# Modulation of gut-immune-brain axis by non-digestible oligosaccharides and omega-3 fatty acids in health and allergic disease

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Modulatie van de darm-immuun-brein as door niet-verteerbare oligosaccharides
en omega-3 vetzuren in gezondheid en bij allergische aandoeningen
Synergie of rivaliteit?

(met een samenvatting in het Nederlands)

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**Kirsten Szklany**
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**Promotoren:**
 Prof. dr. J. Garssen
 Prof. dr. A.D. Kraneveld

**Copromotor:**
 Dr. L.M.J. Knippels

**Beoordelingscommissie:**
 Prof. dr. P. Calder
 Prof. dr. M.J.N.L. Benders
 Prof. dr. R.F. Witkamp
 Prof. dr. G.F. Houben
 Prof. dr. R.J. Pieters

Be curious my dear Else

# CONTENTS

# CHAPTER 1

General introduction

During the first 2-3 years of life a tremendous development takes place. At birth and onwards the microbial colonization of the intestine occurs. The intestinal microbiota develops into a diverse and stable community during the first years of life. It is generally accepted that the development of the intestinal microbiota is closely related to the immune system development as well as neurodevelopment (1). The intestinal microbial development might be disrupted by external factors consequently affecting the immune- and neurodevelopment. One of the factors influencing the intestinal microbiota development is mode of delivery. Caesarean section has been associated with allergies and deficits in social behaviour later in life (2). Another factor disrupting the microbiota development is use of antibiotics. Antibiotics affect the intestinal bacterial composition and might lead to a reduction of the formation of the bacterial-derived short-chain fatty acids (SCFAs) which are essential in the maturation of the intestine, immune system, and brain (3). Other factors altering the intestinal microbiota can be stress and mode of feeding (formula fed versus breastfed) (4).

The prevalence of allergic diseases is increasing in the western world (5, 6). One of the earliest food allergies to develop during the first year of life is cow's milk allergy (CMA) (7). The prevalence of CMA in westernized countries is around 2-3% (8, 9). Symptoms characterizing a CMA reaction are skin rash, gastro-intestinal discomfort and in severe cases anaphylactic shock. Currently, no treatment is available for CMA; avoidance of the allergen is the only option to prevent an allergic reaction. The majority of children diagnosed with CMA outgrow this disorder during the first three years of life. However, later in life, these children are more liable to develop other allergies (8). Besides, children diagnosed with CMA might show more disturbed behaviour like impaired sociability and anxiety-like behaviour than children without CMA (10-14). Several nutritional strategies have been evaluated for their CMA preventive capacities (15). Prebiotics, like short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS), and omega-3 polyunsaturated fatty acids (n-3 PUFAs) are examples of such nutritional strategies possessing promising capacities in preventing CMA development (16-18). Being able to prevent CMA development, would lead to reduced prevalence of CMA and might prevent other allergies to develop later in life.

Human breast milk is the golden standard as the first nutrition after birth (19). Human breast milk contains all necessary components needed by the infant and the composition changes according to the nutritional needs of the infant (1, 20). Protective benefits of human breast milk in early life are stimulation of the maturation of the intestinal microbiota, the immune system as well as the central nervous system (1, 21). One of the major components in human breast milk is human milk oligosaccharides (HMOs). HMOs have prebiotic capacities inducing the growth of beneficial bacteria and thereby stimulating the bacterial colonization of the gut. They prevent exposure of the infant gut to pathogens by improving the intestinal epithelial barrier function. HMOs induce regulatory T cells (Tregs) and regulate the T helper (Th) 1/Th2 balance in mesenteric lymph nodes (22-24). Additionally, HMOs have been reported to improve neurodevelopment and cognition (1). However, human breast milk is not always available. An alternative is

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infant formula containing non-digestible oligosaccharides like scGOS and lcFOS mimicking the prebiotic function of HMOs. The mixture of scGOS:lcFOS have direct and indirect immunomodulatory effects. scGOS:lcFOS, like HMOs, induce Tregs and balance the Th1/Th2 T cell subsets (25-27). In the intestine, mainly in the colon, scGOS:lcFOS stimulates the growth of beneficial bacteria such as *Bifidobacteria* and is fermented into SCFAs through which scGOS:lcFOS indirectly can exert immune modulatory functions (28-32).

Another important component in human breast milk are PUFAs. PUFAs are fatty acids containing one or more double bonds and are categorized into omega-3 (n-3) or omega-6 (n-6) PUFAs depending on the location of the double bond. n-3 and n-6 PUFAs incorporate in cell membranes (33, 34). In early life neurodevelopment n-3 and n-6 PUFAs influence neurogenesis, migration and differentiation of neurons as well as synaptogenesis by inclusion of these fatty acids in membranes of neurons, astrocytes and microglia cells (34, 35). PUFAs also have immunomodulatory functions. They exert their effects in the cell affecting transcription factors through modulation of signalling pathways. Arachidonic acid (AA), an n-6 PUFA, can be converted into several mediators such as prostaglandin $E_2$ (PGE$_2$), PGD$_2$ and leukotriene $B_4$ (LTB$_4$) that are able to execute pro-inflammatory actions (33). On the expense of AA, the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) incorporate into the cell membrane and this results in less available AA and consequently less conversion of AA into pro-inflammatory mediators occurs (36). DHA and EPA can be converted into specialised pro-resolving mediators (SPM) such as resolvins, which are suggested to play a role in inflammation resolution (33). The ratio of n-6 and n-3 PUFAs is important as n-6 PUFAs lead to pro-inflammatory components and n-3 PUFAs to anti-inflammatory components. Decades ago, this ratio used to be around 4:1 and now in westernized countries it is 20:1. This skewing is due to dietary alterations. The consumption of n-3 PUFA containing fatty fish has decreased and the consumption of n-6 PUFA containing meat, corn and safflower oil has increased (34). An enhanced consumption of n-6 PUFAs on the expense of n-3 PUFAs has been associated with increased risk of allergy development in both human and mice (37-39).

## Aim and outline of this thesis

This thesis aims to investigate whether a dietary combination of scGOS:lcFOS and n-3 PUFAs amplifies the individually modulatory effects of scGOS:lcFOS or n-3 PUFAs in early life development. The modulatory effects of the dietary interventions on the intestinal bacterial composition, immune system, brain and behaviour have been evaluated in healthy mice. Additionally, the preventive capacity of scGOS:lcFOS, n-3 PUFAs and the combination thereof on the development of CMA and associated behaviour in both male and female mice has been assessed.

**Chapter 2** presents an overview of promising nutritional approaches to prevent atopic diseases focusing on cow's milk allergy.

To provide further evidence of behavioural modulatory effects of prebiotics, the impact of scGOS:lcFOS on behavioural and intestinal bacterial development in healthy mice is determined in **Chapter 3**. The scGOS:lcFOS supplementation, from the day of birth, improved social behaviour in adulthood and reduced repetitive and anxiety-like behaviour over time. The modulated behaviour was accompanied with distinct differences in the intestinal bacterial composition.

In **Chapter 4,** scGOS:lcFOS is combined with n-3 PUFAs in a dietary intervention from the day of birth in healthy mice, to investigate whether an additional effect occurs compared with scGOS:lcFOS and n-3 PUFAs individually. The dietary compounds, both combined and individually, altered the intestinal bacterial profiles, though the effects on the immune system and behaviour were limited. The combination of scGOS:lcFOS with n-3 PUFAs resulted in no obvious additional effects.

Individually scGOS:lcFOS and n-3 PUFAs have the capacity to prevent CMA development. However, the combination of these dietary components has no additional preventive effect on CMA development neither in male nor female mice as described in **Chapter 5**. The outcome of these data might indicate the occurrence of an interaction between scGOS:lcFOS and n-3 PUFAs. scGOS:lcFOS might chemically and/or physically interact with each other leading to less availability of individual dietary components for intestinal absorption. This hypothesis is evaluated in **Chapter 6**. **Chapter 7** describes another hypothesis of a possible interaction between scGOS:lcFOS and n-3 PUFAs, namely whether n-3 PUFAs interfere with the intestinal bacterial fermentation of scGOS:lcFOS. If true, this would lead to lower levels of SCFAs which might result in less preventive immunomodulation and eventually increase the risk of CMA development.

Finally, in **Chapter 8** a summary of the main findings of the thesis and general discussion together with an outlook for future research are presented.

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# CHAPTER 2

Nutritional intervention to prevent the
development of atopic diseases:
A focus on cow's milk allergy

Kirsten Szklany[1], Aletta D. Kraneveld[1,2], Machteld M. Tiemessen[1,3], Johan Garssen[1,3],
Leon M. J. Knippels[1,3]

[1] Division of Pharmacology, Utrecht Institute of Pharmaceutical Sciences,
   Faculty of Science, Utrecht University, Utrecht, The Netherlands
[2] Veterinary Pharmacology & Therapeutics, Institute for Risk Assessment Sciences,
   Utrecht University, Utrecht, The Netherlands
[3] Department of Immunology, Danone Nutricia Research, Utrecht, The Netherlands

## ABSTRACT

In the western world the prevalence of atopic diseases such as food allergies is increasing highly significantly. One of the earliest and most prevalent food allergies occurring in the first year of life is cow's milk allergy. No treatment is available and only avoidance of the cow's milk allergens prevents the occurrence of an allergic reaction. Since cow's milk allergic children have an increased risk of developing other allergies later in life, investigating nutritional strategies to prevent the development of cow's milk allergy by developing oral tolerance is of high interest. Nutritional components such as prebiotics, probiotics, synbiotics and polyunsaturated fatty acids possess potential to support the maturation of the immune system early in life that might prevent the development of cow's milk allergy. The available research, so far, shows promising results particularly on the development of eczema. However, the preventive effects of the nutritional interventions on the development of food allergy are inconclusive. Future research may benefit from the combination of various dietary components. To clarify the preventive effects of the nutritional components in food allergy more randomized clinical trials are needed.

## INTRODUCTION

In the western world the prevalence of atopic diseases is increasing – with the first manifestations occurring early in childhood. Atopic diseases develop in a characteristic sequential pattern, starting early in life with atopic dermatitis, followed by food allergy, allergic rhinitis, and allergic asthma. This sequential process is known as the allergic march, which is also supported by the discovery that these atopic diseases are linked as infants diagnosed with one atopic disease are more predisposed to develop other allergies later in life (1).

Being the second manifestation of the allergic march, food allergies are potential disorders, which may clearly benefit (and have a need) for therapeutic interventions. One of the earliest and most prevalent food allergies occurring in the first year of life is cow's milk allergy (CMA) affecting around 2-5% of infants in some countries (2, 3). Symptoms of CMA include skin rash, gastro-intestinal discomfort like diarrhoea, vomiting, respiratory problems and in severe circumstances anaphylactic shock. Although CMA is spontaneously remitted at the age of 3 in 79-90% of diagnosed children (4, 5), currently, no treatment is available and only avoidance of the cow's milk allergens prevents the occurrence of an allergic reaction. Since cow's milk allergic children have an increased risk of developing other allergies later in life, investigating nutritional strategies to prevent the development of cow's milk allergy is of high interest. Here we review the preventive capacities of nutritional components in the development of allergic diseases with a focus on cow's milk allergy.

### Development of oral tolerance towards food allergens

At birth the baby's immune system is skewed towards a T helper 2 cell (Th2)-mediated response to prevent fatal immunological reactions between mother and child during pregnancy. If this Th2-skewed immune response is not adequately counterbalanced in a timely manner, Th2-mediated immunological disorders, such as food allergy may arise. Environmental factors progressively educate the immune system towards a more balanced immune system, reflecting in appropriate regulatory T cell (Treg), Th17, Th 1 and Th2 responses, thereby preventing the development of diseases like autoimmunity and allergies (6).

An important environmental factor is exposure to food antigens, which is essential for the maturation of the immune system and the development of oral tolerance towards food allergens. The exact mechanism of immunological oral tolerance induction is unknown, but the differentiation of Tregs plays an important role. The differentiation into Tregs, involved in oral tolerance, takes place in the periphery, and more specifically in the gut-associated lymphoid tissue (GALT). Dendritic cells (DCs) sample antigens in the gut from where they migrate to the GALT. In the GALT the DCs instruct naïve T cells to differentiate into antigen-specific Tregs under the influence of anti-inflammatory and regulatory factors, like transforming growth factor-β (TGF-β) and interleukin (IL)-10 (7).

Next to the exposure to food antigens, the development of the immune system is also dependent on the composition of the intestinal microbiota. It has been suggested that the microbiota plays a role in the development of mucosal immunological tolerance (7). Indeed, the composition of the intestinal microbiota between atopic and healthy children is different, and reduced bacterial diversity and dysbiosis is associated with development of atopic diseases (8, 9). A dysbiosis in allergic infants is characterized by low levels of genera *Bifidobacteria* and *Lactobacilli* compared to healthy infants (10). Furthermore, it has been demonstrated that certain commensal intestinal bacteria, such as *Bacteroides fragilis* and several clostridial species through their ligands and metabolites, can stimulate macrophages and DCs to produce high amounts of TGF-β and IL-10, thereby promoting the increase of Tregs (11-14). In addition, early life antibiotic exposure that has a major effect on the intestinal microbes increases the risk of developing allergic problems later in life (15, 16). Therefore, a dysbiosis in the intestinal microbiota might lead to an inadequately developed immune system associated with reduced number of Tregs, reduced oral tolerance and possibly to the development of food allergy.

The development of the intestinal microbiota can be influenced by several factors during infancy. Some of these factors include the delivery mode (vaginal vs caesarean section), antibiotics usage during early life and most importantly early life diet (breast milk vs formula milk) will have a major impact on the intestinal microbiota. In conclusion, early life food allergies, such as cow's milk allergy, may be the result of intestinal dysbiosis and related derailed mucosal immune system not handling cow's milk proteins in a proper way.

## Dietary interventions for the prevention of cow's milk allergy

During the first years of life, diet affects the composition of the intestinal microbiota and has a major influence on the development of the immune system. We here review the allergy-preventive effects of several nutritional components that modulate the intestinal microbiota and/or the immune system early in life.

### Human milk

Breast milk is the recommended dietary source from the day of birth. It contains dietary nutrients important for the growth and development of the new-born as well as growth factors, antigens and immunomodulatory components like immunoglobulins, polyunsaturated fatty acids (PUFAs), bacteria, non-digestible oligosaccharides and vitamins, all derived from the maternal diet or maternal immune system. These components are essential for shaping the intestinal microbiota composition and for the maturation of the immune system, i.e., to develop oral tolerance in the new-born (17, 18). The 2011 guideline from the World Health Organization (WHO) recommends exclusively breast milk as nutrition for infants during the first 6 months of life (19). Already after 3-4 months of consumption of breast milk the risk of wheeze, asthma and eczema development is decreased; however, the evidence is insufficient to form a conclusion regarding food allergy (20-22).

However, breastfeeding is not always possible and the best available alternative for infants is infant milk formula. To ensure optimal development of the intestinal microbiota and (mucosal) immune system, it is of great importance to identify all beneficial components in breast milk to enable full deployment of their potential when added to infant formula. Therefore, more information on the composition of breast milk and the function of the breast milk components might lead to new strategies to prevent allergy development.

**Human milk oligosaccharides - prebiotics**

Human milk oligosaccharides (HMOs) are one of the major components of breast milk (23). They stimulate the development of the immune system either directly via modulation of immune cells or indirectly by influencing the gut microbiota as a substrate for fermentation (24). Indicative for the importance of HMOs is the finding that the profiles of HMOs in breast milk are associated with food sensitization early in life (25, 26). Since the alternative for breast milk, infant formula, is based on cow's milk, the composition of oligosaccharides in infant formula is very different (low abundance) compared to human milk (27). Therefore, to mimic the composition of human milk, it is favourable to supplement infant formulas with non-digestible oligosaccharides, which show beneficial (prebiotic) properties. The definition of a prebiotic according to the International Scientific Association for Probiotics and Prebiotics (ISAPP) is 'a substrate that is selectively utilized by host micro-organisms conferring a health benefit' (28). Prebiotics stimulate the growth and activity of beneficial commensal intestinal bacteria (29). Studies have shown that prebiotic supplementation of infant formula with a specific mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) results in an intestinal microbiota composition similar to breastfed infants (9, 30) indicating that supplementation of infant formula with certain prebiotics will beneficially affect the intestinal microbiota development. In a randomized double-blind placebo controlled study with formula fed infants at risk of developing atopic manifestations it was shown that scGOS:lcFOS supplementation for 6 months reduced the development of atopic manifestations and infections during the first 6 months of life compared to the control group (31). This protective effect was still observed 2 and 5 years after the prebiotic intervention indicating that next to beneficial effects on microbiota composition and the shown health benefits, immune programming by prebiotics in early life can have a long-term protective effect (32, 33).

