in microbiota are the cause or a consequence of the disease (e.g. caused by differences in diet). The first indication that the microbiota may be implicated in the pathogenesis of

food allergy came from a Canadian birth cohort, where it was found that low richness and an elevated Enterobacteriaceae/Bacteroidaceae ratio at three months of age were associated with increased risk of later sensitisation to a food allergen [118]. However, only 12 infants were sensitised in this cohort.

Direct evidence of the microbiota being implicated in the pathogenesis of food allergy came from faecal microbiota transplant experiments in germ-free mice. Germ-free mice have elevated basal levels of Th2 cytokines and IgE [119,120] and are prone to develop food allergy [121]. First, it was shown that reconstitution of germ-free mice with conventional microbiota from healthy infants partly protected these from allergic reactions to BLG after intra-gastric immunisations [122]. Next, it was shown that reconstituting germ-free mice with the microbiota from sensitised *Il4ra*^{F709} mice, that are genetically modified to become food allergy-prone due to a mutation in the IL-4 receptor gene, transferred the increased susceptibility to the recipient, while conventional microbiota from sensitised wild-type mice was protective [123].

Finally, it was recently shown that reconstituting germ-free mice with the microbiota from infants suffering from CMA [124,125] or any food allergy [126] was not protective against oral sensitisation and anaphylaxis reactions to BLG or egg ovalbumin respectively, while reconstituting with conventional microbiota from age matched healthy controls was protective. However, the study design applied by Feehley and colleagues [124] (which was the first to show this) has been criticised, because mice were administered with the same milk formula as consumed by the infant donor (conventional infant formula for healthy and extensively hydrolysed casein formula for CMA) to promote the microbiota establishment in the recipient. This makes it challenging to separate the effects of the microbiota from the effects of dietary exposure [127].

1.7 Animal models to study cow's milk allergy and tolerance

Animal models are valuable tools for investigating the mechanisms underlying development of allergy versus tolerance to allergens, as well as evaluating the safety and efficacy of preventive and therapeutic interventions [128–131]. In rodents, as in humans, the default response to oral exposure to allergens is the development of oral tolerance. Certain breeds of animals such as C3H/HeJ mice [132] and Brown Norway rats [133] resemble atopic individuals by being genetically predisposed to raise an IgE response upon exposure to allergens. However, even when using such breeds, most models still require a non-physiological method to induce a robust allergic response such as administering allergens orally together with an adjuvant or via alternative routes such as by intraperitoneal (ip) [134] or epicutaneous [135] injection. For sensitisation via the oral route, commonly used mucosal adjuvants include cholera toxin (CT) derived from *Vibrio cholera* [136] and staphylococcal enterotoxin B derived from *Staphylococcus aureus* [137,138]. These adjuvants promote the development of a Th2 skewed immune response [129,130,139].

In the domain of oral tolerance, animal models have been exploited to investigate the underlying mechanisms, to screen for biomarkers of oral tolerance [140] and to test the safety and efficacy of preventive and therapeutic interventions [128]. The primary preventive capacity of processed milk proteins have been investigated as the capacity to reduce specific IgE levels and challenge induced

allergic symptoms by orally pre-administration followed by ip [141] or epicutaneous [142] immunisations. The desensitisation capacity of various types of allergen-specific and non-specific interventions have been evaluated as the capacity to reduce specific IgE levels and to ameliorate challenge induced allergic symptoms e.g. by administration of whole or parts of allergens via the oral, epicutaneous or subcutaneous routes in previously sensitised animals [131].

To investigate the *de novo* immunological responses to food allergens in animal models it is required that the animals are naïve to the studied allergen. To achieve fully naïve animals they must be bred on an allergen-free diet for at least two generations [143]. Similar to human milk, murine milk does not contain any BLG [144] which makes mice and rats applicable models to study CMA.

1.7.1 Microbiota of murine animal models

Although the gut microbiota of murine models and human differ considerably, animal models have been a valuable tool to study host microbiota interactions [145]. While the microbiota is strikingly similar among different mammals at the high taxonomic levels (phylum and higher), pronounced differences are observed at lower taxonomic levels (families and genera) [146]. However, a study that compared mice and human gut metagenomes, found that despite the mice and human gut metagenomes share very few genes (around 4%), the majority (around 95%) of gene ontologies are shared [147]. This means that although the gut microbiota of mice and humans is different in composition, it is rather similar in functionality.

Rats have been a long-lasting animal model in biomedical research, and have been used in studies of the gut microbiota in various diseases [148–153], but mice models are more commonly used. Efforts to characterise the rat microbiome goes back a long way [154], and the rat microbiome has since been characterised both temporally through the lifespan [155] and spatially along the gastrointestinal tract [156]. Few attempts have been made to compare mice and rats as models of the human microbiomes; however, while one study found that mouse microbiota is more similar to that of humans [157], another found the opposite, that (Sprague Dawley) rat microbiome is more similar to that of humans [155]. It has been reported that colonisation of germ-free animals by human microbiota is more efficient and stable in rats than in mice [158]. Comparison between microbiomes of different species may be complicated by many factors such as strain, diet, housing etc., but also possibly by bias in bioinformatics analysis due to bias in existing databases used for taxonomic classification [159].

Even within a species, variation in composition exists between different genotypes [160] as well as between animals from different facilities [161]. To reduce experimental variability, most commercially available specific pathogen free (SPF) animals, are inoculated at birth (by C-section) with the so-called Altered Schaedler Flora, a mixture of eight bacterial strains [162], and these animals can thus be considered gnotobiotic. It is questionable if the SPF microbiota is representative of a functionally normal murine gut flora, and the possible improvement in reduced experimental variability may not weight up for this disadvantage [163,164]. A recent study in mice found that mice with natural microbiota (created by implanting laboratory strain embryos into wild mice) correctly predicted the human response to drug

treatments, for which previous pre-clinical experiments with SPF mice had failed to predict the human responses [165].

1.8 Objectives

The mechanism determining the immunological responses to dietary allergens leading to either oral tolerance or food allergy remains elusive. To guide strategies for CMA prevention in the future, more knowledge regarding the process leading to oral tolerance in general and how it is influenced by both protein and host-related factors in particular is essential. Such strategies may include optimising the processing of cow's milk proteins for special infant formulas targeting CMA prevention, and/or the development of individualised guidelines for CMA prevention in specific groups of infants.

The aims of this project were to investigate whether and how (1) physicochemical characteristics of whey products and (2) gut microbiota composition of the host, independently and in combination, affects oral tolerance development, as well as the underlying mechanisms.

This was investigated by inducing tolerance in Brown Norway rat models through oral administration of whey products with different physicochemical characteristics obtained by applying either heat-treatment (**Manuscript I**) or hydrolysis (**Manuscript III**). The association between microbiota composition and acute immune regulation (**Manuscript II**) as well as tolerance development (**Manuscript III**) was assessed by manipulating the microbiota by administration of the antibiotic amoxicillin.

2 Results

2.1 Manuscript I

Cow's milk allergy prevention and treatment by heat-treated whey – a study in Brown Norway rats

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ABSTRACT

Background: Food processing, including heat-treatment, can affect protein structure and stability, and consequently affect protein immunogenicity and allergenicity. A few studies have shown that structural changes induced by heat-treatment impact the intestinal protein uptake and suggest this as a contributing factor for altered allergenicity.

Objective: To investigate the impact of heat-treatment of a whey-based protein product on allergenicity and tolerogenicity as well as on intestinal uptake in various animal models.

Methods: Immunogenicity and sensitising capacity of the heat-treated whey product was compared to that of the unmodified product by intraperitoneal and oral exposure studies, while tolerogenic properties were assessed by oral primary prevention and desensitisation studies in high-IgE responder Brown Norway rats.

Results: Heat-treatment of whey induced partial protein denaturation and aggregation, which reduced the intraperitoneal sensitising capacity but not immunogenicity. In contrast, heat-treatment did not influence the oral sensitising capacity, but the heat-treated whey showed a significantly reduced eliciting capacity compared to unmodified whey upon oral challenge. Heat-treatment did not reduce the tolerogenic properties of whey, as both products were equally good at preventing sensitisation in naïve rats as well as desensitising already sensitised rats. Results from inhibitory ELISA and immunoblots with sera from sensitised rats demonstrated that heat-treatment caused an altered protein and epitope reactivity. Protein uptake studies showed that heat-treatment changed the route of uptake with less whey being absorbed through the epithelium but more into the Peyer's patches.

Conclusion & Clinical Relevance: These results support the notion that the physicochemical features of proteins affect their route of uptake and that the route of uptake may affect the protein allergenicity. Furthermore, the study highlights the potential for heat-treatment in the production of efficient and safe cow's milk protein-based products for prevention and treatment of cow's milk allergy.

INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy among small children, with a prevalence of approximately 0.5-3%, varying from country to country [6–8]. The optimal way to prevent sensitisation to cow's milk is to exclusively breastfeed infants in their first months of life until introduction of solid foods. However, in many cases, breast milk must be supplemented with or replaced by infant formulas. Infants suffering from CMA or at high risk of developing an allergy, are recommended to use hypoallergenic infant formulas, which are most often based on hydrolysed cow's milk proteins [166]. The risk of eliciting an allergic reaction is reduced because epitopes are degraded by hydrolysis, but this may also compromise the tolerogenic effects of these products [167,168]. Currently, there is no accepted treatment for CMA or any other food allergy, yet immunotherapeutic strategies for desensitising food allergic patients have shown promising results. The only accepted strategy to manage CMA is strict avoidance of foods containing cow's milk allergens [5]. Different types of processing, including heat-treatment of cow's milk proteins, are being investigated with the aim of reducing allergenicity but retaining immunogenicity, with the ultimate goal of producing safe and efficient products for prevention and treatment of CMA [25,169–171].

It is the general perception that heat-treatment reduces cow's milk allergenicity since many milk allergic children can tolerate baked milk [172], yet tolerogenic properties of heat-treated milk are not well characterised. It has been reported that the introduction of baked milk into the diet of cow's milk allergic patients can accelerate the development of tolerance to fresh milk [173–175], and baked milk has been used for oral immunotherapy in human clinical trials [176,177]. However, it has been questioned whether heat-treated milk in fact has a true tolerogenic effect, or whether tolerance to baked milk is just a biomarker for spontaneous resolution of CMA [178] since adverse reactions to baked milk is associated with severe CMA [176].

A direct comparison of allergenic and tolerogenic properties of unmodified and heat-treated milk can be addressed in controlled animal studies. A few animal studies have been performed to assess the sensitising and eliciting capacity of heat-treated milk, but results from these studies are conflicting [179–181]. These studies showed that effects on allergenicity are not only related to masking or exposure of antibody-binding epitopes as have previously been described for cow's milk allergic patients [182–184], but that heat-treatment also impacts on allergenicity by influencing the route of intestinal protein uptake [179]. Evidence of the tolerogenic properties of heat-treated milk proteins from controlled animal studies is very limited [141], but development of oral tolerance will likely also be affected by intestinal uptake.

The present study investigated the impact of heat-treatment on the immunogenicity, sensitising capacity, eliciting capacity, and on the ability to induce tolerance as well as on intestinal uptake of a whey product. This was done with the ultimate goal to guide the future design of modified cow's milk proteins with low allergenicity but retained tolerogenicity. The immunogenicity and sensitising capacity of unmodified and heat-treated whey were assessed by intraperitoneal (ip) and oral exposure studies, while the tolerogenic properties were assessed by oral primary prevention and desensitisation studies with recently established models [168]. The intestinal uptake was evaluated in naïve rats by quantifying protein levels in different intestinal tissues at different time points after oral administration of the whey

products by gavage. In addition, protein uptake was evaluated by two different *in vitro* assays. In all animal studies, high-IgE responder Brown Norway (BN) rats, resembling atopic individuals, were used.

METHODS

Product characterisation

Whey protein isolate (WPI) with approx. 60% β -lactoglobulin (BLG) and a heat-treated (HT) (90°C for 10 min) version of the same WPI were kindly provided by Arla Foods Ingredients (Videbæk, Denmark).

Quantification of whey proteins

To examine native protein composition, TSK gel permeation chromatography (GPC) and high-performance liquid chromatography was performed. Whey products were dissolved in solvent (20 mM NaH₂PO₄, Merck KGaG, Darmstadt, Germany, pH 7.5) to a protein concentration of 0.1 % (w:v), left overnight at 4°C and subsequently filtered with a 0.22 μ m filter. A total of 25 μ L was loaded on two connected TSKgel3000PWxl (7.8mm, 30mm, TOSOH Bioscience GmbH, Stuttgart, Germany) columns equipped with a PWxl precolumn (6 mm, 4 cm, TOSOH Bioscience GmbH) at 25°C. Whey products were separated at a flow rate of 0.4 mL/min with 47% (v:v) acetonitrile (CHEM-LAB NV, Zedelgem, Belgium) in Milli Q water, and proteins were detected at a wavelength of 210 nm. Peak areas were normalised with the peak area of the casein macropeptide (CMP).

Analysis of protein aggregation

To examine protein aggregation status, ethylene bridged hybrid (BEH) GPC and ultra- performance liquid chromatography was performed. Whey products were dissolved in solvent (50 mM NaH₂PO₄, 0.15 M NaCl, Merck KGaG, pH 7.0) to a protein concentration of 0.25% (w:v), left overnight at 4°C and subsequently filtered with a 0.22 μ m filter. Five μ L was loaded on an ACQUITY UPLC protein BEH SEC column (200Å, 1.7 μ m, 4.6 x 150mm, Waters Corporation, Massachusetts, US) with an ACQUITY UPLC protein BEH SEC Guard column (200Å, 1.7 μ m, 4.6 x 30mm, 10K-500K, Waters Corporation) at 25°C. The columns were connected on a Waters ACQUITY UPLC equipped with a Waters 2487 Dual λ Absorbance Detector. The whey products were separated at a flow rate of 0.2 mL/min in solvent and detected at a wavelength of 214 nm.

Native and SDS-PAGE

For analysis of proteins under non-reducing conditions by native polyacrylamide gel electrophoresis (PAGE), 40 μ g of whey product was dissolved in native sample buffer (62.5 mM Tris-HCL pH 6.8, 25% glycerol, 0.01% bromophenol blue; BioRad, Hercules CA, US) and loaded onto a 4-20% TGX polyacrylamide gel (BioRad). Gel electrophoresis was conducted in Tris/Glycin running buffer (25 mM Tris, 192 mM Glycin; BioRad) with 200 V constant current.

For analysis of proteins under reducing conditions, sodium dodecyl sulphate (SDS)-PAGE was conducted with 20 μ g of whey product as previously described [185]. Native and SDS-PAGE gels were washed three times in deionised water, and proteins were stained by Bio-Safe Coomassie stain (BioRad) for 1 h at RT

and washed extensively (>10 times) in deionised water. Stained gels were photographed using Imager ChemiDoc XRS+ (BioRad).

Animals

BN rats from the in-house breeding colony (National Food Institute, Technical University of Denmark, Denmark) were weaned at an age of three weeks. They were housed in macrolon cages (2-3/cage), with a 12 h light:dark cycle, at a temperature of $22 \pm 1^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$. The rats were observed twice daily and clinical signs were recorded. The rats were fed a milk-free diet for ≥ 10 generation to avoid tolerance to the studied allergens. The diet was produced in-house and based on rice flour, potato protein and fish meal as protein sources, as previously described [186], with the exception of maize flakes being substituted with rice flour. Diet and acidified water (pH 3.5) was given *ad libitum*. Animal experiments were carried out at the National Food Institute facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate and authorisation given (2015-15-0201-00553-C1). The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

Intraperitoneal sensitisation experiment

To investigate the inherent sensitising capacity of the two whey products, BN rats (6-8 weeks of age, both sex) were immunised ip with 200 μg of either WPI or HT-WPI in 0.5 mL phosphate buffered saline (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 , 1.8 mmol/L KH_2PO_4 , pH 7.2) (n=8/group). Rats were immunised three times, at day 0, 14 and 28, without any use of adjuvant. They were euthanised at day 35 by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected, converted to serum and stored at -20°C until analysis (overview of animal experimental design in Fig. 2A).

Oral sensitisation experiment

To investigate the oral sensitising capacity of the two whey products, BN rats (4-8 weeks of age, only females) were dosed by gavage with 10 mg of either WPI or HT-WPI together with 20 μg cholera toxin (CT, List Biological Laboratories Inc., Campbell, CA, US) in 0.5 mL PBS (n=12/group). Rats were dosed three times per week (Monday, Wednesday and Friday) for five consecutive weeks. Ten days after the last dosing, blood samples were collected from the sublingual vein, converted to serum and stored at -20°C until analysis (overview of animal experimental design in Fig. 3A).

In vivo tests

Three days after the last dosing, rats were subjected to an oral challenge: The body temperature of each rat was measured by a rectal probe before and 30 min after gavage with 100 mg of the same product that was used for dosing, but without adjuvant, in 1 mL water. Six days after the last dosing, rats were subjected to an ear swelling test: Rats were anaesthetised with hypnorm-dormicum and the thickness of both ears was measured at the same spot before and one hour after intradermal injection of 10 μg of WPI or HT-WPI in 20 μL PBS (one product per ear).

Primary prevention experiment

To investigate *de novo* tolerance inducing capacity, also designated primary preventive capacity, of the two whey products, naïve BN rats (4-6 weeks of age, both sex) were given either water, WPI or HT-WPI *ad libitum* in their drinking bottles (12.5 g protein per litre water) for 21 consecutive days (n=8/group). After one week of rest, rats were post-immunised ip with 100 µg WPI in 0.5 mL PBS once a week for eight weeks. Blood samples from the sublingual vein were collected after tolerance induction and after the fourth post-immunisation. Rats were euthanised one week after the last post-immunisation by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected, converted to serum and stored at -20°C until analysis (overview of animal experimental design in Fig. 4A).

Desensitisation experiment

To investigate the desensitising capacity of the two whey products, three groups of BN rats (4-8 weeks of age, only females, n=12) were orally sensitised to WPI with CT (as described above) and subsequently given either water, WPI or HT-WPI *ad libitum* in their drinking bottles (12.5 g protein per litre water) for 21 consecutive days. After one week of rest, desensitised rats together with naïve control rats were post-immunised ip with 50 µg WPI in 0.5 mL PBS once per week for three weeks. The day after the last post-immunisation, rats were subjected to an ear swelling test with WPI, as described above. Blood samples were collected from the sublingual vein the day after *ad libitum* administration and on the day of second and third post-immunisation. Rats were euthanised by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected, converted to serum and stored at -20°C until analysis (overview of animal experimental design in Fig. 5A).

***In vivo* uptake experiment**

To investigate the intestinal uptake of the two whey products, naïve BN rats (9-16 weeks of age, only females) were gavaged with 100 mg of either WPI or HT-WPI in 1 mL water and euthanised at different time points (n=4/ group/time point). Rats were euthanised 15, 30, 60 or 90 min after gavage by exsanguination using carbon dioxide inhalation as anaesthesia. The small intestine was excised and a 20 cm section (7 cm distal from the stomach) was rinsed with 0.9% (w:v) NaCl and divided into three fractions; Peyer's patches, lamina propria and epithelium; which were individually snap frozen in liquid nitrogen and stored at -80°C until analysis. Total proteins from Peyer's patches and epithelium samples were extracted by homogenisation by rotor stator in 10 µL/mg tissue lysis buffer (150 mM NaCl, 20 mM Tris, 1mM EGTA, 1% Triton X-100, 1 mM EDTA) with 2% (v:v) Halt protease inhibitor cocktail (Thermo Fisher, Hampton, VA, US) on ice. Lamina propria samples were homogenised in lysis buffer, and 2% (v:v) Halt protease inhibitor cocktail (Thermo Fisher) was immediately added to 1 mL of the homogenised tissue. Samples were incubated on ice for 20 min and mixed by vortexing every 5 min, and cleared by centrifugation at 15,000g for 20 min at 4°C. The supernatants were stored at -80°C until analysis. BLG concentrations of supernatants were quantified by a commercial bovine BLG ELISA kit (Bethyl Laboratories, Montgomery, AL, US) in 96-wells MaxiSorp plates (Nunc, Roskilde, Denmark) according to the manufacture's protocol. The ELISA kit was shown to detect WPI and HT-WPI equally well (data not shown).

Analysis of product-specific IgG1 by various ELISAs

For detection of product-specific IgG1 antibodies, indirect ELISA was performed as previously described [187]. For evaluation of the antigen-antibody binding strength, avidity IgG1 ELISA was performed as described [187]. For evaluation of the specificity of the antigen-antibody binding, inhibitory IgG1 ELISA was performed as previously described [188] with the exception that sera were pre-incubated with WPI and HT-WPI inhibitor solutions.

Antibody-capture ELISA for detection of product-specific IgE

For detection of product-specific IgE antibodies, antibody-capture ELISA was performed as previously described [187] with two exceptions: That plates were blocked with 3% (v:v) horse serum (Biowest-bw, Nuaillé, France) in PBS with 0.01% (w:v) Tween 20, and product-specific IgE was detected by 50 µL/well of 0.1 µg/mL of 10:1 digoxigenin (DIG)-coupled WPI or HT-WPI in blocking solution.

Immunoblot

To investigate reactivity of IgG1 antibodies raised against WPI and HT-WPI after ip or oral administration, immunoblots were performed with serum pools as previously described [185] with SDS-PAGE loaded with 5 µg whey product. Serum pools were diluted 1:500 (ip study both groups), 1:800 (oral study, WPI group) or 1:1000 (oral study, HT-WPI group) for optimal visualisation.

***In vitro* epithelial transcytosis**

To evaluate the *in vitro* epithelium permeability, transport of the whey products through a Caco-2 cell layer was examined as previously described [189]. In short, culture media were replaced with fetal bovine serum (FBS) free media on day 20 after seeding. The day after, whey products dissolved in FBS-free culture media were added to make a final concentration of 500 µg/mL in the apical compartment. After 1, 3, 6, and 24 h, 250 µL were sampled from the basolateral compartment and stored at -20°C. Only data from 24 h are shown. Supernatant BLG concentrations were quantified by a commercial bovine BLG ELISA kit (Bethyl Laboratories) in 96-wells MaxiSorp plates (Nunc) according to the manufacturer's protocol. Tight junction integrity was confirmed by TEER (EVOM2, World Precision Instruments, Sarasota, FL, US) before and after the uptake assay. Each condition was performed in triplicate and repeated three times.

***In vitro* dendritic cell uptake**

To evaluate the *in vitro* product uptake by dendritic cells, murine bone marrow-derived dendritic cells (BMDC) were generated as described elsewhere [190] and co-incubated with fluorescein isothiocyanate (FITC)-labelled whey products as previously described [191]. In short, bone marrow cells from femur bones of BALB/c mice were isolated and cultured six days in RPMI medium with 10% (v:v) FBS and 10 ng/mL GM-CSF (R&D, Oxon, UK). To assess protein uptake and endocytosis, 10 µg/mL of FITC-labelled products were incubated with 1×10^6 BMDC/mL in 2.4 mL of cell culture medium in a 37°C water bath. After 0, 5, 15, 30, 45, 60 and 120 min, 300 µL of this cell culture was harvested and immediately washed twice in 1 mL cold PBS and twice in cold FACS buffer (PBS with 2 % (v:v) FBS and 0,05 % (w:v) NaN₃) and centrifuged for 5 min at 250g at 4°C. The percentage of FITC positive cells and mean fluorescence

intensity (MFI) was measured using FACS BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, US). Flow cytometry data were analysed in Cyflogic software (CyFlo Ltd, Turku, Finland). Each condition was performed twice in duplicates.

Statistical analysis

Curve and statistical analyses were made using GraphPad Prism (version 8.1.1 for Windows, GraphPad Software, San Diego, CA, US). For inhibitory ELISA curves, best fit slope estimates (HillSlope) with standard errors were compared by one-way ANOVA. IC50 values were only compared for graphs with slope estimates that were not significantly different.

ELISA results were expressed as log₂ antibody titres. Normal distribution of data was tested by D'Agostino-Pearson normality test. For data that passed the normality test, group means are indicated on graphs and differences between groups were analysed by t-test (two groups) or one-way ANOVA followed by Tukey's post-test for multiple comparison (>2 groups). For data that did not pass the normality test, group medians are indicated on graphs, and group differences were analysed by non-parametric Mann-Whitney test (two groups) or Kruskal-Wallis test followed by Dunn's post-test for multiple comparison (>2 groups). MFI of BMDC was compared between the two groups at each time point by a two-way ANOVA of squarer root transformed data with Sidak's post-test. Asterisks indicate statistically significant difference between two given groups: *: $p \leq 0.05$, **: $p \leq 0.01$, and ***: $p \leq 0.001$, ns: no statistically significant difference.

RESULTS

Characterisation of products

WPI and HT-WPI were analysed for native versus denatured protein composition and aggregation status, which showed that heat-treatment caused changes in the physicochemical features of the WPI. Results from TSK GPC showed that some but not all of the proteins in the HT-WPI were denatured; about 35% of the BLG, 50% of the α -lactalbumin and 100% of glycomacropeptides remained in native form after the heat-treatment (Fig. 1A). Results from BEH GPC showed that heat-treatment of WPI resulted in partial protein aggregation (Fig. 1B). SDS-PAGE confirmed that the protein distribution was the same in the two products (Fig. 1C). However, results from native PAGE revealed that protein conformation differed between the two products; while some distinctive protein bands were detectable for WPI, proteins in HT-WPI were retained in the top of the gel and formed a smear under non-reducing conditions (Fig. 1D).

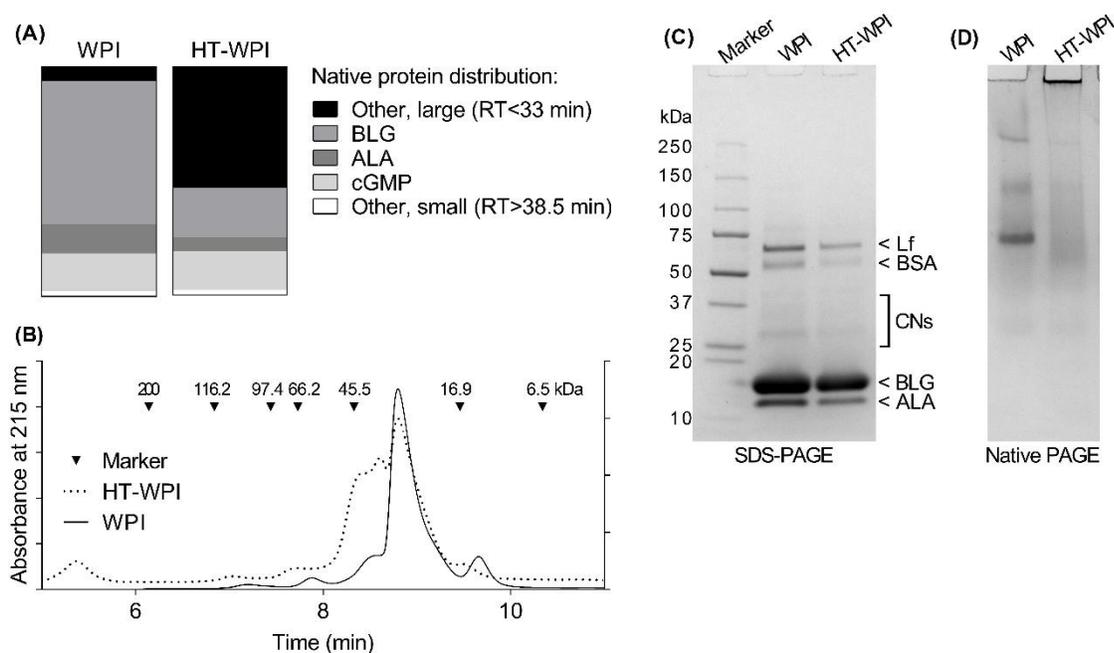


FIGURE 1: Physicochemical characterisation of whey protein isolate (WPI) and heat-treated WPI (HT-WPI). (A) Native protein distribution determined by TSK gel permeation chromatography (GPC), (B) aggregation status determined by ethylene bridged hybrid (BEH) GPC, (C) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and (D) native PAGE. RT, retention time; BLG, β -lactoglobulin; ALA, α -lactalbumin; cGMP, glycomacropeptides; CNs, caseins; Lf, lactoferrin; BSA, bovine serum albumin.

Intraperitoneal sensitisation

To compare the inherent sensitising capacity of the two whey products, serum from rats immunised ip with either WPI or HT-WPI (Fig. 2A) were analysed for specific antibody responses. A statistically significant reduction in the serum level of product-specific IgE was observed in the group of rats ip immunised with HT-WPI compared with those immunised with WPI (Fig. 2B). The reduction in sensitising capacity was not caused by a reduction in immunogenicity, as the serum level of product-specific IgG1 was similar in the two groups (Fig. 2C). The binding strength of the raised IgG1 antibodies were similar in the two groups (Fig. 2D). Results from inhibitory ELISAs demonstrated that the two products differed in competitive capacity; while HT-WPI fully inhibited the binding between WPI and IgG1 antibodies raised against WPI, WPI was not able to fully inhibit the binding between HT-WPI and antibodies raised against HT-WPI, indicating that new epitopes had developed as result of heat-treatment (Fig. 2E). In fact, for antibodies raised against HT-WPI, the IC50 values for HT-WPI were 70 times less than that of WPI, indicating that 70 times more WPI was needed to inhibit 50% of the binding between HT-WPI and antibodies raised against HT-WPI. Immunoblots of SDS-PAGE showed that serum from HT-WPI sensitised rats recognised more protein bands compared with that of WPI sensitised rats (Fig. 2F). Those bands were detectable in both products.

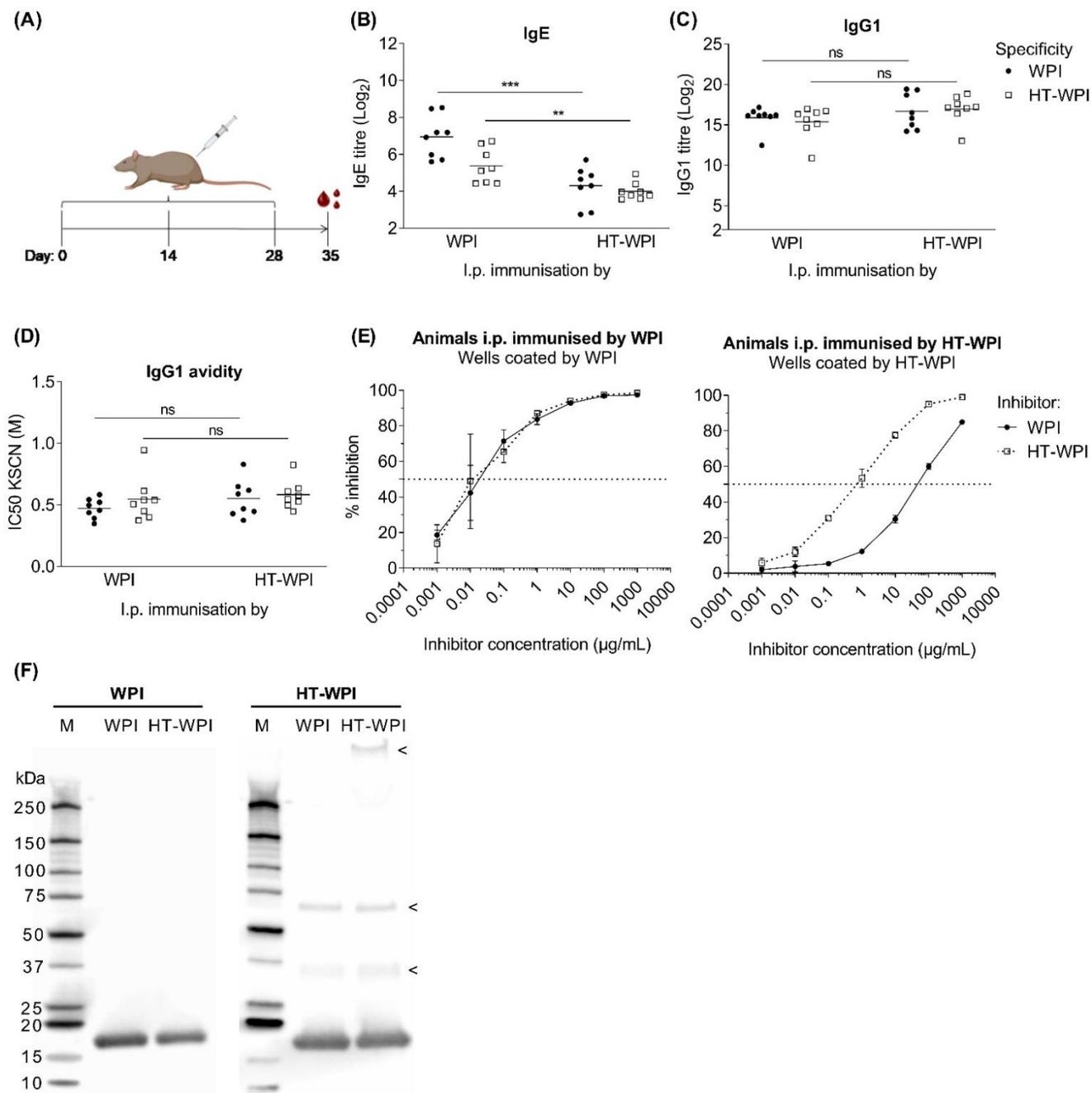


FIGURE 2: Intraperitoneal sensitisation study. (A) Animal experimental design: Groups of eight Brown Norway rats were immunised three times, at day 0, 14 and 28, by intraperitoneal injections of whey protein isolate (WPI) or heat-treated WPI (HT-WPI), and euthanised at day 35. (B) Product-specific IgE titres, (C) product-specific IgG1 titres, (D) binding strength between IgG1 and products, (E) competitive capacity of products by inhibitory IgG1 ELISA, and (F) immunoblotting based on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) with pooled sera for detection of protein reactivity. Differences between the two blots are indicated by <. Analyses were performed on sera from termination. Each symbol represents a single rat, with the exception of inhibitory ELISA where a symbol represents a group of rats and vertical lines indicate standard deviation of three independent technical replicates. Horizontal lines indicate mean value. Statistically significant differences between indicated groups are shown as **: $p \leq 0.01$, ***: $p \leq 0.001$, ns: no statistically significant differences.

Oral sensitisation

To compare the oral sensitising capacity of the two whey products, serum from rats orally gavaged with either WPI or HT-WPI together with the adjuvant CT (Fig. 3A) were analysed for specific antibody responses. In contrast to the ip sensitisation study, oral dosing with WPI or HT-WPI resulted in similar serum levels of product-specific IgE (Fig. 3B) and IgG1 antibodies (Fig. 3C). However, the antigen-IgG1 binding strength was significantly increased in the group of rats that were dosed with the HT-WPI compared with WPI (Fig. 3D). This difference between the binding strength of IgG1 antibodies raised against the two products was observed consistently in both animal experiments in which the products were administered by the oral route (data not shown). Results from inhibitory ELISA confirmed results from the ip sensitisation study; while HT-WPI was able to fully inhibit the binding between WPI and IgG1 antibodies raised against WPI, WPI was not able to fully inhibit the binding between HT-WPI and antibodies raised against HT-WPI (Fig. 3E). However, for antibodies raised orally against HT-WPI, the IC50 values for HT-WPI were only approximately 30 times less than that of WPI; hence, the difference between competitive capacities of the two products was much less for antibodies raised by oral administration compared with antibodies raised by ip immunisations.

To further evaluate the allergenicity, rats were subjected to two elicitation tests: An oral challenge and an ear swelling test. For the oral challenge, the body temperature of the rats was monitored before and 30 min after rats were administered with the same product as they were sensitised to, where a reduction of core body temperature is a sign of anaphylaxis. Despite a similar serum level of specific IgE in the two groups, the relative reduction in body temperature was significantly smaller in the group of rats dosed and challenged with HT-WPI compared with the group dosed and challenged with WPI (Fig. 3F). No statistically significant difference between acute allergic skin responses in the two groups of rats could be observed in the ear swelling test (Fig. 3G). Immunoblots of SDS-PAGE showed that serum from orally WPI sensitised rats recognised more protein bands (Fig. 3H) compared with that of the ip WPI sensitised rats (Fig. 2F). The immunoblots with serum from WPI and HT-WPI orally sensitised rats were overall very similar, but did show different binding pattern in the region between 25-37 kDa, which are likely due to binding of different caseins present in small amounts (Fig. 3H). This showed that intestinal digestion diminished the difference between the products, but that some of the unique epitopes were still present on HT-WPI after ingestion.