The mechanisms by which these prebiotics exert their effects are diverse and not completely clear. Prebiotics stimulate the growth and activity of beneficial commensal intestinal bacteria like *Bifidobacteria* and *Lactobacilli* (29). Increase in number and activity of the beneficial bacteria has antimicrobial effects since they compete with pathogenic bacteria to bind on intestinal epithelium (26). In addition, it is known that HMOs and scGOS:lcFOS are able to cross the intestinal epithelial barrier either through receptor-mediated transcytosis or through paracellular transfer (34, 35). This indicates that these compounds may also have a systemic effect. This is in line with the fact that at least 1% of HMOs is detected systemically (36). In addition to the microbial modulatory capacities of prebiotics, scGOS:lcFOS have been shown to have direct immunomodulatory effects on

the immune system. *In vitro* assays showed that scGOS:lcFOS promotes IL-10 release by DCs and these DCs can upregulate the number of functional suppressive Foxp3 positive T cells (12). In co-culture assays with intestinal epithelial cells and activated peripheral blood mononuclear cells (PBMCs), it is demonstrated that scGOS:lcFOS induces an epithelial cell-dependent development of tolerogenic Treg and Th1 responses (37, 38). Using a murine model of CMA it is demonstrated that the acute allergic response was significantly decreased in mice receiving scGOS:lcFOS during sensitization (39). Moreover, the mixture of scGOS:lcFOS enhanced mucosal IL-10 and TGF-β transcription and induced Tregs response which were essential in allergy prevention since neutralizing TGF-β or IL-10 *in vivo* abrogated the protective effects (40-42).

Despite the documented promising health benefit of prebiotics in several preclinical and clinical studies, some clinical studies show no differences (43, 44). One of these studies, a double-blind, randomized controlled trial comparing prebiotic containing formula with standard formula and breastfeeding, shows no differences in the incidence of allergic manifestations. The lack of difference may be due to the prebiotic mixture used in this trial, as this consisted of GOS and polydextrose (PDX) (43). These results indicate that a careful consideration of which type of prebiotic to use is important. So far, evidence from randomized trials that prebiotics (FOS, GOS and PDX) have a preventive effect on development of allergies is limited (45). More clinical studies are essential to learn more about the possible preventive effects on allergies of specific prebiotics, including HMOs. However, prebiotics are safe to use and the World Allergy Organization (WAO) guideline panel suggests prebiotic supplementation in not-exclusively breastfed infants, both at high and at low risk for developing allergy (conditional recommendation, very low certainty of evidence) (46).

**Live micro-organisms: Probiotics**

The intestinal microbiota play an essential role in the development of the (mucosal) immune system and also in the process of oral tolerance development. Modulation of the microbiota composition via nutrition is an appealing strategy. As discussed in the preceding paragraph, prebiotics are one strategy. Another strategy is to directly supplement diets with live micro-organisms. As live micro-organisms have been isolated from human milk (47, 48), addition of selective bacterial strains to infant formula might further potentiate the beneficial healthy effects of infant formula. The category currently used to refer to these live micro-organisms is probiotics. The definition of a probiotic according to ISAPP is 'live micro-organism that, when administered in adequate amounts, confer a health benefit on the host' (49).

There have been multiple clinical studies in which potential health benefits of probiotics were investigated. In a recently published double-blind placebo-controlled study, children receiving a daily mixture of *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subsp. *lactis* for 6 months (at the age of 8-14 months) developed less eczema compared to the control group. The number of sensitized children was not significantly different between

the 2 groups (50). In another randomized placebo-controlled trial, a positive effect on eczema prevalence was only demonstrated for *L. rhamnosus* and not for *B. animalis* subsp *lactis.* The supplementation was given daily to mothers from gestational week 35 until 6 months after birth (if they were lactating) and to the infants from birth up to 2 years of age. At 6 years of age the incidence of sensitization in the *L. rhamnosus* group was reduced (51); however, this reduction in sensitization was not further specified into the tested allergens, either food (egg, cow's milk and peanut) or aeroallergens (cat, grass pollen or house dust mite). At 11 years of age a significant lower prevalence of eczema was observed in the group receiving *L. rhamnosus* whereas supplementation of the *Bifidobacteria* had no effects (52). These data are in line with the meta-analyses (53, 54) which conclude that probiotic supplementation is beneficial in the prevention of eczema and that there is no proven effect on development of other allergies. It is important to realize that the preventive effect of probiotic supplementation is optimal when supplemented during both the pre- and postnatal period (53). This suggests that a combined strategy (pre- and postnatal) is most effective in prevention of eczema, and also to reduce sensitization. However, timing and duration of the intervention need more investigation and also food challenges are needed as an outcome in clinical trials to achieve more solid evidence of the preventive probiotic effects in food allergies (55, 56). Furthermore, a combination of probiotic strains seemed to lead to a more pronounced effect in the prevention of eczema compared with the use of single strains (54). However, strain-specific differences should be taken into account. The WAO states the following: although the recommendations are supported by weak evidence, there can be a beneficial effect of probiotics in certain cases, i.e. pregnant women having a child at high risk, women who breastfeed children at high risk for developing allergies and infants at high risk for developing allergies (57).

## Synbiotics

As probiotics and prebiotics show some promising effects in allergy management, it is tempting to speculate that a combination of the so-called synbiotics might lead to synergistic effects. Synergy may be achieved by an optimal combination of prebiotics with probiotics, in which the prebiotics selectively promote the growth and activity of the probiotics. A few (pre)clinical studies have evaluated the synbiotic strategy on the development of (food) allergies.

In a mouse model of CMA, a synbiotic diet comprised of a mixture of prebiotics (scGOS:lcFOS, 9:1) in combination with the probiotic strain, *Bifidobacterium breve M-16 V*, significantly reduced the allergic response, and was shown to be more effective in symptom resolution than either the pre- or probiotics singularly (39). Interestingly, next to alleviation of the allergic response, a synbiotic diet (comprised of scFOS:lcFOS and *B. breve M16 V*) was shown to increase tolerance development in a murine CMA preventive model (58). The beneficial effects of the synbiotics in these allergy models can be partly explained by their effects on the intestinal epithelial cells.

The synbiotic intervention increases epithelial-cell-derived galectin-9 (gal-9) levels in the intestine and mesenteric lymph nodes of mice in the CMA model. Moreover, it is demonstrated that in human PBMC assays gal-9 can induce the development of Th1 and Treg responses, which will contribute to amelioration of the allergic (Th2) response (59). Gal-9 is a soluble-type lectin and possesses sugar-binding motifs by which they bind to adaptive immune cells. Gal-9 also binds to IgE, which might prevent IgE cross-linking and consequently prevent degranulation of mast cells and/or basophils (60). In line with these results, the serum level of gal-9 in atopic dermatitis patients and in CMA mice was significantly increased after a synbiotic intervention and associated with amelioration of symptoms (59).

To the best of our knowledge, the preventive effects of a synbiotic strategy on the development of food allergies in clinical studies have not been evaluated. However, several studies investigated the synbiotic effect on the prevention of eczema and they all report significant improvements (61-63). In one of the studies, infants diagnosed with atopic dermatitis (age < 7 months) received a synbiotic supplemented infant formula or a formula without synbiotic for 12 weeks. The synbiotic supplement significantly reduced the severity of eczema, in the infants with IgE-associated eczema (63). After 1 year, asthma-like symptoms and medication use were less in the infants who had received the synbiotic formula (64). In line with the preclinical data, systemic gal-9 levels in children with eczema were increased in the group receiving synbiotic supplementation (59).

In conclusion, limited studies have evaluated the synbiotic intervention as treatment of atopic diseases and the knowledge about the preventive capacities of synbiotics is still limited. Promising results from preclinical studies suggest synbiotics to be of considerable interest in the prevention of allergies.

**Polyunsaturated fatty acids**
Dietary polyunsaturated fatty acids (PUFAs) are important as they are incorporated in the cell membrane and facilitate a favourable environment for immune development and maturation. PUFAs can be divided into omega-3 (n-3 PUFAs) and omega-6 (n-6 PUFAs) fatty acids. The n-6 PUFA arachidonic acid (AA), also found in meat, can be converted into the pro-inflammatory eicosanoids 2 and 4 series like prostaglandin $E_2$. In contrast, the n-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are incorporated in the cell membrane on the expense of AA, which leads to less available AA and therefore less conversion into pro-inflammatory prostaglandins and leukotrienes (65). DHA and EPA also compete with AA as substrates for cyclooxygenase and lipoxygenase by which EPA and DHA can be metabolized into the less pro-inflammatory prostaglandin and thromboxanes 3 and 5 series (66). In addition, cyclooxygenase can also convert DHA and EPA into resolvins, which are suggested to be anti-inflammatory through activation of the resolvins E1 receptor (67).

The skewing towards a high consumption of n-6 PUFAs in the western world has been associated with an increasing prevalence of allergies (68). This was also indicated in a preclinical model of CMA, where CMA mice exposed to n-6 PUFAs containing diet demonstrated more severe allergic symptoms (69, 70). In contrast, a n-3 PUFAs diet prevented the development of the acute allergic response as well as the IgE response and concomitantly increased the number of intestinal Tregs (71, 72). In a rat model of food allergy, it was shown that during two critical periods of immune development (pregnancy and weaning) extra supplementation with n-3 PUFAs was able to steer the immune system towards oral tolerance. Perinatal supplementation (during pregnancy & lactation) of n-3 PUFAs stimulated the maturation of the immune system of the offspring towards a Th1 (interferon-ɣ (IFNɣ)) and Treg (IL-10) response (73). The data from these preclinical studies suggest a promising role for n-3 PUFAs in the prevention of allergies.

Several clinical trials investigated the effect of fish oil, EPA and/or DHA supplementation during pregnancy and/or lactation on atopic disease development (74-79). However, there are differences between studies; which atopic diseases they evaluate, the timing of the intervention and the age of the children at the time of reporting the data. Some report beneficial effects on the development of food allergies and sensitization to allergens (e.g., sensitization to egg) whilst others report no effects on these atopic diseases (75, 78-81). According to a meta-analysis of maternal fish oil supplementation during pregnancy, the infants were at lower risk of developing eczema and a significant reduction in sensitization to egg was demonstrated in the first 12 months. This meta-analysis suggests maternal n-3 PUFAs to have positive effects regarding the prevention of infant allergy development, however, the authors conclude that the link between maternal intake of n-3 PUFAs and allergic disease development in the infants can be neither rejected nor confirmed due to inconsistency in results from the consulted studies (79). Based on current studies there is no clear evidence whether maternal consumption of n-3 PUFAs and/or fish oil prevents development of allergies in offspring, more adequate-designed randomized clinical trials are needed to establish adequate evidence.

The evidence that supplementation of n-3 PUFAs after birth and/or during infancy influences allergy development is limited. In infants receiving fish oil or n-3 PUFAs after birth a lower incidence of diagnosed food sensitization was reported and also a delayed time to first allergic illness (82-84). Mechanistic insights showed that immune cells from infants receiving fish oil displayed a decreased IL-13 production, and increased IFNɣ and tumour necrosis factor α (TNFα) production indicating a favourable shift towards Th1 in the Th1/Th2 balance (84). In contrast, in another study no effect was observed on allergic outcomes in infants receiving n-3 PUFA supplementation after birth (85). A meta-analysis from 2016 concluded that PUFA supplementation during infancy has no effect on the development of food allergy, asthma and eczema (86).

## Avoidance or early life introduction of cow's milk proteins

Historically, allergen avoidance during pregnancy and lactation has been the recommendation to mothers with children at high risk for allergic diseases. Avoidance of food allergens, such as cow's milk, fish, and egg from the maternal diet was hypothesized to prevent and reduce the risk of food allergic reactions in the infants (87). As the ingested allergens have been shown to pass through the placenta and are present in breast milk, this may lead to sensitization of the baby. However, the evidence that avoidance decreases the risk of food allergy is insufficient (88). Moreover, it has been demonstrated that early introduction of peanut could actually prevent peanut allergy in infants at risk (89-92). The increased risk for allergy in infants avoiding allergens, can be explained by the lack of allergen-specific oral tolerance induction due to the absence of the allergen and/or by sensitization towards the allergen via other routes (like the skin or airways) (93, 94).

For infants at risk of developing cow's milk allergy, consumption of infant formulas exposes these infants to the major cow's milk allergens, casein and whey, which may lead to sensitization. To reduce the sensitizing potential of infant formulas, the allergenic load of the formula can be reduced by processes such as hydrolysation, heat-treatment and/or ultra-filtration (95). This leads to reduction in the molecular weight of the cow's milk protein and is expected to reduce sensitization capacities of casein and whey (96). Hydrolysates exist as partial and extensive hydrolysates. Partial hydrolysates are used in the prevention of CMA in high risk infants and extensive hydrolysates are used for infants already diagnosed with CMA (3). The preventive property of the hydrolysates is mainly tested in children at risk, only a few studies were conducted in healthy infants, however, the quality of evidence was very low (97). According to a systematic review, hydrolysates have no effect on the prevention of allergic diseases in non-allergic infants, however the quality of evidence was very low (97). In infants at risk only limited studies have been performed with contradictory results. A recently published systematic review concluded that the use of partial hydrolysates in high risk infants reduces the risk of development of any allergic disease and in particular of eczema (98). The effect of hydrolysed cow's milk formula on allergy prevention has been shown in a cohort of infants at risk of atopic diseases (99). The preventive effect of hydrolysates was particularly shown to reduce the risk of developing atopic dermatitis, which even persisted after 10 years (100). Further investigation into the exact composition of hydrolysates might further contribute to identifying specific tolerizing capacity of the various hydrolysates. It has recently been demonstrated that certain peptides within whey-based hydrolysates can contribute to the development of oral tolerance to whey (101). Moreover, it is tempting to speculate that less processed milk, which is proven to be less allergenic, may have tolerance-promoting capacities (102, 103). Further research is necessary to investigate the role of processing of milk in cow's milk allergy-preventive strategies.

## CONCLUSION

The incidence of food allergies is increasing and there is a need for preventive strategies. Several nutritional components with intestinal microbiota and immune modulatory properties are suggested to have a potential role in the prevention of development of allergies. Next to the components reviewed in this chapter other important nutritional components like vitamins, postbiotics, ferments, and short-chain fatty acids are also known to have potential beneficial effects but could not all be discussed in this overview. The available research, so far, shows promising results particularly on the development of eczema. However, the preventive effects of the nutritional interventions on the development of food allergy are not conclusive. A reason for this can be inconsistency in the used dietary components (e.g., differences in prebiotic mixtures, differences in probiotic strains) and the timing of intervention. Future research may benefit from the combination of various dietary components. To clarify the preventive effects of the nutritional components in food allergy more randomized clinical trials are needed.

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# CHAPTER 3

Supplementation of dietary non-digestible oligosaccharides from birth onwards improve social and reduce anxiety-like behaviour in male BALB/c mice

Kirsten Szklany*[1], Harm Wopereis*[2,3], Cindy de Waard[1], Thecla van Wageningen[1], Ran An[3], Kees van Limpt[2], Jan Knol[2,3], Johan Garssen[1,4], Leon M. J. Knippels[1,4], Clara Belzer[3], Aletta D. Kraneveld[1,4]

[1] Division of Pharmacology, Utrecht Institute of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[2] Department of Gut Biology and Microbiology, Danone Nutricia Research, Utrecht, The Netherlands
[3] Laboratory of Microbiology, Wageningen University & Research, Wageningen, The Netherlands
[4] Department of Immunology, Danone Nutricia Research, Utrecht, The Netherlands
[5] Veterinary Pharmacology & Therapeutics, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
* These authors contributed equally

## ABSTRACT

The intestinal microbiota is acknowledged to be essential in brain development and behaviour. Their composition can be modulated by prebiotics such as short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharide (lcFOS). Several studies reported potential health benefit of prebiotics on behaviour. As the prebiotic mixture of scGOS and lcFOS is included in infant formula, we investigated the effects of dietary supplementation with this specific mixture from the day of birth onwards on behaviour and intestinal microbiota development in mice.

Healthy male BALB/cByJ mice received, from day of birth, a dietary supplement with or without 3% scGOS:lcFOS (9:1). Behavioural tests were performed pre-weaning, in adolescence, early adulthood and adulthood. We assessed faecal microbiota compositions over time, caecal short-chain fatty acids as well as brain mRNA expression of *5-Hydroxytryptamine receptor (Htr) 1a*, *Htr1b* and *Tryptophan hydroxylase 2 (Tph2)* and monoamine levels.

Compared to control fed mice, scGOS:lcFOS fed mice showed reduced anxiety-like and repetitive behaviour over time and improved social behaviour in adulthood. The serotonergic system in the prefrontal cortex (PFC) and somatosensory cortex (SSC) was affected by the scGOS:lcFOS. In the PFC, mRNA expression of brain-derived neurotrophic factor (*Bdnf*) was enhanced in scGOS:lcFOS fed mice. Although the bacterial diversity of the intestinal microbiota was unaffected by the scGOS:lcFOS diet, microbiota composition differed between the scGOS:lcFOS and the control fed mice over time. Moreover, an increased saccharolytic and decreased proteolytic fermentation activity were observed in caecum content.

Supplementing the diet with scGOS:lcFOS from the day of birth is associated with reduced anxiety-like and improved social behaviour during the developmental period and later in life, and modulates the composition and activity of the intestinal microbiota in healthy male BALB/c mice. These data provide further evidence of the potential impact of scGOS:lcFOS on behaviour at several developmental stages throughout life and strengthen the insights in the interplay between the developing intestine and brain.