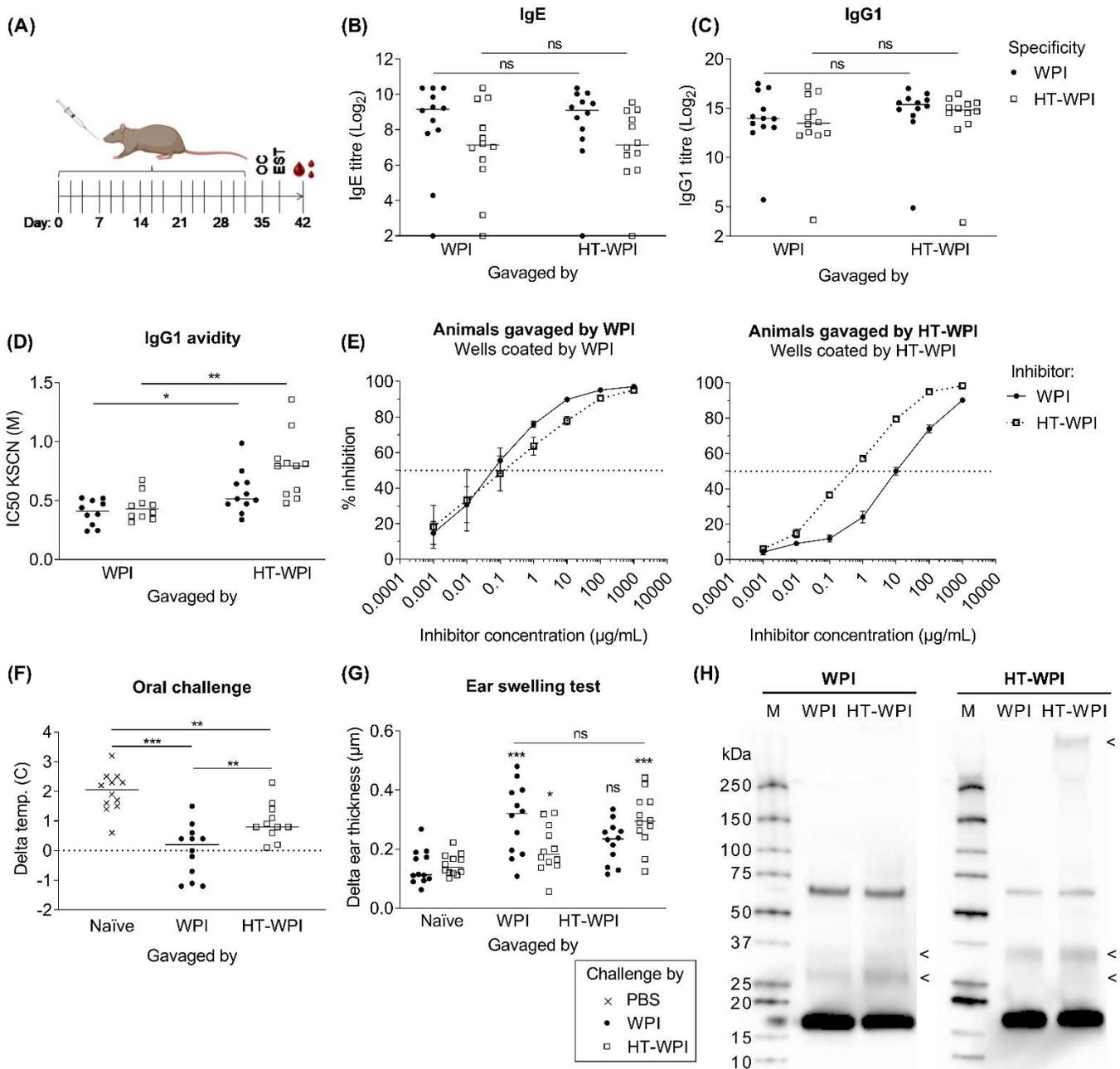


FIGURE 3: Oral sensitisation study. (A) Animal experimental design: Groups of 12 Brown Norway rats were gavaged with whey protein isolate (WPI) or heat-treated WPI (HT-WPI) three times per week for five weeks. At day 35, an oral challenge (OC), and on day 38, an ear swelling test (EST) were performed. (B) Product-specific IgE titres, (C) product-specific IgG1 titres, (D) binding strength between IgG1 and products, (E) competitive capacity of products by inhibitory IgG1 ELISA, (F) changes in core body temperature after OC, (G) acute allergic response from an EST, and (H) immunoblotting based on sodium dodecyl sulphate polyacrylamide gel electrophoresis with pooled sera for detection of protein reactivity. Differences between the two blots are indicated by <. Analyses were performed on sera from day 42. Each symbol represents a single rat, with the exception of inhibitory ELISA where a symbol represents a group of rats and vertical lines indicate standard deviation of three independent technical replicates. Horizontal lines indicate median (B-C) or mean (D, F-G) value. Statistically significant differences between indicated groups (B-D, F-G) or relative to naïve controls (G) are shown as *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ns: no statistically significant differences.

Primary prevention

To compare the *de novo* tolerance inducing properties of the two different products, the primary preventive capacity was evaluated by administering naïve rats with either water, WPI or HT-WPI *ad libitum* in their drinking bottles for 21 consecutive days before the rats were post-immunised ip by WPI (Fig. 4A). Only one rat in each prevention group had a detectable level of WPI-specific IgE after eight post-immunisations while all rats in the control group were sensitised (Fig. 4B). Analysis of WPI-specific IgG1 revealed that less rats had detectable levels of IgG1 after four post-immunisations in the group administered with HT-WPI compared with WPI (Fig. 4C).

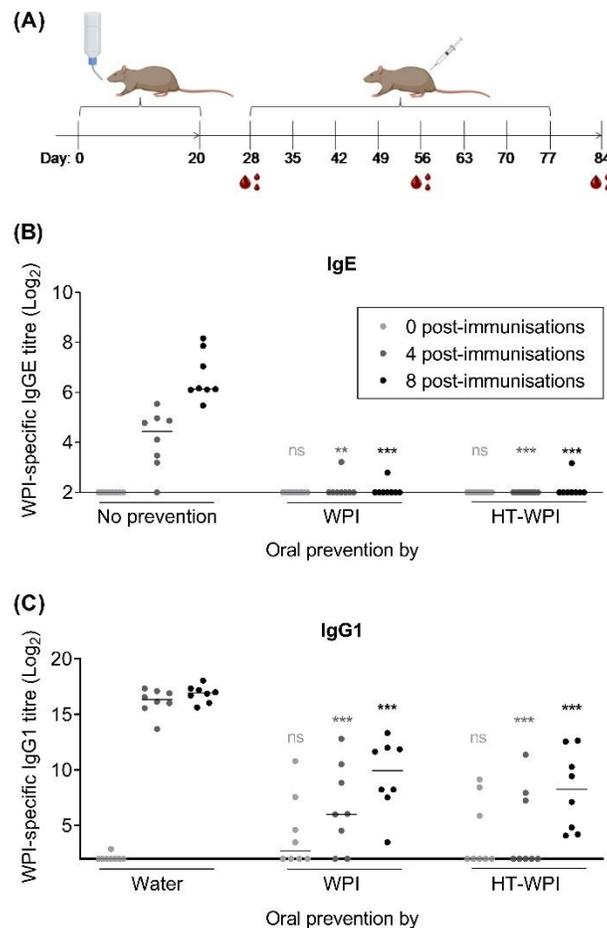


FIGURE 4: Primary prevention study. (A) Animal experimental design: Groups of eight Brown Norway rats were given whey protein isolate (WPI) or heat-treated WPI (HT-WPI) or water *ad libitum* in their drinking bottles for 21 days. After one week of rest, rats were post-immunised by intraperitoneal injection with WPI once a week for eight weeks. Rats were euthanised one week after the last post-immunisation. (B) WPI-specific IgE titres and (C) WPI-specific IgG1 titres. Analyses were performed on sera from day 28, 56 and day 84 (after 0, 4 and 8 post-immunisations, respectively). Each symbol represents a single rat, and horizontal lines indicate group medians. Statistically significant differences relative to the water control group are shown as **: $p \leq 0.01$, ***: $p \leq 0.001$, ns: no statistically significant differences.

Desensitisation

To further investigate the tolerogenic properties of the two different whey products, the desensitising capacity was evaluated by administering rats that were pre-sensitised to WPI with either water, WPI or HT-WPI *ad libitum* in their drinking bottles for 21 consecutive days. Subsequently, the desensitised rats as well as naïve controls were post-immunised three times ip with WPI (Fig. 5A). It was confirmed that serum levels of WPI-specific IgE and IgG1 were not significantly different in the three groups before desensitisation was initiated.

While the natural decrease in WPI-specific IgE in the water control group was minimal, the IgE levels were dramatically decreased in both WPI and HT-WPI groups (Fig. 5B). WPI-specific IgE levels increased slightly with increasing number of ip post-immunisations in both WPI and HT-WPI groups, but remained significantly reduced compared with the water control group at all time points after desensitisation.

In the water control group, WPI-specific IgG1 levels decreased slightly after the desensitisation period and then increased with increasing numbers of immunisations (Fig. 5C). In both WPI and HT-WPI groups, levels remained constant throughout the study. Hence, there was a statistically significant difference in the levels compared with the water control group at all time points after desensitisation. Neither WPI-specific IgE nor IgG1 levels differed significantly between the WPI and HT-WPI groups at any time point. After the third post-immunisation, antibody levels in the WPI and HT-WPI groups did not differ significantly from naïve controls that were just ip immunised (not pre-sensitised and desensitised). Acute allergic skin response, measured by an ear swelling test, was significantly reduced in both WPI and HT-WPI groups compared with the water control group, and the response in the WPI and HT-WPI groups were not different from each other nor from that of naïve rats (Fig. 5D).

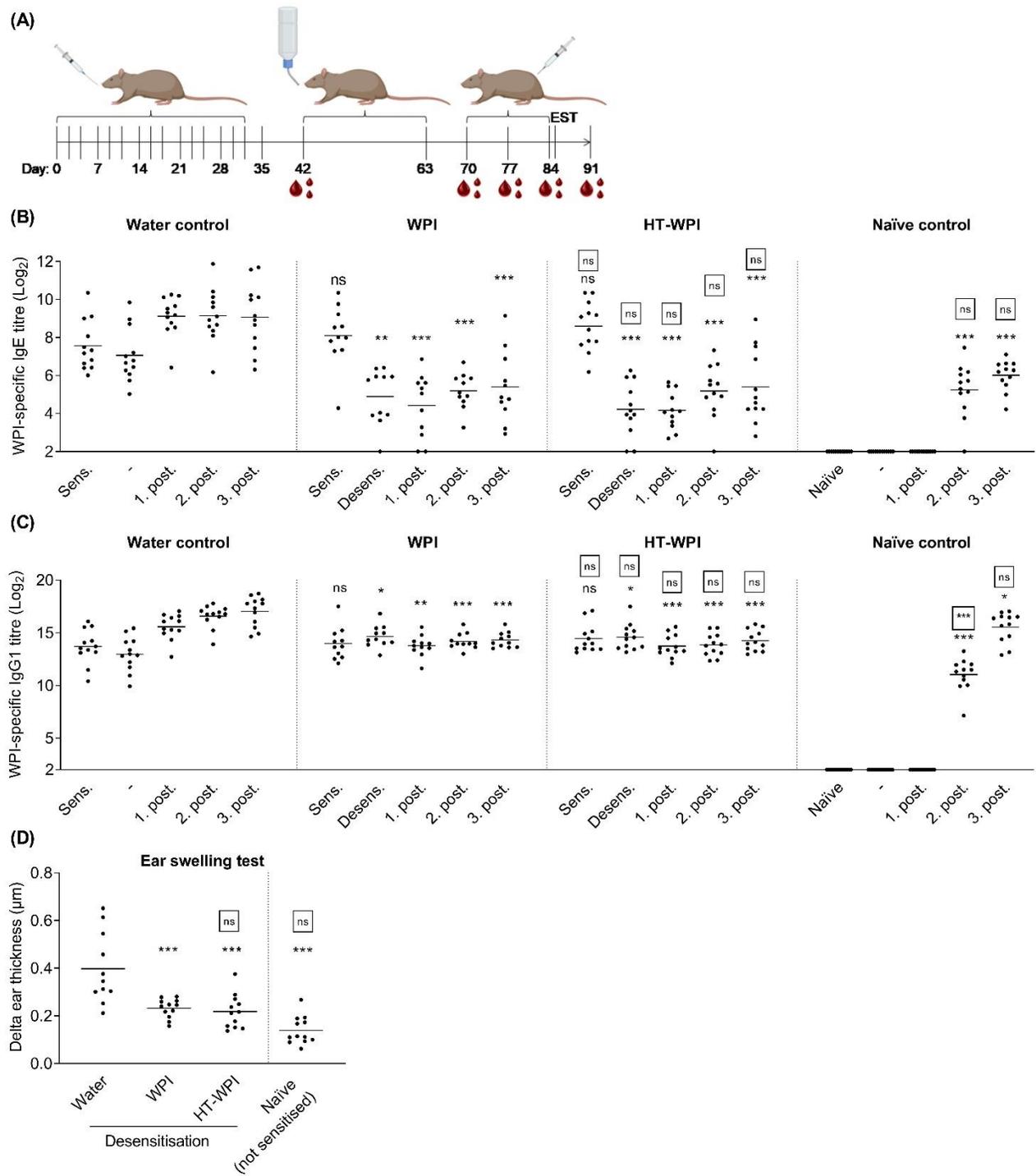


FIGURE 5: Desensitisation study. (A) Animal experimental design: Groups of 12 Brown Norway rats were orally gavaged with whey protein isolate (WPI) three times per week for five weeks. WPI-sensitised rats were given water, WPI or heat-treated WPI (HT-WPI) ad libitum in their drinking bottles for 21 days. After one week of rest, the three groups of desensitised and one group of naïve rats were post-immunised by intraperitoneal injections with WPI once per week for three weeks. The day after the last post-immunisation, rats were subjected to an ear swelling test (EST). Blood samples were taken after sensitisation (day 42) after desensitisation (day 70) and after each post-immunisation (day 77, 84 and 91). (B) WPI-specific IgE titres, (C) WPI-specific IgG1 titres, and (D) acute allergic response from an EST. Each symbol represents a single rat and horizontal lines indicate mean values. Statistically significant differences relative to the water desensitisation control group (unframed) and relative to WPI desensitisation group (framed) are shown as *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ns: no statistically significant differences.

In vivo intestinal uptake

Naïve rats were orally gavaged with WPI or HT-WPI, and euthanised at different time points to examine intestinal protein uptake. BLG was quantified in total protein extracts of three different tissue fractions (Peyer's patches, lamina propria and epithelium) from a piece of the proximal small intestine (Fig. 6A). The distribution of protein between the different tissue fractions differed between the two treatment groups with a larger proportion of BLG being present in epithelium of WPI dosed rats, while a larger proportion of BLG was present in Peyer's patches and lamina propria of HT-WPI dosed rats (Fig. 6B).

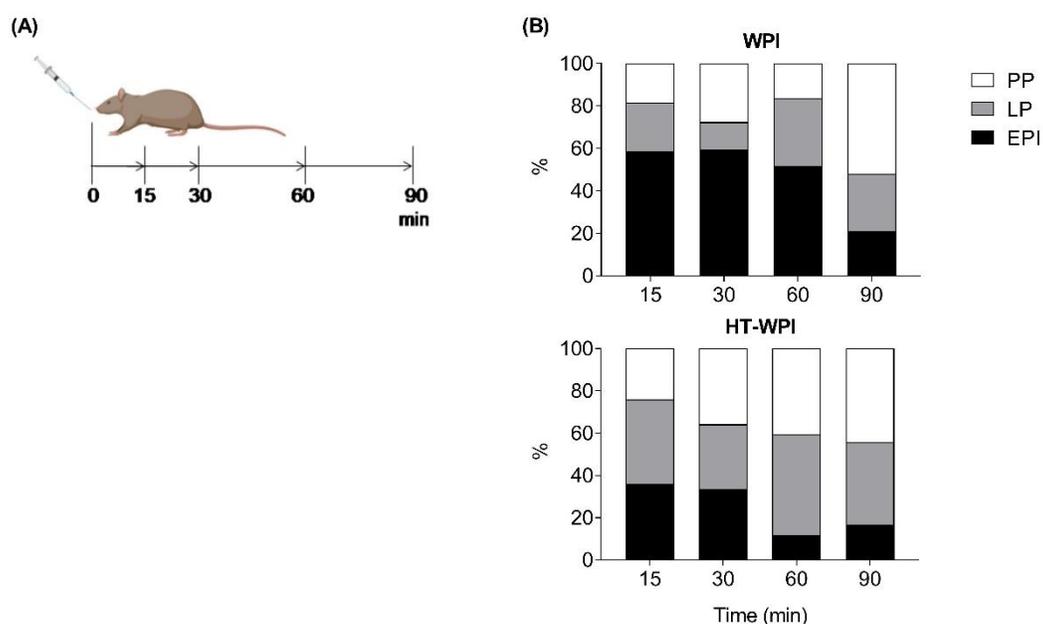


FIGURE 6: *In vivo* intestinal uptake. (A) Animal experimental design: Naïve Brown Norway rats were gavaged with either whey protein isolate (WPI) or heat-treated WPI (HT-WPI) and euthanised at different time points from 15-90 min after dosing (four rats per treatment group per time point). (B) Mean relative distribution of β -lactoglobulin between Peyer's patches (PP), lamina propria (LP), and epithelium (EPI) fractions per tissue weight.

In vitro epithelial transcytosis and uptake by DC

In vitro epithelial transcytosis was assessed by measuring BLG transport across polarised Caco-2 cells grown on semi-permeable membranes. BLG concentrations were quantified in the basolateral compartments 24 h after apical application of the products. This experiment showed a tendency of the basolateral BLG concentration being higher in wells applied with WPI compared with HT-WPI (Fig. 7A). *In vitro* product uptake in DC was assessed by co-incubating murine BMDC with FITC-labelled product preparations. The cells were harvested and washed at different time points, and protein uptake was measured as the level of fluorescence. The experiment showed that BMDC uptake of HT-WPI was more pronounced than the uptake of WPI (Fig. 7B-C).

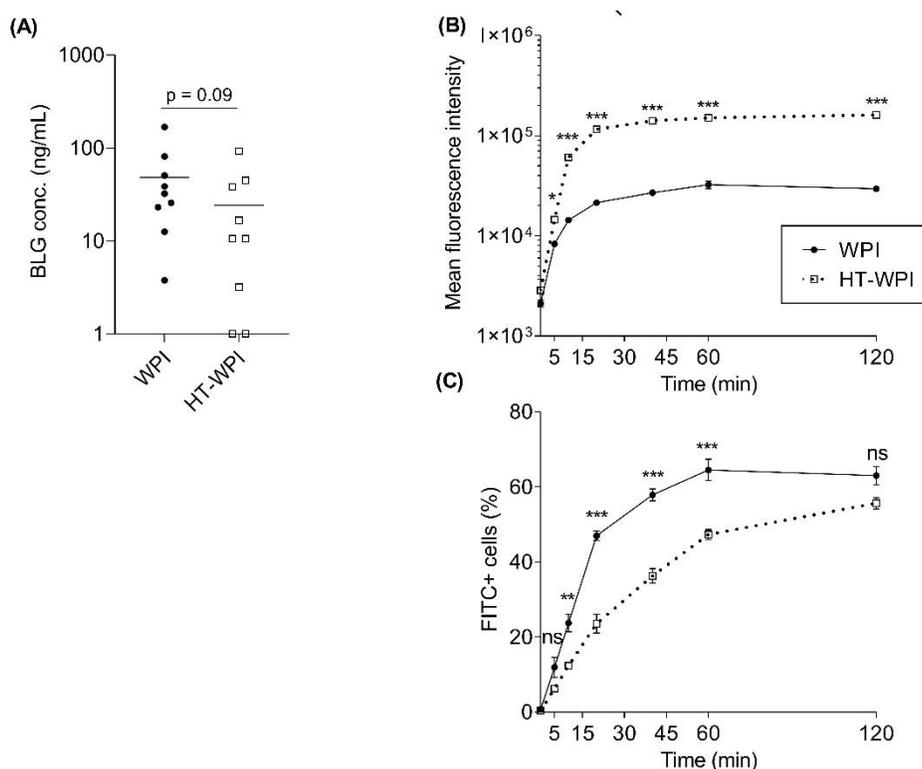


FIGURE 7: *In vitro* epithelial transcytosis and protein uptake by dendritic cells. (A) β -lactoglobulin (BLG) concentrations in basolateral compartment of Caco-2 cells grown on semi-permeable membranes 24 hours after application of whey protein isolate (WPI) or heat-treated WPI (HT-WPI). (B) Mean fluorescent intensity and (C) fraction of fluorescein isothiocyanate positive (FITC+) murine bone marrow derived dendritic cell after incubation with FITC-labelled WPI or HT-WPI. Each symbol represents one replicate (A) or the mean of two replicates (B-C).

DISCUSSION

There is a great interest in producing new and improved hypoallergenic infant formulas for prevention, management, and treatment of CMA. Such infant formulas should have a reduced allergenicity without compromising tolerogenicity. To accomplish this, we need a solid understanding of how structural changes induced by food processing, such as heat-treatment, affect immunological properties of food proteins. This study investigated how heat-treatment affects intestinal uptake, allergenicity and tolerogenicity of a whey protein product.

The whey product was subjected to mild heat-treatment at 90°C for 10 min, which induced partial protein denaturation and aggregation. Heat-treatment disrupts the secondary and tertiary protein structure by breaking hydrogen bonds, and hence cause an unfolding of proteins. In BLG, unfolding has been shown to result in exposure of hydrophobic sulfhydryl groups previously buried inside the folded protein [32]. Interactions of the sulfhydryl group with caseins or other unfolded whey proteins drives aggregation [33]. Conformational changes induced by heat-treatment may influence allergenicity directly by affecting IgE binding epitopes, or indirectly by affecting biophysical properties of proteins such as digestibility and solubility, or the route of intestinal uptake [24,170,192]. The heat-treatment may influence protein epitopes in various ways that either promote or prevent antibody binding: Unfolding may expose epitopes that were previously inaccessible inside the folded protein and/or dissociate conformational epitopes, while aggregation may cause formation of neo epitopes and/or masking of others. Previous studies have indicated that the effect of heat-treatment on epitope accessibility is temperature dependent, as they observed that heat-treatment below 90°C increased BLG antibody reactivity, while heating above 90°C reduced reactivity [34–36].

In the present study, heat-treatment reduced the inherent ip sensitising capacity, while the oral sensitising capacity was unaffected when the products were administered together with the mucosal adjuvant CT. However, HT-WPI induced significantly milder oral symptoms in sensitised rats compared with WPI upon oral challenge without adjuvant. Despite reduced allergenicity, heat-treatment did not reduce the tolerogenic properties as WPI and HT-WPI were equally good at preventing sensitisation of naïve rats and desensitising pre-sensitised rats. In line with our results, it was previously observed that heat-treatment reduced the ip sensitising capacity of BLG [180] and egg ovalbumin [193]. However, in contrast to our results, other studies found that heat-treatment increased the oral sensitising capacity of BLG [179,181], which might be a matter of the exact heat-treatment applied to the products or related to the use of adjuvants.

Tolerogenic properties of heat-treated milk are not well characterised. It has been reported that the introduction of baked milk in the diet of cow's milk allergic patients can accelerate the development of tolerance to fresh milk [173–175], but it has been questioned whether tolerance to baked milk is just a biomarker for spontaneous resolution of milk allergy, or if heat-treated milk has a true tolerogenic effect [178]. Evidence from controlled animal studies is scarce, but one study reported, in line with our results, that feeding of unmodified and heat-treated WPI protected mice against subsequent immunisation with unmodified WPI equally well [141]. To our knowledge, no published animal studies have until now investigated the desensitising effect of heat-treated milk proteins, but results with egg ovomucoid

showed, in line with our results, that heated ovomucoid was as efficacious as unmodified ovomucoid for oral desensitisation of mice [194].

Currently, most hypoallergenic infant formulas are based on hydrolysed milk proteins [166]. Animal studies have shown that hydrolysed products have some capacity to prevent subsequent sensitisation [142,168,195–198]. Evidence on the desensitising capacity of hydrolysed milk products is scarce, but two studies found no desensitising capacity of hydrolysed whey products [168,195]. The present study suggests that heat-treatment could be used as an alternative or supplement to hydrolysis for production of hypoallergenic infant formulas. Future studies should aim for a direct comparison of the allergenic versus tolerogenic properties of milk proteins modified by heat-treatment and hydrolysis to investigate which type of modification is superior.

The present study showed that heat-treated products elicited milder oral symptoms in sensitised animals compared with the unmodified product, which is in agreement with results from previous studies with BLG [179] and egg white proteins [199,200]. This divergence between IgE levels and oral symptoms are hardly related to functionality of the raised IgE antibodies, since no difference in acute allergic skin response was observed, but could be explained by differences in intestinal degradation or intestinal uptake upon oral challenge. BLG is a relatively stable protein resistant to pepsin and acid proteolysis [41]. Previous studies showed that heating increased BLG digestibility [183,201,202] due to the exposure of peptic cleavage sites by unfolding [203]. In the small intestine, food proteins can be taken up through the layer of epithelial cells covering lamina propria by different transport mechanisms, or through M cells into Peyer's patches. The physicochemical features of proteins have been suggested to impact the route of uptake, and the uptake route has been suggested to impact the type of immune response that will be mounted [52,53].

The present study showed that heat-treatment altered the route of uptake, with less being absorbed through the epithelium but more into the Peyer's patches *in vivo* and into BMDC *in vitro* after heat-treatment. This is in line with previous *in vivo* and *in vitro* uptake results for aggregated whey induced by heat-treatment [179] or by cross-linking through laccase treatment [191]. Despite that results from the *in vivo* and *in vitro* intestinal uptake in the present studies were in line with previous studies, this did not increase oral sensitisation as was previously observed [179,191]. This inconsistency could be due to differences in sensitisation study design, in particular dose and potency of CT. The adjuvant function of CT includes induction of the tight junction complex protein claudin-2 on intestinal epithelial cells, which promote antigen transport across the epithelia barrier [204] and could possibly mask differences in intestinal uptake of the products during the sensitisation phase. Alternatively, the inconsistency between the studies might indicate that differences in intestinal uptake are most important in the elicitation phase, for which the results are consistent, while other factors, such as differences in protein epitopes, which depends on the exact heat-treatment [34–36], are critical for sensitisation.

In the present study, results from inhibitory ELISA indicated that antibodies from rats dosed with HT-WPI react to epitopes that were not present or accessible in WPI under physiological conditions. However, considering the results from immunoblotting, performed under reducing conditions, the antibodies from HT-WPI sensitised rats reacted to the same protein bands in the two products, which indicated that the new epitopes were at least partly due to exposure of epitopes previously buried inside the folded

proteins. Results from avidity ELISA suggested that the reactivity towards new epitopes increased the binding strength between HT-WPI and antibodies raised against HT-WPI.

In conclusion, this study showed that heat-treatment overall reduced the allergenicity without compromising immunogenicity and tolerogenic capacity of a whey protein product. This result could be related to differences in intestinal uptake since it was observed that WPI was transported more efficiently through the epithelia cell layer and that heat-treatment increased uptake into Peyer's patches. These results highlight the potential for heat-treatment in the production of efficient and safe whey-based products as an alternative or supplement to whey-based hydrolysates for prevention and treatment of CMA in an attempt to accelerate the outgrowth of allergy and for tolerance development. However, clinical studies are needed to validate the safety and efficacy of heat-treated whey.

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CONFLICT OF INTEREST

L.N.J., H.F.C. and L.V.S. are employees at Arla Foods Ingredients. K.L.B and K.B.G have ongoing collaboration with the company Arla Foods Ingredients, which supplied the whey protein products for the current study. Other authors declare no conflict of interest.

2.2 Manuscript II

Short-term amoxicillin-induced perturbation of the gut microbiota promotes acute intestinal immune regulation in Brown Norway rats

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Keywords: Antibiotics, microbiome, host response, Treg, regulatory T cells, IgA, goblet cells, mucus

Supplementary materials can be found in Appendix A.

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ABSTRACT

The intestinal gut microbiota is essential for maintaining host health. Concerns have been raised about the possible connection between antibiotic use, causing microbiota disturbances, and the increase in allergic and autoimmune diseases observed during the last decades. To elucidate the putative connection between antibiotic use and immune regulation, we have assessed the effects of the antibiotic amoxicillin on immune regulation, protein uptake, and bacterial community structure in a Brown Norway rat model.

Daily intra-gastric administration of amoxicillin resulted in an immediate and dramatic shift in fecal microbiota, characterized by a reduction of within sample (α) diversity, reduced variation between animals (β diversity), increased relative abundance of Bacteroidetes and Gammaproteobacteria, with concurrent reduction of Firmicutes, compared to a water control group. In the small intestine, amoxicillin also affected microbiota composition significantly, but in a different way than observed in feces. The small intestine of control animals was vastly dominated by *Lactobacillus*, but this genus was much less abundant in the amoxicillin group. Instead, multiple different genera expanded after amoxicillin administration, with high variation between individual animals, thus the small intestinal α and β diversity were higher in the amoxicillin group compared to controls.

After one week of daily amoxicillin administration, total fecal IgA level, relative abundance of small intestinal regulatory T cells and goblet cell numbers were higher in the amoxicillin group compared to controls. Several bacterial genera, including *Escherichia/Shigella*, *Klebsiella* (Gammaproteobacteria) and *Bifidobacterium*, for which the relative abundance was higher in the small intestine in the amoxicillin group than in controls, were positively correlated with the fraction of small intestinal regulatory T cells.

Despite of epidemiologic studies showing an association between early life antibiotic consumption and later prevalence of inflammatory bowel diseases and food allergies, our findings surprisingly indicated that amoxicillin-induced perturbation of the gut microbiota promotes acute immune regulation. We speculate that the observed increase in relative abundance of small intestinal regulatory T cells is partly mediated by immunomodulatory lipopolysaccharides derived from outgrowth of Gammaproteobacteria.

INTRODUCTION

The gut associated lymphoid tissue is continuously exposed to a vast amount of bacterial and food derived antigens. Failure to develop tolerance towards these antigens may lead to inflammatory bowel diseases (IBD) or food allergies, both characterized by an adverse immune response directed against either commensal bacteria or dietary components. A healthy gut microbiota is important to avoid detrimental responses against harmless antigens [205], and epidemiological studies suggest that perturbation of the early life gut microbiota by pre- or post-natal exposure to antibiotics increases the risk of food allergies [113–115] and autoimmune disorders including IBD [206–208].

The gut microbiota promotes immune tolerance by stimulating the generation of regulatory T cells (Tregs) [209–211] and by reinforcing the intestinal barrier function e.g. through regulation of tight junction permeability [212]. Additionally, intestinal bacteria influence the secretion of mucus by goblet cells [213] and of immunoglobulin A (IgA) by plasma cells [214,215], both of which contribute to (I) antigen presentation and tolerogenic immune priming [49,84], (II) intestinal barrier function, and (III) balancing the microbial community [103,216]. While multiple studies have assessed the influence of gut microbiota perturbation on immune regulation using broad-spectrum antibiotic cocktails [217–219] and germ-free (GF) animals [119,220], only few studies have investigated how administration of a single, clinically relevant antibiotic affects immune regulation [221,222].

Brown Norway (BN) rats constitute an well-established animal model in food allergy research [168,223], as they mount an immune response resembling that of atopic human individuals [224]. The used BN rats originate from our in-house breeding colony, and in contrast to most animals from commercial vendors they have a natural microbiota, and may thereby better resemble the human immune response [165]. Amoxicillin is a bactericidal β -lactam antibiotic that affects both Gram-positive and Gram-negative bacteria by inhibiting an essential enzyme for bacterial cell wall synthesis [225]. It is the most widely used penicillin in Europe [226], and is frequently prescribed for treatment of pediatric infections [227], and the impact of microbiota alterations caused by this antibiotic on early development of the immune system is therefore highly relevant to address. The gut microbiota of BN rats has not previously been analyzed by NGS based methods. In order to elucidate the interplay between microbiota composition and immune regulation, we characterized the temporal and spatial effects of seven days of amoxicillin administration in BN rats by analyzing the gut microbiota, host immune regulation and intestinal permeability to cow's milk protein. Interestingly, the observed rapid amoxicillin-driven perturbation of the gut microbiota was associated with an acute immune regulatory response.

MATERIALS AND METHODS

Animals

BN rats from the in-house breeding colony (National Food Institute, Technical University of Denmark, Denmark) were housed in macrolon cages and kept at a 12 hour light:dark cycle, at a temperature of $22 \pm 1^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$. Rats were observed twice daily and clinical signs recorded. The rats were fed a milk-free diet for ≥ 10 generation that was produced in-house and based on rice flour, potato protein and fish meal as protein sources, as previously described [186], with the exception of maize flakes being substituted with rice flour. Diet and water were given *ad libitum*. Ethical approval was given by the Danish Animal Experiments Inspectorate and the authorization number given 2015-15-0201-00553-C1. The experiment was overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

Animal experiment

BN rats of both sex were allocated into two groups of 12 rats. Starting from 4-5 weeks of age, rats were daily gavaged with 30 mg amoxicillin in 0.5 mL milli Q water or water alone from Day 0-6. Fecal samples were collected three days before first gavage (Day -3) and daily throughout the study and stored at -80°C for microbiota analysis. From Day -3 and 7 one additional fecal sample was collected and stored on ice for total IgA quantification in fecal water. Intestinal uptake of β -lactoglobulin (BLG) from cow's milk was assessed by dosing animals with 100 mg of whey protein concentrate (kindly provided by Arla Foods Ingredients) in 1 mL milli Q water by oral gavage 15 min prior to sacrifice.

Dissection

Animals were euthanized by exsanguination using carbon dioxide inhalation as anesthesia. Whole blood was collected in sodium-heparin coated tubes (BD Bioscience, Franklin Lakes, NJ, US) that were mixed for 10 min at room temperature (RT) and then stored on ice until processing immediately after the dissection, and in non-coated tubes stored 1 h at RT for preparation of serum. The intestine was excised and divided into multiple fractions for different analyses: mesenteric lymph nodes (mLNs) were stored in phosphate buffered saline (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 , 1.8 mmol/L KH_2PO_4 , pH 7.2) on ice, and processed for flow cytometry immediately after the dissection. From the first 20 cm of the small intestine (7 cm distal from the stomach), content was snap frozen in liquid nitrogen and stored at -80°C for microbiota analysis. The tissue was rinsed with 0.9% (w/v) NaCl before Peyer's patches (PPs) and epithelium was removed, and the tissue was snap frozen in liquid nitrogen and stored at -80°C until analysis of permeability. PPs from the rest of the small intestine were excised, washed in 0.9% (w/v) NaCl and stored in PBS on ice until processing immediately after the dissection. The next 10 cm of the small intestine (without PPs) was rinsed with 0.9% (w/v) NaCl and stored in wash buffer (PBS with 2% (v/v) heat-inactivated fetal calf serum (FCS) and 15 mM HEPES) on ice until processing immediately after the dissection. The next 1 cm piece was rinsed with 0.9% (w/v) NaCl and stored in RNeasy lysis buffer (Qiagen, Crawley, UK) at -20°C until analysis of gene expression. Finally, 1 cm pieces of the small intestine and of colon were fixed overnight in 4% (w/v) paraformaldehyde for

histology. Cecum was weighted, and cecum content was snap frozen in liquid nitrogen and stored at -80°C for microbiota analysis.

Bacterial DNA extraction

DNA was extracted from up to 200 mg of feces and cecum content and up to 250 mg of small intestine content by DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. Mechanical lysis of bacteria was conducted at 30 cycles/s twice for 5 min on bead beater MM300 (Retsch, VWR, Haan, Germany). DNA concentrations were measured by the Qubit ds DNA BR kit (Life Technologies, Carlsbad, CA, US) and DNA was stored at -20°C.

Bacterial load

Bacterial load was estimated by quantification of 16S rRNA gene copies by qPCR as previously described [221]. Briefly, the V3-region of the 16S rRNA gene was amplified in triplicate for each sample, using universal primers HDA1 and HDA2 [228]. Each qPCR reactions contained 5.5 µL LightCycler1 480 II SYBR Green I Master (Roche Applied Science, Penzberg, Germany), 0.2 µM of each primer and 0.2 µL of diluted template DNA in a total reaction volume of 11 µL. Reaction conditions were: Pre-incubation at 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 45 s. Lastly, a melting curve was generated (95°C for 5 s, 68°C for 1 min and increasing the temperature to 98°C with a rate of 0.11°C/s with continuous fluorescence detection). The qPCR was run in 384-well format on a LightCycler® 480 II (Roche Applied Science) and analyzed using the LightCycler® 480 software. Tenfold dilutions of a linearized (SphI-digested) plasmid standard, construction by cloning the 199bp V3region of the 16S rRNA gene of *E. coli* (ATCC 25922) into the pCR14Blunt-TOPO vector (Invitrogen), was used for quantification of 16S rRNA genes.

Amplicon sequencing of the 16S rRNA gene

The bacterial community composition was analyzed by sequencing of the V3-region of the 16S rRNA gene in the extracted DNA. Amplification of the V3-region and subsequent sequencing was performed using the Ion Torrent PGM platform (Life Technologies) as previously described [229]. In short, the V3-region of the 16S rRNA gene was amplified using a universal forward primer (PBU 5'-A-adapter-TCAG-barcode-CCTACGGGAGGCAGCAG-3') with a unique 10–12 bp barcode for each sample (IonXpress barcode as suggested by the supplier, Life Technologies) and a universal reverse primer (PBR 5'-trP1-adapter-ATTACCGCGGCTGCTGG-3'). The PCR reactions were conducted with 4 µL HF-buffer, 0.4 µL dNTP (10 mM of each base), 1 µM forward primer, 1 µM reverse primer, 5 ng template DNA in 1 µL, and 0.2 µL Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, US) in a total reaction volume of 20 µL. Reaction conditions were as follows: Initial 98°C for 30 s followed by 24 (feces and cecum content) or 30 (small intestine content) cycles of 98°C for 15 s and 72°C for 30 s, finally, 72°C for 5 min before cooling to 4°C. PCR products were purified by HighPrep™ PCR Clean-up System (Magbio, Gaithersburg, MD, US) according to the manufacture's protocol, and DNA concentrations were determined with Qubit HS assay. Finally, a library was constructed by mixing an equal amount of PCR products from each sample. Sequencing was performed on a 318-chip for Ion Torrent sequencing using the Ion OneTouch™ 200 Template Kit v2 DL.

Sequence data handling

Sequence data was obtained in FASTQ format and further processed using CLC bio genomic workbench (Qiagen) in order to de-multiplex samples and remove sequencing primers. Further quality trimming using default settings (remove low quality nucleotides pbase-calling error = 0.05, trim ambiguous nucleotides = 2) and filtering only reads with a final length between 125-180bp were exported in FASTQ format. Further quality trimming was performed in Divisive Amplicon Denoising Algorithm 2 (DADA2, Version 1.10.1) with default settings as described elsewhere [230]. Finally, an amplicon sequencing variant (ASV) table was constructed which contains the counts of each sequence variant in each sample. All sequence reads were taxonomically classified using the Ribosomal Database Project Multi-classifier tool [231].

The ASV table was imported into the "Quantitative Insights Into Microbial Ecology" (Qiime) 2 pipeline (Version 2019.1), and α and β diversity metrics were calculated by the function "diversity core-metrics-phylogenetic" based on a rooted phylogenetic tree. For analysis of fecal samples only, samples were rarefied to 20,000 reads to eliminate bias from uneven sampling depth, and for analyses that also included small intestine, samples were rarefied to 10,000 reads.

Preparation of serum and fecal water

To prepare serum, blood samples were allowed to coagulate for 1 h at RT and subsequently overnight at 4°C. The following day the coagulated blood was removed and samples were centrifuged at 1,800g for 20 min at 4°C. The supernatants were transferred to clean tubes and stored at -20°C until analysis.

To prepare fecal water, fecal pellets were mixed with 10 μ L cold PBS with 0.05% (w/v) NaN₃ (Sigma, St. Louis, MO, US) per mg sample in a bead beater MM300 (Retsch) for 15 min at 30 cycles/s followed by centrifugation at 16,000g at 4°C for 10 min. The supernatants were transferred to clean tubes and stored at -20°C until analysis.

Sandwich ELISA for detection of total IgA

For detection of total IgA antibodies sandwich ELISA was performed. Plates (96 wells MaxiSorp, NUNC, Roskilde, Denmark) were coated with 100 μ L/well of mouse anti-rat IgA (MCA191, BioRad, Oxford, UK,) diluted 1:2,000 in carbonate buffer (15 mm Na₂CO₃, 35 mm NaHCO₃, pH 9.6) and incubated overnight at 4°C. To block unspecific binding 200 μ L/well of 3% (w/v) egg white protein (E0500, Sigma) in PBS with 0.01% (w/v) Tween 20 (PBS-T) was added and plates were incubated at 37°C for 1 h. Next, 50 μ L/well of two-fold serial dilutions of serum or fecal water samples and positive and negative control samples diluted in PBS-T were added, and plates were incubated for 1 h at RT. For detection, plates were incubated with 100 μ L/well of horseradish peroxidase-conjugated goat anti-rat IgA (STAR111P, BioRad, UK) diluted 1:10,000 in PBS-T. Between each step, plates were washed five times in PBS-T. After the last wash, plates were additionally washed twice in running tap water. The reaction was visualized by adding 100 μ L/well of 3,3',5,5'-tetramethylbenzidine (TMB)-one substrate (Kem-En-Tec, Taastrup, Denmark) for 12 min and stopped with 100 μ L/well of 0.2 M H₂SO₄. Absorbance was measured at 450 nm with a reference wavelength of 630 nm using a microtiter reader (Gen5, BioTek Instruments, Winooski, VT, US).

Antibody titers were expressed as the log₂ titer values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 standard deviation.

Single cell suspensions of small intestinal and lymphoid tissues

Small intestine lamina propria (LP) samples stored on ice were opened longitudinally and cut into 5 mm pieces. The pieces were washed five times in wash buffer and tissue was digested in RPMI medium (Sigma) with 10% (v/v) FCS (Sigma), 15 mM HEPES (Sigma), 100 units/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), 250 µg/mL collagenase (Sigma) and 1 mg/mL dispase II (Sigma) for 45 min on a shaker at 37°C. Afterwards, the tissue digest was filtered through a 70 µm cell strainer, which was regularly rinsed with FACS buffer (PBS with 2% (v/v) FCS and 0.05% (v/v) NaN₃). PPs and mLNs were minced through 70 µm cell strainers, which were regularly rinsed with FACS buffer. The cell suspensions were centrifuged at 400g (LP and mLNs) or 600g (PP), 4°C for 10 min. The supernatants were discarded, and the cells re-suspended in 1 mL FACS buffer, and cells were counted in NucleoCassettes by use of a NucleoCounter (Chemometec, Allerød, Denmark).

Staining of lymphocytes for flow cytometry

Approximate 10⁶ cells/well were plated in 96-well plates. Non-specific binding was prevented by incubation 5 min on ice with 50 µL blocking solution with 10% (v/v) rat serum and 5 µg/mL anti-CD32 (D34-485, BD Biosciences) in FACS buffer. Cells were surface stained by addition of 50 µL/well antibody cocktail containing 2 µg/mL of each of the following: anti-B220-PE (HIS24, BD Biosciences), anti-CD3-PerCp-Cy5 (eBioG4.18, Thermo Fisher Scientific), anti-CD4-PE-Cy7 (OX-35, BD Biosciences), anti-CD45-APC-Cy7 (OX-1, Thermo Fisher Scientific), anti-CD25-BV421 (OX-39, BD Biosciences) in FACS buffer, and incubated 20 min on ice. Cells were washed with 100 µL FACS buffer and plates were centrifuged at 400g, 4°C for 5 min. Supernatants were discarded and pellets resuspended in 200 µL FACS buffer.

Whole blood samples were diluted two times in FACS buffer and 100 µL were stained with 50 µL of the above described antibody cocktail and incubated for 20 min on ice. Afterwards, 1 mL VersaLyse (Beckman Coulter, Brea, CA, US) was added to lyse the red blood cells. The samples were incubated in dark at RT for 10 min before 2 mL FACS buffer was added and samples were centrifuge at 350g, 4°C for 6 min. Supernatants were discarded and pellets resuspended in 200 µL FACS buffer and transferred to 96-well plates.

For identification of the transcription factors Forkhead box P3 (Foxp3) and Helios, cells were subsequently stained by using the BD Transcription Factor buffer set (BD Biosciences) according to the manufacture's protocol. After fixation and permeabilization, the cells were blocked as described above. Cells were stained with 50 µL of 5 µg/mL anti-Foxp3-FITC (JFK-16s, Thermo Fisher Scientific) and 2 µg/mL anti-Helios-Alexa Flour 647 (22F6, BD Biosciences) in TF Perm/Wash Buffer from the BD Transcription Factor buffer set, and incubated for 40 min on ice. After two washes in TF Perm/Wash Buffer, the cells were resuspended in FACS buffer. The stained cells were stored at 4°C until data was acquired. Just before accusation, 10 µL CountBright counting beads (Thermo Fisher Scientific) was added to each well. Data was acquired on BD FACSCanto II system (BD Bioscience) and analyzed by FlowJo (Version 10.4.2, BD Bioscience).

Intestinal protein uptake

Total proteins were extracted from small intestine tissue samples by mixing with 10 μ L tris-lysis buffer (150 mM NaCl, 20 mM Tris, 1mM EGTA, 1% (v/v) Triton X-100, 1 mM EDTA) with 2% (v/v) Halt protease inhibitor cocktail (Thermo Fisher Scientific) per mg tissue. One stainless steel bead (Qiagen) were added to each sample, and samples were homogenized in buffer by TissueLyser II (Qiagen) at 30 cycles/s for 2 min. Samples were incubated on ice for 20 min and mixed by vortexing every 5 min. Samples were centrifuged at 15,000g for 20 min at 4°C and the supernatants were frozen at -80°C until analysis. Concentrations of the cow's milk protein BLG were quantified in tissue extracts and serum samples by a commercial bovine BLG ELISA kit (Bethyl Laboratories, Montgomery, AL, US) in 96 wells MaxiSorp plates (NUNC) according to the manufacture's protocol with the exception that plates were coated overnight. The concentrations were determined in triplicates and calculated from the duplicated standard curves generated for each plate.

Histology

After overnight fixation in 4% formalin (CellPath, Newtown, UK), sections of small intestine and colon were dehydrated in a graded ethanol series from 77-99% ethanol (VWR Chemicals, Radnor, PS, US). Xylene (VWR Chemicals) was used as clearing agent to replace the ethanol before the tissues were embedded in paraffin (Hounisen, Skanderborg, Denmark). Histological sections of 2 μ m were stained with Hematoxylin (Ampliqon, Odense, Denmark) and Eosin (Merck, Darmstadt, Germany) to identify eosinophils, and Periodic acid–Schiff (PAS) (Merck) to identify goblet cells. The slides were examined using a Leica DMR upright microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the software ImagePro Plus 7.0 (MediaCybernetics, Rockville, MD US) was used for images and measurements. Villus length was measured from the villus tip to the crypt-villus junction and in colon crypt depth was measured, with three villi or crypts measured/counted per animal. Analyses of histological sections were performed blinded.

Tissue RNA extraction, cDNA synthesis and RT-qPCR

Approximately 20-40 mg small intestine tissue, stored in RNAlater, was homogenized using TissueLyser II (Qiagen) and total RNA was extracted with RNeasy Mini Kit (Qiagen) with on-column DNase digestion (RNase Free DNase Kit, Qiagen) according to manufacturer's protocol. RNA quality and quantity were assessed using NanoDrop. cDNA was synthesized from 500 ng RNA with Omniscript RT Kit (Qiagen) in accordance to manufacture's protocol and in addition of Random Primer Mix (BioNordika, Herlev, Denmark) and Anti-RNase (Ambion, Life Technologies, Carlsbad, CA, US). Quantitative RT-PCR (RT-qPCR) reactions were run in technical duplicates using a Quantstudio 7 Flex Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific) in 11 μ L reactions containing 3 μ L diluted cDNA (1:20), TaqMan Fast Universal PCR Master Mix (2x) (Applied Biosystems), and TaqMan gene Expression Assay (Applied Biosystems). TaqMan gene assays used were *Cdh1* (Cadherin-1 Rn00580109_m1), *Ocln* (Occludin Rn00580064_m1), *Cldn2* (Claudin 2 Rn02063575_s1), *Tjp1* (Tight junction protein 1 (ZO-1) Rn02116071_s1), *TSLP* (Thymic stromal lymphopoietin Rn01761072_m1), *Il33* (Interleukin 33 Rn01759835_m1), and *Muc2* (Mucin 2 Rn01498206_m1). Reaction conditions were: An initial cycle at 95°C for 20 s followed by 45 two-step thermal cycles at 95°C for 1 s and at 60°C for 20 s. The relative

gene expression was calculated using the $2^{-\Delta CT}$ method using *B2m* (Beta-2-microglobulin Rn00560865_m1) and *Sdha* (Succinate dehydrogenase complex Rn00590475_m1) as normalization genes. Data was acquired with Quantstudio 7 Flex software (Applied Biosystems, Foster City, CA, US).

Statistics

Differences between β diversity of groups were assessed by applying ANalysis Of Similarities ANOSIM [232] to weighted and unweighted UniFrac distances, and within group dispersion was analyzed with PERMDISP [233] both in Qiime 2. Differential abundant genera between the amoxicillin and control group were determined by ANalysis of COMposition of Microbiomes (ANCOM) [234] in R. This was only applied to small intestine samples, because more than 25% of genera varied between the two groups in cecum and feces. Graphs and additionally statistical analyses were generated in Prism version 8.1.1 (GraphPad, San Diego, CA, US). Difference between initial and later time points within the same group were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Difference between the two groups at the same time point were analyzed by t-test (when data passed D'Agostino-Pearson normality test) or non-parametric Mann-Whitney test (when not normally distributed). Non-parametric Spearman correlations were calculated between all pairs of relative abundance of small intestine genera (present in minimum 10 animals) and selected host response parameters. When indicated, p-values were false discovery rate (FDR) corrected by a two-stage sharpened method [235].

RESULTS

Amoxicillin transiently reduces fecal bacterial load and persistently decreases fecal microbial diversity

BN rats were gavaged daily with either amoxicillin or water for one week, and the temporal effects on fecal bacterial load and composition were followed. The first day after onset, a statistically significant reduction of total bacterial load was observed in the amoxicillin group as compared to both the baseline level (Day -3) and to the level in the control group (Fig 1A). The effect of amoxicillin on bacterial load was transient since no differences were observed compared to the initial level nor to the control group from Day 2 and onwards. In order to assess the overall effect of amoxicillin on gut microbiota, the within sample (α) diversity was estimated by the Faith's phylogenetic diversity index. A statistically significant reduction in diversity was observed in the amoxicillin group as compared to the initial level from Day 1, as well as between the amoxicillin and control group from Day 2 (Fig. 1B).

Amoxicillin rapidly disturbs fecal microbiota and promotes Gammaproteobacteria outgrowth

From Day 1, the mean relative abundance of Firmicutes and Actinobacteria were lower in the amoxicillin group than in the control group, while Proteobacteria, especially Gammaproteobacteria were higher (Fig. 1C). In the amoxicillin group, the fecal microbiota continued changing over the course of the study, with the relative abundance of Bacteroidetes gradually expanding over time. This continuous shift in composition was also observed by principal coordinate analysis of unweighted UniFrac distances measuring the overall diversity between samples, while the microbiota compositions remained relatively stable in the control group (Fig. 1D-E). These observations were confirmed by pairwise comparisons of unweighted UniFrac distances between different days within the same group, which however also revealed a minor temporal effect in the control group (Fig. 1F).

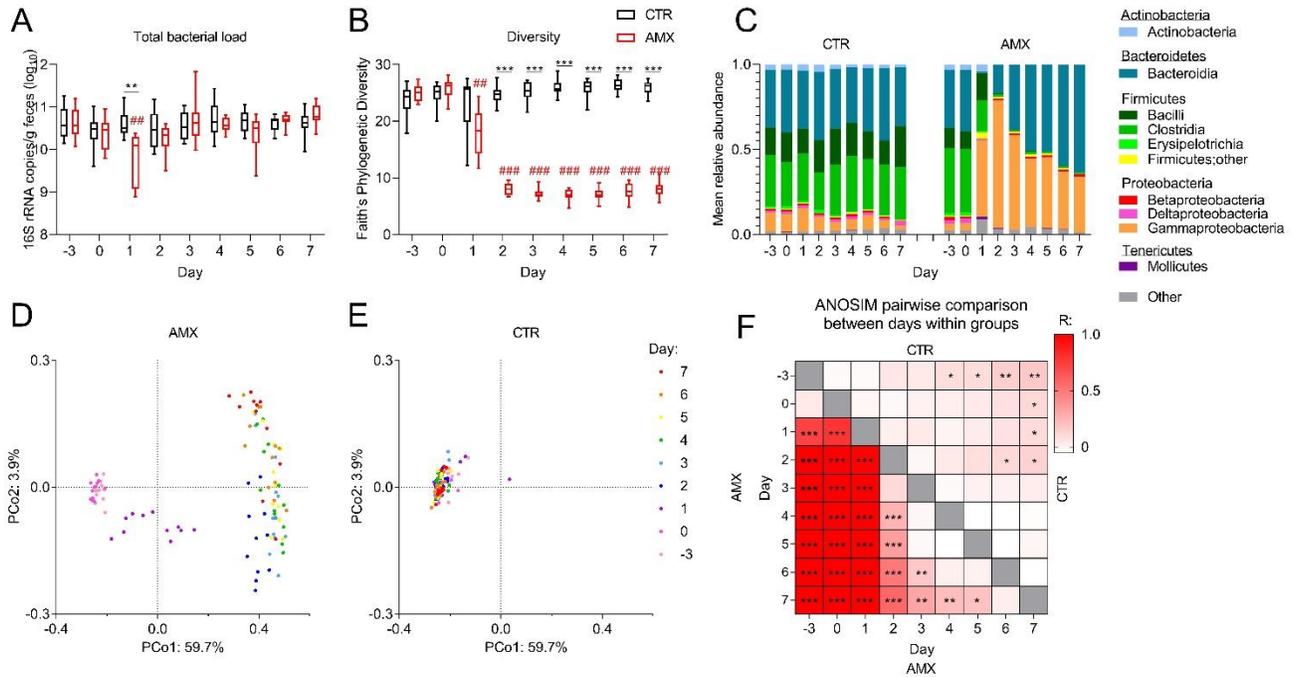


FIGURE 1: Temporal effects of amoxicillin on fecal bacterial load and composition. A-B: Total bacterial load (A) and Faith’s phylogenetic diversity index (B) of control (CTR, black) and amoxicillin (AMX, red) groups. C: Mean relative abundance of bacterial classes. D-E: Principal coordinate analysis (PCoA) plots of unweighted UniFrac distances in the AMX (D) and CTR (E) groups colored according to sample day. E: Heat-map of pairwise comparisons between days within the control group (upper right corner) and within the amoxicillin group (lower left corner) identified by Analysis of similarities (ANOSIM) between unweighted UniFrac distances. Intensity of the color indicates the value of the R statistics, where intense colors indicate large differences. Statistically significant differences between CTR and AMX on the same day, analyzed by multiple t-test and FDR-correction are indicated as **: $q \leq 0.01$ and ***: $q \leq 0.001$, and statistically significant differences relative to the initial level in the same group analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test are indicated as ##: $p \leq 0.01$ and ###: $p \leq 0.001$.

Amoxicillin affects small intestine and cecal/fecal microbiota compositions differently

After seven days of amoxicillin administration, animals were euthanized and the effects on intestinal microbiota in different intestinal compartments were analyzed. Amoxicillin was found to have a profound physiological effect on the ceca, which were dramatically enlarged (Fig. 2A), indicating changes in microbiota composition and activity. Indeed, similarly to the fecal microbiota, the Faith’s phylogenetic diversity of the cecal microbiota was much lower in the amoxicillin group compared to the control group on Day 7 (Fig. 2B). In contrast, the small intestine diversity was higher in the amoxicillin group compared to the control group.

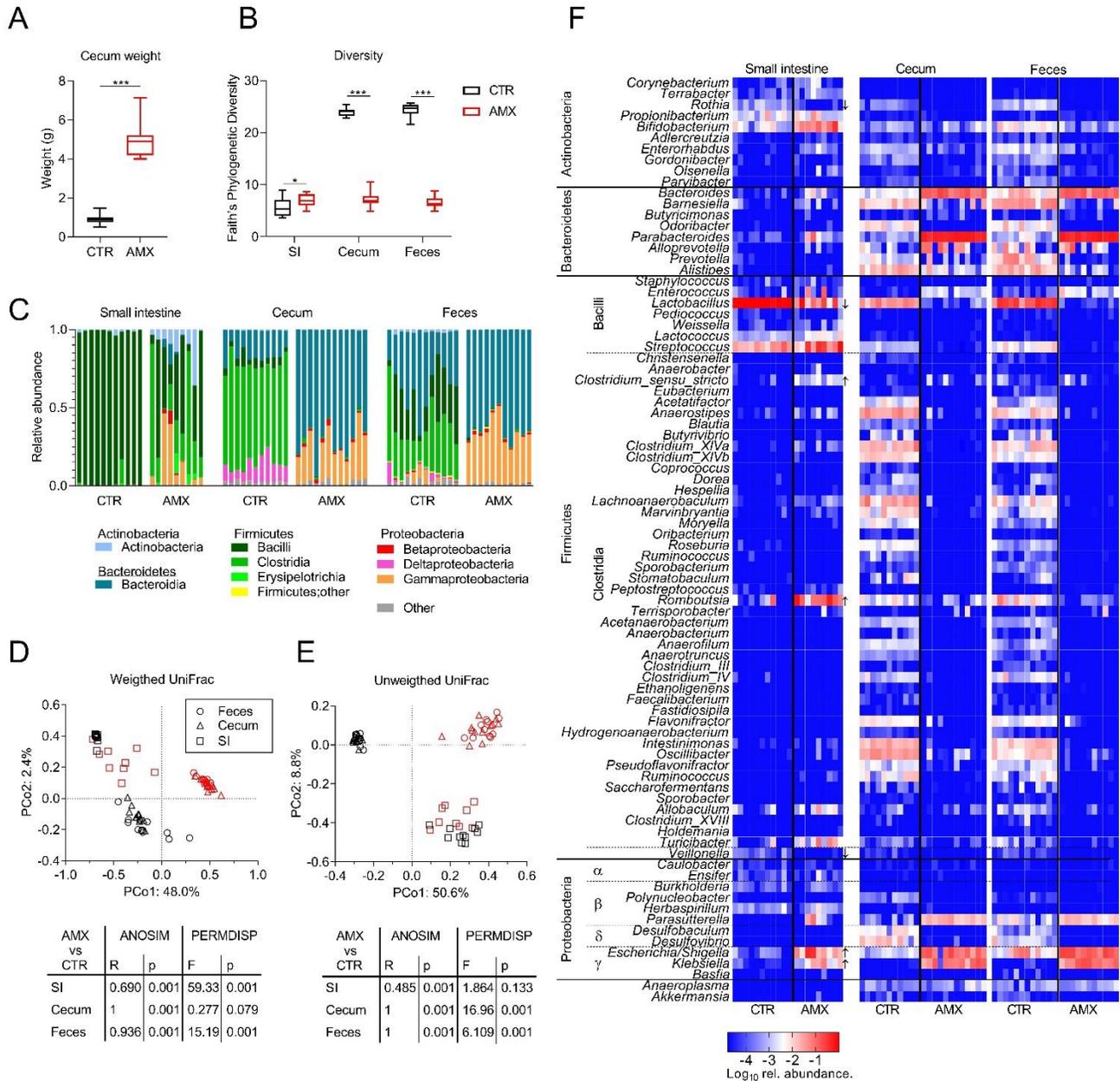


FIGURE 2: Effects of amoxicillin on intestinal microbiota. A-B: Cecum weight (A) and Faith's phylogenetic diversity (B) of control (CTR, black) and amoxicillin (AMX, red) groups. Asterisks indicate statistically significant differences between means in CTR and AMX groups. C: Relative abundance of bacterial classes in individual animals. D-E: Principal coordinate analysis (PCoA) plot of weighted (D) and unweighted (E) UniFrac distances between samples from small intestine (SI) (squares), cecum (triangles) and fecal (circles) content Day 7 colored according to CTR and AMX treatment. F: Relative abundance of bacterial genera in individual animals indicated by the cell color ranging from highly abundant genera in red and less abundant genera in blue. Genera for which the abundance was significantly different between CTR and AMX groups in the small intestine are highlighted with arrows marking whether the genera were higher or lower after amoxicillin administration. Statistically significant differences between groups analyzed by non-parametric Mann-Whitney test (A) or t-tests (B) are indicated as *: $p \leq 0.05$ and ***: $p \leq 0.001$.

The distribution of bacterial classes was found to vary between the small intestinal, cecal and fecal compartments within the control group at Day 7 (Fig. 2C). The small intestine was dominated almost exclusively by Bacilli (lactobacilli), whereas three different classes dominated the cecal (Bacteroidia, Clostridia, and Deltaproteobacteria) and fecal (Bacteroidia, Clostridia, and Bacilli) microbiota. Amoxicillin administration was found to dramatically, and differently alter the microbiota in the different intestinal compartments. In the small intestine, the relative abundance of Bacilli was reduced while Gammaproteobacteria, Clostridia, and Actinobacteria were increased in all animals, but to a varying degree, resulting in a high variation between individual animals in the amoxicillin group. In contrast, the cecal and fecal microbiota composition were found to be uniform among the individual animals in the amoxicillin group, and the distribution of bacterial classes remarkably similar between the cecal and fecal microbiota in the amoxicillin group. In feces and cecum, the relative abundances of Clostridia and Bacilli were lower in the amoxicillin group than in the control group, while the relative abundance of Bacteroidia and Gammaproteobacteria were increased.

In line with the dramatic effect of amoxicillin on bacterial class distribution, the overall bacterial community structure (β diversity) was significantly affected by amoxicillin in both the small intestine, cecum and feces as determined by the ANOSIM method (Fig. 2D-E). Furthermore, analysis of sample dispersion by PERMDISP of weighted UniFrac distances confirmed that the individual animals' small intestinal samples were significantly more dispersed in the amoxicillin group than seen in the controls, while in contrast, cecal and fecal samples were significantly less dispersed in the amoxicillin group than in controls (Fig. 2D). In summary, amoxicillin reduced within sample (α) and between samples (β) diversity with regard to the overall bacterial composition (weighted UniFrac distances) in fecal and cecal samples, but increased α and β diversity in small intestine samples.

Oppositely, analysis of unweighted UniFrac distances based on presence/absence status of ASVs and not relative abundances revealed that the cecal and fecal samples from individual animals were significantly more dispersed in the amoxicillin group than in controls (Fig. 2E). This suggests that while amoxicillin caused the amount of dominating species to become more similar within the group, the presence/absence status of rare species became more dissimilar within the group.

Statistical analysis of compositional changes (ANCOM method) of the microbiota caused by amoxicillin was performed only for the small intestinal compartment due to the very pronounced effects in the cecum and feces. The abundance of *Lactobacillus* was significantly lower in the amoxicillin group, as were *Veillonella* and *Rothia* (Fig. 2F and Table S1). A significant outgrowth of *Escherichia/Shigella* and *Klebsiella* (Gammaproteobacteria) in response to amoxicillin was also observed in the small intestine similar to the cecal and fecal compartments. Furthermore, the relative abundances of the Clostridial genera *Romboutsia* and *Clostridium sensu stricto* were significantly higher within the small intestine in the amoxicillin group than in the control group.

Amoxicillin promotes host mucosal immune regulation

The effects of amoxicillin on humoral and mucosal immune regulation were analyzed after seven days of administration. This revealed that total fecal IgA levels were higher in the amoxicillin group than in the control group, while total serum IgA levels were lower (Fig 3A). Various lymphocyte populations were analyzed in blood, mLNs, small intestine PPs and LP (Fig. S3A-C). Of these, the relative amount of CD25⁺FoxP3⁺Tregs were significantly higher in small intestine LP (both CD4⁺ and CD4⁻Tregs) and PPs (only CD4⁻Tregs) in the amoxicillin group compared to the control group, while amoxicillin had no effect on Tregs in blood and mLNs (Fig 3B-C). Small intestinal uptake of the cow's milk protein BLG was assessed by quantifying BLG in gut tissue and serum 15 min after oral gavage with whey protein. This revealed a tendency for higher uptake in the amoxicillin group ($p=0.183$ and $p=0.139$ for LP and serum respectively; Fig 3D). Finally, mucus-producing goblet cells were quantified in histological slides (representative pictures in Fig. S4). Goblet cell numbers in both the small intestine and colon were elevated in the amoxicillin group compared to controls (Fig. 3E). Relative expression of various immune related host genes were assessed in small intestine LP (Fig. S3D), and a tendency ($p=0.131$) for higher expression of the *Muc2* gene encoding mucin 2 was observed in the amoxicillin group (Fig 3F). A strong positive correlation between small intestine goblet cells and *Muc2* gene expression supported these findings (Fig 3G).

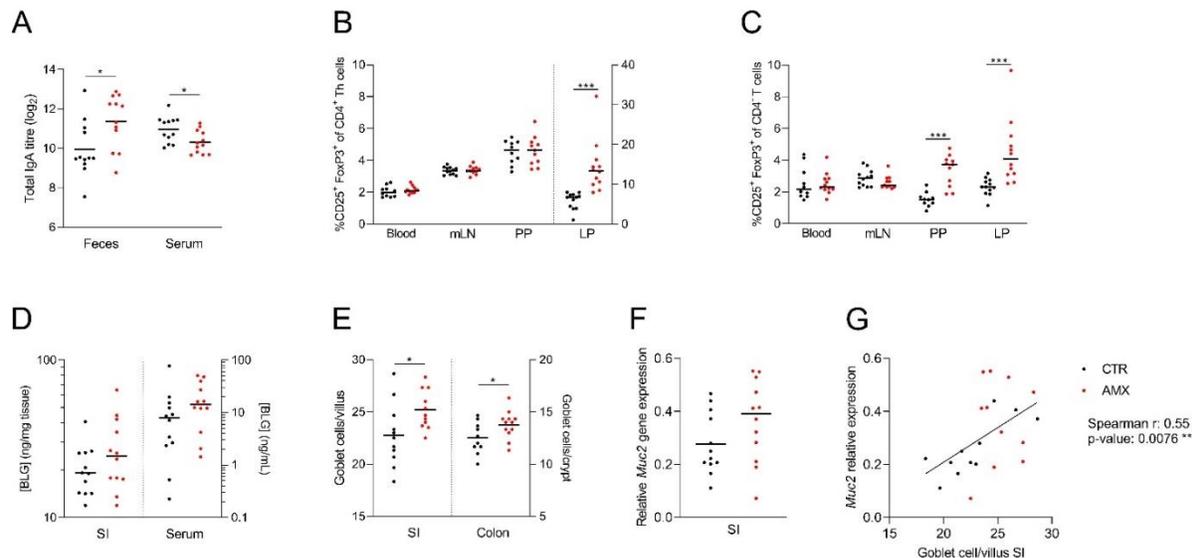


FIGURE 3: Host responses to amoxicillin. A: Total IgA levels in feces and serum. B-C: Relative amounts of CD25⁺FoxP3⁺T regulatory cells of CD4⁺T helper cells (B) and CD4⁻T cells (C) in blood, and small intestine mesenteric lymph nodes (mLN), Peyer's patches (PPs) and lamina propria (LP). Cells in LP are plotted according to the right y-axis while cell from the remaining tissues are plotted according to the left y-axis. D: β -lactoglobulin (BLG) concentration in small intestine LP (left y-axis) and serum (right y-axis). E: Average goblet cells per villi or crypt in histological slides of small intestine (left y-axis) and colon (right y-axis). F: Expression of the mucin 2 (*Muc2*) gene relative to two housekeeping genes. G: Correlation between number small intestine goblet cells and relative expression of *Muc2*. Each symbol represents one animal and horizontal lines indicate mean (A, E-F) or median (B-D). Statistically significant differences between control (CTR, black) and amoxicillin (AMX, red) groups analyzed by t-tests (A, E) or non-parametric Mann-Whitney test (B-C) are indicated as *: $p \leq 0.05$, ***: $p \leq 0.001$.

Specific bacterial genera are linked to immune regulatory responses

To investigate the interplay between the bacterial composition of the small intestine and the host immune response, pairwise correlations between relative abundance of genera and the host immune parameters shown in Fig. 3 and Fig. S3 were performed (Fig. 4A-B). This revealed negative correlations between three bacterial genera with reduced relative abundance in the amoxicillin group, namely *Lactobacillus*, *Rothia* and *Veillonella*, and the fraction of both CD25⁺FoxP3⁺ Tregs in the small intestine LP (both CD4⁺ and CD4⁻ Tregs) and PPs (only CD4⁻ Tregs) as well as with the fraction of B cells of all PPs lymphocytes. Further, a consortium of *Escherichia/Shigella*, *Klebsiella*, *Bifidobacterium* and four Firmicutes genera for which the relative abundance was higher in the amoxicillin group compared to controls were positively correlated with the same lymphocyte populations. These observations were supported by the additional finding of a significant correlation between LP CD4⁺ Tregs and *Escherichia/Shigella* in the control group only ($p=0.035$) and for *Turicibacter* in the amoxicillin group only ($p=0.014$). In addition, a negative correlation was found between the relative abundance of *Propionibacterium* and the expression of the *Cldn2* gene encoding the tight junction protein Claudin 2 in the small intestine tissue, and a positive correlation between the relative abundance of *Weissella* and the fraction of Helios⁻ induced Tregs in circulation.

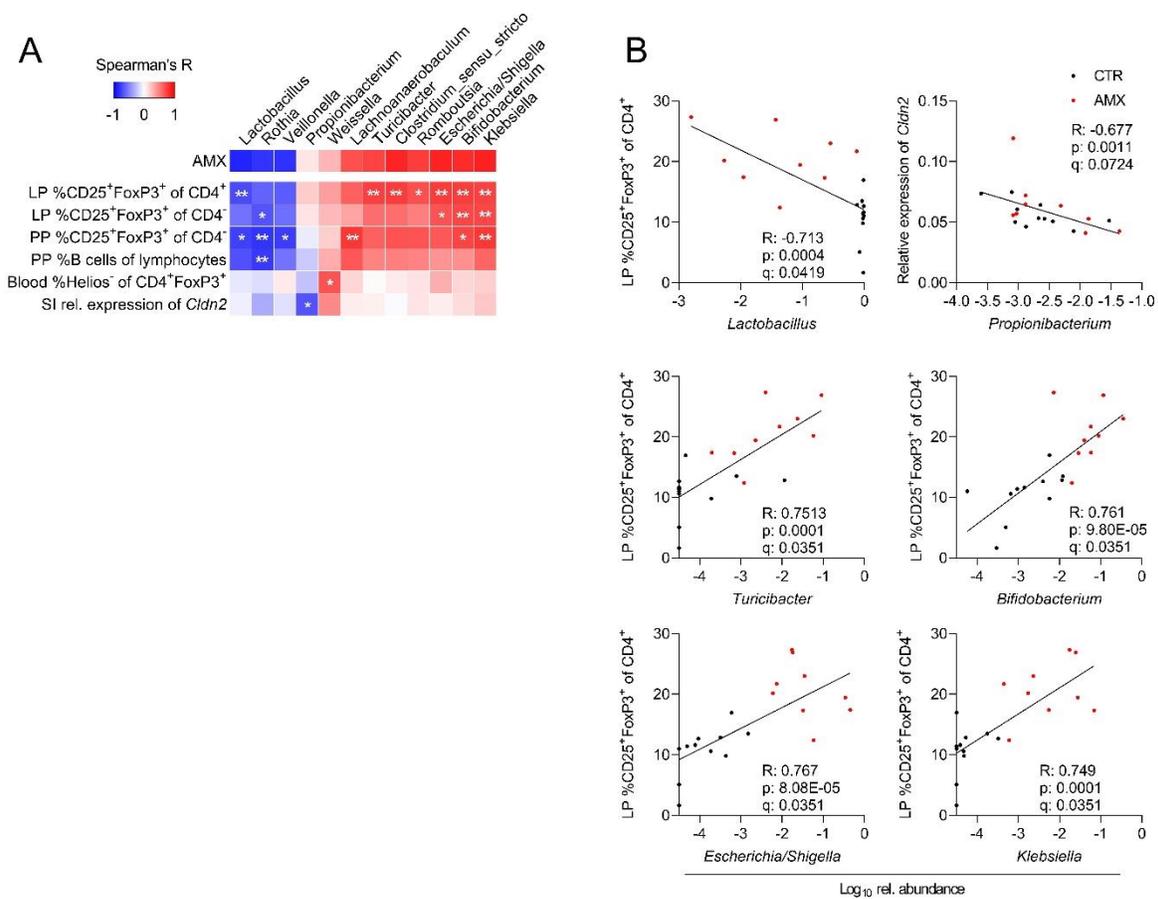


FIGURE 4: Correlations between small intestine bacterial genera and host immune responses. A: Spearman's Rank correlation matrix between relative abundance of small intestinal bacterial genera present in minimum 10 animals, and all host response parameters indicated on Fig. 3 and Fig. S3. Only those parameters for which correlations are significant after applying FDR-correction are included. Spearman's Rank correlation coefficients are indicated by the cell color. Statistically significant differences are indicated as **: $q < 0.05$ and *: $q < 0.1$. B: Visualization of selected correlations between relative abundance of small intestine genera and host responses in control (CTR, black) and amoxicillin (AMX, red) administered group. Each symbol represents one animal.

DISCUSSION

To investigate the putative relationship between microbiota composition and immune regulation, BN rats were orally administered with the antibiotic amoxicillin or water as control for seven days, and the gut microbiota, host immune regulation and intestinal permeability to cow's milk protein was assed. Despite of epidemiologic studies showing an association between early life antibiotic consumption and prevalence of food allergies [113–115] and autoimmune disorders such as IBD [206–208], reflecting a failure to develop appropriate tolerance, the current study surprisingly indicated that a seven days amoxicillin-induced perturbation of the gut microbiota promoted multiple parameters associated with immune regulation. These included higher relative abundance of small intestine Tregs, total fecal IgA and goblet cell numbers in the amoxicillin group compared to controls.

The observed increase in goblet cell numbers is in agreement with a previous study showing that administration the board-spectrum antibiotic ceftriaxone to Wistar rats causes increased goblet cell numbers and size [236]. It is well established that bacterial colonization affects mucin gene expression, and in agreement with the present study, a negative correlation between abundance of *Lactobacillus* spp. and mucin 2 gene expression in newborn mice has previously been reported [237]. Goblet cells promote oral tolerance by delivering luminal antigens to tolerogenic CD103⁺ dendritic cells in the small intestine to generate antigen specific Tregs [49,238] and by producing mucus, which contributes to intestinal barrier function and provides attachment sites and nutrients for commensal gut bacteria [216].

In the current study, the relative abundance of CD25⁺FoxP3⁺ Tregs in the small intestine LP (both CD4⁻ and CD4⁺) and PPs (only CD4⁻ Tregs) were found to be higher in the amoxicillin group than in controls. While conventional CD4⁺ Tregs have been extensively studied, the function of CD8⁺CD4⁻ Tregs remains less characterized, but are thought to play an important role in preventing food allergy [239]. In line with the results from the current study, oral administration of broad-spectrum antibiotic cocktails is often reported to increase the relative abundance of CD4⁺ Treg cells in small intestine [218,219,222,240–243], and to reduce relative abundance of Th1 [217,218] and/or Th17 effector cells [217,218,222,241,243]. This shift in the balance between Tregs and effector T cells has been shown to reduce severity of multiple autoimmune [218,219,222] and allergic diseases [240]. On the other hand, the antibiotic-induced Treg promotion has been shown to prevent the development of proper immune responses to oral vaccination [244] and enteric infections [245,246].

Tregs are central stimulators of mucosal IgA production by plasma cells [247], which is reflected in the current study, since the higher relative abundance of small intestinal Tregs in the amoxicillin group than in controls, was accompanied by higher fecal IgA levels. Mucosal IgA is known to be important for the immunological defense against pathogenic bacteria and toxins, while the role of IgA in immune tolerance is less understood [1]. Still, results from human [248] and animal [249] studies indicate that IgA might play an important role for tolerance development, and alterations in IgA coating patterns of intestinal bacteria have been associated with multiple disorders including atopic diseases [250] and IBD [251], although the results are ambiguous. Oppositely to what was observed for fecal IgA, total serum IgA levels were significantly lower in the amoxicillin group than in controls. This is in line with previous

studies showing that fecal and serum IgA levels are not affected in the same way by oral exposure to allergens [249] and high-fat diet [252] in mice. Amoxicillin-induced perturbation of the microbiota may affect the transport of IgA from circulation to the intestinal lumen and/or tissue specific differences in regulation of IgA secretion.