# INTRODUCTION

The trillions of microbes colonizing our intestine, collectively referred to as the intestinal microbiota, have an essential role in health and disease throughout life (1-3). The establishment of the intestinal microbiota is influenced by several factors, such as gestational age, delivery-mode and nutrition (e.g. breast- or formula-feeding) and early life antibiotic exposure (4). These factors critically influence the optimal maturation of the metabolic, immune and neurological systems (4), i.e. alterations of intestinal microbiota composition (e.g. due to antibiotic-exposure and caesarean section) can increase the risk of developing metabolic-related (e.g. obesity), immune-related (e.g. allergies), but also behaviour-related brain disorders (e.g. autism spectrum disorder) (3, 5).

In both human and mice, microbial compositional changes can lead to behavioural changes (1, 2, 6, 7). The importance of the intestinal microbiota for brain development and behaviour has been demonstrated in animals treated with antibiotics or in germ-free animals. Compared with conventional colonized mice, germ-free mice show social deficits and reduced anxiety (1). In addition, germ-free mice also have altered neurochemical messenger systems (8). Antibiotic-treated mice show cognitive impairments, reduced anxiety and reduced sociability (7). Interestingly, behavioural impairments caused by depletion of the microbiota either with antibiotics or in germ-free mice were ameliorated by bacterial re-colonization or treatment with probiotics, indicating that changes in the microbiota are closely connected to behaviour (1, 7).

Prebiotics modulate the growth and activity of the intestinal microbiota and several studies reported the capability of prebiotics to also modulate behaviour. The prebiotic mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (1:1) reduced anxiety-like and depression-like behaviour in adult mice (2). Moreover, a prebiotic mixture of polydextrose and GOS administered during early life, diminished stress-induced behaviour in juvenile rats, improved recognition memory and increased explorative behaviour in young pigs (9, 10).

Human milk is known to contain substrates with prebiotic function, known as human milk oligosaccharides, to specifically shape the intestinal microbiota of new-borns (11). Also, the human milk oligosaccharide 2'-fucosyllactose (2'FL) possess cognitive modulatory capacities; rat pups exposed to 2'FL during lactation showed improved cognition in adulthood (12).

The specific prebiotic mixture of 90% of the low molecular mass short-chain GOS and 10% of the high molecular mass long-chain FOS (scGOS:lcFOS, 9:1) similar to the molecular size distribution of human milk oligosaccharides is included in infant formula and have been shown to exert similar functionalities as human milk oligosaccharides (11). These prebiotic carbohydrates can be digested by specific bacteria, such as *Bifidobacterium* and *Bacteroides* spp. (4), and subsequently fermented into short-chain fatty acids (SCFAs), which are reported to have neuroactive properties locally and systemically (5, 11). As the behavioural modulatory effects of this specific mixture of scGOS:lcFOS (9:1) is not yet known, we investigated, from the day of birth, in healthy male BALB/c mice, the effects of

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this specific prebiotics mixture scGOS:lcFOS (9:1) on behaviour and intestinal microbiota development, both in taxonomic and in SCFA-composition.

We showed that dietary supplementation with scGOS:lcFOS from birth in healthy male BALB/c mice reduced anxiety-like and stereotypic behaviour over time and improved social behaviour in adulthood. These behavioural improvements were associated with marked differences in microbiota composition and microbiota metabolite composition. In addition, the improved behaviour induced by scGOS:lcFOS was accompanied by altered monoamine levels, mRNA expression of serotonergic markers and brain-derived neurotrophic factor (BDNF) mainly in the prefrontal cortex (PFC).

# MATERIALS AND METHODS

## Animals

Sixteen day pregnant BALB/cByJ mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). From the day of birth of the litter (post-natal day zero, PND0) the dams were allocated to either the control (n = 6, pups n = 17) or the 3% scGOS:lcFOS (n = 5, pups n = 14) enriched diet. After weaning on PND21, the male offspring (n = 10 per group, n = 1-3 from each litter) continued the same diet as allocated to their mother. Additional male offspring (n = 9) from control fed dams were used as interaction mice in the social interaction test (more details in the supplement). All animal experimental procedures were carried out in compliance with national legislation following the EU-Directive for the protection of animals used for scientific purposes, and were approved by the Ethical Committee for Animal Research.

## Diets

Based on the standard AIN-93G control diet, the enriched diet consisted of a 3% (w/w) mixture of short-chain galacto-oligosaccharides (scGOS) (degree of polymerization 2-8) and long-chain fructo-oligosaccharides (lcFOS) (degree of polymerization on average ≥ 23) in a 9:1 (w/w) ratio added in an isocaloric manner (**Table S1**). Both diets were obtained from Research Diet Services (Wijk bij Duurstede, The Netherlands).

## Experimental design

**Figure 1** shows the experimental design. At several ages across lifespan, behavioural tests for anxiety, stereotypic and social behaviour were conducted. In the pre-weaning period, ultrasonic distress vocalization of the male offspring was measured (1.5 weeks old). During adolescence (6 weeks old) and early adulthood (8 weeks old), the marble burying test and self-grooming behaviour were assessed. During adulthood (11 weeks old) the social interaction test was conducted in addition to the marble burying and the self-grooming test. Faecal pellets were collected at 4, 6, 8 and 11 weeks of age and from the dams before starting dietary supplementation. The mice were euthanized by decapitation to collect caecum and brain. Due to limited brain material of the PFC, amygdala and hippocampus n = 5 was used to measure monoamine levels and n = 5 for qRT-PCR.
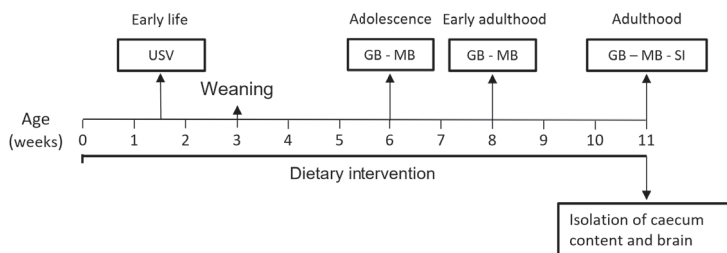
**Figure 1.** Schematic overview of the experimental protocol and the conducted behavioural tests. The mice received either a control diet or a diet enriched with 3% scGOS:lcFOS (9:1) from the day of birth. The ultrasonic distress vocalization test was conducted during infancy. At 3 weeks of age mice were weaned and the male mice continued the allocated diet of the dam. Grooming behaviour and the marble burying test were performed during adolescence, early adulthood and adulthood and the social interaction test was conducted during adulthood. After the last behavioural test organs were collected. UV: ultrasonic distress vocalisation. GB: grooming behaviour. MB: marble burying test. SI: social interaction test.

## Behavioural tests

Ultrasonic distress vocalization (USV) of male offspring was measured to determine anxiety-like behaviour as described previously (13). The marble burying test assesses anxiety-like and stereotypic/repetitive behaviour (14). Mice were scored for spontaneous self-grooming behaviour as previously described (15). The sociability of the mice was assessed with a previously described social interaction test (15). Time spent in the interaction zone near the cage of the unfamiliar target mouse; latency to first approach of the interaction zone and total distance moved were analysed (more details in the supplement).

## Measurement of monoamine levels

The prefrontal cortex (PFC), amygdala (AM), dorsal hippocampus (DH) and the somatosensory cortex (SSC) were, with the previously described HPLC method (16), measured for levels of tryptophan, serotonin (5-hydroxytryptamin, 5-HT) and its metabolite 5-hydroxyindolacetic acid (5-HIAA), noradrenalin (NA), dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) (details in the supplement). The concentrations of each compound were calculated by comparison with both external and internal standards.

## RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated from PFC, SSC, amygdala and hippocampus using the RNeasy isolation kit (Qiagen) and cDNA was synthesised using iScript cDNA synthesis kit (BioRad) following manufacturer's protocol. Quantitative real-time PCR was performed on a CFX96 real-time PCR detection system using iQ SYBR green supermix (BioRad) and primers (Qiagen) for *Rps13* (house-keeping gene), 5-hydroxytryptamin 1a receptor (*Htr1a*), *Htr1b*, brain-derived neurotrophic factor (*Bdnf*) and tryptophan hydroxylase 2 (*Tph2*) (details in the supplement). The mRNA expression of the gene of interest was normalized to the housekeeping gene and data are presented as fold change in expression compared to control mice.

**Short-chain fatty acid (SCFA) levels in caecum content**

The levels of SCFAs: acetic (AA), propionic (PA), butyric (BA), and valeric acid (VA), as well as the branched short-chain fatty acids; isobutyric (iBA) and isovaleric (iVA) acid in the caecum content were quantified with gas chromatography, as described previously (17) (details in the supplement).

**Bacterial DNA extraction from faecal pellets**

Total DNA was extracted from faecal pellets. The pellets were mixed with 350 µL S.T.A.R. buffer (Roche, Basel, Switzerland), followed by three 1-minute rounds of bead beating (25 g of 0.1 mm zirconia beads plus five 2.5 mm glass beads) on a FastPrep instrument (MP Biomedicals, Santa Ana, California, USA) at a power level of 5.5. The sample was heated to 95 °C and mixed by shaking at 100 rpm for 15 min. The homogenate was centrifuged at 4 °C for 5 min at 14,000 $g$ to pellet stool particles. The supernatant, containing DNA, was collected and the pellet was subjected to another extraction round with half the volume of S.T.A.R. buffer. Supernatants were pooled per sample and mixed, and 250 µL was further purified on the Maxwell 16 MDx instrument using the cartridge preparation from the Maxwell 16 tissue LEV total RNA purification kit as described by the manufacturer (Promega, Madison, WI, USA). DNA quality and quantity were assessed with the NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, Massachusetts, USA). DNA extracts were diluted to the same concentration before preparation for 16S rRNA-gene sequencing.

**Gut microbiota profiling by 16S rRNA-gene sequencing**

Extracted faecal DNA was used for microbiota profiling by 16S rRNA-gene sequencing. The V3-V4 region of the 16S rRNA-gene was PCR-amplified with universal primers S-D-Bact-0341-b-S-17 primer (forward 5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 primer (reverse 5'-GACTACHVGGGTATCTAATCC-3') (18) and barcoded using a two-step PCR protocol as described by Van den Bogert *et al.* (19). Sequencing was performed on an Illumina HiSeq sequencing platform (Illumina, USA) in a 300 bp paired-end mode.

**Bioinformatic and analysis of 16S rRNA sequencing data**

The 'Quantitative Insights Into Microbial Ecology' (QIIME) v1.9.0 package was used to analyse sequence data (20). Settings for demultiplexing and merging of paired-end sequences were as recommended by QIIME (according http://qiime.org/tutorials/processing_illumina_data.html). Sequences were clustered into Operational Taxonomic Units (OTUs) based on 97% sequence identity using VSEARCH v2.03 with chimera checking against the RDP gold database (21, 22). Taxonomic assignment was performed using the RDP classifier against the SILVA119 database (23). The species diversity (α-diversity) was calculated using the Chao1 and Shannon index for diversity (24) with correction for the differences in sequencing depths (number of reads per sample) by rarefaction.

**Statistical analysis**

Statistical analyses were performed comparing the control diet group and the scGOS:lcFOS diet group. Fisher's exact test was performed to determine whether the number of pups emitting calls during the USV test differed between the control diet group and the scGOS:lcFOS diet group. The USV, grooming, marble burying and social interaction tests were analysed with linear mixed models, controlled for litter effect and post hoc Sidak's multiple comparison test, repeated measures were added in the analysis of grooming and marble burying tests. All other data were statistically analysed with an unpaired, two-tailed Student's *t*-test. When not normally distributed or unequal variances, data were transformed taking the common logarithm before statistical analysis. The Mann-Whitney test was used to analyse differences between the dietary groups for latency of first approach to the target mouse during the social interaction test as the variance was still unequal after transformation. Correlations were analysed using the Spearman correlation. Results were considered statistically significant when $P < 0.05$. Analyses were performed using SPSS version 24 and GraphPad Prism Software version 7.03 for Windows (GraphPad Prism software, La Jolla, CA. USA).

*16S rRNA-gene sequencing*

The species richness and diversity (α-diversity) indexes calculated in QIIME from the 16S rRNA-gene sequencing data were analysed at one single rarefied sequencing depth. Differences between treatment groups over time were tested by two-way ANOVA with Sidak's multiple comparison test using GraphPad Prism version 7.03 for Windows. The non-rarefied OTU tables obtained from QIIME were aggregated at the bacterial genus level. Genera present in less than 30% of the samples or with an average relative abundance less than 0.005% were omitted from the analysis. Statistical comparisons were conducted by applying a combination of multivariate analysis with Canoco 5 software (25), followed by differential abundance testing using the R-package MetagenomeSeq (26). Firstly, the constrained ordination method, principal response curves (PRC), was used to test time-dependent treatment effects (27). The Monte Carlo Permutation test (MCPT), with 1000 permutations, was used to evaluate statistical significance ($P < 0.05$) of the resulting model. Next, the top 10 responding bacterial taxa identified from the model were evaluated on differential abundances at the different timepoints by metagenomeSeq using the zero-inflation log-normal distribution (FitFeatureModel) as recommended by the developers of the package (28). The Benjamini–Hochberg false-discovery rate (FDR) was used to correct for multiple comparisons in the differential abundance tests (29), and statistical significance was considered for *FDR* < 0.1 when observed for at least two repeated measurements.

# RESULTS

**Ultrasonic distress vocalization**

Ultrasonic distress vocalization was measured 10 days after birth. The number of male pups emitting at least one call was not significantly different between the dietary groups (**Table S2**, $P > 0.05$). The pups emitting at least one call were used for further analysis of

the USV. The total number of calls was significantly reduced in pups from lactating dams receiving the scGOS:lcFOS diet ($P < 0.05$; **Figure 2A**). The average frequency (Hz) showed a trend towards a decrease in the pups from scGOS:lcFOS diet fed dams ($P = 0.06$) and the average time of a call was not significantly different (**Figure S1A, B**).
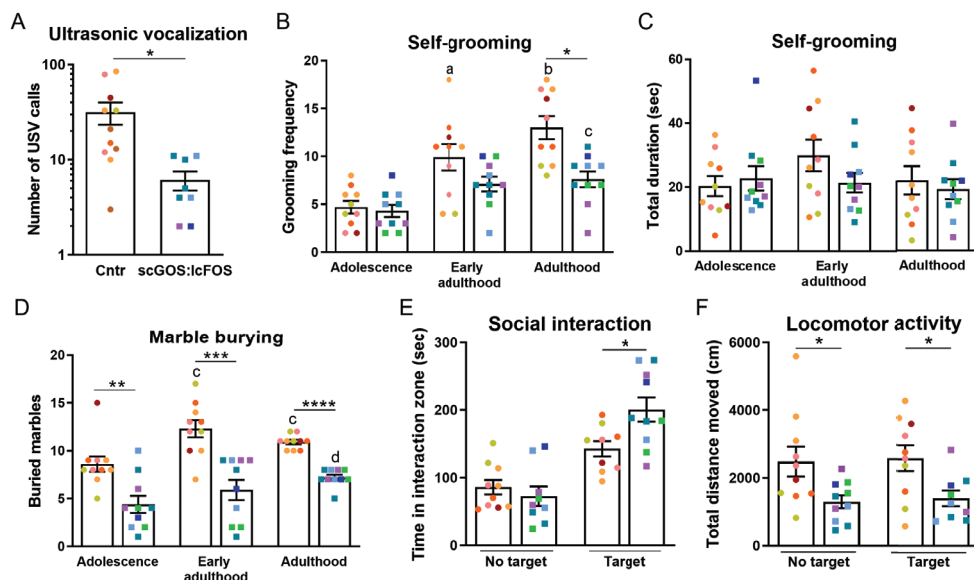


**Figure 2.** Anxiety-like, repetitive and social behavioural effects of scGOS:lcFOS. (A) scGOS:lcFOS receiving mice emitted significantly less ultrasonic vocalisation calls (USV). (B) Grooming frequency significantly increased over time in both control and scGOS:lcFOS receiving mice (diet: $P = 0.08$, age: $P < 0.000001$, interaction: $P < 0.05$). In adulthood the grooming frequency was significantly lower in the scGOS:lcFOS receiving mice compared with the control mice. (C) The grooming duration was unaffected over time and also unaffected by scGOS:lcFOS (diet: ns, age: ns, interaction: ns). (D) The number of buried marbles was increased across age, independent of the dietary supplementation (diet: $P < 0.0001$, age: $P < 0.001$, interaction: $P > 0.05$). In early adulthood and adulthood the scGOS:lcFOS receiving mice buried significantly less marbles compared with control. (E) In the presence of a target mouse the scGOS:lcFOS receiving mice spent more time in the interaction zone compared with control. No differences between the groups in time the mice spent in the zone in absence of target. One scGOS:lcFOS receiving mouse in absence of target and one control mouse in presence of target were excluded as significant outliers (Grubbs test 293 s and 0 s, respectively). (F) The locomotion activity measured by distance moved was decreased in the scGOS:lcFOS receiving mice in both absence and presence of a target mouse. One scGOS:lcFOS receiving mouse in presence of target was excluded as significant outlier (Grubbs test 6200 cm). A-F: The data are shown as individually data points, colour indicating the litter, and mean +/− SEM. A-F were analysed with linear mixed models followed by Sidak's multiple comparison post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, a = *** compared with adolescence within diet group, b = ****** $P < 0.000001$ compared with adolescence within diet group, c = * compared with adolescence within diet group, d = ** compared with adolescence within diet group, ns = not significant. A: control group $n = 11$, scGOS:lcFOS group $n = 7$, B-F: $n = 9$–10 per group.