To investigate the interplay between microbiota composition and immune regulation, the fecal microbiota was analyzed throughout the study, and the small intestinal and cecal microbiota at termination. Amoxicillin was found to dramatically, and differently alter the microbiota in the different intestinal compartments. The small intestine was vastly dominated by *Lactobacillus* in the control group thus this compartment had lower α diversity compared to feces and cecum. This genus was much less abundant in the amoxicillin group, where multiple different genera were more abundant with high variation between individual animals causing a higher α and β diversity compared to controls. This was in contrast to the fecal and cecal microbiota, for which both α and β diversity were significantly lower in the amoxicillin group compared to controls. These differences in response probably result from differences in bacterial load, diversity and composition in the different compartments [253–255]. Differences in the transit time, which is much faster in the small intestine compared to cecum and colon [256], imply a shorter contact time between bacteria and antibiotics, which may also have affected the response. The current study highlights that changes in fecal bacterial population do not provide a good proxy for small intestinal responses to antibiotics.

Common for all analyzed intestinal compartments was the reduction in relative abundance of *Lactobacillus* and increase in relative abundance of Gammaproteobacteria. This was also seen in feces after seven days treatment of healthy adults with amoxicillin in combination with β -lactamase inhibitor clavulanate [257,258]. The elevated level of facultative anaerobes within the Gammaproteobacteria may in part be explained by depletion of butyrate producing clostridia, since reduction in butyrate has been reported to promote expansion of oxygen tolerating species due to increased epithelial oxygenation [259]. In line with this explanation, previous studies report reduced butyrate levels and elevated cecal pH after amoxicillin administration in Wistar rats [221,260].

Multiple studies have revealed that bacterial stimulation is important for mucosal T cell responses and in particular important for balancing the ratio between Tregs and effector T [261]. In the current study, a consortium consisting of *Escherichia/Shigella*, *Klebsiella* (Gammaproteobacteria), *Bifidobacterium* and four genera belonging to Firmicutes, for which the relative abundance in the small intestine were higher in the amoxicillin group than in controls, positively correlated with the fraction of small intestinal Tregs. This is in accordance with previous studies reporting positive effects of administration with various probiotic *Bifidobacterium* spp. on the Treg/T effector cell balance in humans [262] and mice, which thereby ameliorate allergic and autoimmune diseases [263–266]. Furthermore, a growing body of evidence suggests an important immunomodulatory role of hexa-acylated lipopolysaccharide (LPS), a major constituent of the outer membrane of Gammaproteobacteria, which exhibit superior Toll-like receptor 4 (TLR4) stimulatory capacity compared to the less acylated LPS present from other Gram-negative bacteria [267–270]. We speculate that bacterial derived signals such as hexa-acylated LPS from Gammaproteobacteria could directly promote the expansion of the intestinal Tregs, which in turn stimulate mucosal IgA production by local plasma cells (Fig. 5).

Indeed, *in vitro* studies have shown that LPS can activate murine [271] and human [272] Tregs directly via TLR4 activation. Furthermore, animal studies have indicated a link between high dietary LPS levels and higher numbers of CD4⁺ Tregs in mLN, as well as higher CD8⁺ Tregs in mLN and PPs [273]. However, further studies are needed to address this hypothesis, such as applying the same experimental treatment to LPS-nonresponsive animals, to see if Treg and IgA induction fail to appear. One LPS-nonresponsive animal model is TLR4^{-/-}B6129PF mice, which are in fact prone to develop food allergy [274].

In conclusion, this study showed that microbiota perturbation by amoxicillin promotes acute intestinal immune regulation. The observed immune regulation may represent an acute response to overt inflammatory signals derived from the microbiota e.g. released from dead bacteria and/or new or expanded bacterial species. The long-term immunological and clinical consequences of this effect remain to be investigated to determine whether this condition affects development of oral tolerance to luminal antigens and thus prevents inflammatory and allergic diseases.

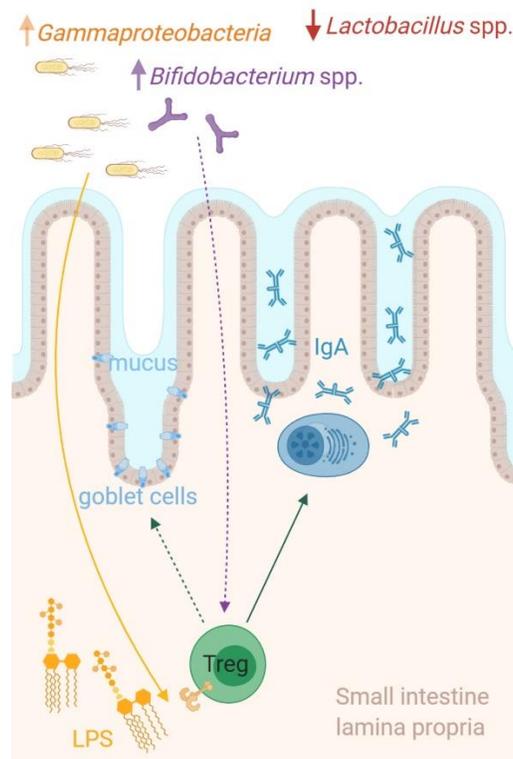


FIGURE 5: Microbiota host interactions in the small intestine. The relative abundance of *Lactobacillus* was dramatically lower in the small intestine of amoxicillin administered rats, while multiple different genera were more abundant. Among these were a consortium including *Escherichia/Shigella*, *Klebsiella* (Gammaproteobacteria) and *Bifidobacterium*, which strongly correlated with the relative abundance of regulatory T cells (Tregs) in the small intestine. We speculate that bacterial derived signals such as hexa-acylated lipopolysaccharide (LPS) from Gammaproteobacteria, could directly promote the expansion of the intestinal Tregs, which in turn stimulate mucosal IgA production by local plasma cells and possibly goblet cell activation and mucus secretion. The figure is created with BioRender.com

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

KB and TL conceived of the study. KG analyzed serum antibody levels, protein uptake and histology. MB and KG analyzed microbiota data. JL analyzed lymphocyte populations and participated in interpretation of the results. AB analyzed gene expression. KG, MB, TL and KB participated in the design of the study and the interpretation of the results. KG drafted the manuscript. All authors made substantial intellectual contributions, revised the manuscript critically, and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The 16S rRNA gene sequence data are deposited in the NCBI Sequence Read Archive with the accession number PRJNA599292.

2.3 Manuscript III

Moderately hydrolysed whey protein has a superior primary preventive capacity compared to intact whey independently of gut microbiota perturbation in Brown Norway rats

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Keywords: Food allergy, cow's milk allergy, allergy prevention, hypoallergenic infant formula, tolerance, antibiotic, amoxicillin, microbiota, extensively hydrolysed, partially hydrolysed, IgA, iTreg, Helios

Supplementary materials can be found in Appendix B.

In preparation.

ABSTRACT

Background: The use of hydrolysed infant formula for allergy prevention in atopic infants is highly debated, but has been suggested as a good option due to the reduced allergenicity. Knowledge on how dietary protein structure influences tolerance development remains scarce. Further, the intestinal microbiota has been suggested to influence tolerance development.

Objective: The aims of the current study were (1) to compare the primary preventive capacity of intact and hydrolysed whey products with different physicochemical characteristics, and (2) to investigate how microbiota composition affects tolerance induction.

Methods: The primary preventive capacity of *ad libitum* administered whey products was investigated in Brown Norway rats, with either conventional or amoxicillin-disturbed gut microbiota. The preventive capacity was assessed as the capacity to reduce whey-specific IgE sensitisation and clinical reactions assessed by an ear-swelling test after four intraperitoneal post-immunisations with intact whey. Additionally, the effect of the whey products on the growth of gut bacteria derived from healthy human infant donors was evaluated in an *in vitro* incubation study.

Results: Moderately (both partially and mildly) hydrolysed whey products had strong primary preventive capacity. The response to intact whey was very diverse, and the level of whey-specific IgE after post-immunisations was not significantly different from the control group that did not receive any whey product for prevention. Daily amoxicillin administration initiated one week prior to tolerance induction significantly disturbed the gut microbiota, but did not affect prevention of sensitisation. *In vitro* incubation of infant faecal microbiota with whey products indicated that moderately hydrolysed whey products promoted the expansion of the genus *Enterococcus*.

Conclusion & Clinical Relevance: Results from the animal study support the recommendation of moderately hydrolysed whey-based infant formulas for primary prevention in atopic infants. However, possible effects of hydrolysed products on infant microbiota composition, indicated by the *in vitro* incubation studies, warrants further investigation.

INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy in infants and young children with a high national variation in prevalence between 0.5 and 3% [6–8]. Non-exclusively breastfed infants suffering from CMA are recommended the use of hypoallergenic infant formula [4], which is most often based on extensively hydrolysed cow's milk proteins. Hydrolysis degrades proteins into peptide fragments and free amino acids and thereby eliminates the antibody-binding epitopes and thereby reduces the risk of inducing an allergic reaction. Hypoallergenic infant formulas based on hydrolysed cow's milk proteins are divided into extensively (eHF) and partially (pHF) hydrolysed infant formula based on the molecular weight of the peptides. There is currently no consensus for a definition of eHF and pHF. The American Academy of Pediatrics has suggested a definition for eHF as containing only peptides with a molecular weight below 3 kDa, and pHF as those that mostly consists of oligopeptides with a molecular weight below 5 kDa [29].

It is generally accepted that the optimal way to prevent CMA is to exclusively breastfeed infants until introduction of solid foods. However, the recommendation for prevention of CMA and other allergic diseases in non-exclusively breastfed atopic infants is highly debated. Two recent systematic reviews conclude that there is not substantial evidence supporting that hydrolysed infant formula prevent CMA or any other allergic disease [275,276]. The European guideline currently recommends the use of hydrolysed infant formulas for food allergy prevention in high-risk infants below the age of 4 months and conventional infant formula thereafter [277], while the American [278] and Australian-Asian [279] guidelines were recently updated to no longer recommend hydrolysed infant formulas for prevention of CMA or other allergic diseases.

Very few clinical studies have evaluated CMA as an endpoint when comparing the effect of pHF to conventional infant formula in high-risk infants [280–282], and results from these studies are varying and not unambiguously in favour of pHF. However, the study designs are problematic, as discussed in the recent Cochrane review [276]. Furthermore, the studies suffer from large heterogeneity in time of initiating intervention and whether product is the sole nutrition or given in combination with breast milk. Results from animal studies indicates that compared to conventional infant formula, the allergenicity of pHF is reduced [283,284], but that the primary preventive capacity may also be reduced [195,197,198], or at best similar to conventional infant formula [168,196].

Variation between studies may be due to the use of different infant formulas with different physicochemical characteristics due to the lack of product standardisation. High variation in physicochemical profiles [30] and peptide sequence signatures [31] has been reported among different commercially available hydrolysed infant formulas based on the same protein source and within the same classification. This implies that different infant formulas may have different immunological effects.

Furthermore, knowledge on how environmental factors influence tolerance induction remains scarce. In recent years the gut microbiota has received increasing focus in the context of food allergy. The increased prevalence of food allergy observed in the last decades has been associated with changes in environmental factors and life style [5], likely related to alterations in the gut microbiota. Environmental factors that may modulate microbiota composition include antibiotic exposure. Epidemiological studies

suggest an association between antibiotic exposure in early life and CMA [113–115]. Recent studies have indicated a causal relationship between gut microbiota and the development of CMA in murine models transplanted with faeces from CMA children [124,126]. In the current study, amoxicillin was used to manipulate the microbiota composition. Amoxicillin is the most widely used penicillin in Europe [226], and is frequently prescribed for treatment of paediatric infections [227].

The aims of the study were (1) to compare the primary preventive capacity of intact and hydrolysed whey products with different physicochemical characteristics, and (2) to elucidate how antibiotic-induced changes in the gut microbiota affects tolerance induction. The study was conducted in Brown Norway (BN) rats, which are high-IgE responders and thereby resembles atopic individuals in their predisposition to develop food allergy [168,223]. To the best of our knowledge, the present study is the first to show that hydrolysed infant formula is superior to conventional infant formula for preventing whey-specific IgE sensitisation.

METHODS

Product characterisation

Four whey-based infant formula protein ingredients with different degrees of hydrolysis were included in the study: Extensively (eHW), partially (pHW) and mildly (mHW) hydrolysed and intact whey (iW). All products were kindly provided by Arla Foods Ingredients and were made from whey protein concentrate.

Degree of hydrolysis

The degree of hydrolysis was determined with an *O*-Phthaldialdehyd-assay as described elsewhere [285]. Briefly, samples were dissolved to a concentration of 0.8 mg/mL protein in milli Q water and filtered through filter paper, and subsequently 20 μ L sample was mixed with 150 μ L OPA reagent (0.08% *O*-Phthaldialdehyd (Merck KGaG, Darmstadt, Germany), 2% (v/v) ethanol (VWR International, Radnor, PA, US), 3.81% di-sodium-tetraborat-decahydrat (Merck KGaG), 0.1% Sodium-dodecyl-sulfat (VWR International), 0.088% DL-Dithiothreitol (Merck KGaG)). Absorbance was measured after 120 seconds at 340 nm on a Lambda 35 UV/VIS Spectrometer (Perkin Elmer, Waltham, MA, US). A calibration curve based on L-serine (Merck KGaG) diluted in milli Q water was prepared in the range of 0.067-0.28 mg/mL.

Amino acid composition

Amino acid analysis was performed by ion exchange chromatography after hydrolysis in HCl, as described elsewhere [286].

Peptide size distribution

Peptide size distribution was determined by TSK gel permeation chromatography (GPC). Samples were dissolved to a concentration of 0.32% (w/v) protein in mobile phase buffer (0.0375 M phosphate buffer (VWR International) with 0.375 M NH_4Cl (VWR International), 0.1% trifluoro acetic acid (Merck KGaG), 25% acetonitrile (Rathburn Chemicals, Walkerburn, Scotland)) to disassociate non-covalent interactions.

Disulphide bonds were reduced by addition of 0.67% of β -mercaptoethanol (Merck, KGaG) followed by a heat treatment in a heating block at 100 °C for 30 min. All samples were filtrated through a 0.45 μ m filter. Chromatographic separation was performed at 25°C on a Thermo Ultimate 3000 LC (Thermo Scientific, Waltham, MA, US) equipped with three TSK G2000 SWXL columns (5 μ m 7.8mm x 300mm, TOSOH Bioscience GmbH, Stuttgart, Germany) bound in series. Samples were separated at a flow rate of 0.7 mL/min with mobile phase buffer. Peptides/proteins were detected at 214 nm. The relative distribution of peptides was calculated based on area under the curve.

Analysis of protein aggregation

To examine protein aggregation status under physiological conditions (solvent: 50 mM NaH₂PO₄, 0.15 M NaCl, pH 7.0), ethylene bridged hybrid (BEH) GPC was performed as previously described [287].

Animals

Brown Norway (BN) rats from the in-house breeding colony (National Food Institute, Technical University of Denmark, Denmark) were housed in macrolon cages and kept at a 12 hour light:dark cycle, at a temperature of 22 \pm 1°C and a relative humidity of 55 \pm 5%. Rats were observed twice daily and clinical signs recorded. The rats were fed a milk-free diet for \geq 10 generation that was produced in-house and based on rice flour, potato protein and fish meal as protein sources, as previously described [186], with the exception of maize flakes being substituted with rice flour. Ethical approval was given by the Danish Animal Experiments Inspectorate (2015-15-0201-00553-C1). The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

Animal experiment

BN rats of both sexes and aged 3½-7½ weeks were allocated into 10 groups of 12 rats. Half of the groups were daily gavaged with 30 mg amoxicillin in 0.5 mL milli Q water and the other half with water alone from Day 0-28 (Fig. 1). From Day 7-28, one amoxicillin and one water group were each given one of the four whey products in their drinking water (12.5 g protein per litre water) *ad libitum* to induce tolerance or water alone for control. One week after the tolerance induction phase ceased, all rats were post-immunised intraperitoneal (ip) with 100 μ g iW in 0.5 mL phosphate buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 1.8 mmol/L KH₂PO₄, pH 7.2) once a week for four weeks (Day 35, 42, 49, and 56). Blood samples from the sublingual vein were collected after tolerance induction (Day 28) and weekly throughout the rest of the study (before dosing). Two faecal samples per rat were collected on Days 0, 7, 28 and 64. One was stored at -80°C for microbiota analysis and one was prepared for IgA quantification. Serum and faecal water was prepared as previously described [288].

Elicitation tests

On Day 63, rats were subjected to an ear-swelling test. The rats were anaesthetised with hypnorm-dormicum and their ear thickness was measured at the same spot before and one hour after intradermal injection of 10 μ g of iW in 20 μ L PBS. In parallel, naïve rats were subjected to the same treatment. On Day 64, rats were dosed with 100 mg of iW in 1 mL milli Q water by oral gavage 15 min prior to termination to assess intestinal uptake of β -lactoglobulin (BLG) as previously described [288].

Dissection

Rats were euthanised one week after the last post-immunisation (Day 64) by exsanguination using carbon dioxide inhalation as anaesthesia. The dissection, storage and preparation of tissues were previously described in details [288]. Briefly, intestinal content from the proximal small intestine was stored at -80°C for microbiota analysis. Small intestinal epithelium and lamina propria (LP) were stored at -80°C for total protein extraction and BLG quantification. Mesenteric lymph nodes (mLN), small intestinal Peyer's patches (PP) and LP were immediately processed for single cell suspensions, together with whole blood for flow cytometry analysis of lymphocyte populations as previously described [288]. Finally, a piece of the small intestine and of colon was fixed in 4% (w/v) paraformaldehyde for histology as previously describe [288].

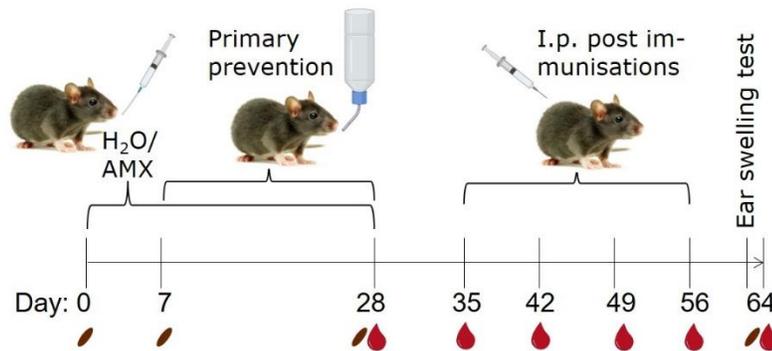


FIGURE 1: Animal experimental design. To create two groups with different microbiota compositions, rats were either gavaged with amoxicillin (AMX) or water (H_2O) as control daily from one week before and during tolerance induction. To induce tolerance, Brown Norway rats were administered one of four different whey products in their drinking water, ad libitum for three weeks. After one week, rats were subsequently post-immunised by weekly intraperitoneal (ip) injection with intact whey (iW) to sensitise the rats. The day before termination, rats were subjected to an ear-swelling test by intradermal injection of iW. The pictures originates from BioRender.com and Colourbox.

Quantification of antibodies by ELISA

Both iW-specific IgG1 and IgA antibodies were quantified in serum by indirect ELISA as previously described [187]. iW-specific IgE antibodies were quantified in serum by antibody-capture ELISA as previously described [187] with the exception that plates were initially blocked with 5% (v/v) rabbit serum (Biowest, Nuaille, France), and iW-specific IgE was detected by $50\ \mu\text{L}/\text{well}$ of $0.2\ \mu\text{g}/\text{mL}$ of 10:1 digoxigenin (DIG)-coupled iW in blocking solution. Total IgA was quantified in serum and faecal water by sandwich ELISA as previously described [288].

DNA extraction and amplicon sequencing of the 16S rRNA gene

DNA extraction and amplicon sequencing of the 16S rRNA gene were performed essentially as previously described [288]. Briefly, DNA was extracted from faeces or small intestine content by DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol.

Mechanical lysis of bacteria was conducted at 30 cycles/s twice for 5 min using bead beater MM300 (Retsch, VWR, Haan, Germany).

The V3-region of the 16S rRNA gene was amplified using a universal forward primer (PBU 5'-A-adapter-TCAG-barcode-CCTACGGGAGGCAGCAG-3') with a unique 10–12 bp barcode for each sample (IonXpress barcode as suggested by the supplier, Life Technologies, Carlsbad, CA, US) and a universal reverse primer (PBR 5'-trP1-adapter-ATTACCGCGGCTGCTGG-3') and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, US). PCR products were purified by HighPrep™ PCR Clean-up System (Magbio, Gaithersburg, MD, US) according to the manufacturer's protocol, and DNA concentrations were determined with Qubit HS assay (Life Technologies). Finally, a library was constructed by mixing an equal amount of PCR products from each sample. Sequencing of all samples was performed on a 318-chip for Ion Torrent sequencing using the Ion OneTouch™ 200 Template Kit v2 DL (Life Technologies).

Sequence data processing and analysis

Raw sequence data in FASTQ format was processed using CLC bio genomic workbench (Qiagen) in order to de-multiplex samples and remove sequencing primers. Further quality trimming using default settings (remove low quality nucleotides pbase-calling error = 0.05, trim ambiguous nucleotides = 2) and filtering only reads with a final length between 125-180bp were exported in FASTQ format. Further quality trimming was performed in Divisive Amplicon Denoising Algorithm 2 (DADA2, Version 1.10.1) with default settings as described elsewhere [230]. Finally, an amplicon sequencing variant (ASV) table was constructed which contains the counts of each sequence variant in each sample. All sequence reads were taxonomically classified using the Ribosomal Database Project Multi-classifier tool [231].

The ASV table was imported into the "Quantitative Insights Into Microbial Ecology" (Qiime) 2 pipeline (Version 2019.1) [289], and α and β diversity metrics were calculated by the function "diversity core-metrics-phylogenetic" based on a rooted phylogenetic tree. Faecal samples were rarefied to 10,000 reads and small intestine samples were rarefied to 3,000 reads to eliminate bias from uneven sampling depth.

***In vitro* incubation experiment**

Collection of infant faecal samples

Faecal samples were obtained from three human infant donors. All donors were 1-4 months old, exclusively breastfed and had never received antibiotics. The study was approved by The Danish National Committee on Health Research Ethics (H-16030078). Faecal samples were collected in the participants' homes in airtight containers and stored at 4°C until delivery to the laboratory, where they were processed immediately. All samples were processed within 12 h. Faecal samples were mixed 1:1 (w/v) with 30% glycerol in saline and frozen at -80°C in aliquots.

Preparation of defined culture mix

Stocks of *Bifidobacterium longum* ssp. *infantis* (NCIMB 702205), *Lactobacillus rhamnosus* (ATCC 53103) and *Enterococcus faecalis* (DSM 20478) were thawed and grown on BSM agar plate for two days (*B. longum*), MRS plate for 2 days (*L. rhamnosus*) or BA agar plate for 1 day (*E. faecalis*). All strains were inoculated in GAM broth and incubated anaerobically overnight. Finally, the three cultures were mixed to obtain an equal OD of each, added 1:1 (v:v) 30% glycerol in saline and frozen at -80°C in aliquots.

Small-scale *in vitro* incubation

A small-scale *in vitro* incubation method [290] was used to assess the effect of whey products on the microbial composition in human faecal samples. Whey products were dissolved in minimal medium to obtain a final protein concentration of 12.5 mg/mL. The minimal basal medium contained: 0.1 g/L of NaCl, 0.04 g/L of K₂HPO₄, 0.04 g/L of KH₂PO₄, 0.01 g/L of MgSO₄·7H₂O, 0.01 g/L of CaCl₂·2H₂O, 2 g/L of NaHCO₃ (all from Merck), 2 g/L of peptone water (Oxoid, Roskilde, Denmark), 1 g/L of yeast extract (Oxoid), 0.5 g/L of bile salts (Oxoid), 2 ml/L of Tween 80 (Sigma) and 0.5 mg/L resazurin (Fluka Honeywell, Charlotte, NC, US) and pH was adjusted to 7. After autoclaving the following sterile filtered ingredients were added: 0.5 g/L of L-cysteine hydrochloride (Sigma), 5 mg/L of hemin (Fluka), 0.001% (v/v) of vitamin K1 (Sigma), and 5 g/L lactose (Merck). The medium was reduced overnight in an anaerobic cabinet.

Faecal samples were defrosted, and 25% (v/v) faecal slurry was prepared by mixing the samples with anoxic sterile PBS. Faecal slurries were centrifuged at 200g for 3 min at 4°C and only the supernatants were used. For each incubation, 80 µL of inoculum (faecal slurry supernatant or defined culture) was inoculated to a total reaction volume of 2 mL in 24-well plates. Incubations with each whey product as well as non-supplemented medium controls were incubated with each inoculum (faecal slurry supernatants or defined culture) in triplicates. Samples were incubated in an anaerobic cabinet at 37°C. After 24 h, the total volume was harvested and centrifuged 10,000g for 8 min at 4°C. Supernatants were discarded and pellets were stored at -20°C until total DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit as described above.

Real-time PCR conditions

Each amplification reaction was done in triplicate for each incubation sample in a final volume of 11 µL containing 5.5 µL LightCycler® 480 SYBR Green I Master (Roche), 2.2 pmol of each of the primers (TAG Copenhagen, Denmark), 2 ng template DNA, and nuclease-free water purified for PCR (Qiagen). The 16S rRNA-targeting primers used in this study are listed in Table 1. The reaction conditions were: Pre-incubation at 95°C for 5 min followed by 45 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 45 s. Lastly, a melting curve was generated (95°C for 5 s, 68°C for 1 min and increasing the temperature to 98°C with a rate of 0.11°C/s with continuous fluorescence detection). The qPCR was run in 384-well format on a LightCycler® 480 II (Roche Applied Science) and analysed using the LightCycler® 480 software.

Table 1. 16S rRNA gene primers used qPCR in this study¹

Target	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size	Reference
<i>Bifidobacterium</i>	GCGTGCTTAACACATGCAAGTC	CACCCGTTTCCAGGAGCTATT	126 bp	[291]
<i>Lactobacillus</i>	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	341 bp	[292,293]
<i>Enterococcus</i>	CCCTTATTGTTAGTTGCCATCATT	ACTCGTTGTACTTCCCATTGT	144 bp	[294]
Universal V3	ACTCCTACGGGAGGCAGCAGT	GTATTACCGCGGCTGCTGGCAC	200 bp	[228]

¹All primers were previously validated for use in gut communities [295].

Real-time PCR data handling

For each incubation replicate, the mean threshold cycle (Ct) value between qPCR triplicates was used to calculate the relative abundance of the genera *Bifidobacterium*, *Lactobacillus* and *Enterococcus* relative to the total bacteria ($n_{\text{target}}/n_{\text{total}}$) using $2^{\Delta\text{Ct}}$ as described elsewhere [296]. ΔCt is the Ct value of the bacterial target normalised to the Ct value of the total bacterial population in the same incubation sample. Furthermore, the ratio between the relative abundance of a bacterial target in incubation with different whey products relative to iW ($n_{\text{treated}}/n_{\text{iW}}$) were calculated using $2^{\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct}$ is the ΔCt value of a given sample normalised to the median ΔCt of three iW samples.

Statistics

Graphs and statistically analyses were generated in Prism version 8.1.1 (GraphPad, San Diego, CA, US). ELISA results were expressed as log2 antibody titres. Normal distribution of data was tested by D'Agostino-Pearson normality test. For data that passed the normality test, group means are indicated on graphs and differences between groups were analysed by t-test (two groups) or one-way ANOVA followed by Tukey's post-test for multiple comparison. For data that did not pass the normality test, group medians are indicated on graphs, and group differences were analysed by non-parametric Mann-Whitney test (two groups) or Kruskal-Wallis test followed by Dunn's post-test for multiple comparison.

Differences between β diversity of groups were assessed by applying ANalysis Of Similarities ANOSIM [232] to weighted UniFrac distances in Qiime 2. Differential abundant genera between the amoxicillin and conventional group were determined by ANalysis of COMposition of Microbiomes (ANCOM) [234].

RESULTS

Physicochemical characterisation of whey products

Four whey-based infant formula protein ingredients with different degree of hydrolysis were included in the study (extensively (eHW, 27%), partially (pHW, 22.4%), mildly (mHW, 7.2%) hydrolysed, and intact whey (iW)). Only eHW had been filtered following hydrolysis to remove the smallest and largest molecules. Thus, only eHW had a slightly different amino acid composition compared to pHW, mHW and iW, which all were found to have similar amino acid compositions (Fig. 2A).

Each product had a unique peptide size distribution profile (Fig. 2B). eHW and pHW were most similar but different concerning the peptides below 375 Da (corresponding to 2-5 amino acids) and the peptides above 2500 Da (corresponding to more than 20 amino acids). For mHW notable differences were observed between peptide size distributions analysed under reduced and non-reduced conditions (Fig. 2C). This indicated that complexes are formed by disulphide bonds between multiple small peptides, especially those below 375 Da in that product. The presence of protein aggregates/complexes in mHW was confirmed by GPC under physiological conditions, which also revealed the formation of small peptide complexes with a weight above 3500 Da in both eHW and pHW (Fig. 2D).

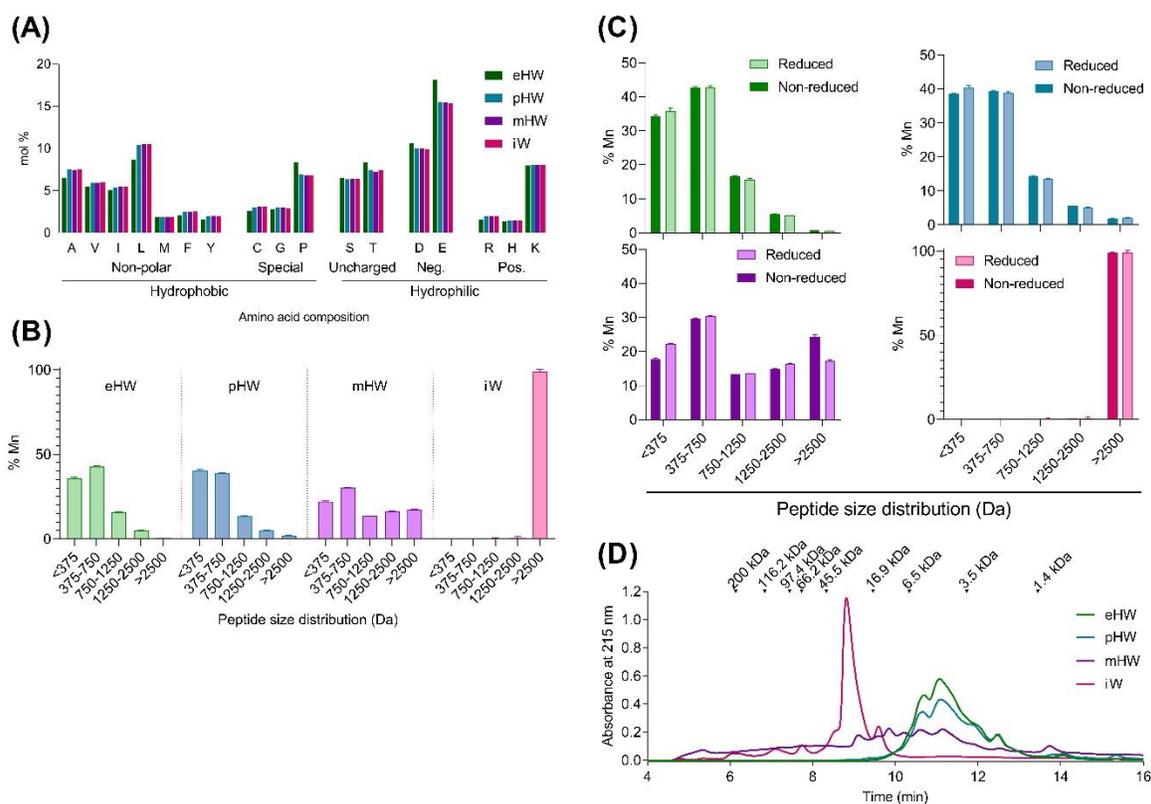


FIGURE 2: Physicochemical characteristics of whey products. Amino acid distribution (A), peptide size distribution under reduced (disulphide bonds and non-covalent interactions disassociated) (B-C) and non-reduced (non-covalent interactions disassociated) (C) conditions, and aggregation status under physiological conditions (D) of extensively (eHW), partially (pHW), mildly (mHW) hydrolysed and intact (iW) whey products. Mean and standard derivation of triplicates (B-C). Mn, number average molecular mass.

Moderately hydrolysed whey products induce sustained tolerance

The four different whey products were administered to naïve BN rats (with a conventional microbiota) in their drinking water for three weeks. Rats were subsequently ip post-immunised with intact whey once a week for four weeks to assess the capacity of the products to reduce whey-specific IgE sensitisation and clinical reactions (Fig. 1). Whey-specific IgE and IgG1 levels were quantified in serum after tolerance induction and after each post-immunisation.

Control rats that received no product for prevention in the drinking water were readily sensitised to whey after two to three ip post-immunisations (Fig. 3A). In the eHW group a transient reduction in whey-specific IgE level compared to the control group was observed after three post-immunisations ($p=0.025$ by t-test between eHW and control group). In both pHW and mHW groups strong prevention was observed, and whey-specific IgE level in those groups remained significantly lower than in the control group after each post-immunisation. There were no significant difference between the pHW and mHW groups. Interestingly, the whey-specific IgE response in the iW group was very diverse and was not significantly different from the level in the control group. In the iW group, some rats had detectable levels of whey-specific IgE already after the first post-immunisation, indicating that oral exposure to iW had already primed an IgE immune response below detection level.

Analysis of whey-specific IgG1 levels after tolerance induction and first post-immunisation revealed that the immunogenicity varied between the products; the highest IgG1 level was observed in the iW and mHW groups, a lower level in the pHW group and lowest in the eHW group (Fig. 3B). This finding is in line with the notion that hydrolysis eliminates the antigenic epitopes of whey.

One week after the fourth post-immunisation, clinical reactions were assessed by an ear-swelling test by intradermal injection of iW. This revealed that the iW and mHW groups had the lowest ear-swelling, and these two groups were the only ones significantly lower than the control group (Fig 3C). The ear-swelling in the mHW group was not even significantly different from the non-specific swelling observed in naïve rats subjected to the same ear-swelling test.

The ear-swelling results (Fig. 3C) reflect the IgE sensitisation results (Fig. 3A) for all groups except for the iW group, which showed little clinical reactions in ear-swelling despite some rats having high IgE levels. This could indicate that the raised IgE antibodies had decreased functionality, or that IgE function was blocked by other antibody classes.

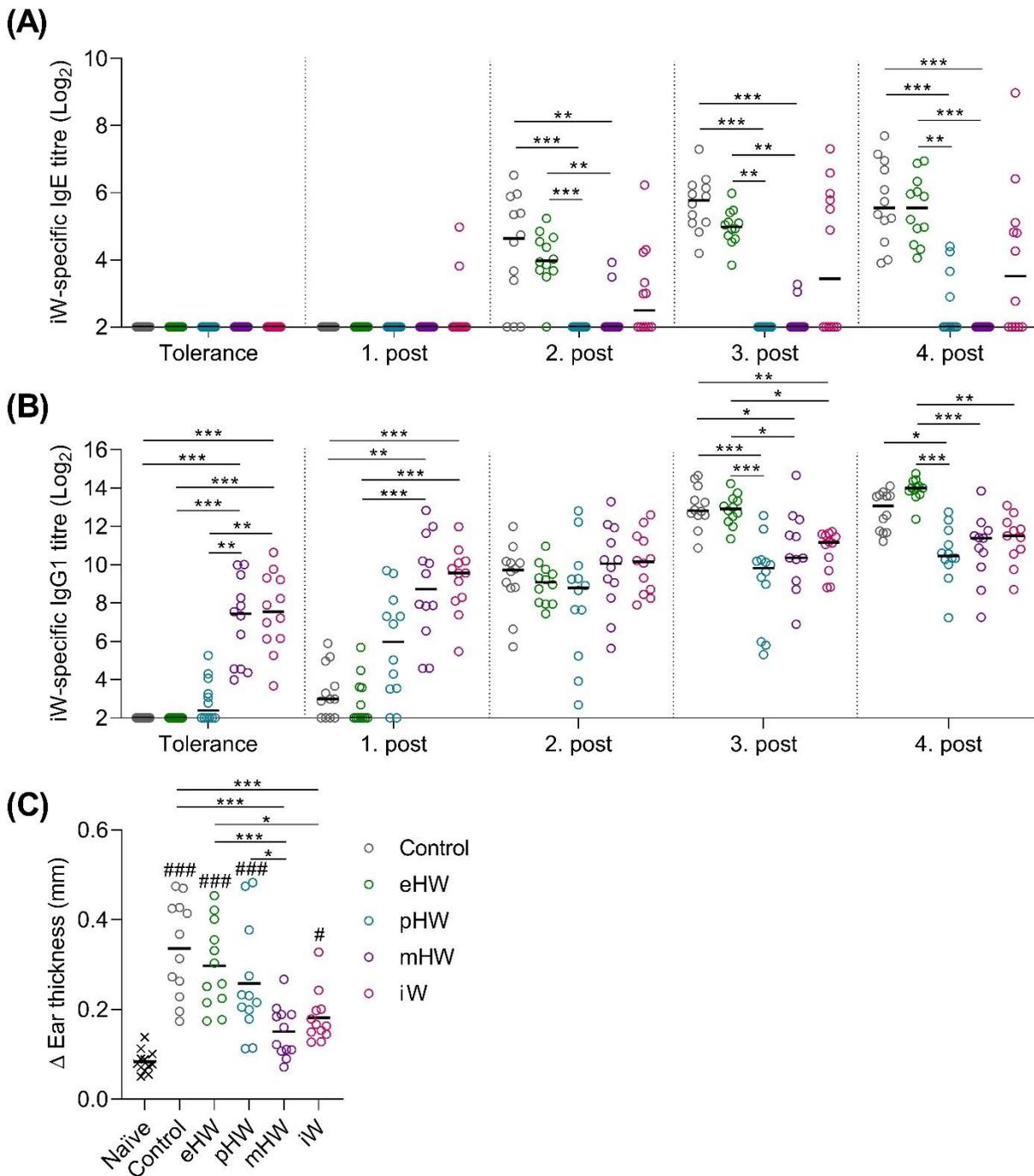


FIGURE 3: Primary preventive capacity of the whey protein products. Intact whey (iW)-specific IgE titres (A), iW-specific IgG1 titres (B), and ear-swelling after ear-swelling test (C) in rats that received extensively (eHW), partially (pHW), mildly (mHW) hydrolysed and intact (iW) whey products in drinking water or just water as control. Analyses were performed on sera from Day 35 (tolerance) and after the post immunisations on Day 42, 49, 56 and 64. Each symbol represents a single rat and horizontal lines indicate median (A-B) or mean (C) values. Statistically significant differences between product groups are indicated as *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, and between product groups and naïve controls as #: $p \leq 0.05$ and ### ≤ 0.001 .

Amoxicillin reduces microbial diversity and eradicates commensal species

To investigate whether perturbation of the gut microbiota composition affects tolerance induction, parallel groups of rats were administered with the antibiotic amoxicillin by daily oral gavage from one week before and during the tolerance induction phase (Fig. 1). Faecal samples were collected during the study and small intestinal content at termination, and analysed by 16S rRNA gene sequencing. The effect of amoxicillin on microbiota composition was analysed irrespective of whey product group.

Within sample (α) microbiota diversity as determined by the Shannon diversity index was significantly lower in faeces (Fig. 4A) and the small intestine (Fig. 4B) in the amoxicillin group compared to water group (conventional group). In faecal samples the effect was most pronounced at Day 7, and gradually decreased hereafter, but remained significant throughout the experiment, which ended five weeks after cessation of amoxicillin administration (Day 64) (Fig. 4A).

On Day 7 and 28, the faecal mean relative abundances of Firmicutes and Actinobacteria were lower in the amoxicillin group than in the conventional group, while the relative abundances of Proteobacteria, especially Gammaproteobacteria, and Bacteroidetes were higher (Fig. 4C). Five weeks after cessation of amoxicillin administration (Day 64), the overall microbial composition in faeces (Fig. 4C) and small intestinal content (Fig. 4D) was similar to that of the conventional group. The small intestine was dominated by *Lactobacillus* (Bacilli), and this genus constituted averagely 84.1 and 76.4% of bacteria in the water and amoxicillin groups, respectively, however with a great variation between individual rats.

In line with the observed longitudinal effects of amoxicillin on bacterial phylum distribution, the between sample (β) diversity assessed by ASV-based principal coordinate analysis (PCoA) of unweighted UniFrac distances revealed a temporal shift in faecal microbial composition of the amoxicillin group during the study (Fig. 4E). On Day 64, the microbial composition of amoxicillin rats approached that of Day 0, but did not revert completely, and remained significantly different from the conventional group as determined by ANOSIM in the faecal (Fig. 4E) and the small intestinal (Fig. 4F) microbiota on Day 64. This persistent difference between the amoxicillin and conventional groups was mostly due to some ASVs that were absent after cessation of antibiotic treatment (Fig. 4G). Out of the most prevalent ASVs (present in 75% of all samples at Day 0), four ASVs were present in less than 2% of samples from the amoxicillin group (Fig. 3H). Three of these were assigned to the family Porphyromonadaceae (Bacteroidetes) of which one was further assigned to the genus *Barnesiella*. One ASV was assigned to the phylum *Candidatus* Saccharibacteria ("Other"). Numerous ASVs were differently abundant in the amoxicillin and conventional groups in the faecal microbiota (Table S1), while only two ASVs were differently abundant in the small intestinal microbiota (Table S2) as determined by ANCOM. One ASV that was differently abundant in both faeces and small intestines was assigned to *Lactobacillus intestinalis* (Fig. 4I-J).

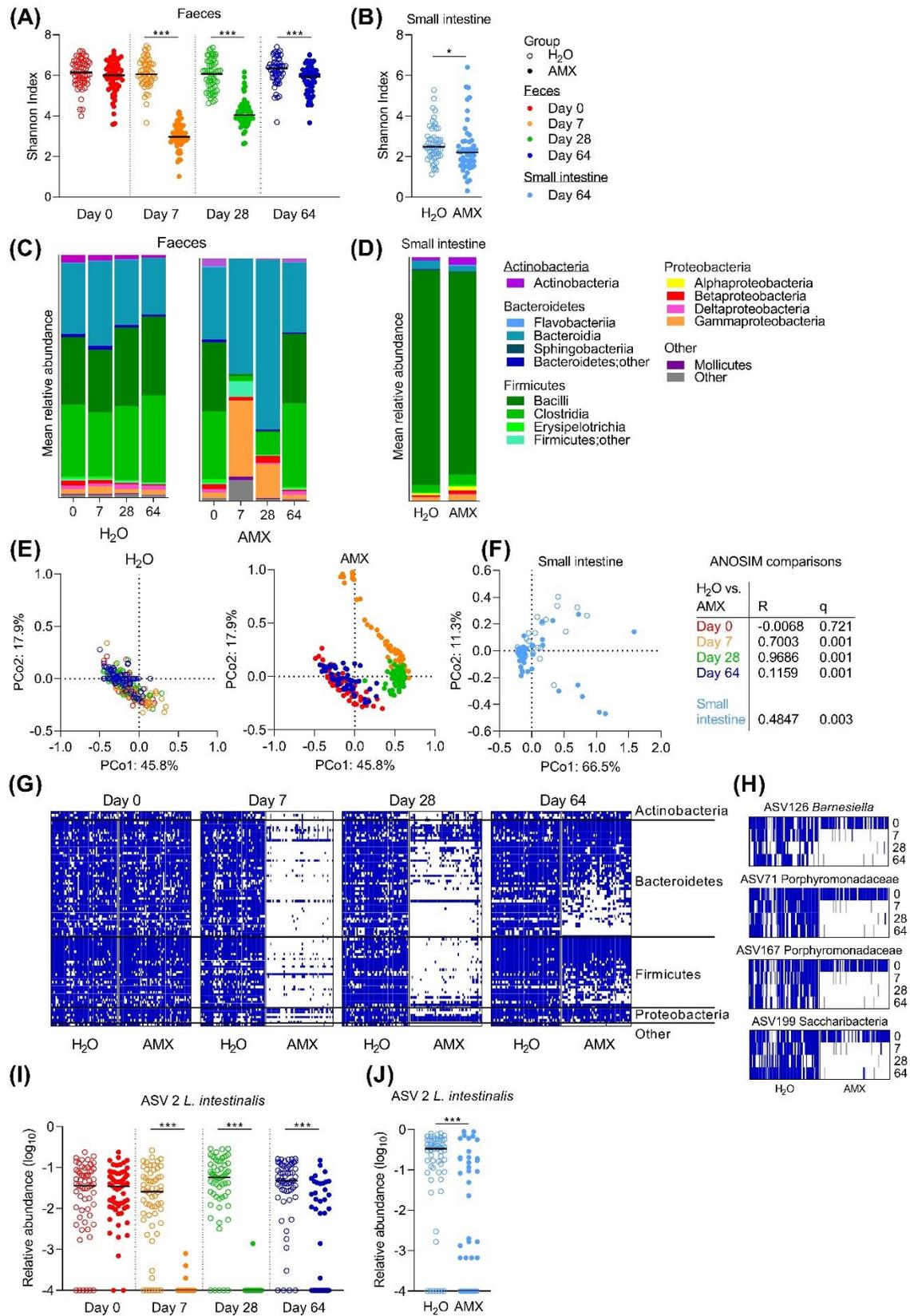


FIGURE 4: Effects of amoxicillin on faecal and small intestine microbiota. Shannon diversity index of faecal (A) and small intestinal (B) microbiota of conventional (H₂O, open symbols) and amoxicillin (AMX, closed symbols) groups. Mean relative abundance of bacterial classes in faecal (C) and small intestinal (D) microbiota. Principal coordinate analysis (PCoA) plots of weighted UniFrac distances in faecal (E) and small intestinal (F) samples coloured according to sample day. Pairwise comparisons between the H₂O and AMX group at different days in faeces and in small intestine by analysis of similarities (ANOSIM). (G) Presence/absence status of the most prevalent amplicon sequencing variant (ASVs) (those that were present in the more than 75% of the rats at Day 0) in the faecal samples at different days. Presence (blue)/ absence (white)/missing data (grey) status of four ASVs at different days (H). Relative abundance of *Lactobacillus intestinalis* in faecal (I) and small intestinal (J) microbiota. Each symbol represents a single rat and horizontal lines indicate median (A-B, H-I) values. Statistically significant differences between groups are indicated as *: $p \leq 0.05$, ***: $p \leq 0.001$.

Amoxicillin promotes Helios⁺ regulatory T cells and acute faecal IgA responses

The effect of amoxicillin on host intestinal immune regulation was assessed by quantifying IgA in faeces and sera throughout the experiment and by profiling various lymphocyte populations irrespective of whey product group. The level of total faecal IgA was affected by amoxicillin administration in a time-dependent manner; on Day 7, the level was significantly higher in the amoxicillin group than in conventional group, whereas on Day 28, it was significantly lower (Fig. 5A), indicating that acute changes in microbiota composition regulate local IgA secretion. Five weeks after amoxicillin cessation (Day 64), a difference in total faecal IgA level was no longer observed. Total serum IgA level was not affected by amoxicillin administration (Fig. 5B). There was no significant effect on whey-specific serum IgA level on Day 28 (Fig. 5C).

We have previously observed an acute induction of faecal IgA and LP regulatory T cells (Tregs) after 7 days of amoxicillin administration [288]. In the current study, we found no difference in Treg frequencies, nor in the distribution of helper T cells, cytotoxic T cells, or B cells in blood, mLN and small intestinal PP and LP five weeks after cessation of amoxicillin administration (Day 64) (Fig. S1), suggesting a return to homeostatic levels at this time point. However, the fraction of Helios⁺ Tregs within the CD25⁺FoxP3⁺ Treg population was significantly higher in blood and mLN in the amoxicillin group than in the conventional group (Day 64) (Fig. 5D), indicating a long-lasting effect of amoxicillin-induced changes in the microbiota on the phenotype of systemic Tregs.

To account for the small variation between the product groups, flow cytometry and total IgA data were normalised to the water groups of each product group before testing the difference between conventional and amoxicillin groups. This led to a similar result (Fig. S2).

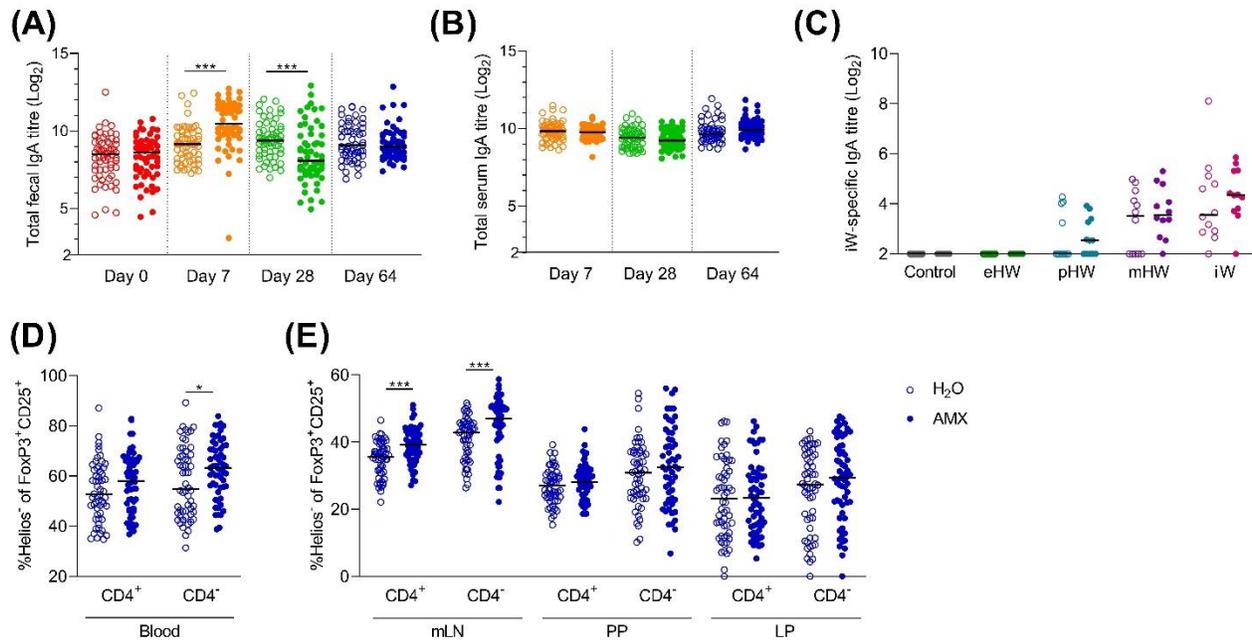


FIGURE 5: Effect of amoxicillin administration on host immune regulation. Total faecal (A) and total serum (B) IgA titres in conventional (H₂O, open symbols) and amoxicillin (AMX, closed symbols) groups. Intact whey (iW)-specific serum IgA titres at Day 28 in rats administered with extensively (eHW), partially (pHW), mildly (mHW) hydrolysed and intact (iW) whey products or water as control in their drinking water (D). Fraction of Helios⁺ Tregs out of the CD25⁺FoxP3⁺ Treg populations (both CD4⁺ and CD4⁻) in blood (D), and mesenteric lymph nodes (mLN), Peyer's patches (PP) and lamina propria (LP) at Day 64 (E). Each symbol represents a single rat and horizontal lines indicate median values. Statistically significant differences between groups are indicated as *: $p \leq 0.05$, ***: $p \leq 0.001$.

Amoxicillin promotes tolerance induction by extensively hydrolysed whey

Despite the fact that amoxicillin administration disturbed the gut microbiota and affected immune regulation, no significant differences in preventing whey-specific IgE sensitisation were observed between amoxicillin and conventional groups for rats receiving any of the whey products (Fig. S3). However, in the eHW group, amoxicillin administered rats were found to have reduced clinical reactions measured by the ear-swelling test compared to conventional rats (Fig. 6A). Surprisingly, this may indicate that amoxicillin administration promoted tolerance induction in that group. No differences between amoxicillin and water groups were observed for any of the other product groups (Fig. 6A). In line with this, a tendency ($p=0.079$ and $p=0.257$ for EPI and LP, respectively) for reduced protein uptake in the small intestine at the oral challenge with iW 15 min prior to termination was observed (Fig. 6B), which may indicate reduced oral allergic reactions. Despite the observed differences in clinical reactions, the whey-specific IgE (Fig. 6C) or IgG1 (Fig. 6D) levels were not significantly different between the amoxicillin and conventional groups.

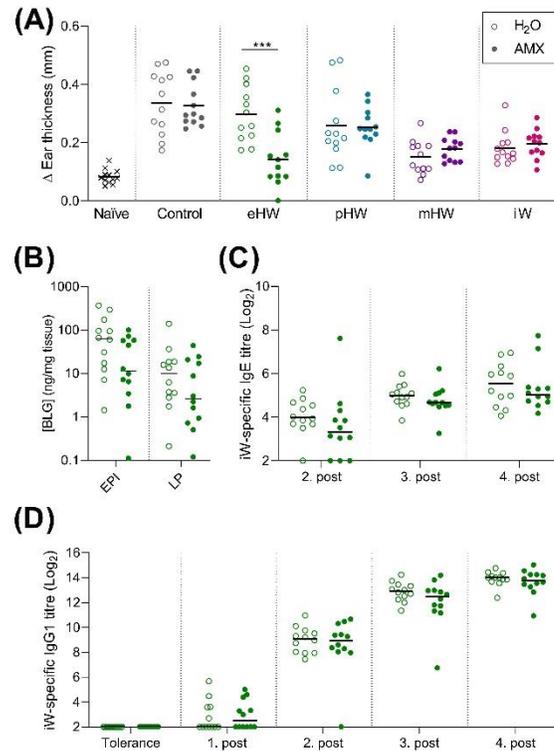


FIGURE 6: Effect of amoxicillin on the primary preventive capacity of the whey products. Ear-swelling after intradermal injection of intact whey product in water (H₂O, open symbols) and amoxicillin (AMX, closed symbols) rats administered with extensively (eHW), partially (pHW), mildly (mHW) hydrolysed or intact (iW) whey products or water as control (A). β -lactoglobulin (BLG) concentration in small intestine epithelium (EPI) and lamina propria (LP) after oral challenge with iW protein (B), iW-specific IgE titres (C), and iW-specific IgG1 titres (D). Sub-figure B-D only show results for the eHW group. Each symbol represents a single rat and horizontal lines indicate mean (A) or median (B-D) values. Statistically significant differences are indicated as ***: $p \leq 0.001$.

Immunological and morphological effects of amoxicillin in the extensively hydrolysed whey group

Because it was observed that amoxicillin promoted tolerance induction by eHW group, the effects amoxicillin administration on host immune regulation was assessed by reanalysing data for immune regulation in this group separately. The result for total faecal IgA level (Fig. 7A), total serum IgA level (Fig. 7B) and Helios⁻ Tregs frequency (Fig. 7C-D) in the eHW group alone confirmed the observation across all whey product groups (Fig. 5). Furthermore, a significant negative correlation between clinical reactions measured by the ear-swelling test and Helios⁻ CD4⁺Tregs frequency in mLN (Fig. 7E) was observed, indicating that tolerance induction may involve activation of Helios⁻ Tregs by amoxicillin.

Additionally, possible morphological effects of amoxicillin were assessed by histological analysis. No effects of amoxicillin on small (Fig. 7F) or large intestinal (Fig. 7I) tissue morphology, eosinophil (Fig. 7G,J) or goblet cell (Fig. 7H,K) count was observed five weeks after cessation of amoxicillin administration (Day 64).

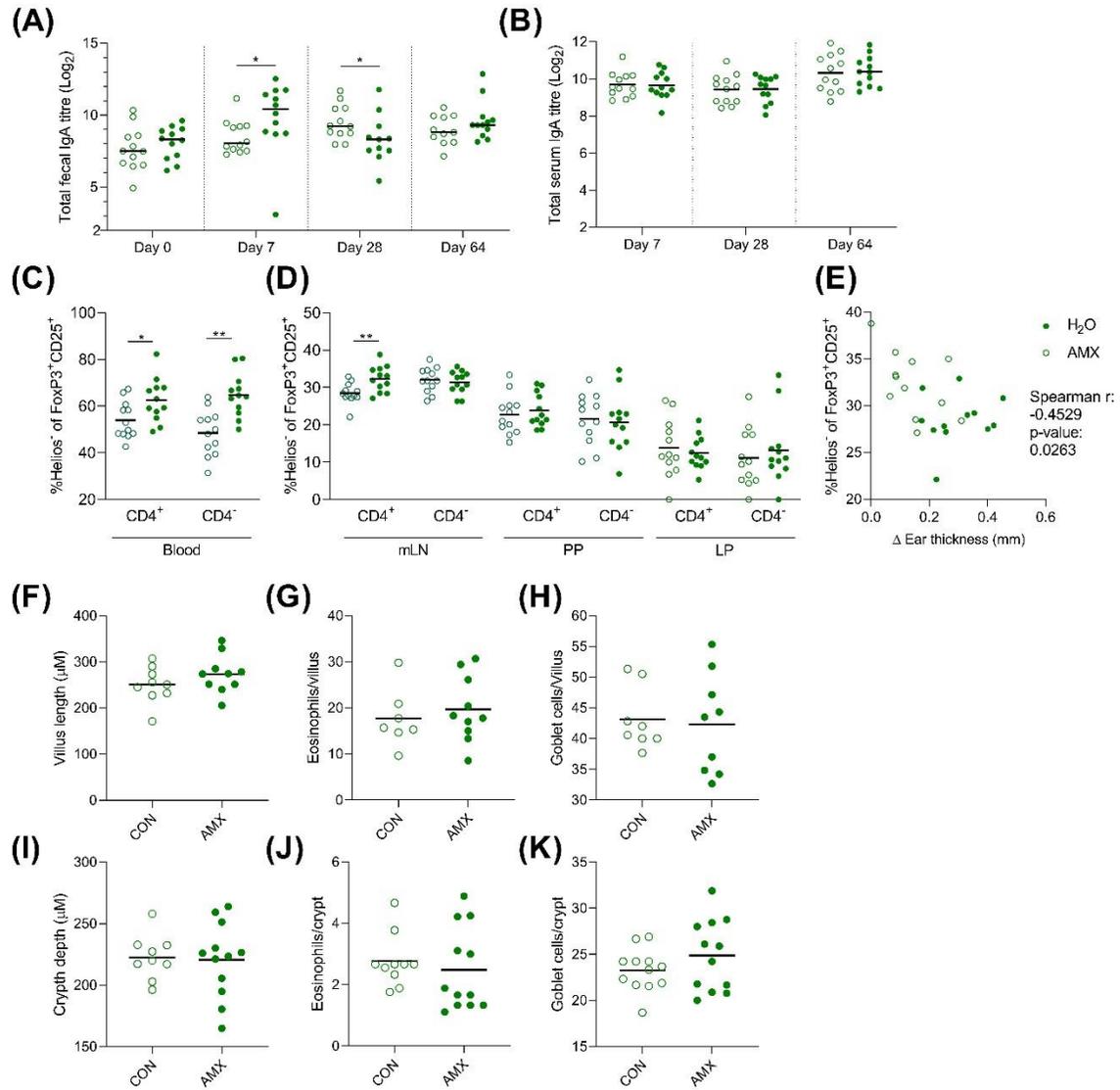


FIGURE 7: Effect of amoxicillin administration on immune regulation in the extensively hydrolysed whey group. Total faecal (A) and total serum (B) IgA titres in water (H₂O, open symbols) and amoxicillin (AMX, closed symbols) groups. Fraction of Helios⁺ Tregs out of the CD25⁺FoxP3⁺ Treg populations (both CD4⁺ and CD4⁻) in blood (C), and mesenteric lymph nodes (mLN), Peyer's patches (PP) and lamina propria (LP) (D). Correlation between fraction of Helios⁺ Tregs out of the CD4⁺ Treg populations and ear-swelling after intradermal injection of intact whey (E). Average villus length (F), eosinophil (G) and goblet cell (H) count per villus in small intestine, and average crypt depth (I), eosinophil (J) and goblet cell (K) count per crypt in colon. Each symbol represent one rat and horizontal lines indicate median (A) or mean (B-K) values. Statistically significant differences between groups are indicated as *: $p \leq 0.05$, **: $p \leq 0.01$.

Moderately hydrolysed whey promote *in vitro* expansion of *Enterococcus*

To investigate whether the different products affect gut microbiota composition, faecal samples from three healthy infant donors and one defined mixed culture made from equal amounts of three common infant gut bacteria (*Bifidobacterium longum* ssp. *Infantis*, *Lactobacillus rhamnosus*, *Enterococcus faecalis*), were anaerobically incubated in minimal medium supplemented with the four different whey products. The relative growth characteristics of selected bacterial genera were assessed by use of qPCR.

The three healthy, exclusively breastfed infant donors each had different amounts of the three analysed bacterial genera (Fig. 8A). The response was very variable across the different donors for *Bifidobacterium* and *Lactobacillus*, while for *Enterococcus* the response was uniform across the three donors (Fig. S4). Across the donors and defined mixed culture, no significant effect on the relative abundance of *Bifidobacterium* was observed of any product (Fig. 8B). The relative abundance of *Lactobacillus* was lower in incubations supplemented with mHW compared to all other products (Fig. 8C). The relative abundance of *Enterococcus* was higher in incubations supplemented with pHW and mHW compared to both eHW and iW. For *Enterococcus*, no difference between the products was observed for the defined culture (Fig. S4F), hence it is likely not the growth of *E. faecalis* specifically that was affected by the products.

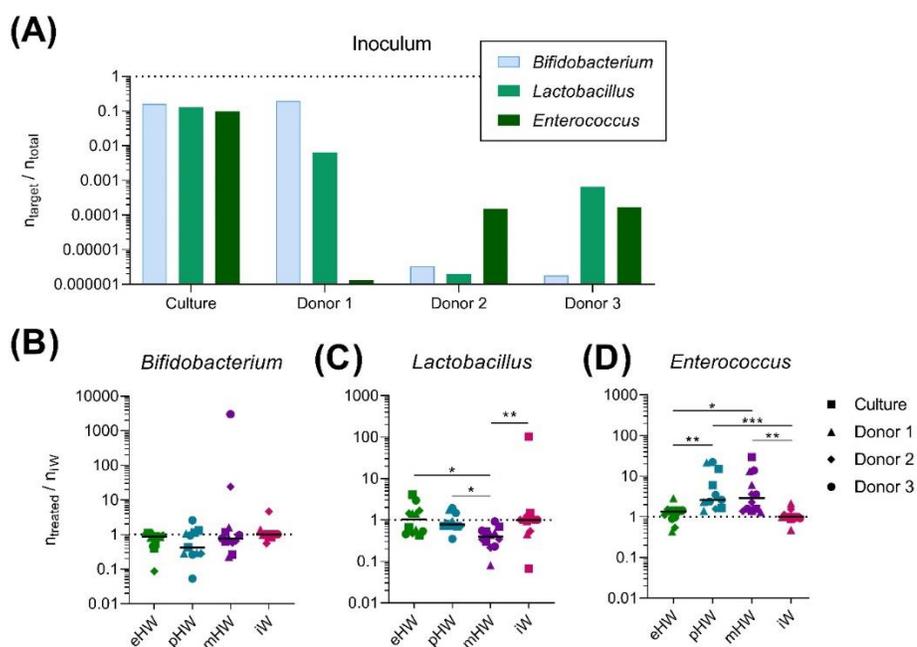


FIGURE 8: Effect of whey products on *in vitro* expansion of bacterial genera derived from healthy infants. The relative abundance of selected bacterial genera before (A) and after (B-D) *in vitro* incubations supplemented with extensively (eHW), partially (pHW), mildly (mHW) hydrolysed and intact (iW) whey products. The results after *in vitro* incubations (B-D) are normalised to the median of the iW group. Each bar represents one donor/culture (A), each symbol represents an *in vitro* incubation replicate, which is the average of three technical qPCR replicates and horizontal lines indicate median values (B-D). The symbol shape indicates the inoculum source. Statistically significant differences between groups are indicated as *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

DISCUSSION

The use of hydrolysed infant formulas for allergy prevention has been suggested as a good option for atopic infants due to the reduced allergenicity, but is still highly debated. The knowledge on how protein and host factors influence tolerance development remains scarce. To address this, the aims of the current study were (1) to investigate the capacity of intact and hydrolysed whey products, with different physicochemical characteristics, to induce oral tolerance, and (2) to investigate how microbiota composition affects tolerance induction.

Moderately hydrolysed whey products induce sustained tolerance

The tolerance inducing capacity of whey products was investigated with a previously established oral primary prevention BN rat model [168]. The study revealed that pHW and mHW with a degree of hydrolysis of 22.4% and 7.2%, respectively, and very different peptide size distribution and aggregation profiles, both were very efficient in inducing tolerance and i.e. protecting against subsequent whey-specific IgE sensitisation and clinical reactions to iW. In contrast, eHW with degree of hydrolysis of 27% and a peptide size distribution that only marginally differed from that of pHW had poor primary preventive capacity. The reduced preventive capacity may be related to filtration of eHW. In the eHW group, a statistically significant reduction in IgE level compared to the control group was observed only transiently, and no difference in IgE level nor ear-swelling was observed at the end of the study. Similarly, previous animal studies have shown that eHF retains little capacity to induce tolerance, however much less than pHF [168,195,196]

In line with our results, two previous reports indicate that administration of partially hydrolysed whey products can completely prevent epicutaneous [196] and ip sensitisation [168]. In contrast, other studies found that partially hydrolysed whey products reduced oral [197,198] or ip [195] sensitisation but not as efficiently as intact products. However, in many of these studies the used partially hydrolysed whey products were not well-characterised.

In the current study, a very diverse whey-specific IgE response was observed in the iW group. Some rats had detectable levels of whey-specific IgE already after the first post-immunisation, indicating that oral exposure to intact whey had primed an IgE immune response, while other rats were well protected and never raised detectable levels even after four post-immunisations. The whey-specific IgE level was not significantly different between the iW and control group. To our knowledge, the current study is the first to show that hydrolysed whey is superior to intact for preventing whey-specific IgE sensitisation. These results support the recommendation of partially or mildly hydrolysed whey with reduced sensitising capacity to infants at high risk of developing CMA.

Even though some rats in the iW group had high IgE levels, their response in the ear-swelling test was minimal. The inconsistency between IgE levels and acute allergic skin response in this group indicates that the raised IgE antibodies had decreased functionality, or that IgE function was blocked by other antibody classes.

Amoxicillin reduces microbial diversity and eradicates commensal species

To investigate how gut microbiota composition affects tolerance induction, parallel groups of rats were administered daily with the antibiotic amoxicillin by oral gavage daily starting one week before and continuing throughout tolerance induction. Amoxicillin induced a major shift in the faecal microbiota, characterised by reduction in Shannon diversity index and reduction in relative abundance of Firmicutes and Actinobacteria, and increased relative abundance of Gammaproteobacteria and Bacteroidetes. These effects are in accordance with our previous observation of the effect of seven-days amoxicillin administration in BN rats [288], as well as the reported effect in healthy adults [257,258].

Five weeks after cessation of amoxicillin administration, the Shannon diversity index as well as the β diversity assessed by weighted UniFrac distances was similar to that of non-treated conventional microbiota rats, but significant differences remained. Observations from human studies suggest that faecal microbiome perturbations after antibiotic administration recovered to near baseline within 1-2 weeks, but that differences in certain taxa persisted for six months to two years after antibiotic cessation [108–111]. Further, Palleja *et al.* (2018) reported that recovery rates were strain specific by observing differences in recovery of different strains of the same species. Only few studies have investigated the long-term consequences of intervention with amoxicillin specifically. A recent large study in healthy adults reported that gut microbiota did recover to baseline by means of beta diversity (weighted UniFrac distances) already one week after amoxicillin cessation, however noted that three operational taxonomic units were still different from the baseline relative abundance two weeks after cessation. These were assigned to the genus *Lactococcus*, family Lachnospiraceae and order Clostridiales [258]. The more persistent effects of amoxicillin treatment observed in the current study on overall composition and individual taxa may in part be explained by the animals' young age. In support of this, the microbiota of children is known to take a longer while to return to baseline after antibiotic treatment than that of adults [112].

In the current study, numerous ASVs still differed notable in prevalence and/or abundance between the amoxicillin and conventional groups five weeks after cessation of amoxicillin administration. Among the most differently prevalent ASVs (absent in the amoxicillin group) only one were classified to genus level (*Barnesiella*). One ASV was classified to the phylum *Candidatus Saccharibacteria* (formerly known as candidate division TM7), which is an candidate phylum present in many environmental samples also in the human microbiome [297,298]. Among the most differently abundant ASVs (lowly abundant in the amoxicillin group), one was classified as *Lactobacillus intestinalis*. This facultative heterofermentative species is commonly associated with the intestines of healthy mice and rats [299]. This species has been shown to decrease drastically in response to high-fat diet and to be negatively correlated with body weight and fat mass in an obese rat model [300].

Amoxicillin-induced perturbation of microbiota promotes tolerance development

Even though daily amoxicillin administration from one week before and during tolerance induction significantly disturbed the gut microbiota and affected immune regulation, no significant differences in prevention from whey-specific IgE sensitisation was observed between amoxicillin and water groups for rats receiving any of the whey products. In the group administered with the extensively hydrolysed

wey product, amoxicillin treated rats were actually better protected against allergic reactions (measured by an ear-swelling test) than those with a conventional microbiota. This surprisingly suggests that amoxicillin promoted tolerance induction.

Previous studies in mice indicate that microbiota depletion with broad-spectrum antibiotic cocktails promotes sensitisation [274,301,302]. Most sensitisation models require non-physiological methods of sensitisation to allergens such as damaging the gastro-intestinal tract with toxins such as cholera toxin, for which the mucosal adjuvant function has been shown to be microbiota dependent [303]. A strength of the current study is that the effect of microbiota perturbation on tolerance induction was investigated under physiologically relevant conditions by administering products in the drinking water similarly to feeding of infant formula [168]. Only two studies have previously assessed the influence of antibiotic induced microbiota perturbation on tolerance development both with hen's egg allergens [304,305]. In accordance with our results, one study found that administration of amoxicillin before and during tolerance induction by ovalbumin either slightly promoted or did not affect tolerance development [304].

It is well recognised that Tregs play an important role for tolerance development [1]. Tregs are either produced in the thymus (natural, nTreg) or induced in the peripheral tissues (iTreg). The iTreg population can be distinguished from nTregs by their low expression of the transcription factor Helios. Recent studies suggested that commensal bacteria-induced Helios⁻ROR- γ ⁺ iTregs play an important role in oral tolerance development and the prevention of food allergy in human and murine models [126]. Previous studies found that Helios⁻ROR- γ ⁺ iTregs were dramatically reduced in mice treated by a broad-spectrum antibiotic cocktail [306,307], but were less reduced or unaffected by each individual antibiotic [306], implying some degree of redundancy in microbial activation of Helios⁻ROR- γ ⁺ iTregs.

Results from the current study indicate that the microbiota perturbation induced by administration of the single, clinically relevant antibiotic amoxicillin actually increased the frequency of Helios⁻ iTregs in mLNs and blood. This was associated with acute increased total faecal IgA after amoxicillin administration, and is in line with our previous observation of acute upregulation of a Treg-IgA axis after amoxicillin administration [288]. The tolerance promoting effect of amoxicillin in the eHW group is likely related to the upregulation of the iTreg-IgA axis locally in the gut, but other factors not investigated in the study, such as effects of microbiota-perturbation on digestion and intestinal uptake of proteins during tolerance induction, may possibly also have contributed to the result. However, our previous results suggests that amoxicillin does not affect intestinal uptake of cow's milk proteins in naïve rats [288].

Mildly and partially hydrolysed wey promote *in vitro* expansion of *Enterococcus*

Even though products based on hydrolysed wey are commonly used for infants suffering from CMA or at high risk of developing CMA, only few studies have addressed the effect of hydrolysed cow's milk protein-based infant formula on gut microbiota [308,309]. To address this an *in vitro* incubation study was conducted in which faecal samples from three healthy infant donors and one defined culture of three common infant gut bacteria, were anaerobically incubated in minimal medium supplemented with each of the wey products. Growth of selected bacterial genera was assessed by qPCR.

While the three infant donor microbiotas were very different, they responded quite uniformly to the *in vitro* incubations, especially with regards to the relative abundance of *Enterococcus*. The relative abundance of *Enterococcus* was higher in the incubations with pHW and mHW than in eHW and iW. This was surprisingly in the light of *Enterococcus* species are generally considered as having very efficient proteolytic systems, however, there are strain specific differences in the expression of proteases and transport proteins [310]. The response was similar for all three donors, while no response was observed for the defined culture, probably because the specific strain of *Enterococcus* in the synthetic culture mix was different from those actually present in the donor microbiotas.

Species belonging to *Enterococcus* are common gut bacteria in early life and has been detected in both meconium of healthy infants and in human breast milk [311]. However, children sensitised to food allergens have been reported to have higher relative abundances of *Enterococcus* than healthy children [312], and high relative abundance of *Enterococcus* in early life has been associated with maternal eczema and later respiratory problems in infants [313]. Furthermore, higher relative abundance of *Enterococcus* has been observed in infants after exposure to antibiotic intra-partum [314] or post-partum [315]. The current study thus indicates that caution should be taken not to negatively influence the gut microbiota in early life by hypoallergenic infant formula.

Collectively our results support the recommendation of mildly or partially hydrolysed whey-based infant formula for tolerance induction in infants predisposed to develop CMA, regardless of their microbiota status. However, the effect of hydrolysed products on infant microbiota composition, revealed by the *in vitro* incubation study with infant-derived faecal samples, warrants further investigation.

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3 General discussion

Food allergies, including CMA, are a health problem of growing concern [5]. The increased prevalence of food allergies observed in the last decades has been associated with changes in environmental and life style factors [89], which are likely related to alterations in the gut microbiota.

Since CMA is associated with the risk of severe allergic reactions [10], impaired growth [11,12] and comorbidities [13], it is essential to prevent the development of CMA. In order to prevent CMA, the ideal infant formula should possess the following characteristics: (1) low allergenicity i.e. low sensitising capacity, (2) high tolerogenicity i.e. high primary preventive capacity, and (3) should support the development of a healthy gut microbiota. Products for treatment/desensitisation should likewise possess low allergenicity i.e. low eliciting capacity and high tolerogenicity i.e. high desensitising capacity.

3.1 Protein processing and tolerogenicity

The allergenicity and tolerogenicity of cow's milk proteins can be modified through processing such as hydrolysis and heat-treatment [24,25]. Most hypoallergenic infant formula are based on hydrolysed cow's milk proteins [4]. The destruction of epitopes by hydrolysis is largely considered as a trade-off between allergenicity and tolerogenicity, which means: the higher degree of hydrolysis the lower allergenicity but also the lower tolerogenicity. Interestingly, results presented in **Manuscript III** showed that although the sensitising capacity of moderately hydrolysed whey products was lower than the intact parent-product, the primary preventive capacity was not compromised.