## Self-grooming and marble burying

The grooming frequency trended towards an overall effect by diet (control vs scGOS:lcFOS) ($F_{(1, 7.241)}$ = 4.055, $P$ = 0.083) and overall age was significantly affected ($F_{(2, 18)}$ = 32.667, $P$ < 0.000001) (**Figure 2B**). The change in grooming frequency over time was dependent on the interaction effect between diet and age ($F_{(2, 18)}$ = 4.347, $P$ < 0.05). The grooming frequency increased over time from adolescence to adulthood in control diet fed mice (adolescence vs early adulthood $P$ < 0.001, adolescence vs adulthood $P$ < 0.000001). A significant increase in grooming frequency was only observed between adolescence and adulthood in the scGOS:lcFOS fed mice ($P$ < 0.05). Although no significant differences were observed between the diets in grooming frequency in adolescence and early adulthood, in adulthood the mice receiving the scGOS:lcFOS diet showed a significantly lower grooming frequency compared with mice receiving the control diet ($P$ < 0.05). However, the scGOS:lcFOS diet displayed no effect on self-grooming duration neither over time nor at any age (**Figure 2C**).

The number of buried marbles was significantly affected by diet (control vs scGOS:lcFOS) ($F_{(1, 13.337)}$ = 39.208, $P$ < 0.0001) and age ($F_{(2, 18)}$ = 9.64, $P$ < 0.001). The number of marbles buried over time was independent on the diet (interaction effect between diet and age ($F_{(2, 18)}$ = 2.505, $P$ > 0.05). Mice receiving the control diet buried significantly more marbles in early adulthood and adulthood compared to adolescence ($P$ < 0.05). The scGOS:lcFOS fed mice buried significantly more marbles in adulthood compared with adolescence ($P$ < 0.01). From adolescence and onwards the mice exposed to the scGOS:lcFOS diet demonstrated a significant decrease in the number of buried marbles compared to control mice (adolescence $P$ < 0.01, early adulthood $P$ < 0.001, adulthood $P$ < 0.0001) (**Figure 2D**).

## Social interaction

The scGOS:lcFOS fed adult mice spent as much time in the interaction zone as the control mice in absence of a target mouse ($P$ > 0.05). However, in presence of a target mouse mice exposed to the scGOS:lcFOS diet spent significantly more time in the interaction zone than did the control mice ($P$ < 0.05) (**Figure 2E**). The latency of first approach to the target mouse and the frequency a mouse entered the interaction zone did not significantly differ between the two dietary groups with either the absence or presence of a target mouse ($P$ > 0.05) (**Figure S1C, D**). The locomotion activity measured as total distance moved through the arena was significantly less in the scGOS:lcFOS receiving mice in both absence and presence of a target mouse ($P$ < 0.05) (**Figure 2F**).
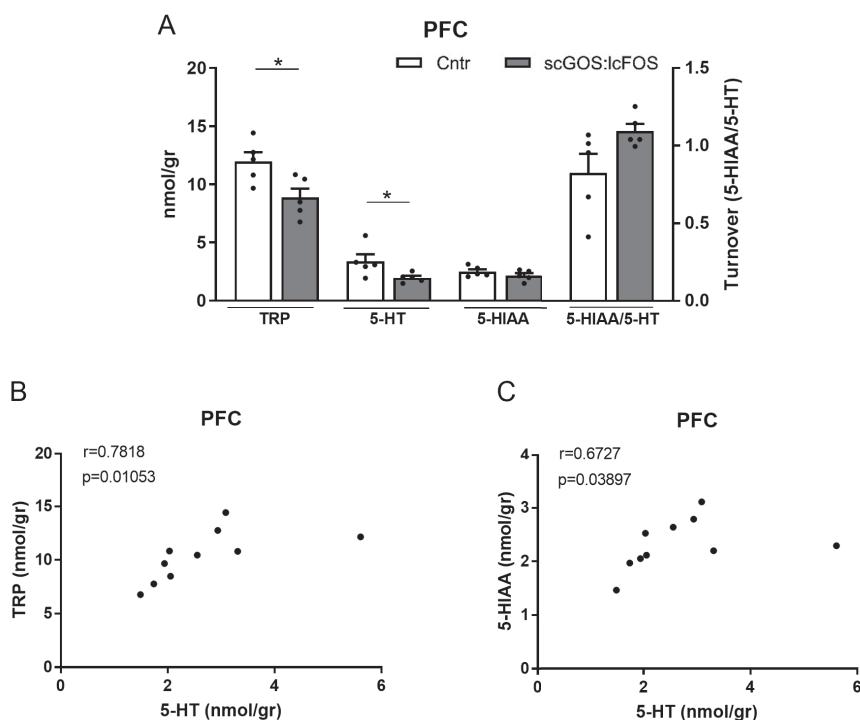
**Figure 3.** The PFC levels of tryptophan, 5-HT, the 5-HT metabolite 5-HIAA and the serotonin turnover (5-HIAA/5-HT). (A) The tryptophan level was significantly decreased in the scGOS:lcFOS receiving mice. The serotonin level was significantly decreased in the scGOS:lcFOS receiving mice. The 5-HIAA level and the serotonin turnover were not significantly different. The tryptophan levels and the serotonin levels (B) as well as the 5-HT and the 5-HIAA levels (C) correlated significantly. A: Data shown as individually data points and mean +/− SEM. A: Analysed by Student's $t$-test. B,C: Analysed by spearman correlation. * $P < 0.05$. A–C: $n = 5$ per group. TRP: tryptophan, 5-HT: 5-hydroxytryptamine (serotonin), 5-HIAA: 5-hydroxyindolacetic acid.

## Monoamine levels in several brain regions

In the PFC, tryptophan ($P < 0.05$) and 5-HT ($P < 0.05$) levels were significantly decreased in the scGOS:lcFOS dietary group compared with the control group (**Figure 3A**) and the NA, DA and DA metabolites levels were unchanged (**Figure S2A-C**). In the SSC the individual monoamines were unchanged; however, the 5-HT turnover indicated by 5-HIAA/5-HT was significantly higher in the scGOS:lcFOS fed group ($P < 0.05$) (**Figures 4A** and **S2D-F**). The tryptophan and 5-HT levels as well as the 5-HT and the 5-HIAA levels correlated significantly in the PFC (Spearman correlation were respectively $r = 0.782$, $P < 0.05$ and $r = 0.673$, $P < 0.05$) (**Figures 3B**, **C**), whereas no significant correlations between these monoamines were present in the SCC (**Figures 4B**, **C**). In the AM and the DH no significant differences in the monoamine levels and their metabolites were observed between the control and scGOS:lcFOS diet groups (**Figure S3**).
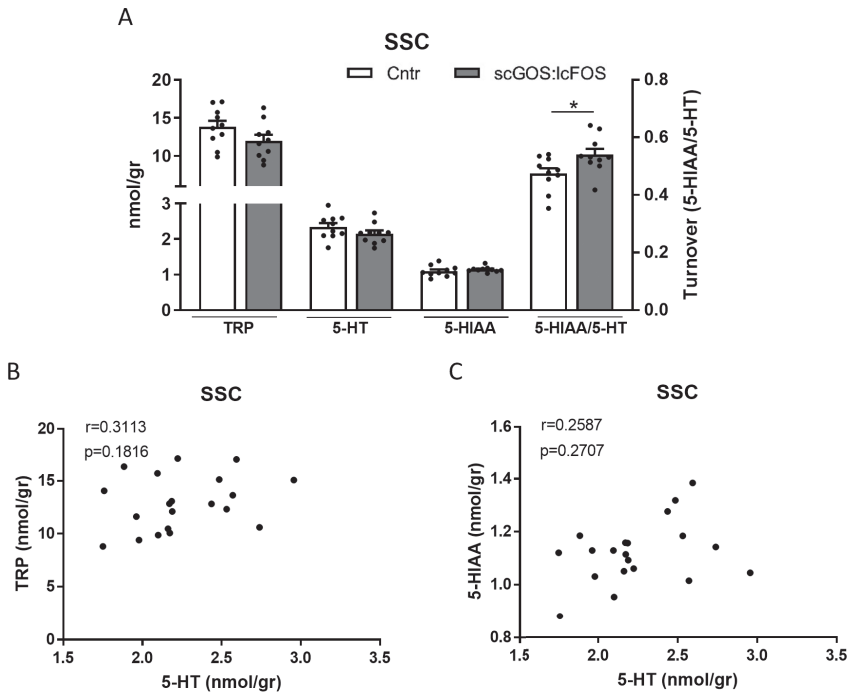
**Figure 4.** The SSC levels of tryptophan, 5-HT, the 5-HT metabolite 5-HIAA and the serotonin turnover (5-HIAA/5-HT). (A) The tryptophan, 5-HT and 5-HIAA levels were not significantly different between the scGOS:lcFOS and control diet receiving mice. The serotonin turnover was significantly increased in the scGOS:lcFOS receiving mice. The tryptophan and the 5-HT levels (B) as well as the 5-HT and the 5-HIAA levels (C) did not correlate. A: Data shown as individually data points and mean +/− SEM. A: Analysed by Student's *t*-test. B,C: Analysed by spearman correlation. * $P < 0.05$. A: $n = 10$ per group. B, C: $n = 10$ per group. TRP: tryptophan, 5-HT: 5-hydroxytryptamine (serotonin), 5-HIAA: 5-hydroxyindolacetic acid.

## Serotonergic system

The mRNA expression of *Htr1a* was significant decreased in the PFC ($P < 0.01$) in the scGOS:lcFOS receiving mice, whereas the expression of *Htr1b* was unchanged. As the tryptophan and the 5-HT levels in the PFC were decreased in the mice receiving the scGOS:lcFOS diet, scGOS:lcFOS might influence the 5-HT synthesis from tryptophan. This is regulated by tryptophan hydroxylase 2 (TPH2). However, the mRNA expression of *Tph2* was unaffected by the dietary intervention (**Figures 5A-C**). In the SSC the mRNA expression of *Htr1a* trended towards a decrease ($P < 0.1$) in the scGOS:lcFOS receiving mice, whereas the expression of *Htr1b* and *Tph2* were unchanged (**Figures 5E-G**). In the AM and the DH, the mRNA expression of the tested markers was unchanged between the dietary groups (**Figure S4A-F**). These data are preliminary as the sample size was small.
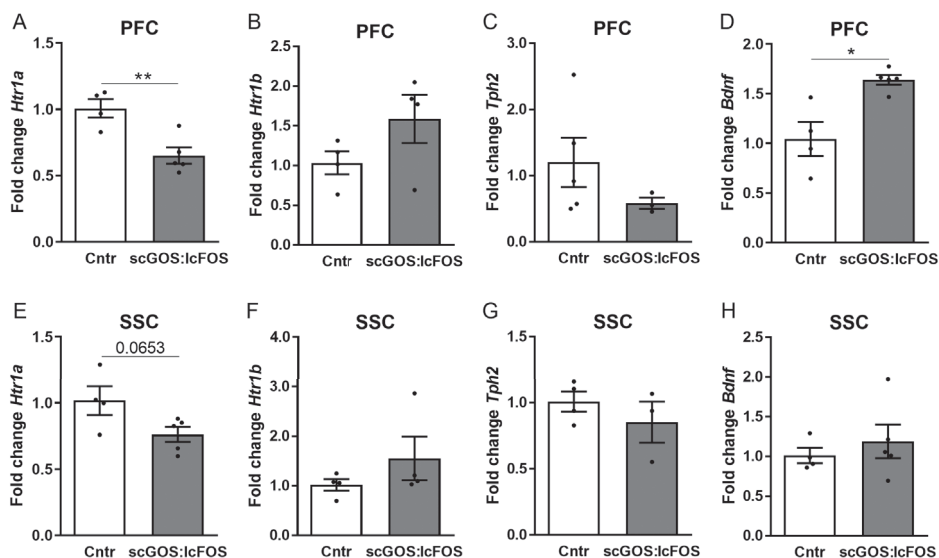
**Figure 5.** The mRNA expression of *Htr1a*, *Htr1b*, *Tph2 and Bdnf* in the PFC and SSC. (A) In the PFC the mRNA expression of *Htr1a* was significantly decreased in the scGOS:lcFOS receiving mice. The *Htr1b* (B) and *Tph2* (C) mRNA expressions were unchanged. The mRNA expression of *Bdnf* was significantly increased in the scGOS:lcFOS receiving mice (D). In the SSC the mRNA expression of *Htr1a* trended towards a decrease in the scGOS:lcFOS group (E). The *Htr1b* (F), *Tph2* (G) and *Bdnf* (H) mRNA expression was not significantly changed between the groups. A-H: Data shown as individually data points and mean +/− SEM. A-H: Analysed by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$. A–H: $n = 3–5$ per group. PFC: prefrontal cortex, SSC: somatosensory cortex, *Htr1a*: serotonin receptor 1a, *Htr1b*: serotonin 1b receptor, *Tph2*: tryptophan hydroxylase 2, *Bdnf*: Brain derived neurotrophic factor.

## Brain-derived neurotrophic factor (Bdnf)

The mRNA expression of *Bdnf* significantly increased in the PFC ($P < 0.05$) (**Figure 5D**), but not in the SSC (**Figure 5H**) or AM (**Figure S4G**) of the scGOS:lcFOS mice.

## Caecal SCFA and branched SCFA levels

The absolute levels of the bacterial fermentation end-products, AA ($P < 0.05$), PA ($P < 0.01$) and BA ($P < 0.001$) were increased in the caecum content of the mice fed the scGOS:lcFOS diet (**Figures 6A-C**). In contrast, compared to the control group, the levels of protein-derived VA (not significant, **Figure 6D**), and the branched SCFAs iBA and iVA all decreased in the scGOS:lcFOS group ($P < 0.0001$ for both, **Figures 6E-F**).
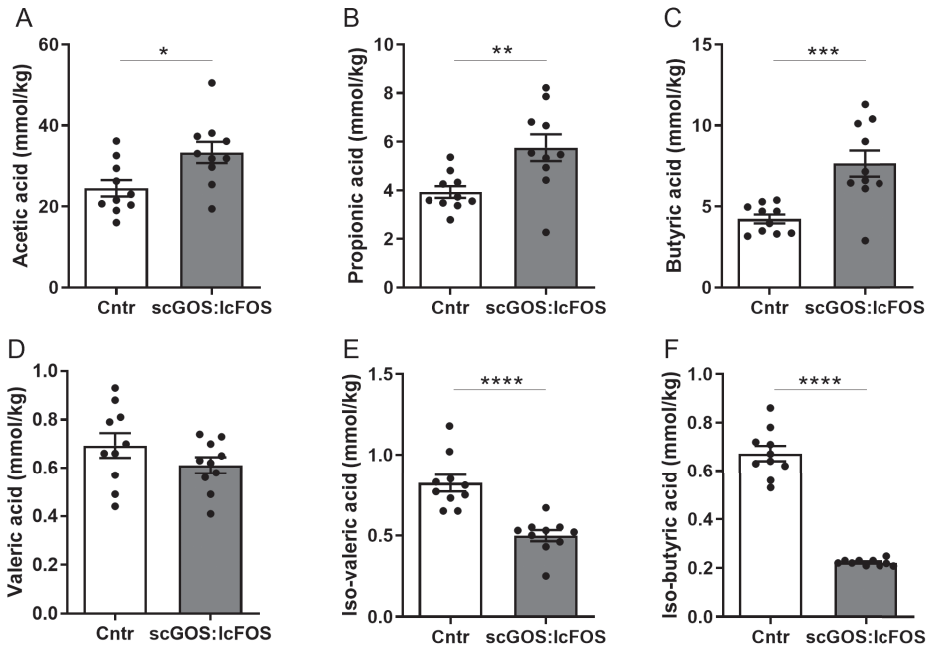
**Figure 6.** Caecal SCFAs. The levels of the SCFAs acetic (A), propionic (B) and butyric (C) acids were significantly increased in the scGOS:lcFOS group. (D) The level of valeric acid was unaffected by scGOS:lcFOS and the levels of the branched SCFAs iso-valeric acid (E) and iso-butyric acid (F) were significantly decreased in the scGOS:lcFOS group. A–F: Data shown as individually data points and mean +/− SEM. A-F: Analysed by Student's $t$-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. A–F: $n = 10$ per group. SCFAs: short-chain fatty acids.