Heat-treatment is an alternative processing method that can be applied to change the structure of proteins and thereby the allergenicity/tolerogenicity. Heat-treatment may not only affect the epitopes (which either promote or prevent antibody binding depending on the exact heating conditions [34–36]), but equally important may affect digestibility [183,201,202] and intestinal uptake [179]. In line with previous studies [179], it was observed in **Manuscript I** that heat-treatment of a whey product, which resulted in partial denaturation and aggregation of the proteins, promoted uptake into Peyer's patches and reduced uptake through the epithelial cell layer in naïve rats.

In **Manuscript I**, it was shown that heat-treatment reduced the ip sensitising capacity, but did not affect oral sensitisation in a model where protein products were administered by gavage along with the mucosal adjuvant CT. However, in line with previous studies [179], heat-treated whey gave rise to reduced allergic reactions (measured by body temperature) when administered to sensitised rats by gavage without any adjuvant. This indicates that the oral eliciting capacity was reduced by heat-treatment. The adjuvant function of CT includes induction of the tight junction protein claudin-2 on intestinal epithelial cells, upon which the paracellular permeability increases [204]. Thus, CT may have overridden differences in intestinal uptake of the products in the sensitisation phase, and thereby obscured the detection of potential differences in sensitising capacity.

Knowledge on how protein structures (such as aggregates) affect intestinal uptake and thereby allergenicity may be exploited in the design of ingredients for infant formula intended for CMA

prevention and desensitisation by specifically targeting uptake via Peyer's patches and not via epithelial cells. Other processes than heat-treatment may also be applied to achieve these characteristics. In **Manuscript III**, protein aggregates were likewise observed in mildly hydrolysed whey, which had good primary preventive capacity. It was not investigated in that study whether the presence of aggregates affected intestinal uptake.

3.1.1 What are the applications of heat-treated and moderately hydrolysed proteins?

It is highly debated what type of infant formula should be recommended for non-exclusively breastfed infants at high risk of developing allergy. Until recently, the recommendation of hypoallergenic infant formula based on hydrolysed proteins was generally accepted because of the reduced allergenicity [277]. However, recent systematic reviews conclude that there is not sufficient evidence to support the recommendation of hypoallergenic infant formula for the prevention of CMA or any other allergic disease [275,276]. In line with that, two of the major medical societies have recently updated their guidelines to no longer recommend hypoallergenic infant formula for prevention [278,279]. Due to this controversy, it remains questionable if protein ingredients for infant formula targeting prevention should be benchmarked against extensively hydrolysed proteins (high safety) or against intact proteins as in conventional formula (high efficacy). This question is critical for the interpretation of the results of the current study: Compared to extensively hydrolysed proteins, the primary preventive capacity of moderately hydrolysed proteins is higher (efficacy is higher) but the sensitising capacity is also higher (thus safety is lower). However, compared to intact proteins, the allergenicity of heat-treated and moderately hydrolysed proteins is higher (safety is higher) while the primary preventive capacity is unaffected (efficacy is not compromised). Thus, in the later context, the results of the current study suggest both heat-treatment and moderate hydrolysis as potential methods for producing efficient and safe(r) products for prevention of CMA.

In addition to primary prevention, another important tolerogenic characteristic is desensitising capacity. Results presented in **Manuscript I** indicate, that the capacity to desensitise already sensitised rats was not compromised by heat-treatment. Most children suffering from CMA tolerate baked milk [172], and the gradual introduction of baked milk into the diet has been described to accelerate the outgrowth of CMA [173–175]. Similarly, heat-treated and moderately hydrolysed whey may have potential for desensitisation as a convenient and standardised alternative to baked milk.

3.2 Hypoallergenic infant formula and the gut microbiota

In recent years, evidence of the microbiota being involved in the pathogenesis of food allergy have accumulated [124,126]. In that light, the optimal infant formula for CMA prevention should support the development of a healthy gut microbiota. Considering specifically the protein fraction, which was the focus of the current project, this implies that the protein ingredients should have no adverse effect on the gut microbiota. Only very few studies have investigated whether hydrolysed cow's milk protein-based infant formula affect the gut microbiota composition [308,309].

To address this, an *in vitro* incubation study was conducted with stool samples from three healthy infant donors (**Manuscript III**). This revealed that the relative abundance of *Enterococcus* was higher after incubations with the moderately hydrolysed whey protein products compared to the intact parent-product. Although species of *Enterococcus* are common gut bacteria in early life, high abundance of these has been associated with allergic conditions [312,313]. Thus, this observation warrants further investigation. The *in vitro* incubation protocol presented in **Manuscript III** was found to be applicable to screen the effect of ingredients for infant formula on the growth of bacteria derived from faecal sample in a simplified system, however the results should be confirmed by *in vivo* studies.

One of the few studies that have previously investigated the influence on hydrolysed versus intact proteins on gut microbiota composition, reported that the introduction of intact proteins in the diet of children who were previously drinking extensively hydrolysed infant formula (along with their complementary diet free of cow's milk), promoted the expansion of lactobacilli [308]. However, the authors speculate that the effect may actually be related to the absence of lactose in the hydrolysed infant formula rather than related to the protein content. While knowledge on the influence of the protein part of infant formula on gut microbiota composition is scarce, much more is known about the influence of the carbohydrate component. The primary carbohydrate in human milk is the disaccharide lactose which is known to promote the growth of (beneficial) lactic acid bacteria including lactobacilli [316]. In hypoallergenic infant formula, lactose has increasingly been substituted with other carbohydrates such as maltodextrin [317], despite the lack of any scientific evidence to support this. In human milk, another type of carbohydrates that promotes the growth of commensal gut bacteria, especially bifidobacteria, are non-digestible HMOs [100–102]. Two synthetic HMOs have currently been approved for addition to infant formula [318], thus closing the gap between infant formula and human milk. The inclusion of lactose, HMOs, other prebiotic carbohydrates and/or live probiotic bacteria [319] may be included in hypoallergenic infant formula to counterbalance possible adverse effects of hydrolysed proteins on infant microbiota composition.

3.3 Microbiota and immune regulation

In order to support the development of a healthy gut microbiota with the aim to promote tolerance development, a better understanding of microbiome-host interactions is needed. This was addressed in **Manuscript II-III**, where amoxicillin was administered to rats to manipulate their gut microbiota. Epidemiological studies suggest an association between antibiotic exposure in early life and CMA [113–115]. Thus, we expected amoxicillin-induced perturbation of the microbiota to negatively influence tolerance development. Surprisingly, we observed that amoxicillin-induced perturbation of the gut microbiota promoted acute immune regulation (**Manuscript II**) and tolerance development by extensively hydrolysed whey (the product with the poorest primary preventive capacity) (**Manuscript III**). This definitely highlights the need for more knowledge on what are the characteristics of a protective versus non-protective microbiota.

3.3.1 Acute versus prolonged effects of amoxicillin-induced perturbation of gut microbiota

It is well established that the gut microbiota may affect immune regulation through multiple different mechanisms, most notably via the activation of Tregs [209,211], which in turn stimulate mucosal IgA production [78–80]. In **Manuscript II**, it was observed that the total faecal IgA level and the fractions of Tregs in the small intestinal lamina propria were higher after one week of amoxicillin administration compared to controls. These observations are likely explained by bacteria-derived signals, prompted by the rapid and dramatic change in microbiota composition, activating the propagation of Tregs already present locally in the lamina propria and through that the activation of IgA secretion by plasma cells (Fig 3.1 A).

Five weeks after cessation of prolonged amoxicillin administration, it was observed that the level of total faecal IgA and Tregs was normalised to the homeostatic level, but that Helios⁺ iTregs were higher in mesenteric lymph nodes and in circulation in amoxicillin administered rats (**Manuscript III**). This may in contrast, represent the *de novo* differentiation of naïve T cells to become Helios⁺FoxP3⁺ iTreg via antigen-specific stimulation in the mesenteric lymph nodes (as outlined in section 1.5) (Fig 3.1 B). It is the general perception that the microbial-dependent activation of Tregs is antigen-independent [205], thus some of these newly differentiated iTregs may be whey-specific (this depends on what antigen the tolerogenic DC was loaded with). The specificity of the Tregs was not analysed in the current study. However in support of this notion, a trend for increased whey-specific IgA in the serum of amoxicillin administered animals was observed in **Manuscript III**.

Five weeks after cessation of amoxicillin administration, the microbiota composition almost returned to the baseline (with the exception of some minor taxa that failed to repopulate), and hence the bacteria-derived signals responsible for the acute local effect observed in **Manuscript II** were naturally ceased. The elevated levels of Helios⁺ iTregs five weeks after cessation may be because the *de novo* induction of Tregs is a lengthier process (iTreg induction has been shown to peak after 9-14 days [320]) and therefore just delayed compared to the bacterial stimuli. Alternatively, this could indicate that the *de novo* induction of Tregs depends on different bacterial signals (for which difference may persist) than the acute activation of local Tregs.

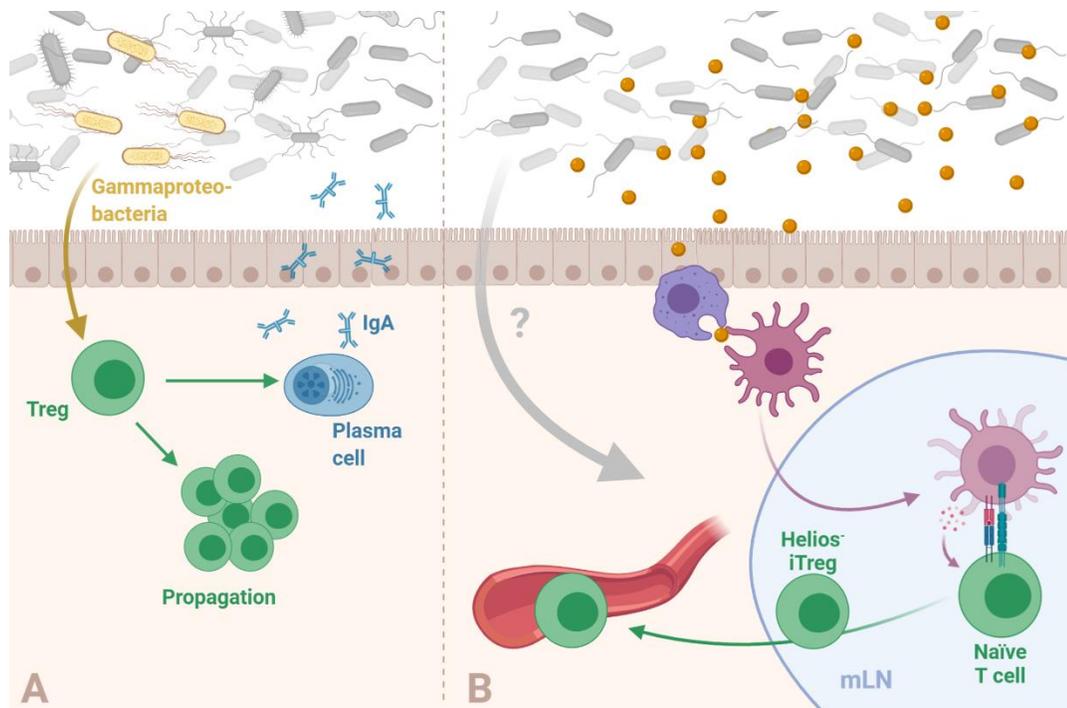


FIGURE 3.1: Acute and prolonged effect of amoxicillin induced microbiota perturbation on host immune regulation. A: In Manuscript II, it was observed that the total faecal IgA level and lamina propria Tregs were higher in response to acute changes in the gut microbiota caused by amoxicillin such as the expansion of Gammaproteobacteria. B: In Manuscript III, five weeks after cessation of prolonged amoxicillin administration, the microbiota composition had almost returned to the baseline and total faecal IgA and lamina propria Tregs was normalised to the homeostatic level. Interestingly, Helios⁺ induced iTregs were higher in mesenteric lymph nodes (mLN) and in circulation in amoxicillin administered rats. This likely represent the *de novo* differentiation of naïve T cells via antigen-specific stimulation in the mesenteric lymph nodes. The figure was created with Biorender.com

3.3.2 What bacterial signals promote immune regulation?

It remains elusive which signals drive bacterial-dependant Treg-mediated protection against food allergy [205]. In **Manuscript II**, a strong correlation between Gammaproteobacteria and frequencies of lamina propria Tregs was observed, and based on that, we speculate that Gammaproteobacteria-derived lipopolysaccharide (LPS) stimulation of host Toll-like receptor 4 (TLR4) may play a role for the observed upregulation of Tregs. In line with this notion, previous studies have found that administration of LPS enables tolerance development in germ-free mice [321] and that TLR4 knockout mice fail to develop tolerance upon oral administration of peanut allergen [274]. Furthermore, there is evidence from *in vitro* studies that LPS can activate murine [271] and human [272] Tregs directly via TLR4 interaction.

Numerous studies have otherwise pinpointed clostridia as being highly important for microbiota-dependent Treg induction [209–211] and protection against allergy [124,126,301]. Many clostridia are notable producers of the short chain fatty acid butyrate [322], which may in part explain the beneficial effect of clostridia on Treg induction [209]. However, in one of the recent studies showing that the reconstitution of germ-free mice with a defined consortium of six species of Clostridiales (which were

differentially abundant between healthy and food allergic infants) was protective against sensitisation to ovalbumin, reported that supplementation with butyrate alone did not prevent sensitisation [126]. Additionally, they reported that a taxonomically distinct consortium of five Bacteroidales strains was equally protective as the Clostridiales strains. This indicates that there may be some redundancy in what bacterial stimuli is required for microbiota-dependant Treg-mediated protection against food allergy. Interestingly, they furthermore reported that a consortium of four Delta and Gammaproteobacteria promoted Tregs (both nTregs and iTregs), but in contrast to the Clostridiales/ Bacteroidales strains (which only promoted iTreg not nTregs) this did not prevent sensitisation [126]. This could indicate that the acute activation of Tregs (not iTregs) in relation to the expansion of Gammaproteobacteria observed in **Manuscript II** may not be protective against food allergy. Further studies are needed to improve the understanding of the underlying mechanisms of microbiota-dependent protection against food allergy in order to elucidate what specific bacteria-derived signals promote tolerance development.

3.3.3 Amoxicillin-induced perturbation and intestinal protein uptake

Additionally, it is well known that bacteria-derived signals can affect the intestinal barrier function and either reduce [301] or increase [204,323] paracellular permeability. The paracellular pathway is generally not considered relevant for the uptake of antigen under healthy conditions. However, during overt inflammation the paracellular “leak pathway” may promote sensitization to food allergens [301]. Additionally, bacterial stimuli may promote uptake via microfold cell (however this may be bacterial specific) [48], and stimulate the uptake via goblet cell associated antigen passage [324].

In **Manuscript II**, BLG was quantified in small intestinal lamina propria and serum 15 min after intra-gastric administration of whey in naïve rats, and revealed a non-significant tendency for higher uptake in the amoxicillin group compared to those with a conventional microbiota. We did not find any difference in uptake into Peyer’s patches. In **Manuscript III**, we found the opposite tendency for decreased intestinal uptake in the amoxicillin group, however this is likely related to the reduced allergic response in the amoxicillin administered group (as outlined in section 1.4.1) rather than a direct effect of microbial derived signals.

4 Conclusion

The results of the current project suggest both heat-treatment and moderate hydrolysis as potential methods for producing efficient products for CMA prevention (and desensitisation), as a safer alternative to conventional formula. Additionally, studies suggest that the presence of protein aggregates, which can be obtained by heat-treatment but also by other processing methods, affects intestinal uptake of proteins, and thereby reduces their eliciting capacity. This knowledge may be applied in the production of protein ingredients for infant formula.

Despite the gut microbiota becoming increasingly appreciated in food allergy research, there is a huge knowledge gap in the understanding of how hypoallergenic infant formula affects the infant gut microbiota. Results from an *in vitro* incubation study revealed that moderately hydrolysed whey products promote the growth of *Enterococcus*, which may be associated with an increased risk of allergic diseases. Thus, this finding warrants further investigation to ensure that infant formula targeting CMA prevention does not negatively influence the gut microbiota.

Finally, results of the project suggest that amoxicillin-induced perturbation of the gut microbiota does not impair tolerance development. Actually, amoxicillin administration was found to promote acute immune regulation and tolerance development by extensively hydrolysed whey (the product with the poorest primary preventive capacity). In order to design infant formula targeting CMA prevention by supporting the development of a healthy gut microbiota, more studies are needed to elucidate which bacteria-derived signals promote tolerance development.

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References

1. Tordesillas L, Berin MC. Mechanisms of Oral Tolerance. *Clin Rev Allergy Immunol*. 2018;55:107–17.
2. Yu W, Freeland DMH, Nadeau KC. Food allergy: Immune mechanisms, diagnosis and immunotherapy. *Nat Rev Immunol*. 2016;16:751–65.
3. Tordesillas L, Berin MC, Sampson HA. Immunology of Food Allergy. *Immunity*. 2017;47:32–50.
4. Fiocchi A, Schünemann HJ, Brozek J, Restani P, Beyer K, Troncone R, et al. Diagnosis and rationale for action against Cow's milk allergy (DRACMA): A summary report. *J Allergy Clin Immunol*. 2010;126:1119-1128.e1-e12.
5. Sicherer SH, Sampson HA. Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *J Allergy Clin Immunol*. 2018;141:41–58.
6. Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, et al. The prevalence of food allergy: A meta-analysis. *J Allergy Clin Immunol*. 2007;120:638–46.
7. Nwaru BI, Hickstein L, Panesar SS, Roberts G, Muraro A, Sheikh A. Prevalence of common food allergies in Europe: A systematic review and meta-analysis. *Allergy Eur J Allergy Clin Immunol*. 2014;69:992–1007.
8. Schoemaker AA, Sprickelman AB, Grimshaw KE, Roberts G, Grabenhenrich L, Rosenfeld L, et al. Incidence and natural history of challenge-proven cow's milk allergy in European children - EuroPrevall birth cohort. *Allergy Eur J Allergy Clin Immunol*. 2015;70:963–72.
9. Wood RA, Sicherer SH, Vickery BP, Jones SM, Liu AH, Fleischer DM, et al. The natural history of milk allergy in an observational cohort. *J Allergy Clin Immunol*. 2013;131:805-812.e4.
10. Castro A, Dias D, Ferreira L, Yonamine G, Beck C, Gushken A, et al. Anaphylaxis in infants with cow's milk protein allergy: clinical aspects. *Clin Transl Allergy*. 2013;3:119.
11. Thomassen RA, Kvammen JA, Eskerud MB, Júlíusson PB, Henriksen C, Rugtveit J. Iodine Status and Growth in 0-2-Year-Old Infants with Cow's Milk Protein Allergy. *J Pediatr Gastroenterol Nutr*. 2017;64:806–11.
12. Flammarion S, Santos C, Guimber D, Jouannic L, Thumerelle C, Gottrand F, et al. Diet and nutritional status of children with food allergies. *Pediatr Allergy Immunol*. 2011;22:161–5.
13. Tran MM, Lefebvre DL, Dharma C, Dai D, Lou WYW, Subbarao P, et al. Predicting the atopic march: Results from the Canadian Healthy Infant Longitudinal Development Study. *J Allergy Clin Immunol*. 2018;141:601-607.e8.
14. Wal JM. Bovine milk allergenicity. *Ann Allergy, Asthma Immunol*. 2004;93:S2–11.
15. Hochwallner H, Schulmeister U, Swoboda I, Balic N, Geller B, Nystrand M, et al. Microarray and allergenic activity assessment of milk allergens. *Clin Exp Allergy*. 2010;40:1809–18.
16. Wal JM, Bernard H, Creminon C, Hamberger C, David B, Peltre G. Cow's milk allergy: The humoral immune response to eight purified allergens. *Adv Exp Med Biol*. 1995;371:879–81.

17. Sawyer L, Kontopidis G. The core lipocalin, bovine β -lactoglobulin. *Biochim Biophys Acta - Protein Struct Mol Enzymol*. 2000;1482:136–48.
18. Papiz MZ, Sawyer L, Eliopoulos EE, North ACT, Findlay JBC, Sivaprasadarao R, et al. The structure of β -lactoglobulin and its similarity to plasma retinol-binding protein. *Nature*. 1986;324:383–5.
19. McKenzie HA, Ralston GB, Shaw DC. Location of Sulfhydryl and Disulfide Groups in Bovine β -Lactoglobulins and Effects of Urea. *Biochemistry*. 1972;11:4539–47.
20. D’Auria E, Agostoni C, Giovannini M, Riva E, Zetterström R, Fortin R, et al. Proteomic evaluation of milk from different mammalian species as a substitute for breast milk. *Acta Paediatr Int J Paediatr*. 2005;94:1708–13.
21. Jakobsson I, Lindberg T, Benediktsson B, Hansson B. Dietary bovine beta-lactoglobulin is transferred to human milk. *Acta Paediatr Scand*. 1985;74:342–5.
22. American Academy of Pediatrics. Committee on Nutrition. Hypoallergenic infant formulas. *Pediatrics*. 2000;106:346–9.
23. Barlow DJ, Edwards MS, Thornton JM. Continuous and discontinuous protein antigenic determinants. *Nature*. 1986;322:747–8.
24. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, et al. Food processing and allergenicity. *Food Chem Toxicol*. 2015;80:223–40.
25. Golkar A, Milani JM, Vasiljevic T. Altering allergenicity of cow’s milk by food processing for applications in infant formula. *Crit Rev Food Sci Nutr*. 2019;59:159–72.
26. Pahud JJ, Monti JC, Jost R. Allergenicity of whey protein: Its modification by tryptic in vitro hydrolysis of the protein. *J Pediatr Gastroenterol Nutr*. 1985;4:408–13.
27. Zheng H, Shen X, Bu G, Luo Y. Effects of pH, temperature and enzyme-to-substrate ratio on the antigenicity of whey protein hydrolysates prepared by Alcalase. *Int Dairy J*. 2008;18:1028–33.
28. Guo M, Ahmad S. Formulation guidelines for infant formula. *Hum Milk Biochem Infant Formula Manuf Technol*. 2014. p. 141–71.
29. Greer FR, Sicherer SH, Burks AW, Baker RD, Bhatia JJS, Daniels SR, et al. Effects of early nutritional interventions on the development of atopic disease in infants and children: The role of maternal dietary restriction, breastfeeding, timing of introduction of complementary foods, and hydrolyzed formulas. *Pediatrics*. 2008;121:183–91.
30. Nutten S, Maynard F, Järvi A, Rytz A, Simons PJ, Heine RG, et al. Peptide size profile and residual immunogenic milk protein or peptide content in extensively hydrolyzed infant formulas. *Allergy Eur J Allergy Clin Immunol*. 2019;
31. Lambers TT, Gloerich J, van Hoffen E, Alkema W, Hondmann DH, van Toll EAF. Clustering analyses in peptidomics revealed that peptide profiles of infant formulae are descriptive. *Food Sci Nutr*. 2015;3:81–90.
32. Croguennec T, O’Kennedy BT, Mehra R. Heat-induced denaturation/aggregation of β -lactoglobulin A and B: Kinetics of the first intermediates formed. *Int Dairy J*. 2004;14:399–409.
33. Raikos V. Effect of heat treatment on milk protein functionality at emulsion interfaces. A review.

Food Hydrocoll. 2010;24:259–65.

34. Bu G, Luo Y, Zheng Z, Zheng H. Effect of heat treatment on the antigenicity of bovine α -lactalbumin and β -lactoglobulin in whey protein isolate. *Food Agric Immunol.* 2009;20:195–206.

35. Kleber N, Krause I, Illgner S, Hinrichs J. The antigenic response of β -lactoglobulin is modulated by thermally induced aggregation. *Eur Food Res Technol.* 2004;219:105–10.

36. Kleber N, Hinrichs J. Antigenic response of beta-lactoglobulin in thermally treated bovine skim milk and sweet whey. *Milchwissenschaft.* 2007;62:121–4.

37. Erickson R, Kim YS. Digestion And Absorption Of Dietary Protein. *Annu Rev Med.* 1990;41:133–9.

38. Husby S, Foged N, Høst A, Svehag SE. Alimentary tract and pancreas passage of dietary antigens into the blood of children with coeliac disease. Quantification and size distribution of absorbed antigens. *Gut.* 1987;28:1062–72.

39. Warshaw AL, Walker WA, Isselbacher KJ. Protein Uptake by the Intestine: Evidence for Absorption of Intact Macromolecules. *Gastroenterology.* 1974;66:987–92.

40. Astwood JD, Leach JN, Fuchs RL. Stability of Food Allergens to Digestion in Vitro. *Nat Biotechnol.* 1996;14:1269–73.

41. Bøgh KL, Madsen CB. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? *Crit Rev Food Sci Nutr.* 2016;56:1545–67.

42. Mohan Reddy I, Kella NKD, Kinsella JE. Structural and Conformational Basis of the Resistance of β -Lactoglobulin to Peptic and Chymotryptic Digestion. *J Agric Food Chem.* 1988;36:737–41.

43. Kondoh M, Takahashi A, Yagi K. Spiral progression in the development of absorption enhancers based on the biology of tight junctions. *Adv Drug Deliv Rev.* 2012;64:515–22.

44. Watson CJ, Hoare CJ, Garrod DR, Carlson GL, Warhurst G. Interferon- γ selectively increases epithelial permeability to large molecules by activating different populations of paracellular pores. *J Cell Sci.* 2005;118:5221–30.

45. Reitsma M, Westerhout J, Wichers HJ, Wortelboer HM, Verhoecx KCM. Protein transport across the small intestine in food allergy. *Mol Nutr Food Res.* 2014;58:194–205.

46. Walker WA, Isselbacher KJ, Bloch KJ. Immunologic control of soluble protein absorption from the small intestine: a gut surface phenomenon. *Am J Clin Nutr.* 1974;27:1434–40.

47. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. *Nat Rev Immunol.* 2014;14:667–85.

48. Rios D, Wood MB, Li J, Chassaing B, Gewirtz AT, Williams IR. Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria. *Mucosal Immunol.* 2016;9:907–16.

49. McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, et al. Goblet cells deliver luminal antigen to CD103 + dendritic cells in the small intestine. *Nature, Nat.* 2012;483:345–9.

50. Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, et al. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol.*

2001;2:361–7.

51. Mazzini E, Massimiliano L, Penna G, Rescigno M. Oral Tolerance Can Be Established via Gap Junction Transfer of Fed Antigens from CX3CR1+ Macrophages to CD103+ Dendritic Cells. *Immunity*. 2014;40:248–61.

52. Knoop KA, Miller MJ, Newberry RD. Transepithelial antigen delivery in the small intestine: Different paths, different outcomes. *Curr Opin Gastroenterol*. 2013;29:112–8.

53. Perrier C, Corthésy B. Gut permeability and food allergies. *Clin Exp Allergy*. 2011;41:20–8.

54. Järvinen KM, Konstantinou GN, Pilapil M, Arrieta MC, Noone S, Sampson HA, et al. Intestinal permeability in children with food allergy on specific elimination diets. *Pediatr Allergy Immunol*. 2013;24:589–95.

55. Dupont C, Barau E, Molkhou P, Raynaud F, Barbet JP, Dehennin L. Food-Induced alterations of intestinal permeability in children with cow's milk-sensitive enteropathy and atopic dermatitis. *J Pediatr Gastroenterol Nutr*. 1989;8:459–65.

56. Andre C, Andre F, Colin L, Cavagna S. Measurement of intestinal permeability to mannitol and lactulose as a means of diagnosing food allergy and evaluating therapeutic effectiveness of disodium cromoglycate. *Ann Allergy*. 1987;59:127–30.

57. Yu LCH, Yang PC, Berin MC, Di Leo V, Conrad DH, McKay DM, et al. Enhanced transepithelial antigen transport in intestine of allergic mice is mediated by IgE/CD23 and regulated by interleukin-4. *Gastroenterology*. 2001;121:370–81.

58. Berin MC, Kiliaan AJ, Yang PC, Groot JA, Kitamura Y, Perdue MH. The influence of mast cells on pathways of transepithelial antigen transport in rat intestine. *J Immunol*. 1998;161:2561–6.

59. Jacob C, Yang PC, Darmoul D, Amadesi S, Saito T, Cottrell GS, et al. Mast cell tryptase controls paracellular permeability of the intestine: Role of protease-activated receptor 2 and β -arrestins. *J Biol Chem*. 2005;280:31936–48.

60. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature*. 1998;392:245–52.

61. Schulz O, Jaensson E, Persson EK, Liu X, Worbs T, Agace WW, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med*. 2009;206:3101–14.

62. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007;204:1775–85.

63. Coombes JL, Siddiqui KRR, Arancibia-Cárcamo C V., Hall J, Sun CM, Belkaid Y, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF- β -and retinoic acid-dependent mechanism. *J Exp Med*. 2007;204:1757–64.

64. Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, et al. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med*. 2006;203:519–27.

65. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, et al. Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from Peripherally Induced

- Foxp3 + T Regulatory Cells. *J Immunol.* 2010;184:3433–41.
66. Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto De Lafaille MA. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest.* 2005;115:1923–33.
67. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, et al. Intestinal Tolerance Requires Gut Homing and Expansion of FoxP3+ Regulatory T Cells in the Lamina Propria. *Immunity.* 2011;34:237–46.
68. Esterházy D, Canesso MCC, Mesin L, Muller PA, de Castro TBR, Lockhart A, et al. Compartmentalized gut lymph node drainage dictates adaptive immune responses. *Nature.* 2019;569:126–30.
69. Kraus TA, Brimnes J, Muong C, Liu JH, Moran TM, Tappenden KA, et al. Induction of mucosal tolerance in Peyer’s patch-deficient, ligated small bowel loops. *J Clin Invest.* 2005;115:2234–43.
70. Spahn TW, Weiner HL, Rennert PD, Lgering N, Fontana A, Domschke W, et al. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer’s patches. *Eur J Immunol.* 2002;32:1109–13.
71. Cassani B, Villablanca EJ, Quintana FJ, Love PE, Lacy-Hulbert A, Blaner WS, et al. Gut-tropic T cells that express integrin $\alpha 4\beta 7$ and CCR9 Are required for induction of oral immune tolerance in mice. *Gastroenterology.* 2011;141:2109–18.
72. Noval Rivas M, Chatila TA. Regulatory T cells in allergic diseases. *J Allergy Clin Immunol.* 2016;138:653.
73. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. *J Immunol.* 2008;180:5916–26.
74. Zheng Y, Chaudhry A, Kas A, DeRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control TH2 responses. *Nature.* 2009;458:351–6.
75. Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting Edge: Direct Suppression of B Cells by CD4 + CD25 + Regulatory T Cells. *J Immunol.* 2005;175:4180–3.
76. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, et al. CD4+CD25+ Regulatory T Cells Suppress Mast Cell Degranulation and Allergic Responses through OX40-OX40L Interaction. *Immunity.* 2008;29:771–81.
77. Read S, Malmström V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. *J Exp Med.* 2000;192:295–302.
78. Cazac BB, Roes J. TGF- β receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity.* 2000;13:443–51.
79. Borsutzky S, Cazac BB, Roes J, Guzmán CA. TGF- β Receptor Signaling Is Critical for Mucosal IgA Responses. *J Immunol.* 2004;173:3305–9.
80. Cong Y, Feng T, Fujihashi K, Schoeb TR, Elson CO. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci U S A.* 2009;106:19256–61.
81. Chen Y, Inobe JI, Kuchroo VK, Baron JL, Janeway CA, Weiner HL. Oral tolerance in myelin basic protein T-cell receptor transgenic mice: Suppression of autoimmune encephalomyelitis and dose-

dependent induction of regulatory cells. *Proc Natl Acad Sci U S A*. 1996;93:388–91.

82. Chen Y, Inobe JI, Marks R, Gonnella P, Kuchroo VK, Weiner HL. Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature*. 1995;377:257–257.

83. Brandtzaeg P, Johansen FE. Mucosal B cells: Phenotypic characteristics, transcriptional regulation, and homing properties. *Immunol Rev*. 2005;206:32–63.

84. Weltzin R, Lucia-Jandris P, Michetti P, Fields BN, Kraehenbuhl JP, Neutra MR. Binding and transepithelial transport of immunoglobulins by intestinal M cells: Demonstration using monoclonal IgA antibodies against enteric viral proteins. *J Cell Biol*. 1989;108:1673–85.

85. Baumann J, Park CG, Mantis NJ. Recognition of secretory IgA by DC-SIGN: Implications for immune surveillance in the intestine. *Immunol Lett*. 2010;131:59–66.

86. Savilahti E, Tainio VM, Salmenpera L, Arjomaa P, Kallio M, Perheentupa J, et al. Low colostral IgA associated with cow's milk allergy. *Acta Paediatr Scand*. 1991;80:1207–13.

87. Jävinen KM, Laine ST, Jävenpää AL, Suomalainen HK. Does low IgA in human milk predispose the infant to development of cow's milk allergy? *Pediatr Res*. 2000;48:457–62.

88. Strait RT, Mahler A, Hogan S, Khodoun M, Shibuya A, Finkelman FD. Ingested allergens must be absorbed systemically to induce systemic anaphylaxis. *J Allergy Clin Immunol*. 2011;127:982-989.e1.

89. Platts-Mills TAE. The allergy epidemics: 1870-2010. *J Allergy Clin Immunol*. 2015;136:3–13.

90. Strachan DP. Hay fever, hygiene, and household size. *Br Med J*. 1989;299:1259–60.

91. Liu AH. Revisiting the hygiene hypothesis for allergy and asthma. *J Allergy Clin Immunol*. 2015;136:860–865.

92. Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? *Res Microbiol*. 2008;159:187–93.

93. Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, et al. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr Microbiol*. 2005;51:270–4.

94. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe*. 2015;17:690–703.

95. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med*. 2016;8:343ra82.

96. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A*. 2010;107:11971–5.

97. Koplein J, Allen K, Gurrin L, Osborne N, Tang MLK, Dharmage S. Is caesarean delivery associated with sensitization to food allergens and IgE-mediated food allergy: a systematic review. *Pediatr Allergy Immunol*. 2008;19:682–7.

98. Mitselou N, Hallberg J, Stephansson O, Almquist C, Melén E, Ludvigsson JF. Cesarean delivery, preterm birth, and risk of food allergy: Nationwide Swedish cohort study of more than 1 million children. *J Allergy Clin Immunol*. 2018;142:1510-1514.e2.

99. Jones KJ, Sitarik AR, Fujimura K, Johnson CC, Havstad S, Kim H, et al. Breastfeeding Is Associated with Infant Gut Microbial Composition. *J Allergy Clin Immunol*. 2015;135:AB169–AB169.
100. Borewicz K, Gu F, Saccenti E, Arts ICW, Penders J, Thijs C, et al. Correlating Infant Fecal Microbiota Composition and Human Milk Oligosaccharide Consumption by Microbiota of 1-Month-Old Breastfed Infants. *Mol Nutr Food Res*. 2019;63:1801214.
101. Moossavi S, Atakora F, Miliku K, Sepehri S, Robertson B, Duan QL, et al. Integrated analysis of human milk microbiota with oligosaccharides and fatty acids in the child cohort. *Front Nutr*. 2019;6:58.
102. Wang M, Li M, Wu S, Lebrilla CB, Chapkin RS, Ivanov I, et al. Fecal microbiota composition of breastfed infants is correlated with human milk oligosaccharides consumed. *J Pediatr Gastroenterol Nutr*. 2015;60:825–33.
103. Donaldson GP, Ladinsky MS, Yu KB, Sanders JG, Yoo BB, Chou WC, et al. Gut microbiota utilize immunoglobulin a for mucosal colonization. *Science (80-)*. 2018;360:795–800.
104. Sitarik AR, Bobbitt KR, Havstad SL, Fujimura KE, Levin AM, Zoratti EM, et al. Breast Milk Transforming Growth Factor β Is Associated with Neonatal Gut Microbial Composition. *J Pediatr Gastroenterol Nutr*. 2017;65:e60–7.
105. Hunt KM, Foster JA, Forney LJ, Schütte UME, Beck DL, Abdo Z, et al. Characterization of the diversity and temporal stability of bacterial communities in human milk. *PLoS One*. 2011;6:e21313.
106. Miliku K, Robertson B, Sharma AK, Subbarao P, Becker AB, Mandhane PJ, et al. Human milk oligosaccharide profiles and food sensitization among infants in the CHILD Study. *Allergy Eur J Allergy Clin Immunol*. 2018;73:2070–3.
107. Seppo AE, Autran CA, Bode L, Järvinen KM. Human milk oligosaccharides and development of cow's milk allergy in infants. *J Allergy Clin Immunol*. 2017;139:708-711.e5.
108. Raymond F, Ouameur AA, Déraspe M, Iqbal N, Gingras H, Dridi B, et al. The initial state of the human gut microbiome determines its reshaping by antibiotics. *ISME J*. 2016;10:707–20.
109. Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One*. 2010;5:e9836.
110. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J*. 2007;1:56–66.
111. Palleja A, Mikkelsen KH, Forslund SK, Kashani A, Allin KH, Nielsen T, et al. Recovery of gut microbiota of healthy adults following antibiotic exposure. *Nat Microbiol*. 2018;3:1255–65.
112. Yassour M, Vatanen T, Siljander H, Hamalainen A-M, Harkonen T, Ryhanen SJ, et al. Natural history of the infant gut microbiome and impact of antibiotic treatment on bacterial strain diversity and stability. *Sci Transl Med*. 2016;8:343ra81.
113. Metsälä J, Lundqvist A, Virta LJ, Kaila M, Gissler M, Virtanen SM. Mother's and Offspring's Use of Antibiotics and Infant Allergy to Cow's Milk. *Epidemiology*. 2013;24:303–9.
114. Mitre E, Susi A, Kropp LE, Schwartz DJ, Gorman GH, Nylund CM. Association between use of acid-suppressive medications and antibiotics during infancy and allergic diseases in early childhood. *JAMA Pediatr*. 2018;172:e180315.