## Faecal microbiota composition

The 16S rRNA-gene sequencing of the collected faecal pellets resulted in an average sequence depth of 351,548 (SD = 220,402) reads per sample (**Table S3**). Species richness by Chao1 index was significantly decreased in scGOS:lcFOS vs control at week 4 ($P < 0.01$). The species diversity (based on Shannon index) was not significantly different between the dietary groups at the different ages (**Figure 7B**). The PRC ordination method (27) was used to assess the age dependent effects on the intestinal microbiota composition in scGOS:lcFOS fed mice compared to control fed mice. The microbial composition over time was significantly dependent on the diet (MCPT interaction, $P = 0.001$). The top 10 associated bacterial genera from this PRC analysis were further evaluated by differential abundance testing, which confirmed that 9 of the 10 identified genera were differentially abundant for at least 2 repeated measurements (**Figure 7C** and **Table S4**). In sum, an increase of 3 genera within the order of *Bacteroidales* was observed for scGOS:lcFOS compared to control, as well as an increase of the genus *Dorea* within the family of *Lachnospiraceae* and an unknown genus of the family *Erysipelotrichaeae*. In contrast, a decrease was observed for 3 unknown genera within the family of *Peptostreptococcaceae,* and the genus *Anaerovorax* within the *Clostridiales* family XIII (**Figures 7A, C**).
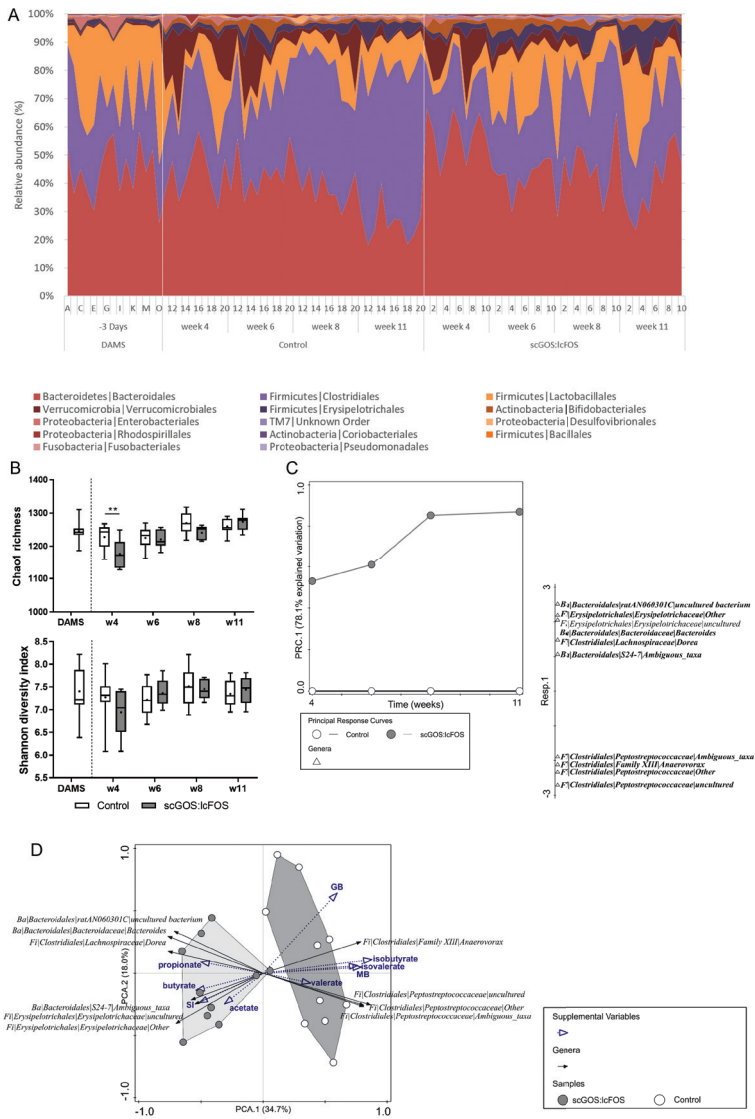
**Figure 7.** Faecal microbiota. (A) Taxa composition of each mouse per group and age at the bacterial phylum and order level. Only taxa with a relative abundance > 0.1% on average (based on number of reads per total reads) are plotted in the stacked area chart. Sorted from largest to smallest relative abundance. (B) Chao1 richness and Shannon species diversity plotted as box plots with whiskers showing the range, boxes the interquartile range and the line at median. The '+' shows the average Chao1 and Shannon index per group and age. Statistics performed with two-way ANOVA with Sidak's multiple comparisons test comparing scGOS:lcFOS with control per timepoint (** $P$ < 0.01). Rarefaction depth used for summary of diversity: 27,242 reads per sample. (C) PRC with the top 10 responding bacterial genera for treatment over time with adjustment for age. The treatments are presented as a single response curve over time (on the horizontal axis) with the control-group as reference with zero PRC values (on the vertical axis) and so its curve lays over the horizontal axis. The top 10 bacterial genera are plotted on the separate vertical (one-dimensional) plot based on best

fit with the first PRC axis. Monte Carlo Permutation Test (MCPT) with 1,000 permutations showed a significant interaction ($P = 0.001$) of treatment with time on the 1st PRC axis (78.1% explained variation). Differential abundance testing was performed with the metagenomeSeq-package on the top 10 identified bacterial taxa. The taxa that were confirmed to be significantly different at 2 or more timepoints (with correction for multiple testing by false discovery rate < 0.1) are shown in bold. The bacterial genera are detailed at the phylum level (Ba = Bacteroidetes, Fi = Firmicutes), the order, family and genus level. (D) PCA tri-plot of faecal microbiota composition of all mice in adulthood coloured by membership to the scGOS:lcFOS diet group and control group, respectively. Individual samples (dots) are plotted on the first two axes of the PCA-analysis with more similar compositions closer to each other. The bacterial genera that were identified from the PRC analysis (the top 10) are plotted as the black biplot arrows. The behavioural readouts and the SCFAs are supplemented as blue biplot arrows to visualize the correlations in adulthood. The bacterial genera are detailed at the phylum level (Ba = Bacteroidetes, Fi = Firmicutes), the order, family and genus level. PRC: Principal Response Curve, PCA: Principal Component Analysis, SI: social Interaction, seconds in interaction zone in presence of target, MB: number of buried marbles, GB: grooming frequency.

A principal component analysis (PCA) was used to evaluate the microbiota compositional differences in adulthood between the dietary groups and to correlate these with the adulthood behavioural assessments as well as the caecal concentrations of SCFAs (**Figure 7D**). The PCA analysis confirmed the distinct microbiota composition of the two dietary groups. The compositional differences between scGOS:lcFOS and control in adulthood correlated with increased sociability, lower number of marbles buried and lower frequency in grooming behaviour. Moreover, as measured by the caecal levels of SCFAs, the microbial composition also correlated positively with the changes in microbial activity.

## DISCUSSION

Prebiotic fibres are known to be effective modulators of the intestinal microbiota composition and activity and may thereby positively influence health through immune- and neuromodulation (30). However, little is known about the effect of dietary prebiotic fibres on the development of the nervous system (31). Here, we demonstrated that dietary supplementation with scGOS:lcFOS in healthy male BALB/cByJ, started on the day of birth, was associated with altered intestinal microbiota composition and increased saccharolytic fermentation and decreased proteolytic fermentation activity. These microbial changes were accompanied with reduced stereotypic and anxiety-like behaviour throughout life and improved social behaviour in adulthood.

The first effect of scGOS:lcFOS on behaviour in our study was already observed in early life in the ultrasonic distress vocalization test. The pups from dams fed the scGOS:lcFOS diet, emitted fewer USV calls compared to control pups. This indicates reduced anxiety-like behaviour of the pups (32) and might be a result of modulation of beneficial intestinal bacteria by scGOS:lcFOS leading to improved behaviour. On the other hand, a decreased number of USV calls by pups might indicate better maternal responsiveness (33), which may lead to improved behaviour in the pups later in life. Possibly, the maternal effects of scGOS:lcFOS on the neurodevelopment of the pups could be additive to the direct effects of scGOS:lcFOS on the brain of the pups or more relevant. To confirm this, maternal behaviour has to be measured in future studies.

During adolescence, early adulthood and adulthood, anxiety-like behaviour was reduced in mice exposed to dietary scGOS:lcFOS. This is in line with the observation that a lower number of neonatal USV calls is related to reduced anxiety-like behaviour later in life (34). In adulthood, the reduced anxiety-like behaviour was accompanied by improved social interaction in the scGOS:lcFOS receiving mice. During the social interaction test, locomotion activity was reduced in mice fed the scGOS:lcFOS diet in presence and absence of target mouse. The reduced locomotion did not confound the time spent in the interaction zone. We used male BALB/c mice because behaviour-related brain disorders like autism spectrum disorder is more prevalent in males (35) and the BALB/c behavioural phenotype is represented by low sociability and high levels of anxiety-like behaviour (36). Our findings that scGOS:lcFOS reduced anxiety-like behaviour and improved sociability is in agreement with a recently published study by Burokas *et al.* using a similar prebiotic combination (2). In their research, the prebiotic combination GOS:FOS (1:1) given during adulthood for 10 weeks reduced the anxiety-like behaviour in healthy mice (2). In contrast to our study, prebiotic supplementation had no effect on social interaction, however, the GOS:FOS receiving mice showed significantly increased number of prosocial events (2). Other prebiotic mixtures also have behavioural modulatory capacities; juvenile rats and young pigs exposed to a prebiotic mixture of polydextrose and GOS showed reduced stress-induced behaviour and improved explorative behaviour, respectively (9, 10). These behavioural changes were accompanied with increased *Lactobacillus* spp. (9). Together these results suggest the capability of prebiotic fibres to modulate behaviour.

In addition to the behavioural changes, scGOS:lcFOS also influenced the neurochemistry of the brain in adulthood. It significantly affected the serotonergic system mainly in the PFC indicated by reduced tryptophan and 5-HT levels and lower mRNA expression of the serotonin receptor 1A, however these are preliminary data due to small sample size. In the GOS:FOS study by Burokas *et al*. (2), the plasma level of tryptophan was lowered after GOS:FOS administration. Together with our data this may indicate that the combination of GOS and FOS may be able to stimulate the growth and/or function of tryptophan utilizing intestinal bacteria. Several intestinal bacteria, for example *Eschericheria coli, Lactobaccillus* and *Bacteroides* (37) possess tryptophanase, an enzyme that converts tryptophan into metabolites like indole (38). The scGOS:lcFOS induced changes of the microbiota could lead to lower availability of tryptophan for the brain leading to lower levels of 5-HT. The decreased level of 5-HT is in contrast with the monoamine theory stating that higher levels of 5-HT and tryptophan reduce depressive and anxiety symptoms (39). However, the monoamine theory is based on the available monoamine levels in the synaptic cleft and in this study, we measured total 5-HT levels including unreleased 5-HT in the presynaptic neurons. In other murine prebiotic studies, the total 5-HT level was either increased or unaffected in the PFC (2) or the frontal cortex (40). However, in a comprehensive meta-study by Ruhé *et al.* (41) systemic depletion of tryptophan and 5-HT did not lead to decreased mood in healthy human controls. Considering the available preliminary data, the causal role of changed 5-HT and tryptophan levels on behaviour in a healthy situation is inconclusive.

The 5-HT1A receptor has an essential role in anxiety (42). Activating the receptor with a 5-HT1A agonist induces anxiogenic effects which are inhibited by a 5-HT1A antagonist (43). In our study preliminary data showed that decreased (mRNA) *Htr1a* expression in the PFC was associated with reduced anxiety-like behaviour. Due to limited brain material the mRNA levels were measured in a small sample size and therefore a limitation of this study. During adolescence, signalling of 5-HT through the 5-HT1A receptor is important and its disruption can lead to an increase in anxiety later in life (44). The serotonin signalling during adolescence might be stimulated by scGOS:lcFOS supplementation and consequently lead to reduced anxiety-like behaviour later in life.

The neurotrophin BDNF is important in neural circuit development and in regulation of mature neural circuits (45). Higher BDNF levels in the hippocampus, striatum and hypothalamus are associated with improved social interaction in mice (46, 47). *Bdnf* mRNA and protein expression were enhanced in the hippocampus of adult mice and rats after prebiotic supplementation (2, 48). Although the hippocampal (mRNA) *Bdnf* level was unavailable in our study due to insufficient material, the (mRNA) *Bdnf* level in the PFC was increased in scGOS:lcFOS fed mice. This is in line with a study in healthy rats, in which a mix of prebiotics including GOS resulted in an increase of (mRNA) *Bdnf* in the PFC (9), indicating a beneficial effect of specific prebiotics in behaviour development.

The prebiotics modulated the intestinal microbiota in the mice and led to differences in bacterial richness and marked differences in taxonomic composition. These included increased relative abundances of the genus *Dorea* and several genera of *Bacteroidales* and *Erysipelotrichales*, and decreased relative abundances of the genus *Anaerovorax* and several genera of *Peptostreptococcacae* (all within the order of *Clostridiales*). The prebiotic effects observed in these mice differ markedly from observations in human infants, in which prebiotics predominantly leads to increased levels of bifidobacteria (11). Although the mice receiving scGOS:lcFOS showed enhanced levels of bifidobacteria compared to control, the differences were not significant (**Figure S5**). The modulatory effects of scGOS:lcFOS likely depends on the already resident mouse microbiota. This is supported by the findings in another mouse study, in which only the combination of scGOS:lcFOS with the probiotic *Bifidobacterium breve* M-16 V showed a bifidogenic effect in contrast to the intervention containing the prebiotic components only (49). Possibly, the mice specific bifidobacterial species are inefficient in utilizing scGOS:lcFOS or are outcompeted by other bacterial species. Indeed, we observed that the supplementation with scGOS:lcFOS mainly led to increased relative abundances of the more dominant *Bacteroidales* spp., which are known to degrade similar complex carbohydrates as used in this experiment (50, 51). Similarly to our study, Burokas *et al*. found increased levels of the *Bacteroidales S24-7* group, which is a prominent but yet uncultured group in the murine gut that may have the machinery to degrade scGOS:lcFOS (52). However, the other bacterial groups reported to respond to the scGOS:lcFOS intervention were distinct from our observations. This could be due to several factors such as the different genetic background of the mice (BALB/c vs C57BL/6J), supplier, housing (group vs individual), diets and ratio of used prebiotic mixture, and location of the laboratory. Moreover, the selective

effects of the prebiotics on the *Bacteroidales* spp. may have stimulated the putative butyrate-producing microbial groups of *Erysipelotrichales* (53) through cross-feeding mechanisms (4). Therefore, investigating the composition of intestinal microbiota as a whole in relation to in depth metabolomics is important to obtain functional information on precisely how the intestinal microbiota is influenced by scGOS:lcFOS and influences brain and behaviour.

In contrast to the distinct bacterial composition between our and the Burokas study, the bacterial activity assessed by caecal SCFAs were similar. The acetate and propionate levels were increased, and the iso-butyrate level was decreased, and this was also observed by Burokas *et al*. (2). Additionally, in our study, the butyrate level was increased and the iso-valerate level was decreased. Overall, the decrease of branched SCFAs (iso-butyrate and iso-valerate), which are fermentation products from degradation of proteins and amino acids (54, 55), indicates that the scGOS:lcFOS diet shifted the intestinal microbiota from a more proteolytic profile to a more saccharolytic profile. The latter is typically associated with higher levels of acetate, propionate and butyrate, and metabolite profiles considered to be more beneficial for colonic health (56).

Changes in intestinal microbiota can affect brain and behaviour through the gut-brain axis through several mechanisms, including microbe-derived molecules like SCFAs, and tryptophan metabolites neuroactive molecules like neurotransmitters and neuronal signalling by stimulation of the vagus nerve (57). As SCFAs are fermentation products from scGOS:lcFOS and SCFAs have been shown to reduce anxiety-like and depressive-like behaviour in mice (58), these mediators might play a role in the behavioural changes observed in our study. How this communication between the SCFAs and the brain occurs is unknown, however, signalling through the vagus nerve (10) or improved integrity of the blood brain barrier could play a role (59). In the central nervous system, SCFAs (among others) play a role in the homeostasis of microglia cells, macrophage-like cells in the brain. Studies in germ-free mice indicated that microglia cells are affected by the intestinal microbiota (60) indicating that microglial function might be influenced by scGOS:lcFOS in an immunomodulatory way.

In conclusion, scGOS:lcFOS from the day of birth modulates the composition and activity of the intestinal microbiota, and is associated with reduced anxiety-like and improved social behaviour in healthy male BALB/c mice. To evaluate the exact effects in early life, it is essential to investigate brain neurochemistry at several time points throughout phases of development. Here, we provide further evidence of the potential beneficial effects of scGOS:lcFOS on behaviour at several developmental stages throughout life and strengthen the insights in the interplay between the developing intestine and brain.

## ACKNOWLEDGMENTS

## SUPPLEMENTARY INFORMATION

### Animals

Dams were housed solitary and males (after weaning) were housed in groups all in Makrolon IIL cages containing standard wooden chip bedding (Tecnilab-BMI) and *ad libitum* access to food and water in conventional room. A 12 h light/dark cycle was followed and experimental procedures were conducted during the light phase. Cages were cleaned weekly on the same day of the week. Sample size estimation was based on previous data of time spent in interaction zone in the social interaction test.

### Behavioural tests

*USV*

An ultrasonic vocalization testing cylinder with a lid containing the UltraSound Advice microphone (London, United Kingdom), was connected to an amplifier and a computer containing the SonoTrack software (Metris B.V.; Hoofddorp, The Netherlands). The aluminium bottom of the cylinder was connected to a Julabo F12-ED heating circulator (Sigma-Aldrich; Zwijndrecht, The Netherlands) to maintain the temperature at 19 °C. A pup was separated from the dam and littermates and placed in the cylinder for three min; the number of USV calls was measured, together with the average time of a call and the average frequency wavelength of a call. Dam and littermates were housed in the same room during this test.

*Marble burying*

A home cage (L35xW20xH15 cm) was filled with 2 cm of standard chip bedding, lightly tamped down to make a flat, even surface. Twenty black marbles (15 mm in diameter) were cleaned with 70% ethanol and placed on the surface in a regular pattern. Each mouse was placed individually in a cage for 30 min. The number of marbles that were at least ⅔ buried with bedding was counted.

*Self-grooming*

Mice were scored for spontaneous self-grooming behaviour. Each mouse was individually placed in an empty home cage without bedding, cleaned with 70% ethanol. Mice were recorded with a Sony Handycam DCR-SR72 video camera for 10 min of which the first 5 min were considered as habituation period. The video recordings were analysed blindly using ObserverXT software (Noldus, The Netherlands) for frequency and cumulative time spent grooming.

*Social interaction*

Mice were placed in a L45 x W45 cm open field, that was cleaned with 70% ethanol. In the open field a perforated plexiglas cage (10 cm diameter) was placed against a side, allowing for visual and olfactory interaction. The test mice were habituated to the open

field for 5 min. An age- and gender matched unfamiliar target mouse was placed in the plexiglas cage for an additional 5 min. The social interaction was recorded with a Sony Handycam DCR-SR72 video camera and analysed using tracking software (Ethovision 3.1.16; Wageningen, The Netherlands) for time spent in the interaction zone (25.3 cm x 18.8 cm) near the cage of the unfamiliar target mouse; latency until first entrance of the interaction zone and total distance moved across the open field.

## Monoamine levels and qRT-PCR

*Isolation of brain regions*

After decapitation the brains were rapidly isolated, frozen in isopentane and stored at -70 °C until further analysis. The frozen brains were coronally sliced into 500 μm thick sections using a cryostat (Model 700, Lameris Instruments, Utrecht, The Netherlands) and the PFC, SSC, amygdala and the hippocampus were isolated.

*Measurements of monoamine levels*

The brain tissues were homogenised by sonication in 50-100 μL solution containing 0.6 μM N-methylserotonin (NMET, internal standard) and 5 μM clorgyline. Afterwards 12.5 μL 2M $HClO_4$ was added to 50 μL of the homogenate and mixed. After 15 min in ice-water the homogenates were centrifuged for 10 min at 15,000 $g$, 4 °C. The separation on the HPLC was performed at 40 °C using a flow rate of 0.8 mL/min. The limit of detection (signal/noise ratio 3:1) was 0.9 nM. The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 μL/L dibutylamine, and 77 mg/L 1-octanesulfonic acid sodium salt, 10% methanol (pH = 3.3). The concentration of each compound was calculated by comparison with both external and internal standards as nmol/gram tissue weight.

*RNA isolation*

Brain tissue from the PFC, SSC, amygdala and hippocampus was homogenised and total RNA was isolated using the RNeasy mini isolation kit (Qiagen). 1 volume of EtOH was added to the lysate and mixed thoroughly by pipetting. Afterwards 700 μL sample was transferred to the RNeasy Mini spin column placed in a collection tube and centrifuged for 15 s at 14,000 rpm and the flow-through was discarded. 80 μL of RNase-Free DNase was added to the column and incubated for 15 min. Then 350 μL RW-buffer was added and the columns were centrifuged for 15 s at 14,000 rpm. Next 500 μL RPE was added to the column and centrifuged for 15 s at 14,000 rpm and this step was repeated once. After each centrifugation the flow-through was discarded. Finally, 30 μL RNase-free water was added to the column and centrifuged for 1 minute at 14,000 rpm.

*cDNA synthesis and qRT-PCR*

cDNA was synthesised using 500 ng total RNA and 4 μL iScript reaction mix and 1 μL reversed transcriptase from the iScript cDNA synthesis kit (Bio Rad). qRT-PCR was performed using 2 μL cDNA, 6 μL iQ SYBR green supermix (Bio Rad) and 0.6 μL qPCR primers.

## SCFA measurements

The caecal contents, which were stored until analysis at -80 °C, were thawed on ice, weighed and ten times diluted in PBS buffer (150 mM NaCl, 10 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, pH 7.4). The suspensions were homogenised and centrifuged for 10 min at 14,000 $g$. Next, the supernatant was heated for 10 min at 100 °C to inactivate all enzymes and centrifuged again. The SCFAs acetic, propionic, n-butyric, isobutyric and n-valeric acids were quantitatively determined by gas chromatography as described previously (17) using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and column selective for organic acids (ZB-FFAP) (Phenomenex, Torrance, USA).

**Table S1**. Diet composition based on AIN-93G

|  | Control | scGOS:lcFOS |
|---|---|---|
|  | g/kg diet | g/kg diet |
| **Protein** |  |  |
| Casein | 200.0 | 200.0 |
| **Carbohydrates** |  |  |
| Corn starch | 397.5 | 370.0 |
| Maltodextrin | 132.0 | 120.0 |
| Dextrose monohydrate phase | 100.0 | 92.0 |
| GOS sirop Vivinal (45% GOS) | 0.0 | 60.0 |
| Inulin HP (lcFOS) (97% FOS) | 0 | 3.0 |
| Cellulose | 50.0 | 50.0 |
| **Fat** |  |  |
| Soybean oil | 70.0 | 70.0 |
| **Others** |  |  |
| Mineral mix AIN-93G | 35.0 | 35.0 |
| Vitamin mix AIN-93G | 10.0 | 10.0 |
| L-cysteine | 3.0 | 3.0 |
| Tert-butylhydroquinone | 0.014 | 0.014 |

**Table S2**. Number of pups emitting calls or not

|  | Control diet | scGOS:lcFOS enriched diet |
|---|---|---|
| No calls | 6 | 6 |
| At least 1 call | 11 | 8 |

**Table S3**. Summary of sequence depths from 16S rDNA-sequencing

| Sequence depth | Dams | Pups | Grand total |
|---|---|---|---|
| **Control** | | | |
| N | 9 | 40 | **49** |
| Mean | 348,459 | 333,932 | **336,600** |
| StdDev | 179,397 | 228,942 | **219,050** |
| **scGOS:lcFOS** | | | |
| N | 6 | 40 | **46** |
| Mean | 358,701 | 368,786 | **367,471** |
| StdDev | 234,510 | 224,462 | **223,132** |
| **Total N** | **15** | **80** | **95** |
| **Total mean** | **352,556** | **351,359** | **351,548** |
| **Total StdDev** | **195,086** | **225,955** | **220,402** |

**Table S4.** Relative abundances with SD (‰) of top 10 responding bacterial genera for treatment over time with adjustment for age identified by the Principal Response Curves analysis. Monte Carlo Permutation Test (MCPT) with 1,000 permutations showed a significant interaction ($P = 0.001$) of treatment with time for the PRC-analysis. Differential abundance testing was performed with the metagenomeSeq-package on the top 10 identified bacterial taxa. The treatment differences that were found significant at a specific timepoint (with correction for multiple testing by false discovery rate < 0.1) are shown in bold. Test = scGOS:lcFOS group.

| Taxa | Test vs Control | week 4 | | week 6 | | week 8 | | week 11 | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | Test | Control | Test | Control | Test | Control | Test |
| Ba\|Bacteroidales\|ratAN060301C\|uncultured bacterium | increased | **8.17** **(1.84)** | **23.22** **(18.40)** | 15.50 (11.46) | 37.16 (20.65) | **14.83** **(13.02)** | **42.27** **(26.56)** | 10.42 (7.03) | 35.60 (29.20) |
| Fi\|Erysipelotrichales\|Erysipelotrichaceae\|Other | increased | 4.98 (5.01) | 4.79 (3.21) | 6.01 (3.84) | 13.15 (6.76) | **1.63** **(0.80)** | **7.53** **(4.95)** | **1.45** **(0.31)** | **8.13** **(4.14)** |
| Fi\|Erysipelotrichales\|Erysipelotrichaceae\|uncultured | increased | 0.80 (0.39) | 1.59 (1.99) | 0.92 (0.62) | 2.27 (2.87) | 0.67 (0.35) | **2.66** **(2.59)** | 0.53 (0.18) | 2.33 (2.17) |
| Ba\|Bacteroidales\|Bacteroidaceae\|Bacteroides | increased | **42.90** **(11.24)** | **102.18** **(89.88)** | 50.06 (25.39) | 73.56 (24.45) | **26.85** **(19.75)** | **66.28** **(27.82)** | **17.28** **(8.73)** | **52.30** **(30.71)** |
| Fi\|Clostridiales\|Lachnospiraceae\|Dorea | increased | **8.85** **(2.41)** | **17.14** **(7.02)** | 13.70 (14.09) | 16.87 (10.33) | **8.56** **(3.19)** | **17.88** **(8.17)** | **10.93** **(3.12)** | **23.04** **(8.34)** |
| Ba\|Bacteroidales\|S24-7\|Ambiguous_taxa | increased | **39.48** **(10.86)** | **74.87** **(26.64)** | 36.67 (11.00) | 43.25 (14.10) | **39.58** **(6.32)** | **60.94** **(17.80)** | 46.22 (20.28) | 62.09 (16.12) |
| Fi\|Clostridiales\|Peptostreptococcaceae\|Ambiguous_taxa | decreased | 0.05 (0.04) | 0.01 (0.01) | 0.06 (0.07) | 0.02 (0.01) | **0.11** **(0.08)** | **0.02** **(0.00)** | **0.18** **(0.13)** | **0.02** **(0.01)** |
| Fi\|Clostridiales\|Family XIII\|Anaerovorax | decreased | **0.16** **(0.07)** | **0.04** **(0.03)** | 0.14 (0.10) | 0.04 (0.02) | **0.26** **(0.22)** | **0.05** **(0.02)** | **0.23** **(0.11)** | **0.05** **(0.02)** |
| Fi\|Clostridiales\|Peptostreptococcaceae\|Other | decreased | **0.73** **(0.52)** | **0.14** **(0.03)** | 0.72 (0.45) | 0.19 (0.06) | **1.64** **(1.11)** | **0.23** **(0.08)** | **2.25** **(1.36)** | **0.37** **(0.11)** |
| Fi\|Clostridiales\|Peptostreptococcaceae\|uncultured | decreased | 26.94 (21.80) | 5.81 (1.35) | 45.16 (34.91) | 7.87 (1.14) | **60.12** **(40.51)** | **7.60** **(1.01)** | **91.14** **(55.89)** | **10.32** **(2.11)** |

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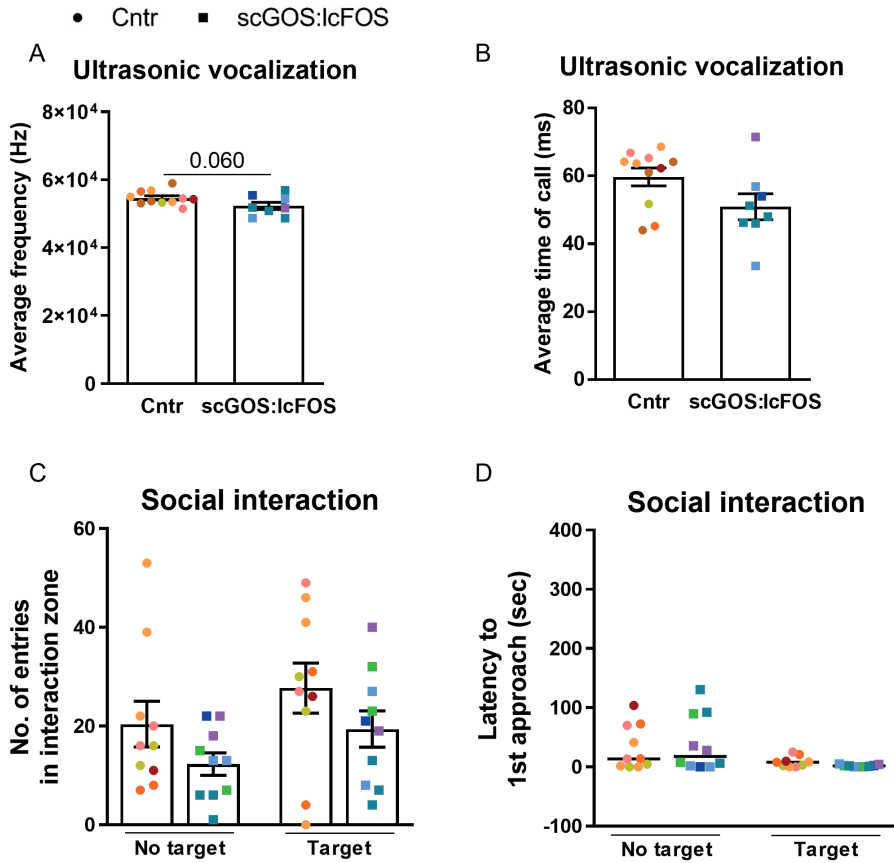
**Figure S1.** Ultrasonic vocalization and social interaction. The average frequency of a USV call (A) trended towards a decrease in the scGOS:lcFOS group and no difference in the average time between the dietary groups (B). (C) The number of times a mouse entered the interaction zone was not different between the control and the scGOS:lcFOS group neither in presence nor absence of a target mouse. (D) Also the latency to first approach was not different between the diets neither in presence nor absence of a target mouse. In presence of target one control mouse and one scGOS:lcFOS receiving mouse were excluded as significant outliers (Grubbs test 300 s and 22.2 s, respectively). A-C: Data shown as individually data points, colour indicating the litter, and mean +/- SEM. D: Data shown as median. A-C: Analysed with linear mixed models followed by Sidak's multiple comparison post hoc test. D: Analysed by Mann Whitney. A: control group $n$ = 11, scGOS:lcFOS group $n$ = 7. C,D: $n$ = 10 per group.
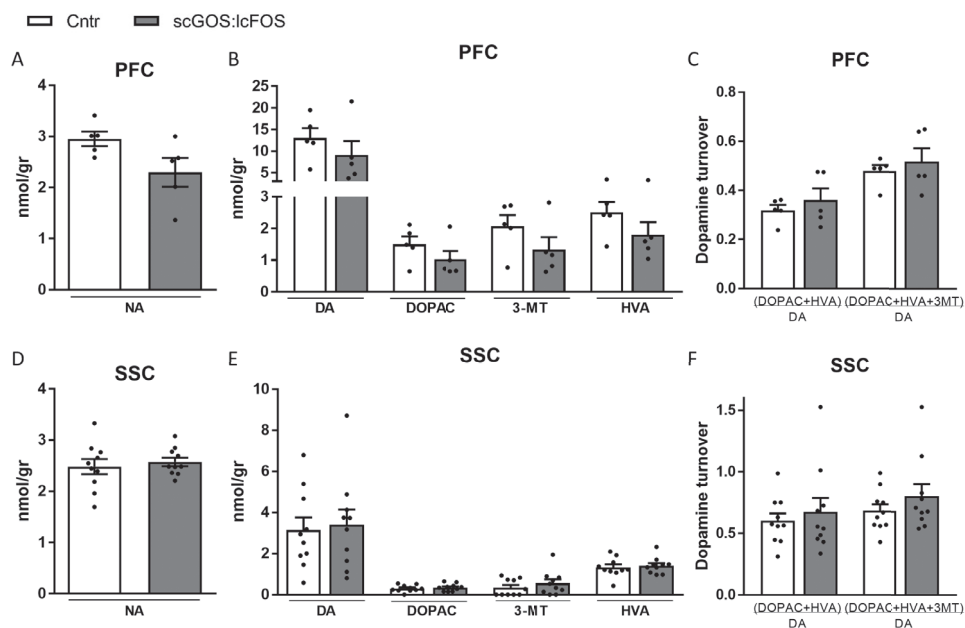
**Figure S2**. The PFC levels of noradrenaline (A), dopamine, dopamine metabolites (B) and the dopamine turnover (C) were not different between the dietary groups. The SCC levels of noradrenaline (D), dopamine and dopamine metabolites (E) and the dopamine turnover (F) were also not different between the dietary groups. A-F: Data shown as individually data points and mean +/- SEM. A-F: Analysed by Student's *t*-test. A-C: *n* = 5 per group; D-F: *n* = 10. NA: noradrenaline; DA: dopamine; DOPAC: 3,4 dihydroxyphenylacetic acid; 3-MT: 3-methoxytyramine; HVA: homovanillic acid.
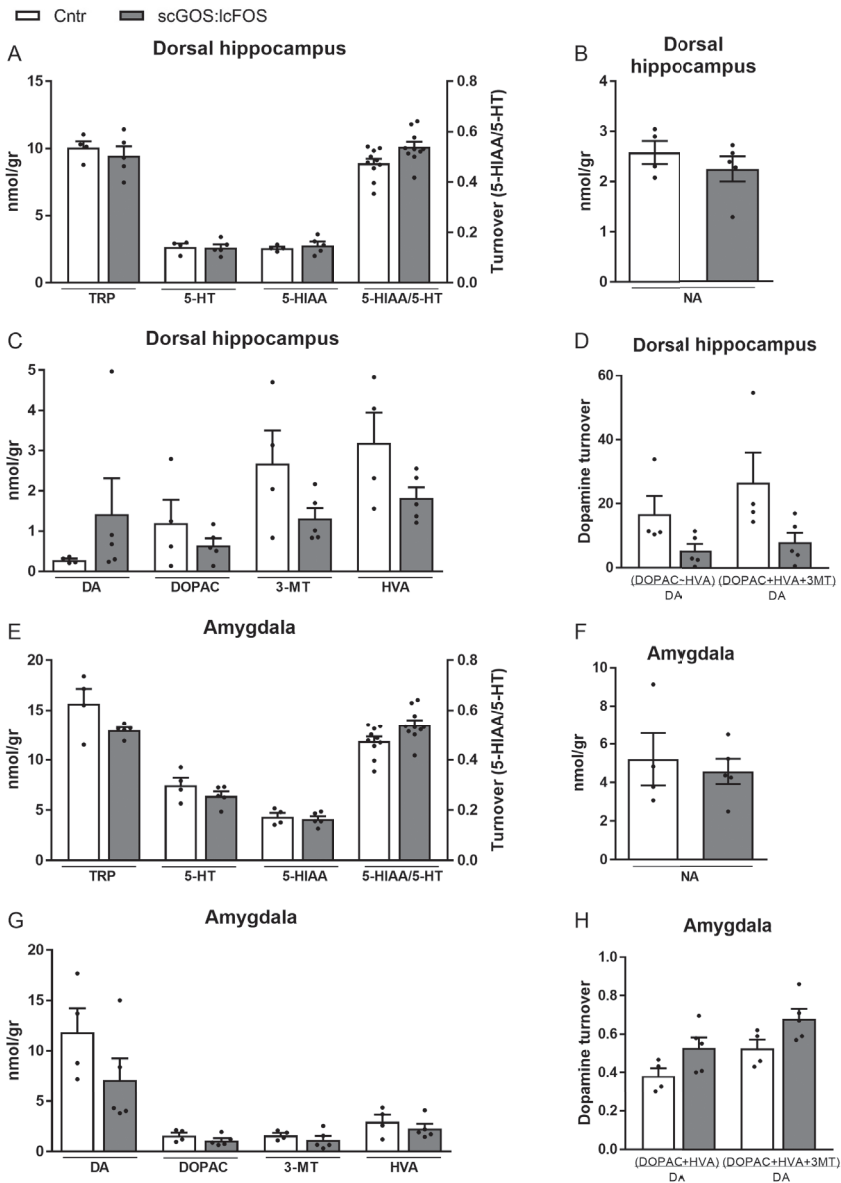
**Figure S3**. The hippocampal levels of tryptophan, 5-HT, the 5-HT metabolite 5-HIAA, serotonin turnover (5-HIAA/5-HT) (A), noradrenaline (B), dopamine, dopamine metabolites (C) and dopamine turnover (D) were not different between the dietary groups. The amygdala levels of tryptophan, 5-HT, the 5-HT metabolite 5-HIAA serotonin turnover (5-HIAA/5-HT) (E), noradrenaline, dopamine, dopamine metabolites (G) and the dopamine turnover (H) were also not different between the dietary groups. A-H: Data shown as individually data points and mean +/- SEM. A-H: Analysed by Student's $t$-test. A-H: $n$ = 4-5 per group. TRP: tryptophan, 5-HT: 5-hydroxytryptamine, 5-HT: serotonin, 5-HIAA: 5-hydroxyindolacetic acid, NA: noradrenaline; DA: dopamine; DOPAC: 3,4 dihydroxyphenylacetic acid; 3-MT: 3-methoxytyramine; HVA: homovanillic acid.
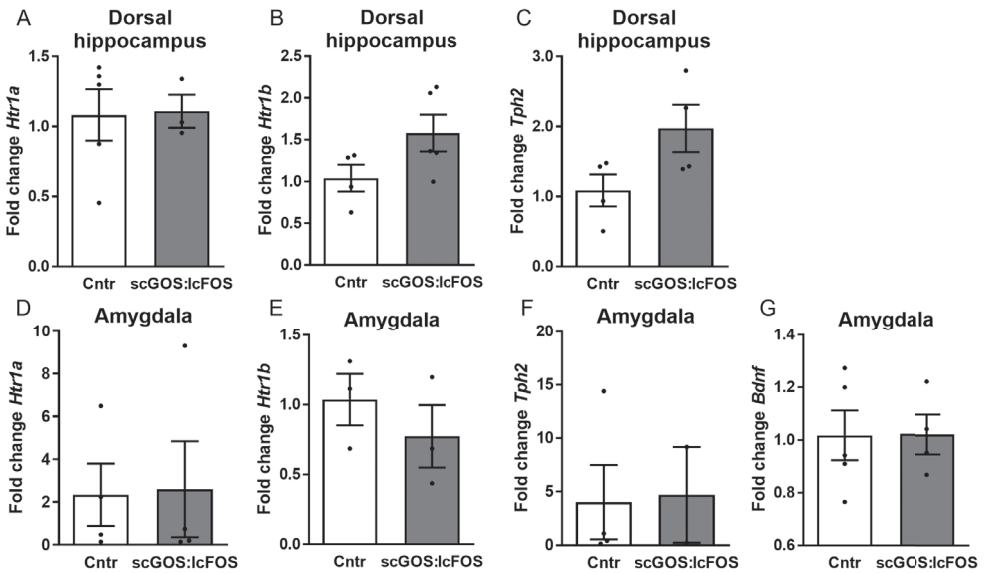
**Figure S4.** The mRNA expression of *Htr1a*, *Htr1b, Tph2* and *Bdnf* in the hippocampus and amygdala. In the hippocampus the mRNA expression of *Htr1a* (A), *Htr1b* (B) and *Tph2* (C) were unchanged. In the amygdala the mRNA expression of *Htr1a* (D), *Htr1b* (E) and *Tph2* (F) were unchanged and also *Bdnf* mRNA expression was not affected by scGOS:lcFOS. A-F: Data shown as individually data points and mean +/- SEM. A-F: Analysed by Student's *t*-test. A-F: *n* = 2-5 per group. *Htr1a*: serotonin receptor 1a, *Htr1b*: serotonin 1b receptor, *Tph2*: tryptophan hydroxylase 2, *Bdnf*: brain derived neurotrophic factor.
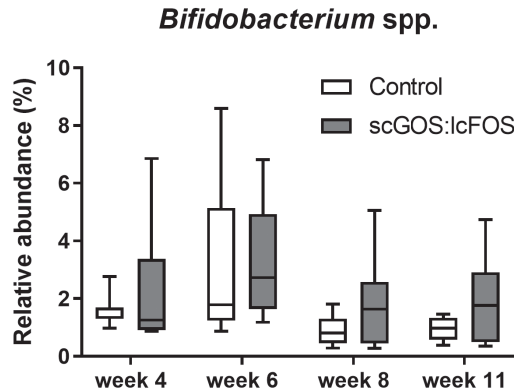