115. Hirsch AG, Pollak J, Glass TA, Poulsen MN, Bailey-Davis L, Mowery J, et al. Early-life antibiotic use and subsequent diagnosis of food allergy and allergic diseases. *Clin Exp Allergy*. 2017;47:236–44.
116. Thompson-Chagoyan OC, Fallani M, Maldonado J, Vieites JM, Khanna S, Edwards C, et al. Faecal microbiota and short-chain fatty acid levels in faeces from infants with cow's milk protein allergy. *Int Arch Allergy Immunol*. 2011;156:325–32.
117. Ling Z, Li Z, Liu X, Cheng Y, Luo Y, Tong X, et al. Altered fecal microbiota composition associated with food allergy in infants. *Appl Environ Microbiol*. 2014;80:2546–54.
118. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, et al. Infant gut microbiota and food sensitization: associations in the first year of life. *Clin Exp Allergy*. 2015;45:632–43.
119. Cahenzli J, Köller Y, Wyss M, Geuking MB, McCoy KD. Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe*. 2013;14:559–70.
120. Hong SW, Eunju O, Lee JY, Lee M, Han D, Ko HJ, et al. Food antigens drive spontaneous IgE elevation in the absence of commensal microbiota. *Sci Adv*. 2019;5:aaw1507.
121. Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol*. 1997;159:1739–45.
122. Rodriguez B, Prioult G, Hacini-Rachinel F, Moine D, Bruttin A, Ngom-Bru C, et al. Infant gut microbiota is protective against cow's milk allergy in mice despite immature ileal T-cell response. *FEMS Microbiol Ecol*. 2012;79:192–202.
123. Noval Rivas M, Burton OT, Wise P, Zhang Y, Hobson SA, Garcia Lloret M, et al. A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. *J Allergy Clin Immunol*. 2013;131:201–12.
124. Feehley T, Plunkett CH, Bao R, Choi Hong SM, Cullen E, Belda-Ferre P, et al. Healthy infants harbor intestinal bacteria that protect against food allergy. *Nat Med*. 2019;25:448–53.
125. Mauras A, Wopereis H, Yeop I, Esber N, Delannoy J, Labellie C, et al. Gut microbiota from infant with cow's milk allergy promotes clinical and immune features of atopy in a murine model. *Allergy Eur J Allergy Clin Immunol*. 2019;74:1790–3.
126. Abdel-Gadir A, Stephen-Victor E, Gerber GK, Noval Rivas M, Wang S, Harb H, et al. Microbiota therapy acts via a regulatory T cell MyD88/ROR γ t pathway to suppress food allergy. *Nat Med*. 2019;25:1164–74.
127. Bunyavanich S, Berin MC. Food allergy and the microbiome: Current understandings and future directions. *J Allergy Clin Immunol*. 2019;144:1468–77.
128. Oyoshi MK, Oettgen HC, Chatila TA, Geha RS, Bryce PJ. Food allergy: Insights into etiology, prevention, and treatment provided by murine models. *J Allergy Clin Immunol*. 2014;133:309–17.
129. Bøgh KL, van Bilsen J, Głogowski R, López-Expósito I, Bouchaud G, Blanchard C, et al. Current challenges facing the assessment of the allergenic capacity of food allergens in animal models. *Clin Transl Allergy*. 2016;6:21.
130. Liu T, Navarro S, Lopata AL. Current advances of murine models for food allergy. *Mol Immunol*. 2016;70:104–17.

131. Larsen JM, Bøgh KL. Animal models of allergen-specific immunotherapy in food allergy: Overview and opportunities. *Clin Exp Allergy*. 2018;48:1255–74.
132. Li XM, Schofield BH, Huang CK, Kleiner GI, Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol*. 1999;103:206–14.
133. Knippels LMJ, Penninks AH, Spanhaak S, Houben GF. Oral sensitization to food proteins: A Brown Norway rat model. *Clin Exp Allergy*. 1998;28:368–75.
134. Dearman RJ, Kimber I. A mouse model for food allergy using intraperitoneal sensitization. *Methods*. 2007;41:91–8.
135. Birmingham NP, Parvataneni S, Hassan HMA, Harkema J, Samineni S, Navuluri L, et al. An adjuvant-free mouse model of tree nut allergy using hazelnut as a model tree nut. *Int Arch Allergy Immunol*. 2007;144:203–10.
136. Pierce NF, Gowans JL. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. *J Exp Med*. 1975;142:1550–63.
137. Ganeshan K, Neilsen C V., Hadsaitong A, Schleimer RP, Luo X, Bryce PJ. Impairing oral tolerance promotes allergy and anaphylaxis: A new murine food allergy model. *J Allergy Clin Immunol*. 2009;123:231-238.e4.
138. Yang PC, Xing Z, Berin CM, Soderholm JD, Feng BS, Wu L, et al. TIM-4 Expressed by Mucosal Dendritic Cells Plays a Critical Role in Food Antigen-Specific Th2 Differentiation and Intestinal Allergy. *Gastroenterology*. 2007;133:1522–33.
139. Berin MC, Shreffler WG. TH2 adjuvants: Implications for food allergy. *J Allergy Clin Immunol*. 2008;121:1311–20.
140. Ponce M, Diesner SC, Szépfalusi Z, Eiwegger T. Markers of tolerance development to food allergens. *Allergy Eur J Allergy Clin Immunol*. 2016;71:1393–404.
141. Enomoto A, Konishi M, Hachimura S, Kaminogawa S. Milk whey protein fed as a constituent of the diet induced both oral tolerance and a systemic humoral response, while heat-denatured whey protein induced only oral tolerance. *Clin Immunol Immunopathol*. 1993;66:139–42.
142. Fritsché R, Pahud JJ, Pecquet S, Pfeifer A. Induction of systemic immunologic tolerance to β -lactoglobulin by oral administration of a whey protein hydrolysate. *J Allergy Clin Immunol*. 1997;100:266–73.
143. Brix S, Christensen HR, Barkholt V, Frøkiær H. Effect of maternal dietary cow's milk on the immune response to β -lactoglobulin in the offspring: A four-generation study in mice. *Int Arch Allergy Immunol*. 2005;136:250–7.
144. Simons JP, McLenaghan M, Clark AJ. Alteration of the quality of milk by expression of sheep β -lactoglobulin in transgenic mice. *Nature*. 1987;328:530–2.
145. Fritz J V., Desai MS, Shah P, Schneider JG, Wilmes P. From meta-omics to causality: Experimental models for human microbiome research. *Microbiome*. 2013;1:14.
146. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on humang-microbe mutualism and disease. *Nature*. 2007;449:811–8.

147. Xiao L, Feng Q, Liang S, Sonne SB, Xia Z, Qiu X, et al. A catalog of the mouse gut metagenome. *Nat Biotechnol.* 2015;33:1103–8.
148. Larrosa M, Yañez-Gascón MJ, Selma MV, González-Sarrías A, Toti S, Cerón JJ, et al. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. *J Agric Food Chem.* 2009;57:2211–20.
149. Duca FA, Sakar Y, Lepage P, Devime F, Langelier B, Doré J, et al. Replication of obesity and associated signaling pathways through transfer of microbiota from obese-prone rats. *Diabetes.* 2014;63:1624–36.
150. Dalby AB, Frank DN, St. Amand AL, Bendele AM, Pace NR. Culture-independent analysis of indomethacin-induced alterations in the rat gastrointestinal microbiota. *Appl Environ Microbiol.* 2006;72:6707–15.
151. Mell B, Jala VR, Mathew A V., Byun J, Waghulde H, Zhang Y, et al. Evidence for a link between gut microbiota and hypertension in the Dahl rat. *Physiol Genomics.* 2015;47:187–97.
152. Patterson E, Marques TM, O’Sullivan O, Fitzgerald P, Fitzgerald GF, Cotter PD, et al. Streptozotocin-induced type-1-diabetes disease onset in Sprague–Dawley rats is associated with an altered intestinal microbiota composition and decreased diversity. *Microbiol (United Kingdom).* 2015;161:182–93.
153. Li J V., Ashrafian H, Bueter M, Kinross J, Sands C, Le Roux CW, et al. Metabolic surgery profoundly influences gut microbial - Host metabolic cross-talk. *Gut.* 2011;60:1214–23.
154. Brooks SPJ, McAllister M, Sandoz M, Kalmokoff ML. Culture-independent phylogenetic analysis of the faecal flora of the rat. *Can J Microbiol.* 2003;49:589–601.
155. Flemer B, Gaci N, Borrel G, Sanderson IR, Chaudhary PP, Tottey W, et al. Fecal microbiota variation across the lifespan of the healthy laboratory rat. *Gut Microbes.* 2017;8:428–39.
156. Li D, Chen H, Mao B, Yang Q, Zhao J, Gu Z, et al. Microbial Biogeography and Core Microbiota of the Rat Digestive Tract. *Sci Rep.* 2017;8:45840.
157. Nagpal R, Wang S, Solberg Woods LC, Seshie O, Chung ST, Shively CA, et al. Comparative microbiome signatures and short-chain fatty acids in mouse, rat, non-human primate, and human feces. *Front Microbiol.* 2018;9:2897.
158. Wos-Oxley ML, Bleich A, Oxley APA, Kahl S, Janus LM, Smoczek A, et al. Comparative evaluation of establishing a human gut microbial community within rodent models. *Gut Microbes.* 2012;3:234–49.
159. Werner JJ, Koren O, Hugenholtz P, Desantis TZ, Walters WA, Caporaso JG, et al. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *ISME J.* 2012.
160. Hildebrand F, Nguyen TLA, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, et al. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol.* 2013;14:R4.
161. Chang HYS, Mitzner W, Watson J. Variation in airway responsiveness of male C57BL/6 mice from 5 vendors. *J Am Assoc Lab Anim Sci.* 2012;51:401–6.
162. Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, et al. Phylogeny of the defined murine microbiota: Altered Schaedler flora. *Appl Environ Microbiol.* 1999;65:3287–92.

163. Laukens D, Brinkman BM, Raes J, De Vos M, Vandenaabeele P. Heterogeneity of the gut microbiome in mice: Guidelines for optimizing experimental design. *FEMS Microbiol Rev.* 2015;40:117–32.
164. Dobson GP, Letson HL, Biros E, Morris J. Specific pathogen-free (SPF) animal status as a variable in biomedical research: Have we come full circle? *EBioMedicine.* 2019;41:42–3.
165. Rosshart SP, Herz J, Vassallo BG, Hunter A, Wall MK, Badger JH, et al. Laboratory mice born to wild mice have natural microbiota and model human immune responses. *Science (80-).* 2019;365:461.
166. Bahna SL. Hypoallergenic formulas: Optimal choices for treatment versus prevention. *Ann Allergy, Asthma Immunol.* 2008;101:453–9.
167. Cabana MD. The Role of Hydrolyzed Formula in Allergy Prevention. *Ann Nutr Metab.* 2017;70:38–45.
168. Jensen LH, Larsen JM, Madsen CB, Laursen RR, Jacobsen LN, Bøgh KL. Preclinical Brown Norway Rat Models for the Assessment of Infant Formulas in the Prevention and Treatment of Cow's Milk Allergy. *Int Arch Allergy Immunol.* 2019;178:307–14.
169. Bogahawaththa D, Chandrapala J, Vasiljevic T. Modulation of milk immunogenicity by thermal processing. *Int Dairy J.* 2017;59:159–72.
170. Rahaman T, Vasiljevic T, Ramchandran L. Effect of processing on conformational changes of food proteins related to allergenicity. *Trends Food Sci Technol.* 2016;49:24–34.
171. Bu G, Luo Y, Chen F, Liu K, Zhu T. Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. *Dairy Sci Technol.* 2013;93:211–23.
172. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, et al. Tolerance to extensively heated milk in children with cow's milk allergy. *J Allergy Clin Immunol.* 2008;122:342-347.e2.
173. Esmailzadeh H, Alyasin S, Haghghat M, Nabavizadeh H, Esmailzadeh E, Mosavat F. The effect of baked milk on accelerating unheated cow's milk tolerance: A control randomized clinical trial. *Pediatr Allergy Immunol.* 2018;29:747–53.
174. Kim JS, Nowak-Wegrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA. Dietary baked milk accelerates the resolution of cow's milk allergy in children. *J Allergy Clin Immunol.* 2011;128:125-131.e2.
175. Weinbrand-Goichberg J, Benor S, Rottem M, Shacham N, Mandelberg A, Levine A, et al. Long-term outcomes following baked milk-containing diet for IgE-mediated milk allergy. *J Allergy Clin Immunol Pract.* 2017;5:1776-1778.e1.
176. Goldberg MR, Nachshon L, Appel MY, Elizur A, Levy MB, Eisenberg E, et al. Efficacy of baked milk oral immunotherapy in baked milk-reactive allergic patients. *J Allergy Clin Immunol.* 2015;136:1601–6.
177. Amat F, Kouche C, Gaspard W, Lemoine A, Guiddir T, Lambert N, et al. Is a slow-progression baked milk protocol of oral immunotherapy always a safe option for children with cow's milk allergy? A randomized controlled trial. *Clin Exp Allergy.* 2017;47:1491–6.
178. Upton J, Nowak-Wegrzyn A. The Impact of Baked Egg and Baked Milk Diets on IgE- and Non-IgE-Mediated Allergy. *Clin Rev Allergy Immunol.* 2018;55:118–38.
179. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, et al. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy.*

2008;63:882–90.

180. Rytönen J, Karttunen TJ, Karttunen R, Valkonen KH, Jenmalm MC, Alatossava T, et al. Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats: Denatured betaLG induces a more intensive local immunologic response than native betaLG. *Pediatr Allergy Immunol*. 2002;13:269–77.

181. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J, et al. Milk processing increases the allergenicity of cow's milk—Preclinical evidence supported by a human proof-of-concept provocation pilot. *Clin Exp Allergy*. 2019;49:1013–25.

182. Taheri-Kafrani A, Gaudin JC, Rabesona H, Nioi C, Agarwal D, Drouet M, et al. Effects of heating and glycation of β -lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *J Agric Food Chem*. 2009;57:4974–82.

183. Morisawa Y, Kitamura A, Ujihara T, Zushi N, Kuzume K, Shimanouchi Y, et al. Effect of heat treatment and enzymatic digestion on the B cell epitopes of cow's milk proteins. *Clin Exp Allergy*. 2009;39:918–25.

184. Ehn BM, Ekstrand B, Bengtsson U, Ahlstedt S. Modification of IgE Binding during Heat Processing of the Cow's Milk Allergen β -Lactoglobulin. *J Agric Food Chem*. 2004;52:1398–403.

185. Maryniak NZ, Hansen EB, Ballegaard ASR, Sancho AI, Bøgh KL. Comparison of the allergenicity and immunogenicity of Camel and cow's milk—A study in brown Norway rats. *Nutrients*. 2018;10:1903.

186. Bøgh KL, Kroghsbo S, Dahl L, Rigby NM, Barkholt V, Mills ENC, et al. Digested Ara h 1 has sensitizing capacity in Brown Norway rats. *Clin Exp Allergy*. 2009;39:1611–21.

187. Bøgh KL, Barkholt V, Madsen CB. The sensitising capacity of intact β -lactoglobulin is reduced by co-administration with digested β -lactoglobulin. *Int Arch Allergy Immunol*. 2013;161:21–36.

188. Bøgh KL, Barkholt V, Madsen CB. Characterization of the Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates - A Study in Brown Norway Rats. *Scand J Immunol*. 2015;81:274–83.

189. Diesner SC, Bergmayr C, Wang XY, Heiden D, Exenberger S, Roth-Walter F, et al. Characterization of *Vibrio cholerae* neuraminidase as an immunomodulator for novel formulation of oral allergy immunotherapy. *Clin Immunol*. 2018;192:30–9.

190. Muccioli M, Pate M, Omosibi O, Benencia F. Generation and Labeling of Murine Bone Marrow-derived Dendritic Cells with Qdot Nanocrystals for Tracking Studies. *J Vis Exp*. 2011;2785.

191. Stojadinovic M, Pieters R, Smit J, Velickovic TC. Cross-linking of β -lactoglobulin enhances allergic sensitization through changes in cellular uptake and processing. *Toxicol Sci*. 2014;140:224–35.

192. Davis PJ, Williams SC. Protein modification by thermal processing. *Allergy Eur J Allergy Clin Immunol*. 1998;53:102–5.

193. Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, Srutkova D, et al. Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. *PLoS One*. 2012;7:e37156.

194. Leonard SA, Martos G, Wang W, Nowak-Wegrzyn A, Berin MC. Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. *J Allergy Clin Immunol*. 2012;129:1579-1587.e1.

195. Peng HJ, Su SN, Tsai JJ, Tsai LC, Kuo HL, Kuo SW. Effect of ingestion of cow's milk hydrolysed formulas on whey protein-specific Th2 immune responses in naïve and sensitized mice. *Clin Exp Allergy*. 2004;34:663–70.
196. Matsubara T, Iwamoto H, Nakazato Y, Okamoto T, Ehara T, Izumi H, et al. Ingestion of partially hydrolyzed whey protein suppresses epicutaneous sensitization to β -lactoglobulin in mice. *Pediatr Allergy Immunol*. 2018;29:433–40.
197. Chikhi A, Elmecherfi KE, Bernard H, Cortes-Perez N, Kheroua O, Saidi D, et al. Evaluation of the efficiency of hydrolyzed whey formula to prevent cow's milk allergy in the BALB/c mouse model. *Pediatr Allergy Immunol*. 2019;30:370–7.
198. van Esch BCAM, Schouten B, de Kivit S, Hofman GA, Knippels LMJ, Willemsen LEM, et al. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3+regulatory T-cells in the mesenteric lymph nodes. *Pediatr Allergy Immunol*. 2011;22:820–6.
199. Pablos-Tanarro A, Lozano-Ojalvo D, Martínez-Blanco M, López-Fandiño R, Molina E. Sensitizing and Eliciting Capacity of Egg White Proteins in BALB/c Mice As Affected by Processing. *J Agric Food Chem*. 2017;65:4500–8.
200. Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC, Nowak-Węgrzyn A. Mechanisms underlying differential food allergy response to heated egg. *J Allergy Clin Immunol*. 2011;127:990-997.e2.
201. Peram MR, Loveday SM, Ye A, Singh H. In vitro gastric digestion of heat-induced aggregates of β -lactoglobulin. *J Dairy Sci*. 2012;96:63–74.
202. Rahaman T, Vasiljevic T, Ramchandran L. Digestibility and antigenicity of β -lactoglobulin as affected by heat, pH and applied shear. *Food Chem*. 2017;217:517–23.
203. Bertrand-Harb C, Baday A, Dalgalarondo M, Chobert JM, Haertlé T. Thermal modifications of structure and co-denaturation of α -lactalbumin and β -lactoglobulin induce changes of solubility and susceptibility to proteases. *Nahrung - Food*. 2002;46:283–9.
204. Liu X, Yang G, Geng X-R, Cao Y, Li N, Ma L, et al. Microbial Products Induce Claudin-2 to Compromise Gut Epithelial Barrier Function. *PLoS One*. 2013;8:e68547.
205. Stephen-Victor E, Chatila TA. Regulation of oral immune tolerance by the microbiome in food allergy. *Curr Opin Immunol*. 2019;60:141–7.
206. Kronman MP, Zaoutis TE, Haynes K, Feng R, Coffin SE. Antibiotic exposure and IBD development among children: A population-based cohort study. *Pediatrics*. 2012;130:X17.
207. Shaw SY, Blanchard JF, Bernstein CN. Association between the use of antibiotics and new diagnoses of Crohn's disease and ulcerative colitis. *Am J Gastroenterol*. 2011;106:2133–42.
208. Hviid A, Svanström H, Frisch M. Antibiotic use and inflammatory bowel diseases in childhood. *Gut*. 2011;60:49–54.
209. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*. 2013;504:446–50.
210. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*. 2013;500:232–6.

REFERENCES

211. Atarashi K. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science* (80-). 2011;331:337–41.
212. Peng L, Li Z-R, Green RS, Holzman IR, Lin J. Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly via Activation of AMP-Activated Protein Kinase in Caco-2 Cell Monolayers. *J Nutr*. 2009;139:1619–25.
213. Jung TH, Park JH, Jeon WM, Han KS. Butyrate modulates bacterial adherence on LS174T human colorectal cells by stimulating mucin secretion and MAPK signaling pathway. *Nutr Res Pract*. 2015;9:343–9.
214. Moreau MC, Ducluzeau R, Guy-Grand D, Muller MC. Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. *Infect Immun*. 1978;21:532–9.
215. Shroff KE, Meslin K, Cebra JJ. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. *Infect Immun*. 1995;63:3904–13.
216. Martens EC, Roth R, Heuser JE, Gordon JI. Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. *J Biol Chem*. 2009;284:18445–57.
217. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, et al. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. *Mucosal Immunol*. 2010;3:148–58.
218. Ochoa-Repáraz J, Mielcarz DW, Ditrio LE, Burroughs AR, Foureau DM, Haque-Begum S, et al. Role of Gut Commensal Microflora in the Development of Experimental Autoimmune Encephalomyelitis. *J Immunol*. 2009;183:6041–50.
219. Nakamura YK, Metea C, Karstens L, Asquith M, Gruner H, Moscibrocki C, et al. Gut microbial alterations associated with protection from autoimmune uveitis. *Investig Ophthalmol Vis Sci*. 2016;57:3747–58.
220. Ishikawa H, Tanaka K, Maeda Y, Aiba Y, Hata A, Tsuji NM, et al. Effect of intestinal microbiota on the induction of regulatory CD25 + CD4+ T cells. *Clin Exp Immunol*. 2008;153:127–35.
221. Tulstrup MVL, Christensen EG, Carvalho V, Linnings C, Ahrné S, Højberg O, et al. Antibiotic Treatment Affects Intestinal Permeability and Gut Microbial Composition in Wistar Rats Dependent on Antibiotic Class. *PLoS One*. 2015;10:e0144854.
222. Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal $\gamma\delta$ T cells. *Nat Med*. 2016;22:516–23.
223. Abril-Gil M, Garcia-Just A, Pérez-Cano FJ, Franch À, Castell M. Development and characterization of an effective food allergy model in Brown Norway rats. *PLoS One*. 2015;10:e0125314.
224. Knippels LMJ, Penninks AH, Van Meeteren M, Houben GF. Humoral and cellular immune responses in different rat strains on oral exposure to ovalbumin. *Food Chem Toxicol*. 1999;37:881–8.
225. Bodey GP, Nance J. Amoxicillin: in vitro and pharmacological studies. *Antimicrob Agents Chemother*. 1972;8:563–73.
226. European Centre for Disease Prevention and Control. Antimicrobial consumption Annual

- Epidemiological Report for 2017. *Eur. Cent. Dis. Prev. Control.* 2018.
227. Dolk FCK, Pouwels KB, Smith DRM, Robotham J V., Smieszek T. Antibiotics in primary care in England: Which antibiotics are prescribed and for which conditions? *J Antimicrob Chemother.* 2018;73:ii2–10.
228. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, et al. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol.* 2000;66:297–303.
229. Christensen EG, Licht TR, Leser TD, Bahl MI. Dietary Xylo-oligosaccharide stimulates intestinal bifidobacteria and lactobacilli but has limited effect on intestinal integrity in rats. *BMC Res Notes.* 2014;7:660.
230. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods.* 2016;13:581–3.
231. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol.* 2007;73:5261–7.
232. Clarke KR. Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol.* 1993;18:117–43.
233. Anderson MJ, Ellingsen KE, McArdle BH. Multivariate dispersion as a measure of beta diversity. *Ecol Lett.* 2006;9:683–93.
234. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Heal Dis.* 2015;26:27663.
235. Benjamini Y, Krieger AM, Yekutieli D. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika.* 2006;93:491–507.
236. Holota Y, Dovbynychuk T, Kaji I, Varenjuk I, Dzyubenko N, Chervinska T, et al. The long-term consequences of antibiotic therapy: Role of colonic short-chain fatty acids (SCFA) system and intestinal barrier integrity. *PLoS One.* 2019;14:e0220642.
237. Bergström A, Kristensen MB, Bahl MI, Metzendorff SB, Fink LN, Frøkiær H, et al. Nature of bacterial colonization influences transcription of mucin genes in mice during the first week of life. *BMC Res Notes.* 2012;5:402.
238. Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. *Science.* 2013;342:447–53.
239. Yamada A, Ohshima Y, Yasutomi M, Ogura K, Tokuriki S, Naiki H, et al. Antigen-primed splenic CD8+ T cells impede the development of oral antigen-induced allergic diarrhea. *J Allergy Clin Immunol.* 2009;123:889–94.
240. Strzępa A, Majewska-Szczepanik M, Lobo FM, Wen L, Szczepanik M. Broad spectrum antibiotic enrofloxacin modulates contact sensitivity through gut microbiota in a murine model. *J Allergy Clin Immunol.* 2017;140:121-133.e3.
241. Ivanov II, Frutos R de L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, et al. Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. *Cell Host Microbe.* 2008;4:337–49.

242. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci U S A*. 2011;108:5354–9.
243. Janelins BM, Lu M, Datta SK. Altered inactivation of commensal LPS due to acyloxyacyl hydrolase deficiency in colonic dendritic cells impairs mucosal Th17 immunity. *Proc Natl Acad Sci U S A*. 2014;192:MUC4P.825.
244. Hall JA, Bouladoux N, Sun CM, Wohlfert EA, Blank RB, Zhu Q, et al. Commensal DNA Limits Regulatory T Cell Conversion and Is a Natural Adjuvant of Intestinal Immune Responses. *Immunity*. 2008;29:637–49.
245. Sekirov I, Finlay BB. The role of the intestinal microbiota in enteric infection. *J Physiol*. 2009;587:4159–67.
246. Thackray LB, Handley SA, Gorman MJ, Poddar S, Bagadia P, Briseño CG, et al. Oral Antibiotic Treatment of Mice Exacerbates the Disease Severity of Multiple Flavivirus Infections. *Cell Rep*. 2018;22:3440-3453.e6.
247. Feng T, Elson CO, Cong Y. Treg cell-IgA axis in maintenance of host immune homeostasis with microbiota. *Int Immunopharmacol*. 2011;11:589–92.
248. Kukkonen K, Kuitunen M, Haahtela T, Korpela R, Poussa T, Savilahti E. High intestinal IgA associates with reduced risk of IgE-associated allergic diseases. *Pediatr Allergy Immunol*. 2010;21:67–73.
249. Frossard CP, Hauser C, Eigenmann PA. Antigen-specific secretory IgA antibodies in the gut are decreased in a mouse model of food allergy. *J Allergy Clin Immunol*. 2004;114:377–82.
250. Dzidic M, Abrahamsson TR, Artacho A, Björkstén B, Collado MC, Mira A, et al. Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development. *J Allergy Clin Immunol*. 2017;139:1017-1025.e14.
251. Van Der Waaij LA, Kroese FGM, Visser A, Nelis GF, Westerveld BD, Jansen PLM, et al. Immunoglobulin coating of faecal bacteria in inflammatory. *Eur J Gastroenterol Hepatol*. 2004;16:669–74.
252. Luck H, Khan S, Kim JH, Copeland JK, Revelo XS, Tsai S, et al. Gut-associated IgA+ immune cells regulate obesity-related insulin resistance. *Nat Commun*. 2019;10:3650.
253. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y. Molecular analysis of jejunal, ileal, caecal and rectosigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *J Med Microbiol*. 2005;54:1093–101.
254. Wang M, Ahrné S, Jeppsson B, Molin G. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS Microbiol Ecol*. 2005;54:219–31.
255. Seekatz AM, Schnizlein MK, Koenigsnecht MJ, Baker JR, Hasler WL, Bleske BE, et al. Spatial and Temporal Analysis of the Stomach and Small-Intestinal Microbiota in Fasted Healthy Humans. *mSphere*. 2019;4:e00126-19.
256. Wang YT, Mohammed SD, Farmer AD, Wang D, Zarate N, Hobson AR, et al. Regional gastrointestinal transit and pH studied in 215 healthy volunteers using the wireless motility capsule: Influence of age, gender, study country and testing protocol. *Aliment Pharmacol Ther*. 2015;42:761–72.

257. Kabbani TA, Pallav K, Dowd SE, Villafuerte-Galvez J, Vanga RR, Castillo NE, et al. Prospective randomized controlled study on the effects of *Saccharomyces boulardii* CNCM I-745 and amoxicillin-clavulanate or the combination on the gut microbiota of healthy volunteers. *Gut Microbes*. 2017;8:17–32.
258. MacPherson CW, Mathieu O, Tremblay J, Champagne J, Nantel A, Girard SA, et al. Gut Bacterial Microbiota and its Resistome Rapidly Recover to Basal State Levels after Short-term Amoxicillin-Clavulanic Acid Treatment in Healthy Adults. *Sci Rep*. 2018;8:11192.
259. Rivera-Chávez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, et al. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. *Cell Host Microbe*. 2016;19:443–54.
260. Tulstrup MV-L, Roager HM, Thaarup IC, Frandsen HL, Frøkiær H, Licht TR, et al. Antibiotic treatment of rat dams affects bacterial colonization and causes decreased weight gain in pups. *Commun Biol*. 2018;1:145.
261. Lee N, Kim WU. Microbiota in T-cell homeostasis and inflammatory diseases. *Exp Mol Med*. 2017;49:e340.
262. Konieczna P, Groeger D, Ziegler M, Frei R, Ferstl R, Shanahan F, et al. Bifidobacterium infantis 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: Potential role for myeloid and plasmacytoid dendritic cells. *Gut*. 2012;61:354–66.
263. Lyons A, O’Mahony D, O’Brien F, MacSharry J, Sheil B, Cedia M, et al. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. *Clin Exp Allergy*. 2010;40:811–9.
264. Kim WG, Kang GD, Kim HI, Han MJ, Kim DH. Bifidobacterium longum IM55 and Lactobacillus plantarum IM76 alleviate allergic rhinitis in mice by restoring Th2/Treg imbalance and gut microbiota disturbance. *Benef Microbes*. 2019;10:55–67.
265. Liu Q, Jing W, Wang W. Bifidobacterium lactis Ameliorates the Risk of Food Allergy in Chinese Children by Affecting Relative Percentage of Treg and Th17 Cells. *Can J Infect Dis Med Microbiol*. 2018;2018:4561038.
266. Zuo L, Yuan KT, Yu L, Meng QH, Chung PCK, Yang DH. Bifidobacterium infantis attenuates colitis by regulating T cell subset responses. *World J Gastroenterol*. 2014;20:18316–29.
267. Larsen JM, Steen-Jensen DB, Laursen JM, Søndergaard JN, Musavian HS, Butt TM, et al. Divergent pro-inflammatory profile of human dendritic cells in response to commensal and pathogenic bacteria associated with the airway microbiota. *PLoS One*. 2012;7:e31976.
268. Brix S, Eriksen C, Larsen JM, Bisgaard H. Metagenomic heterogeneity explains dual immune effects of endotoxins. *J Allergy Clin Immunol*. 2015;135:277–80.
269. Larsen JM, Musavian HS, Butt TM, Ingvorsen C, Thysen AH, Brix S. Chronic obstructive pulmonary disease and asthma-associated Proteobacteria, but not commensal Prevotella spp., promote Toll-like receptor 2-independent lung inflammation and pathology. *Immunology*. 2015;144:333–42.
270. Li Y, Wang Z, Chen J, Ernst RK, Wang X. Influence of lipid acylation pattern on membrane permeability and innate immune stimulation. *Mar Drugs*. 2013;11:3197–208.

271. Caramalho I, Rodrigues-Duarte L, Perez A, Zelenay S, Penha-Gonçalves C, Demengeot J. Regulatory T cells contribute to diabetes protection in lipopolysaccharide-treated non-obese diabetic mice. *Scand J Immunol.* 2011;74:585–95.
272. Lewkowicz N, Mycko MP, Przygodzka P, Ćwiklińska H, Cichalewska M, Matysiak M, et al. Induction of human IL-10-producing neutrophils by LPS-stimulated Treg cells and IL-10. *Mucosal Immunol.* 2016;9:364–78.
273. Hrnčir T, Stepankova R, Kozakova H, Hudcovic T, Tlaskalova-Hogenova H. Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: Studies in germ-free mice. *BMC Immunol.* 2008;9:65.
274. Bashir MEH, Louie S, Shi HN, Nagler-Anderson C. Toll-Like Receptor 4 Signaling by Intestinal Microbes Influences Susceptibility to Food Allergy. *J Immunol.* 2004;172:6978–87.
275. Boyle RJ, Ierodiakonou D, Khan T, Chivinge J, Robinson Z, Geoghegan N, et al. Hydrolysed formula and risk of allergic or autoimmune disease: Systematic review and meta-analysis. *BMJ.* 2016;352:i974.
276. Osborn DA, Sinn JKH, Jones LJ. Infant formulas containing hydrolysed protein for prevention of allergic disease. *Cochrane Database Syst Rev.* 2018;2018:CD003664.
277. Muraro A, Halken S, Arshad SH, Beyer K, Dubois AEJ, Du Toit G, et al. EAACI Food Allergy and Anaphylaxis Guidelines. Primary prevention of food allergy. *Allergy Eur J Allergy Clin Immunol.* 2014;69:590–601.
278. Greer FR, Sicherer SH, Wesley Burks A, Abrams SA, Fuchs GJ, Kim JH, et al. The effects of early nutritional interventions on the development of atopic disease in infants and children: The role of maternal dietary restriction, breastfeeding, hydrolyzed formulas, and timing of introduction of allergenic complementary foods. *Pediatrics.* 2019;143:e20190281.
279. Joshi PA, Smith J, Vale S, Campbell DE. The Australasian Society of Clinical Immunology and Allergy infant feeding for allergy prevention guidelines. *Med J Aust.* 2019;210:89–93.
280. Lowe AJ, Hosking CS, Bennett CM, Allen KJ, Axelrad C, Carlin JB, et al. Effect of a partially hydrolyzed whey infant formula at weaning on risk of allergic disease in high-risk children: A randomized controlled trial. *J Allergy Clin Immunol.* 2011;128:360-365.e4.
281. Oldæus G, Anjou K, Björkstén B, Moran JR, Kjellman NIM. Extensively and partially hydrolysed infant formulas for allergy prophylaxis. *Arch Dis Child.* 1997;77:4–10.
282. Vandenplas Y, Hauser B, Van den Borre C, Sacre L, Dab I. Effect of a whey hydrolysate prophylaxis of atopic disease. *Ann Allergy.* 1992;68:419–24.
283. Niggemann B, Nies H, Renz H, Herz U, Wahn U. Sensitizing capacity and residual allergenicity of hydrolyzed cow's milk formulae: Results from a murine model. *Int Arch Allergy Immunol.* 2001;125:316–21.
284. van Esch BCAM, Knipping K, Jeurink P, van der Heide S, Dubois AEJ, Willemsen LEM, et al. In vivo and in vitro evaluation of the residual allergenicity of partially hydrolysed infant formulas. *Toxicol Lett.* 2011;201:264–9.
285. Nielsen PM, Petersen D, Dambmann C. Improved method for determining food protein degree of hydrolysis. *J Food Sci.* 2001;66:642–6.

286. Barkholt V, Jensen AL. Amino acid analysis: Determination of cysteine plus half-cysteine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. *Anal Biochem.* 1989;177:318–22.
287. Graversen KB, Ballegaard AR, Kræmer LH, Hornslet SE, Sørensen L V, Christoffersen HF, et al. Cow's milk allergy prevention and treatment by heat-treated whey – a study in Brown Norway rats. *Clin Exp Allergy.* 2020;Accepted.
288. Graversen KB, Bahl MI, Larsen JM, Ballegaard AR, Licht TR, Bøgh KL. Short-term amoxicillin-induced perturbation of the gut microbiota promotes acute intestinal immune regulation in Brown Norway rats. *Front Microbiol.* 2020;Accepted.
289. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* 2019;37:852–7.
290. Vignæs LK, Holck J, Meyer AS, Licht TR. In Vitro fermentation of sugar beet arabino-oligosaccharides by fecal microbiota obtained from patients with ulcerative colitis to selectively stimulate the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. *Appl Environ Microbiol.* 2011;77:8336–44.
291. Penders J, Vink C, Driessen C, London N, Thijs C, Stobberingh EE. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett.* 2005;243:141–7.
292. Heilig HGJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, De Vos WM. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl Environ Microbiol.* 2002;68:114–23.
293. Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* Species in Human Feces by Using Group-Specific PCR Primers and Denaturing Gradient Gel Electrophoresis. *Appl Environ Microbiol.* 2001;67:2578–85.
294. Rinttilä T, Kassinen A, Malinen E, Krogus L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol.* 2004;97:1166–77.
295. Bergström A, Licht TR, Wilcks A, Andersen JB, Schmidt LR, Grønlund HA, et al. Introducing GUT Low-Density Array (GULDA) - a validated approach for qPCR-based intestinal microbial community analysis. *FEMS Microbiol Lett.* 2012;337:38–47.
296. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001;29:e45.
297. Soro V, Dutton LC, Sprague S V., Nobbs AH, Ireland AJ, Sandy JR, et al. Axenic culture of a candidate division TM7 bacterium from the human oral cavity and biofilm interactions with other oral bacteria. *Appl Environ Microbiol.* 2014;80:6480–9.
298. He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, et al. Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. *Proc Natl Acad Sci U S A.* 2015;112:244–9.
299. Fujisawa T, Itoh K, Benno Y, Mitsuoka T. *Lactobacillus intestinalis* (ex Hemme 1974) sp. nov., nom.