**Figure S5.** The relative abundance (percentage) of *Bifidobacterium* spp. at different timepoints. The mice receiving scGOS:lcFOS showed enhanced levels of *Bifidobacterium* spp. compared to control, however, the differences were not significant. Data shown as box plots with whiskers showing min-max, boxes the interquartile range and line at median. Analysed by two-way ANOVA and Sidak's multiple comparisons post hoc test. *n* = 10 mice per group.

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# CHAPTER 4

Dietary supplementation throughout life
with non-digestible oligosaccharides and/or
omega-3 polyunsaturated fatty acids
in healthy mice modulates the gut
– immune system – brain axis

Kirsten Szklany[1], Philip Engen[2], Ankur Naqib[2], Stefan J. Green[3], Ali Keshavarzian[2,4], Alejandro Lopez Rincon[1], Cynthia J. Siebrand[1], Mara A. P. Diks[1], Melanie van de Kaa[1], Johan Garssen[1,5], Leon M. J. Knippels[1,5], Aletta D. Kraneveld[1,6]

[1] Division of Pharmacology, Utrecht Institute of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[2] Rush Medical College, Rush Center for Integrated Microbiome and Chronobiology Research, Rush University Medical Center, Chicago, IL, USA
[3] Genomics and Microbiome Core Facility, Rush University Medical Center, Chicago, IL, USA
[4] Department of Medicine & Physiology, Rush University Medical Center, Chicago, IL, USA
[5] Danone Nutricia Research, Utrecht, The Netherlands
[6] Veterinary Pharmacology & Therapeutics, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

## ABSTRACT

The composition and activity of the intestinal microbial community structures can be beneficially modulated by nutritional components such as non-digestible oligosaccharides and omega-3 polyunsaturated fatty acids (n-3 PUFAs). These components affect immune function, brain development and behaviour. We investigated the additive effect of a dietary combination of short-chain galacto-oligosaccharides(scGOS):long-chain fructo-oligosaccharides (lcFOS) mixture and n-3 PUFAs on caecal content microbial community structures and development of the immune system, brain and behaviour from day of birth to early adulthood in healthy mice. Male BALB/cByJ mice received a control or enriched diet with a combination of scGOS:lcFOS (9:1) and 6% tuna oil (n-3 PUFAs) or individually scGOS:lcFOS (9:1) or 6% tuna oil (n-3 PUFAs). Behaviour, caecal content microbiota composition, short-chain fatty acid levels, brain monoamine levels, enterochromaffin cells and immune parameters in the mesenteric lymph nodes (MLN) and spleen were assessed. Caecal content microbial community structures displayed differences between the control and dietary groups, and between the dietary groups. Compared to control diet, the scGOS:lcFOS and combination diets increased caecal saccharolytic fermentation activity. The diets enhanced the number of enterochromaffin cells. The combination diet had no effects on the immune cells. Although the dietary effect on behaviour was limited, serotonin and serotonin metabolite levels in the amygdala were increased in the combination diet group. The combination and individual interventions affected caecal content microbial profiles, but had limited effects on behaviour and the immune system. No apparent additive effect was observed when scGOS:lcFOS and n-3 PUFAs were combined. The results suggest that scGOS:lcFOS and n-3 PUFAs together create a balance – the best of both in a healthy host.

# INTRODUCTION

The gut microbiota is the total collection of microbial organisms within a community. The microbiota evolves and adapts to its host over a lifetime and microbiota activities have significant consequences for the host in terms of health and disease. The development of the intestinal microbiota during early life appears to be essential in the maturation of the immune system, adaptation of intestinal tissue morphology, as well as the development of the brain and behaviour (1-3). In the first phase of life, microbiota development can be modified by several factors, such as mode of delivery (caesarean section versus vaginal delivery), antibiotic use and mode of feeding (formula versus breastfeeding) (4). Consequently, impaired early life development of the gut microbiota could lead to a disturbed maturation of the immune system and brain, possibly resulting in increased risk of developing immune and brain disorders, respectively (1). Therefore, healthy development of the intestinal microbiota is important.

For the maturation of the immune system, exposure to environmental factors such as food antigens is essential. This environmental exposure is necessary for the immune system to develop tolerance towards harmless components. The precise mechanism of tolerance development is unknown, but the differentiation of regulatory T cells (Tregs) in Peyer's patches and mesenteric lymph nodes (MLNs) could play an important role (5). As mentioned above, the maturation of the immune system is also influenced by the intestinal microbiota. Short-chain fatty acids (SCFAs) play a role in the development of mucosal tolerance of food antigens. The SCFAs are metabolites from the intestinal microbiota fermentation of carbohydrates. They have immunomodulatory capacities and are able to induce the differentiation and activity of Tregs, which in turn can result in tolerance development (6, 7). In the absence of an intestinal commensal microbiota, the immune system is deprived. This deprivation is manifested by functional defect Tregs and an exaggerated systemic type-2 immune response (8, 9). These data indicate the essential role of the intestinal microbiota in immune system development.

It is widely accepted that the intestinal microbiota plays an important role in the development of the brain and behaviour. Animals without intestinal microbes, such as germ-free mice and antibiotic-treated animals, have shown reduced anxiety-like behaviour and impaired social behaviour (10-12). These altered behaviours in germ-free mice are accompanied by disturbed neurotransmission in the central nervous system, where serotonin levels are increased compared to conventional mice (12, 13). To emphasise the importance of the intestinal microbiota in behaviour, these behavioural deficits were alleviated after microbial re-colonisation (10-12). To imply causality of the modulation of behaviour by intestinal microbiota in neurobehavioural disorders, faecal microbiota transplant (FMT) has been used. FMT from attention-deficit hyperactivity disorder and autism spectrum disorder (ASD) individuals into germ-free mice, resulted in increased anxiety, repetitive and impaired social behaviour (14, 15). Transfer of anxiety and depression behavioural traits also occurred via the intestinal microbiota in both mice and rats (16). Moreover, in preclinical models of autism spectrum, depression and anxiety disorders, administration of specific bacteria restored the behavioural deficits (16).

Tryptophan is an essential amino acid that is metabolised into indole, serotonin (5-HT) and kynurenine. In the intestine, tryptophan first encounters bacteria, some of these bacteria are tryptophan utilising and metabolise tryptophan into 5-HT and/or indole (17, 18). Indole is able to modulate host physiology, for example integrity of intestinal epithelial barrier (19). Then, tryptophan encounters intestinal epithelial cells, where tryptophan in enterochromaffin cells is converted into 5-HT. The basolateral released 5-HT interacts with the enteric nervous system and through vagal afferent nerve signalling reaches the central nervous system (17). The secretion of 5-HT from enterochromaffin cells can be mediated by SCFAs and thus indirectly by intestinal bacteria (20). Lastly, most of the tryptophan available is metabolised through the kynurenine pathway. Kynurenine is further metabolised into neuroprotective kynurenic acid and neurotoxic quinolinic acid (18). The intestinal microbiota might indirectly affect the kynurenine pathway (19); for example, *L. reuteri* was associated with decreased circulating kynurenine and normalised stress-induced behaviour (21). In germ-free mice, the levels of plasma 5-HT and tryptophan are elevated, indicating the reduced metabolism of tryptophan (18, 19).

It is well acknowledged that diet plays a significant role in shaping the intestinal microbiota. Microbial community structures can be beneficially modulated by nutritional components such as non-digestible oligosaccharides (NDOs) with prebiotic function and omega-3 polyunsaturated fatty acids (n-3 PUFAs) (22-26). These dietary components have been shown to be important in immune function as well as brain development and behaviour (27-31).

Specific NDOs in, called human milk oligosaccharides (HMOs), are the third most abundant milk solid component in human breast milk. These prebiotics are essential in the development of the immune system (32). In infant formula, which is the alternative to breastfeeding, NDOs such as a mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) are added to mimic the modulatory effects of HMOs. scGOS:lcFOS modulates the activity and growth of intestinal bacteria to shape a healthy intestinal microbiota (23, 26, 33). Additionally, scGOS:lcFOS modulates the immune system directly, i.e., by upregulating IL-10 generation by dendritic cells (DCs) and inducing Tregs (34, 35). This is important to stimulate development of tolerance and for skewing from a T helper 2 (Th2) response at birth towards a Th1 response (36, 37). This skewing might decrease the risk of developing chronic inflammatory diseases such as allergies. Prebiotics also have the capacity to influence brain development and behaviour (31, 38-40). Specific prebiotics are indicated to affect the serotonergic system and to modulate behaviour in *in vivo* models where social behaviour is improved and anxiety-like and depression-like behaviour are reduced (31, 38, 40).

n-3 PUFAs play an important role in the development of the immune system and the brain and, according to International Scientific Association for Probiotics and Prebiotics (ISAPP), are prebiotic candidates (41). These fatty acids are known to exert anti-inflammatory effects and also induce the skewing from a Th2 to a Th1 immune response early in life, which, as mentioned above, is important to reduce the risk of developing allergies (42-

44). n-3 PUFAs are essential in the development of the brain. They are incorporated in the neuronal cell membrane and play an essential role in supporting brain function throughout life (45, 46). Two of the most important n-3 PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Deficiencies in n-3 PUFAs in mice have resulted in depression-like behaviour and impaired social behaviour (25, 47). Moreover, both EPA and DHA supplementation in rats have led to reduced anxiety-like behaviour (48). Although there is limited evidence, n-3 PUFAs are probably able to modulate intestinal microbiota composition. Dietary supplementation with n-3 PUFAs resulted in changed faecal bacterial taxa depicting lower abundances of the genus *Coprococcus* and higher abundances of the genera *Bifidobacterium*, *Oscillospira* and *Lactobacillus* (25, 49). These data might indicate that, in addition to affecting the immune system and the brain development directly, n-3 PUFAs are able to influence these systems through microbial modulation of the intestinal microbiota (25, 49).

Based on the data that both scGOS:lcFOS and n-3 PUFAs modulate the intestinal microbiota and beneficially affect both the immune system and brain development, we hypothesised that a combination of scGOS:lcFOS and n-3 PUFAs can result in an additive effect. To evaluate this hypothesis, we investigated the effect of a combined dietary mixture of scGOS:lcFOS and n-3 PUFAs on caecal content microbiota and activity and the development of the immune system, the brain and behaviour from day of birth in healthy mice.