- rev., isolated from the intestines of mice and rats. *Int J Syst Bacteriol.* 1990;40:302–4.
300. Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, et al. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. *PLoS One.* 2015;10:e0126931.
301. Stefka AT, Feehley T, Tripathi P, Qiu J, McCoy K, Mazmanian SK, et al. Commensal bacteria protect against food allergen sensitization. *Proc Natl Acad Sci U S A.* 2014;111:13145–50.
302. Boutin RCT, Dwyer Z, Farmer K, Rudyk C, Forbes MR, Hayley S. Perinatal antibiotic exposure alters composition of murine gut microbiota and may influence later responses to peanut antigen. *Allergy, Asthma Clin Immunol.* 2018;14:42.
303. Kim D, Kim YG, Seo SU, Kim DJ, Kamada N, Prescott D, et al. Nod2-mediated recognition of the microbiota is critical for mucosal adjuvant activity of cholera toxin. *Nat Med.* 2016;22:524–30.
304. Nutten S, Schumann A, Donnicola D, Mercenier A, Rami S, Garcia-Rodenas CL. Antibiotic administration early in life impairs specific humoral responses to an oral antigen and increases intestinal mast cell numbers and mediator concentrations. *Clin Vaccine Immunol.* 2007;14:190–7.
305. Lambert SE, Kinder JM, Then JE, Parliament KN, Bruns HA. Erythromycin treatment hinders the induction of oral tolerance to fed ovalbumin. *Front Immunol.* 2012;3:203.
306. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, et al. Individual intestinal symbionts induce a distinct population of ROR γ + regulatory T cells. *Science (80-).* 2015;349:993–7.
307. Ohnmacht C, Park JH, Cording S, Wing JB, Atarashi K, Obata Y, et al. The microbiota regulates type 2 immunity through ROR γ t+ T cells. *Science (80-).* 2015;349:989–92.
308. Guadamuro L, Diaz M, Jiménez S, Molinos-Norniella C, Pérez-Solis D, Rodríguez JM, et al. Fecal Changes Following Introduction of Milk in Infants With Outgrowing Non-IgE Cow's Milk Protein Allergy Are Influenced by Previous Consumption of the Probiotic LGG. *Front Immunol.* 2019;10:1819.
309. Kok CR, Brabec B, Chichlowski M, Harris C, Moore N, Wampler J, et al. Stool Microbiota in Infants Receiving Extensively Hydrolyzed Formula, Amino Acid Formula, or Human Milk Through Two Months of Age (FS04-07-19). *Curr Dev Nutr.* 2019;3:119.
310. Foulquié Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. *Int J Food Microbiol.* 2006;106:1–24.
311. Martín R, Langa S, Reviriego C, Jiménez E, Marín ML, Olivares M, et al. The commensal microflora of human milk: New perspectives for food bacteriotherapy and probiotics. *Trends Food Sci Technol.* 2004;15:121–7.
312. Chen C-C, Chen K-J, Kong M-S, Chang H-J, Huang J-L. Alterations in the gut microbiotas of children with food sensitization in early life. *Pediatr Allergy Immunol.* 2016;27:254–62.
313. Gosalbes MJ, Llop S, Vallès Y, Moya A, Ballester F, Francino MP. Meconium microbiota types dominated by lactic acid or enteric bacteria are differentially associated with maternal eczema and respiratory problems in infants. *Clin Exp Allergy.* 2013;43:198–211.
314. Azad MB, Konya T, Persaud RR, Guttman DS, Chari RS, Field CJ, et al. Impact of maternal intrapartum antibiotics, method of birth and breastfeeding on gut microbiota during the first year of life: A prospective cohort study. *BJOG An Int J Obstet Gynaecol.* 2016;123:983–93.

315. Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunol Med Microbiol*. 2009;56:80–7.
316. Francavilla R, Calasso M, Calace L, Siragusa S, Ndagijimana M, Vernocchi P, et al. Effect of lactose on gut microbiota and metabolome of infants with cow's milk allergy. *Pediatr Allergy Immunol*. 2012;23:420–7.
317. Green Corkins K, Shurley T. What's in the Bottle? A Review of Infant Formulas. *Nutr Clin Pract*. 2016;31:723–9.
318. EFSA Panel on Dietetic Products N and A (NDA). Safety of 2'-O-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 258/97. 2015;13:4184.
319. Braegger C, Chmielewska A, Decsi T, Kolacek S, Mihatsch W, Moreno L, et al. Supplementation of infant formula with probiotics and/or prebiotics: A systematic review and comment by the ESPGHAN committee on nutrition. *J Pediatr Gastroenterol Nutr*. 2011;52:238–50.
320. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*. 2005;6:1219–27.
321. Wannemuehler MJ, Kiyono H, Babb JL, Michalek SM, McGhee JR. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J Immunol*. 1982;129:959–65.
322. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett*. 2009;294:1–8.
323. Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM, et al. Proinflammatory Cytokines Disrupt Epithelial Barrier Function by Apoptosis-Independent Mechanisms. *J Immunol*. 2003;171:6164–72.
324. Knoop KA, McDonald KG, McCrate S, McDole JR, Newberry RD. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol*. 2015;8:198–210.

Appendix

Appendix A: Supplementary material for Manuscript II

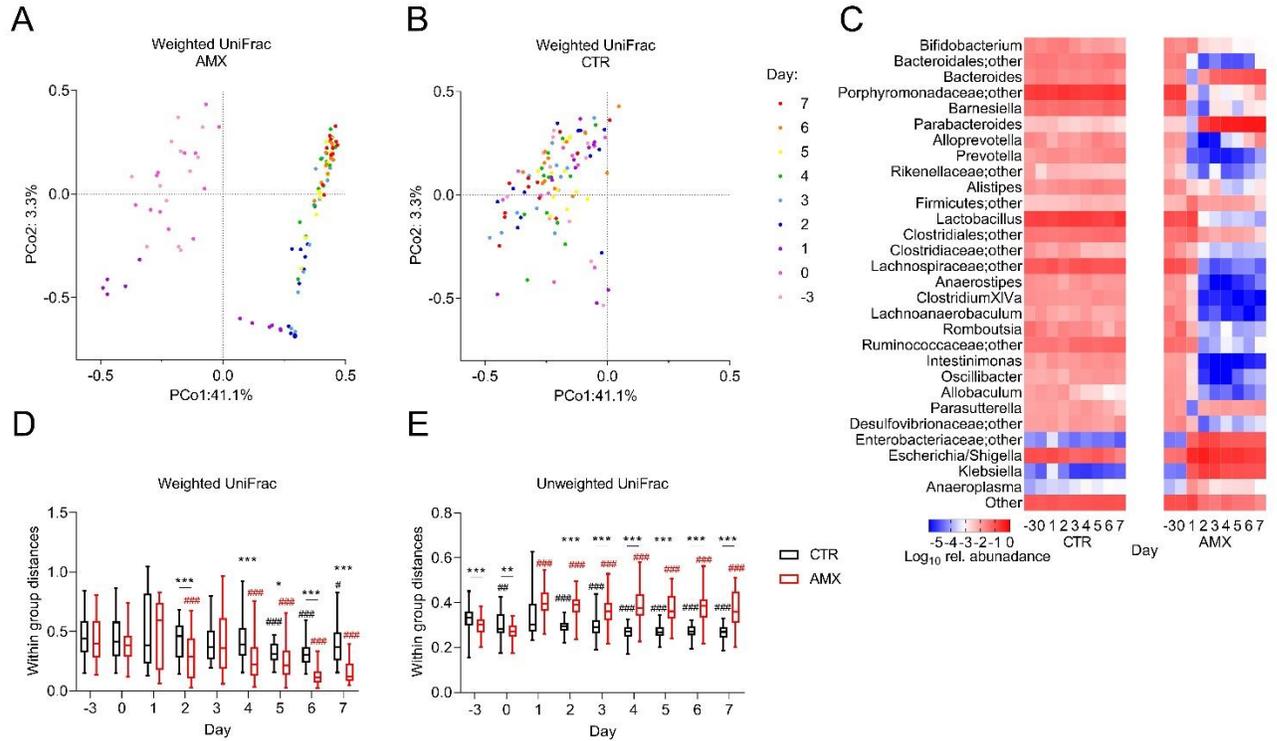


FIGURE S1: A-B: Principal coordinate analysis (PCoA) plots of weighted UniFrac distances in control (CTR, A) and amoxicillin (AMX) group (B) colored according to sample day. C: Heat-map colored according to mean relative abundance of bacterial genera at different days. D-E: Within group distances based on weighted (D) and unweighted (E) UniFrac distances.

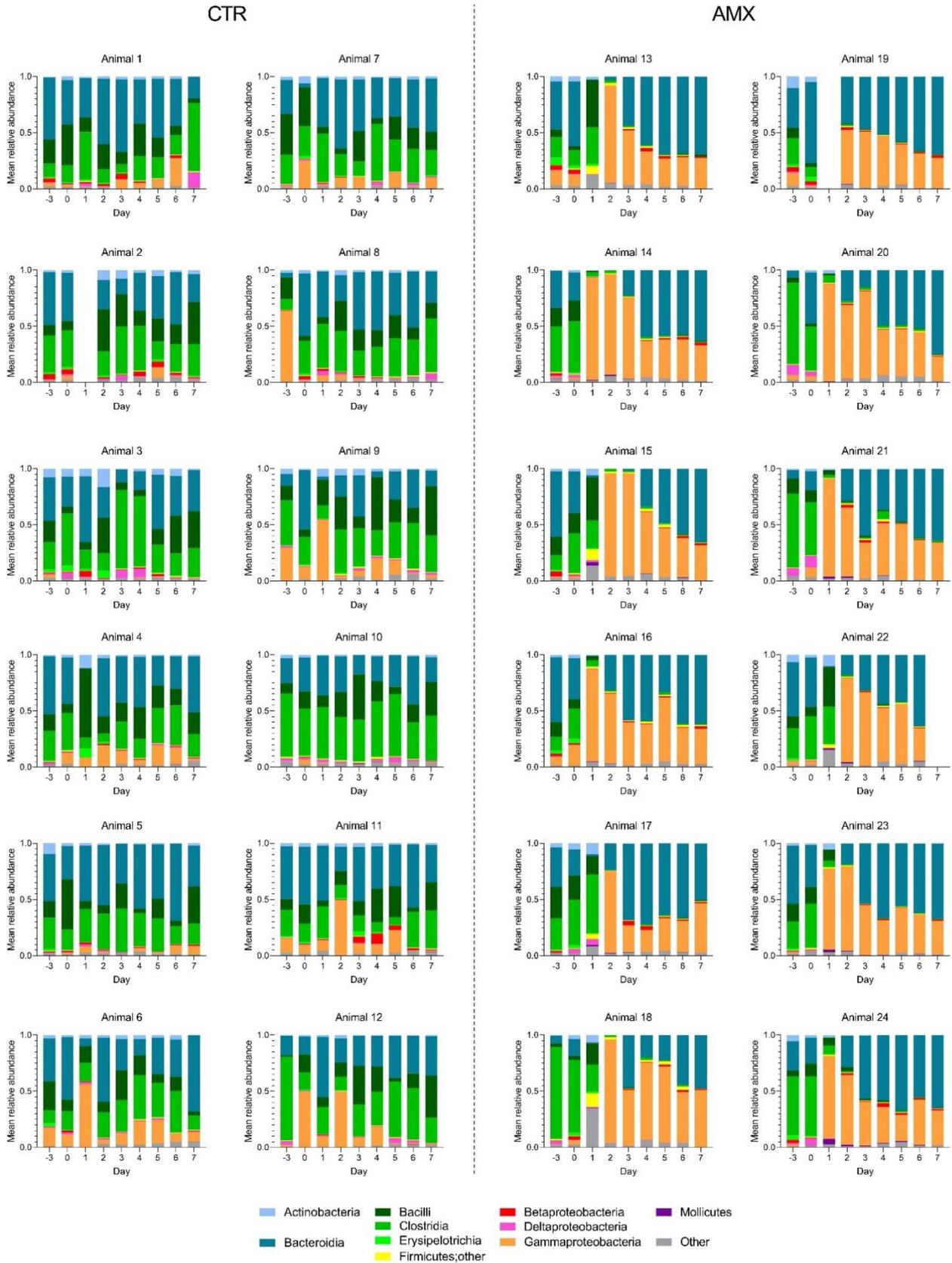


FIGURE S2: Temporal effects of amoxicillin on relative abundance of bacterial classes in individual animals.

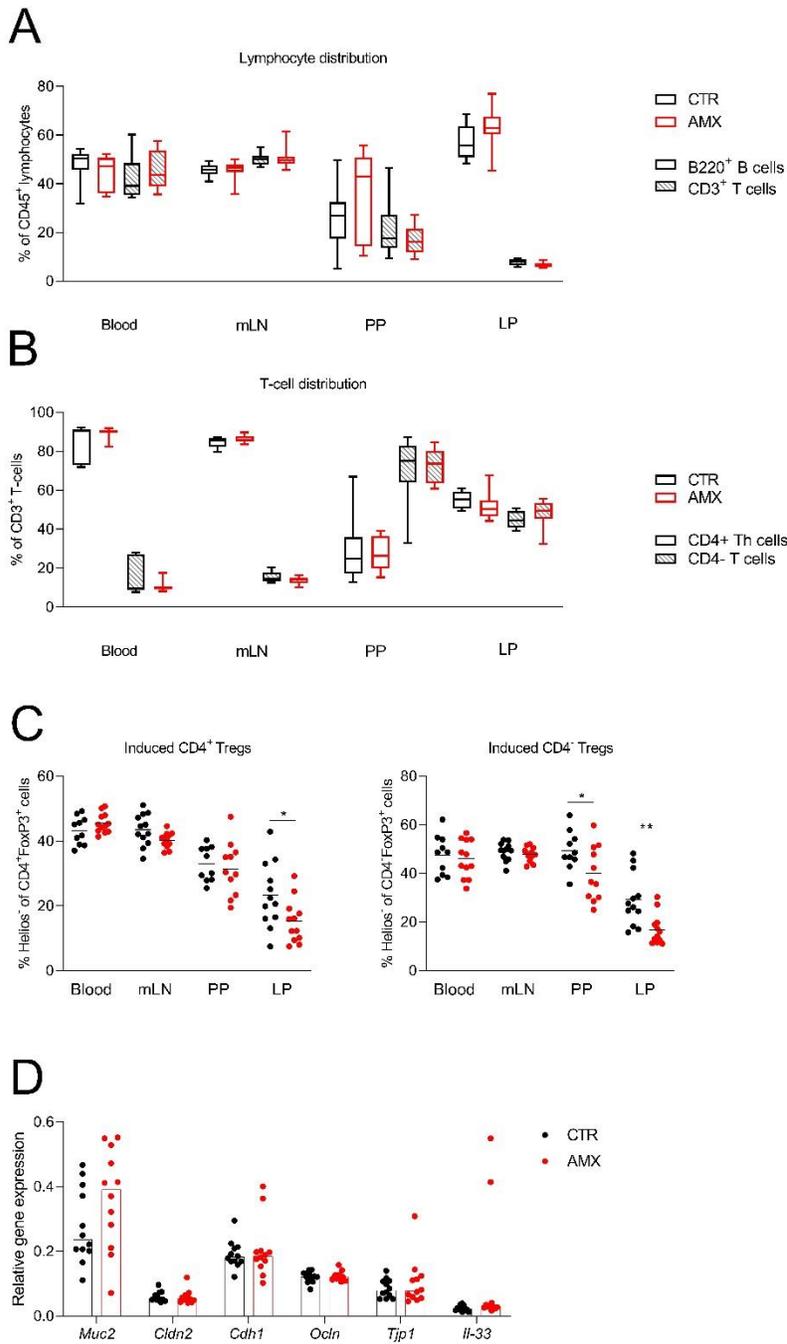


FIGURE S3: Relative distribution of lymphocyte populations in blood, mesenteric lymph nodes (mLN), lamina propria (LP) and Peyer’s patches (PP) (A) and relative gene expression in small intestine LP of control (red) and amoxicillin administered (red) animals. A: Percentage B220⁺ B cells (empty bars) and CD3⁺ T cells (hatched bars) of all lymphocytes. B: Percentage CD4⁺ T helper cells (empty bars) and CD4⁻ T cells (hatched bars) of all CD3⁺ T cells. C: Percentage Helios⁻ cells of all CD4⁺ and CD4⁻ FoxP3⁺ T regulatory (Treg) cells. D: Gene expression of mucin 2 (*Muc2*), claudin 2 (*Cldn2*), cadherin 1 (*Cdh1*), occluding (*Ocln*), zonula occludens/tight junction protein 1 (*Tjp1*) and interleukin 33 (*Il33*) relative to housekeeping genes.

A CTR AMX

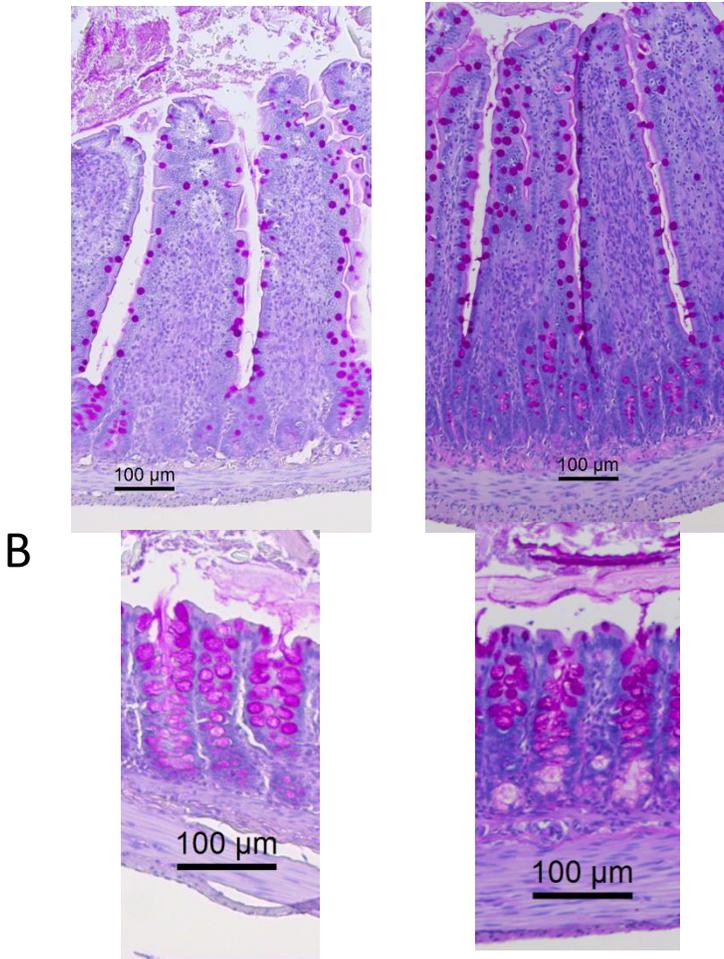


FIGURE S4: Representable pictures of goblet cell staining by Periodic acid-Schiff (PAS) in small intestinal (A) and colon (B) sections.

Table S1: Differentially abundant genera between small intestine of control and amoxicillin administered rats analysed by analysis of composition of microbiomes (ANCOM).

	GENERA	W	CLR
STATISTICALLY SIGNIFICANT	<i>Lactobacillus</i>	81	-3.9796866193279854
	<i>Escherichia/Shigella</i>	78	4.3408022010976515
	<i>Rothia</i>	78	-2.673502453407982
	<i>Romboutsia</i>	74	4.830549788692652
	<i>Klebsiella</i>	69	3.236854263583691
	<i>Veillonella</i>	63	-1.967662858164571
BORDERLINE STATISTICALLY SIGNIFICANT	<i>Clostridium_sensu_stricto</i>	63	2.09302398153761
	<i>Bifidobacterium</i>	55	2.4258928100122437

Appendix B: Supplementary material for Manuscript III

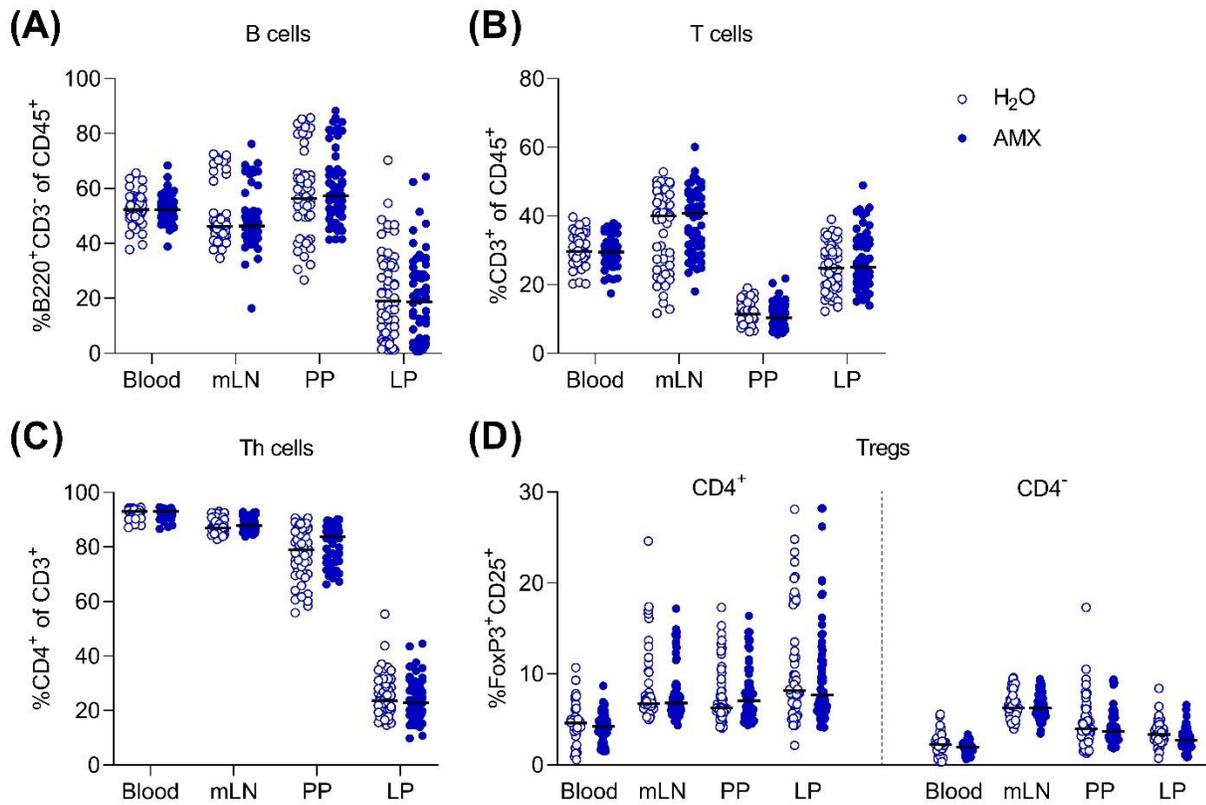


FIGURE S1: Effect of amoxicillin administration on lymphocyte populations. Total B cells (A), T cells (B), T helper (Th) cells (C) and regulatory T cells (Tregs) (D) in blood, mesenteric lymph nodes (mLN), Peyer's patches (PP) and lamina propria (LP) of water (H₂O, open symbols) and amoxicillin (AMX, closed symbols) administered rats (Day 64). Each symbol represents a single rat and horizontal lines indicate median values.

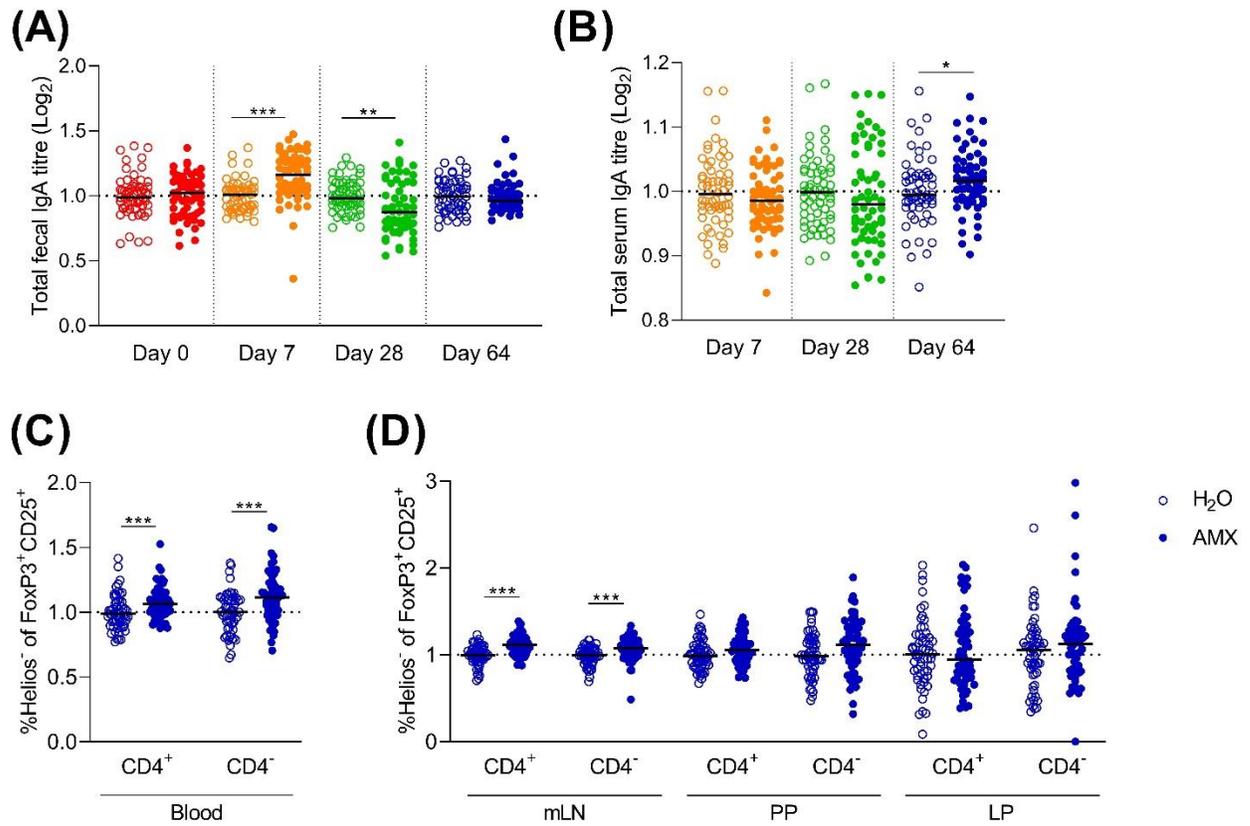


FIGURE S2: Normalised host immune regulation. Total faecal (A) and total serum (B) IgA titres and fraction of Helios⁻ Tregs out of the CD25⁺FoxP3⁺ Treg populations (both CD4⁺ and CD4⁻) in blood (C), and mesenteric lymph nodes (mLN), Peyer’s patches (PP) and lamina propria (LP) (D) of water (H₂O, open symbols) and amoxicillin (AMX, closed symbols) administered rats (Day 64). To account for the small variation between the product groups, data are normalised to the water groups of each product group. Each symbol represents a single rat and horizontal lines indicate median values. Statistically significant differences between groups are indicated as *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001.

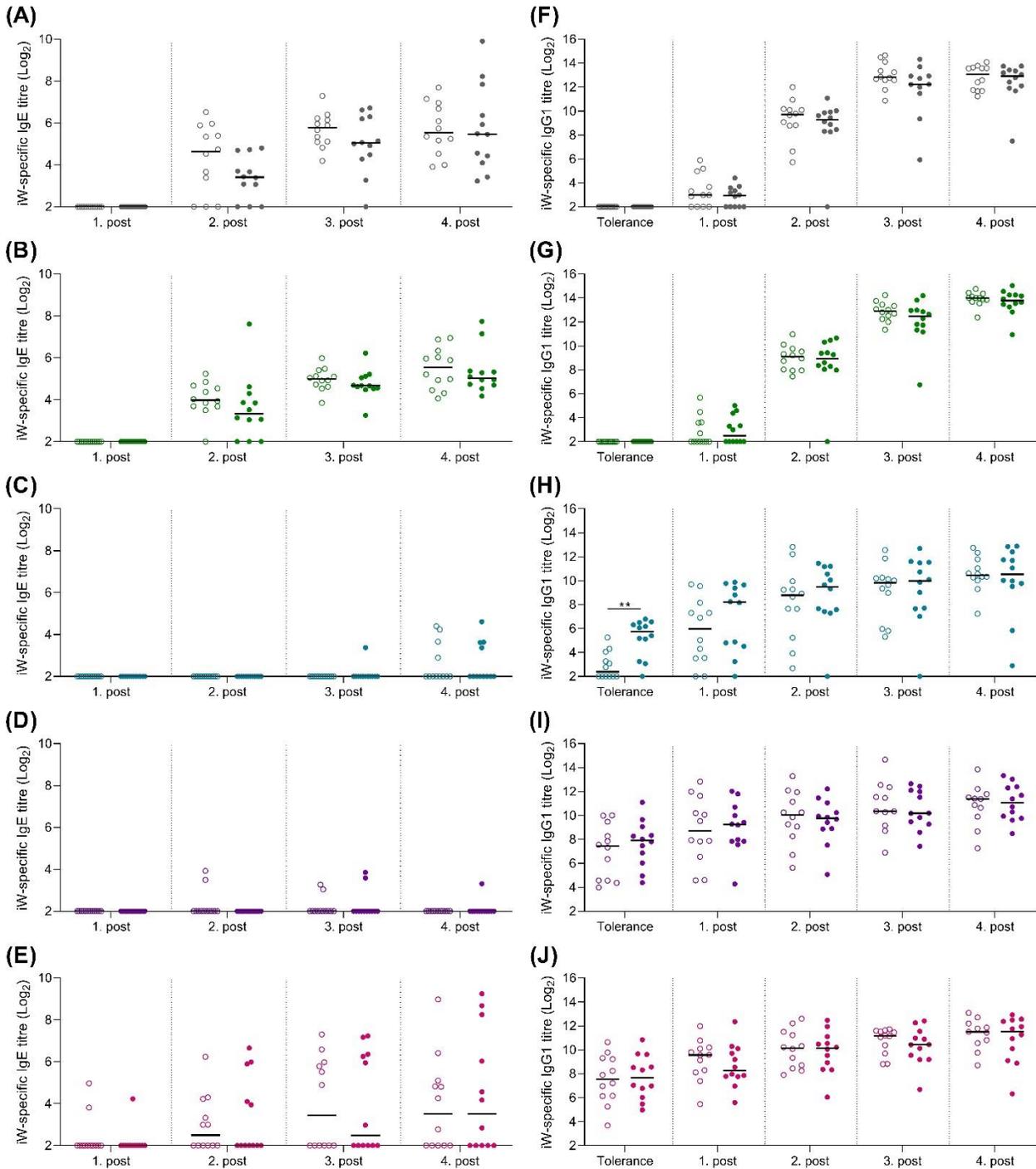


FIGURE S3: Effect of amoxicillin on the primary preventive capacity of the whey protein products. Intact whey (iW)-specific IgE titres (A-E) and iW-specific IgG1 titres (F-J) in water (H₂O, open symbols) and amoxicillin (AMX, closed symbols) rats administered with extensively (B,G), partially (C,H), mildly (D,I) hydrolysed or intact (E,J) whey products or water as control (A,F). Each symbol represents a single rat and horizontal lines indicate median values. Statistically significant differences are indicated as **: $p \leq 0.01$.

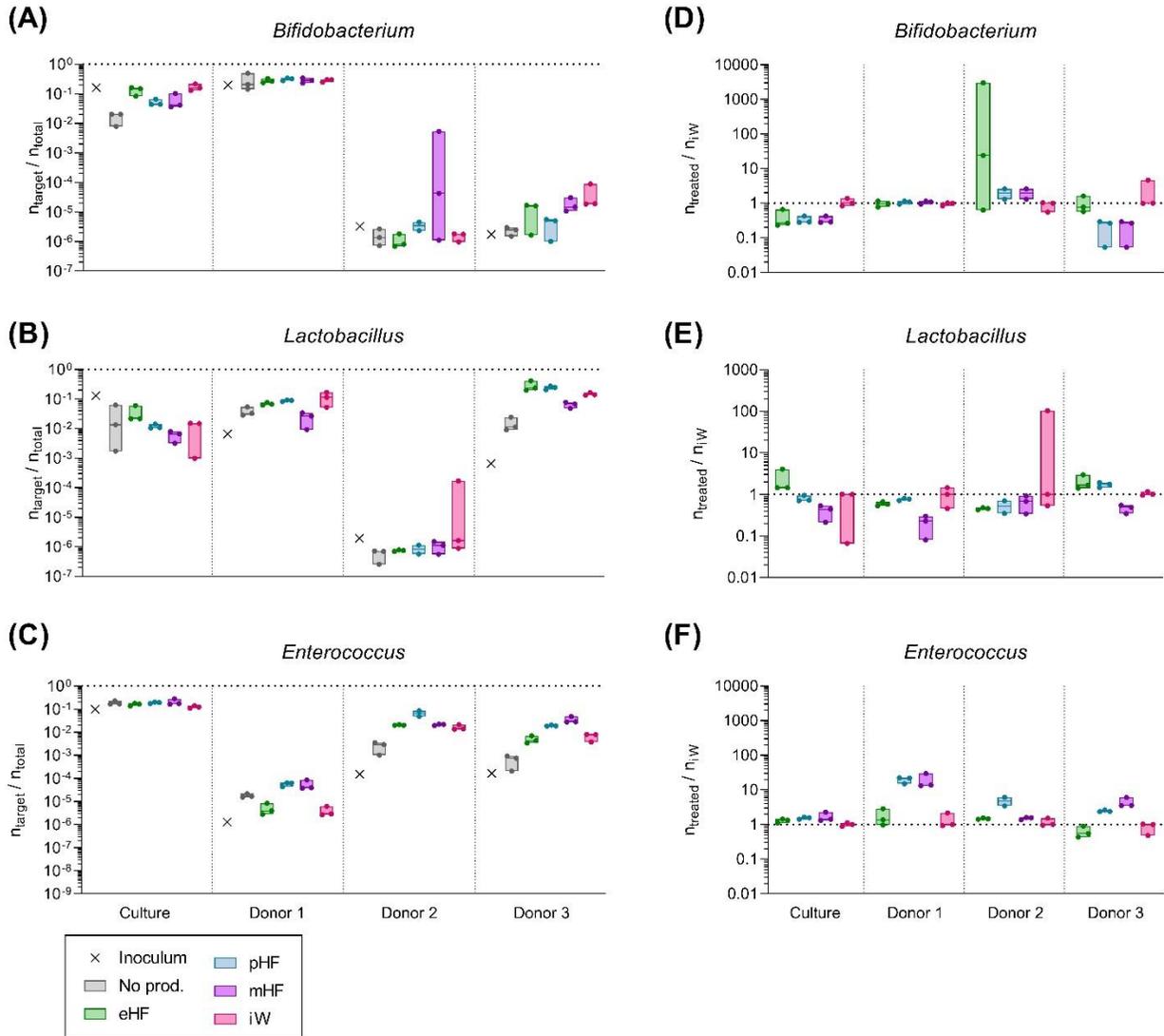


FIGURE S4: Effect of whey protein products on in vitro expansion of bacterial genera derived from healthy infants. The relative abundance of *Bifidobacterium* (A, D), *Lactobacillus* (B, E) and *Enterococcus* (C, E) after in vitro incubations with extensively (eHW), partially (pHW), mildly (mHW) hydrolysed or intact (iW) whey products. Results for each bacteria is shown relative to the total bacteria ($n_{\text{target}}/n_{\text{total}}$) (A-C) and normalised to the median ΔCt of three iW samples ($n_{\text{treated}}/n_{\text{iW}}$) (D-F).

Table S1: Differentially abundant ASVs in faecal samples (Day 64) of amoxicillin administered rats compared to water administered (conventional) rats as determined by ANCOM analysis.

	Taxonomy	W
↓	Bacteria;__Bacteroidetes	1575
	Bacteria;__Firmicutes;__Bacilli;__Lactobacillales;__Lactobacillaceae;__Lactobacillus;__intestinalis	1573
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1571
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales	1568
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1554
	Bacteria;__Candidatus_Saccharibacteria	1553
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1546
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1546
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1537
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae;__Barnesiella	1536
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1535
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales	1532
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1520
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1517
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1506
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1504
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae;__Barnesiella	1501
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1499
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales	1498
	Bacteria;__Firmicutes;__Bacilli;__Lactobacillales;__Lactobacillaceae;__Lactobacillus;__reuteri	1496
	Bacteria;__Firmicutes;__Bacilli;__Lactobacillales;__Lactobacillaceae;__Lactobacillus	1492
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1492
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1489
	Bacteria;__Firmicutes	1484
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae;__Barnesiella	1476
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae;__Odoribacter	1467
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Ruminococcaceae	1464
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1460
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales	1456
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Ruminococcaceae	1450
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Lachnospiraceae	1447
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Lachnospiraceae	1441
	Bacteria;__Firmicutes	1436
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales	1432
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Ruminococcaceae	1432
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Lachnospiraceae	1423
↑	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Rikenellaceae	1580
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales	1535
	Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Bacteroidaceae;__Bacteroides	1534
	Bacteria;__Firmicutes;__Clostridia;__Clostridiales	1515

Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae;__Barnesiella	1488
Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Bacteroidaceae;__Bacteroides	1480
Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Bacteroidaceae;__Bacteroides;__acidifaciens	1473
Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae	1460
Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Lachnospiraceae;__Lachnoanaerobaculum	1452
Bacteria;__Firmicutes;__Clostridia;__Clostridiales;__Ruminococcaceae	1448
Bacteria;__Bacteroidetes;__Bacteroidia;__Bacteroidales;__Porphyromonadaceae;__Parabacteroides;__goldsteinii	1441

Table S2: Differentially abundant ASVs in small intestinal samples of amoxicillin administered rats compared to water administered (conventional) rats as determined by ANCOM analysis.

	Taxonomy	W
↓	Bacteria;__Firmicutes;__Bacilli;__Lactobacillales;__Lactobacillaceae;__Lactobacillus;__intestinalis	1022
	Bacteria;__Firmicutes;__Bacilli;__Lactobacillales;__Lactobacillaceae;__Lactobacillus;__reuteri	1021