## MATERIALS AND METHODS

### Animals

Sixteen day pregnant BALB/cByJ were purchased from Charles River Laboratories (Maastricht, The Netherlands) and housed individually. The dams were allocated to the control (n = 3, male pups n = 8), scGOS:lcFOS (n = 4, male pups n = 10), n-3 PUFAs (n = 3, male pups n = 11) or the combination (n = 4, male pups n = 15) diet groups on the day their pups were born (postnatal day 0). The male mice were weaned on postnatal day 21 and continued the same diet as their mother until the end of the experiment (control n = 8, the supplementary diet groups n = 10, n = 1-5 from each litter). Fourteen age-matched male BALB/cByJ were purchased from Charles River to be used as interaction mice in the social interaction behavioural test (see behavioural tests). The male mice were housed in groups after weaning. All mice were housed in Makrolon IIL cages and had *ad libitum* access to food and water. A light/dark cycle of 12 h was followed and the experimental procedures, including the behavioural tests, were performed during the light phase. All animal experimental procedures were carried out in compliance with national legislation following the EU-Directive for the protection of animals used for scientific purposes and were approved by the Ethical Committee for Animal Research of Utrecht University (Approval number DEC 2014.I.12.090).

**The diets**

The enriched diets were based on AIN-93G diets with a fat percentage of 10%. The scGOS:lcFOS diet consisted of 3% scGOS (degree of polymerization 2-8, Friesland Campina, The Netherlands) and lcFOS (degree of polymerization on average ≥ 23, Orafti, Wijchen, The Netherlands) in a 9:1 (w/w) ratio. In the n-3 PUFA diet, 6% of the soybean oil was substituted with 6% tuna oil. The composition of the experimental diets is shown in **Table S1**. The tuna oil was a kind gift from Bioriginal (Den Bommel, The Netherlands). The combination diet contained 3% of the scGOS:lcFOS mixture and 6% tuna oil. The supplementations were added in an isocaloric manner. The diets were obtained from Ssniff Spezialdiäten gmbH (Soest, Germany).

**Experimental design**

A schematic overview of the experimental design is illustrated in **Figure 1**. Behavioural tests for social, explorative, stereotypic and anxiety-like behaviour were conducted during adolescence (6 weeks old) and early adulthood (8 weeks old). After decapitation, the brain, intestines, caecum content, MLNs and spleen were isolated for further analysis. For MLN isolation, the abdominal cavity was opened, the caecum was lifted, and the superior mesenteric lymph nodes were isolated. Working towards the middle of the mesentery, the inferior lymph nodes were isolated.
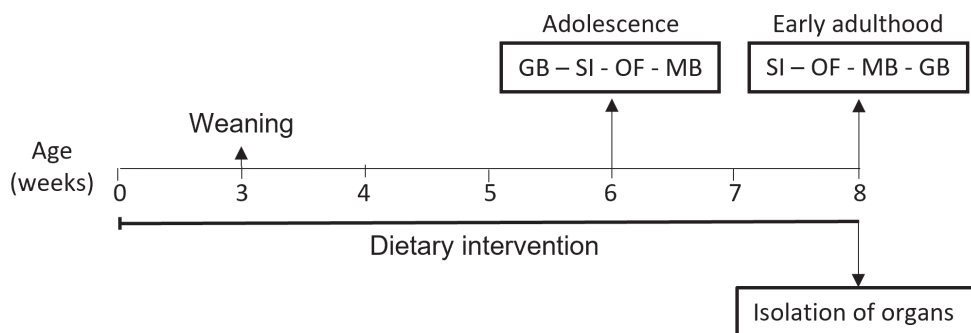


**Figure 1.** Schematic overview of the experimental protocol and the executed behavioural tests. From day of birth, the dams received a control diet, a 3% scGOS:lcFOS (9:1) enriched diet, a n-3 PUFAs diet or a combination diet containing 3% scGOS:lcFOS and n-3 PUFAs. The pups were weaned when 3 weeks old and continued on the allocated diet to the end of the experiment. During adolescence and early adulthood, a battery of behavioural tests was conducted. Organs were collected after the last behavioural test. GB: grooming behaviour, SI: social interaction test, OF: open field test, and MB: marble burying test.

**Microbiota profiling and bioinformatics analyses**

Total DNA was extracted from mice caecal contents utilising the FastDNA bead-beating Spin Kit for Soil (MP Biomedicals, Solon, OH, USA), and DNA concentrations were measured via fluorometric quantitation (Qubit 1.0, Life Technologies, Grand Island, NY, USA). Primers 515F (Caporaso)–806R (Caporaso) targeting the variable region four (V4)

of microbial small subunit (SSU or 16S) ribosomal RNA (rRNA) genes were used for PCR (50), and prepared for high-throughput amplicon sequencing using a modified two-step targeted amplicon sequencing (TAS) approach (51). Negative controls were used with each set of amplifications, which indicated no contamination. Samples were pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The library pool was purified using an AMPure XP cleanup protocol (0.6x, *v/v*; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq (Illumina, San Diego, CA, USA) mid-output flow cell (2 x 153 paired-end reads) and sequenced using Fluidigm sequencing primers. Based on the distribution of reads per barcode, the amplicons (before purification) were re-pooled to generate a more balanced distribution of reads. The re-pooled and re-purified libraries were then sequenced on a high-output MiniSeq run (2 x 153 paired-end reads). Library preparation, pooling, and sequencing were performed at the Genome Research Core (GRC) at the University of Illinois at Chicago (UIC). Raw sequence data (FASTQ files) were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), under the BioProject identifier PRJNA701436.

Raw FASTQ files for each sample were merged using the software package PEAR (Paired-end-read merger) (v0.9.8) (Dalhousie University, Halifax, Nova Scotia, NS, Canada (http://www.exelixis-lab.org/web/software/pear (accessed on 28 December 2021)) (52, 53). Merged reads were quality trimmed, and primer sequences removed. Sequences shorter than 250 bases were discarded (CLC Genomics Workbench, v10.0, CLC Bio, Qiagen, Boston, MA, USA). Sequences were screened for chimeras (usearch8.1 algorithm) (54), and putative chimeric sequences were removed from the dataset (QIIME v1.8, Quantitative Insights Into Microbial Ecology, Knight Lab at the University of Colorado at Boulder, Boulder, CO, USA) (55). Each sample was rarefied (45,000 sequences/sample) and data were pooled, renamed, and clustered into operational taxonomic units (OTU) at 97% similarity (usearch8.1 algorithm). Representative sequences from each OTU were extracted and classified using the uclust consensus taxonomy assigner (Greengenes 13_8 reference database). A biological observation matrix (BIOM) (56) was generated at each taxonomic level from phylum to species ("make OTU table" algorithm) and analysed and visualised using the software packages Primer7 (57) (PRIMER-E Ltd., Lutton, UK) and the R programming environment (58).

**Caecum short-chain fatty acids levels**
The levels of SCFAs were analysed as previously described (59). In short, the caecal contents were stored at -80 °C until analysis. After being defrosted on ice the samples were diluted in PBS and homogenised followed by centrifugation for 10 min at 14,000 *g*. Next, the supernatant was heated for 10 min at 100 °C to inactivate all enzymes and centrifuged again. The SCFAs acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids were quantitatively determined by gas chromatography using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) as described previously (59).

## Immunohistochemistry analysis of 5-HT positive cells in jejunum, ileum and colon

The intestinal 5-HT-positive cells were determined following the protocol previously described (60). After isolation jejunal, ileal and colonic tissue (n = 6 of each tissue) were opened longitudinally, rolled in the direction from distal to proximal and fixed in 10% formalin for at least 24 h and embedded in paraffin. The 5 µm tissue sections (8 tissue sections from each swiss roll) were incubated in 0.3% $H_2O_2$ in methanol for 30 min to block endogenous peroxidase activity. Sections were rehydrated in ethanol and incubated with Proteinase K (DAKO, Code S3020). Non-specific staining was blocked with 5% goat serum, and sections were incubated overnight at 4 °C with the primary antibody mouse anti-5-HT (DAKO, Code M0758) 1:100. The following day, sections were incubated with biotinylated goat anti-mouse (DAKO, Code E0433) 1:200, followed by incubation with sABC complex 1:100. Staining was visualised by incubating the sections in the dark with 1x DAB solution for 10 min at room temperature. Nuclear staining with Mayer's haematoxylin has been performed for 10 s. Digital images were captured using the software Image Pro (Media Cybernetics, Rockville, MD, USA) and an Olympus BX50 light microscope with a Leica DFC 320 digital camera. In colonic tissue, 5-HT positive cells in the epithelial layer were counted in 10 consecutive crypts on five different places per tissue section. These colonic crypts covered 461 x 187 µm (8.6 x 104 µm$^2$). For tissue sections of the jejunum and ileum, 5-HT positive cells were counted in the same area size of 461 x 187 µm (8.6 x 104 µm$^2$) on five different places per section. Due to differences in crypt sizes, using a consistent area size was the most practical way to make comparable measurements.

## Cell isolation from MLNs and spleen

Single-cell suspensions of the MLNs and spleen were obtained by crushing the organs through a 70 µm cell strainer. The splenocytes were incubated with a lysis buffer (8.3 g/L $NH_4Cl$, 1 g/L $KHCO_3$, and 37.2 mg/L EDTA) to lyse the red blood cells. The cell suspensions were resuspended in PBS + 1% BSA (Sigma-Aldrich, St. Louis, MO, USA).

## Flow cytometry analysis of immune cells of MLNs and spleen

MLN and spleen single-cell suspensions were prepared for flowcytometry analysis as previously described (61). The cell suspensions were incubated with anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences, Franklin Lake, NJ, USA) in PBS + 1% BSA for 15 min on ice to block unspecific binding sites. Afterwards, cells were stained with the following surface markers CD4-PerCp-Cy5.5, CD69-PE-Cy7, CXCR3-PE, CD25-AlexaFluor488, CD25-PE, (all purchased from eBioscience) or T1ST2-FITC (MD Bioproducts, St Paul, MN, USA) for 30 min on ice. Fixable Viability dye eFluor 780 (eBioscience, San Diego, CA, USA) was used to exclude non-viable cells. Next, the cells were fixed and permeabilised with the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol and then stained with the intracellular markers Foxp3-PE-Cy7, RORγt-PE, IRF4-FITC, Tbet-eFluor660 or Gata3-eFluor660 (all purchased from eBioscience). Cells were measured on BD FACSCanto II flow cytometer, and results were analysed with FlowLogic software (Inivai Technologies, Mentone, Vic, Australia). The used gating strategy is shown in **Figure S1, S3** and **S4**.

## Monoamine levels in the amygdala, dorsal hippocampus and prefrontal cortex

The levels of the monoamines noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), and tryptophan (TRP) were measured in the amygdala, prefrontal cortex (PFC) and dorsal hippocampus (DH) by HPLC with electrochemical detection as previously described (60, 62). In these brain regions 5-HT has a role in regulating anxiety and social behaviour (63, 64). Samples were pooled in pairs to reach detection minimum. Each sample contained two left brains (n = 4-5 per group). The frozen tissue samples were homogenised in an ice-cold solution containing 5 µM pargyline and 0.6 µM N-methylserotonin (NMET, internal standard). To 50 µL homogenate, 12.5 µL 2 M $HClO_4$ was added, mixed, and placed in ice-water. Thereafter, the homogenates were centrifuged for 15 min at 15,000 $g$ (4 °C). The supernatants were diluted 10 times with mobile phase, which contained 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 µL/L dibutyl amine, 77 mg/L 1-octanesulfonic acid sodium salt, 10% methanol. The pH of the buffer was adjusted to 3.4 with NaOH. The settings of the HPLC system were previously described in de Theije *et al.* (60). Separation was conducted at 40 °C using a flow rate of 0.8 mL/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The detection limit was 0.9 nM (signal/noise ratio 3:1). The turnovers were calculated by dividing the metabolite concentration by the monoamine concentration (5-HIAA/5-HT and (DOPAC + 3-MT + HVA)/DA).

## Behavioural tests

Anxiety-like and repetitive behaviours were assessed by the marble burying test as previously described (31, 65). Briefly, twenty black marbles were placed on the bedding in a cage (L35 x W20 x H15 cm) and the mice were placed individually. After 30 min the number of marbles buried for $^2/_3$ in the bedding was counted. Self-grooming was determined as previously described (66). To measure self-grooming, in short, each mouse was placed individually in an empty cage for 10 min (first 5 min were considered as habituation period) and the cumulative time spent grooming and the frequency of grooming were analysed. Social behaviour was determined as previously described (66). In brief, in an open field of L45 x W45 cm, a perforated plexiglass cage allowing visual and olfactory interaction was placed against a side. The mice were habituated for 5 min in the open field, followed by 5 min with an age- and sex-matched unfamiliar mouse in the plexiglass cage. Time in interaction zone and distance moved were analysed. The open field test was used to evaluate explorative behaviour, and the procedure was adapted from Seibenhener et al. (67, 68). The mice were individually placed in the centre of an open field (L45 x W45 cm), cleaned with 70% alcohol, and recorded for 5 min with a Sony Handycam DCR-SR72 video camera. The time spent in the open field and locomotor activity were blindly analysed using the tracking software (Ethovision 3.1.16; Wageningen, The Netherlands).

**Statistical analysis**

α-diversity indices (within-sample) and β-diversity (between-sample) were used to examine changes in microbial community structures between control and different dietary mice samples. α-diversity metrics (i.e., Shannon index, richness and evenness) were calculated from rarefied datasets (45,000 sequences/sample) using the package 'vegan' implemented in the R programming language (https://cran.r-project.org (accessed on 28 December 2021), https://github.com/vegandevs/vegan (accessed on 28 December 2021)). Differences in α-diversity indices between groups were assessed for significance using one-way analysis of variance (ANOVA) test and Sidak's post hoc test. To examine β-diversity differences in microbial community composition between samples, the pairwise Bray-Curtis dissimilarity (non-phylogenetic) metric was generated using the Primer7 software package and used to perform analysis of similarity (ANOSIM) calculations. ANOSIM was performed at the taxonomic level of genus, using square root transformed data, with 999 permutations, and data were visualised using non-multi-dimensional scaling (nMDS) incorporating taxa with strong Pearson's correlation (R > 0.6).

β-diversity differences in relative abundance of individual taxa, between mice group samples, were assessed for significance using Kruskal-Wallis test controlling for false-discovery rate (FDR) using the Benjamini-Hochberg correction, implemented within the software package QIIME1.8. Taxa with an average abundance of (< 1%) across the sample set were removed from the analysis. Furthermore, microbial relative abundances and Firmicutes-to-Bacteroidetes (F/B) ratios between conditions were studied. Additionally, an inferred 16S rRNA bacterial taxa model of the caecal content SCFA metabolite measurements were examined depicting the percent relative abundances of acetate, propionate, and butyrate-producing genera taxa (69, 70), as well as the SCFAs and branch-chain fatty acids (BCFAs). Based on the Shapiro-Wilks normality test, these analyses used either the parametric one-way ANOVA and Sidak's post hoc test or the non-parametric Kruskal-Wallis and Dunn's post hoc tests.

Furthermore, statistical analysis was performed by comparing the control diet group to the enriched diets and by comparing the scGOS:lcFOS and the n-3 PUFA groups to the combination diet group. All data except the behavioural data were analysed using one-way ANOVA and Sidak's multiple comparison post hoc test for selected comparisons. When not normally distributed or unequal variances, the data were transformed; if this failed, the non-parametric Kruskal-Wallis test was applied followed by Dunn's multiple comparison post hoc test for selected comparisons. Marble burying, open field, self-grooming and social interaction were analysed using mixed models, controlled for repeated measures, litter effect and Sidak's multiple comparisons test as post hoc analysis. Only grooming duration is not corrected for litter effect as the variance between dams is larger than the variance of the data. These data were considered statistically significant at ($P$ < 0.05).

**Recursive ensemble feature selection**

In order to achieve insight into which microbial and immune features influence specific behaviours, we have analysed the whole dataset (38 samples and 87 features) regarding the marble burying (number of marbles buried) and open field (frequency in zone) tests at the age of 8 weeks (early adulthood). For marble burying data, we assigned the label '0' for < 10 and '1' for ≥ 10 number of buried marbles. For the open field data, we assigned the label '0' for ≥ 10 and '1' for <10 number of entries into the zone. A feature selection algorithm for recursive ensemble feature selection (REFS) algorithm (71) was run based on the Borda Method (72). In this algorithm, 8 different classifiers were used from the sci-kit learning toolbox (73): Bagging, Random Forest, Logistic Regression, Gradient Boosting, Support Vector, Stochastic Gradient Descent, Passive Aggressive and Ridge. The algorithm assigns rankings to the features, depending on how they are used by the classifiers. Finally, this procedure was repeated in 10 cycles, where at each run, we reduce the number of features to 80%.

## RESULTS

**Caecum microbiota profiling and short-chain fatty acid concentrations**

Analysis of caecum content microbial communities at 8 weeks, using 16S ribosomal RNA gene amplicon sequencing, revealed that the microbial α-diversity indices were not significantly different between the control and three dietary groups, at the taxonomic level of genus (Shannon index: **Figure 2A**; evenness: **Figure 2B**). However, α-diversity was significantly higher in the combination diet mice compared to the scGOS:lcFOS mice (Shannon index ($P$ < 0.05): **Figure 2A**) and (evenness ($P$ < 0.01): **Figure 2B**). No significant differences in richness (observed species in a sample) between the four mice groups were observed (**Figure 2C**).
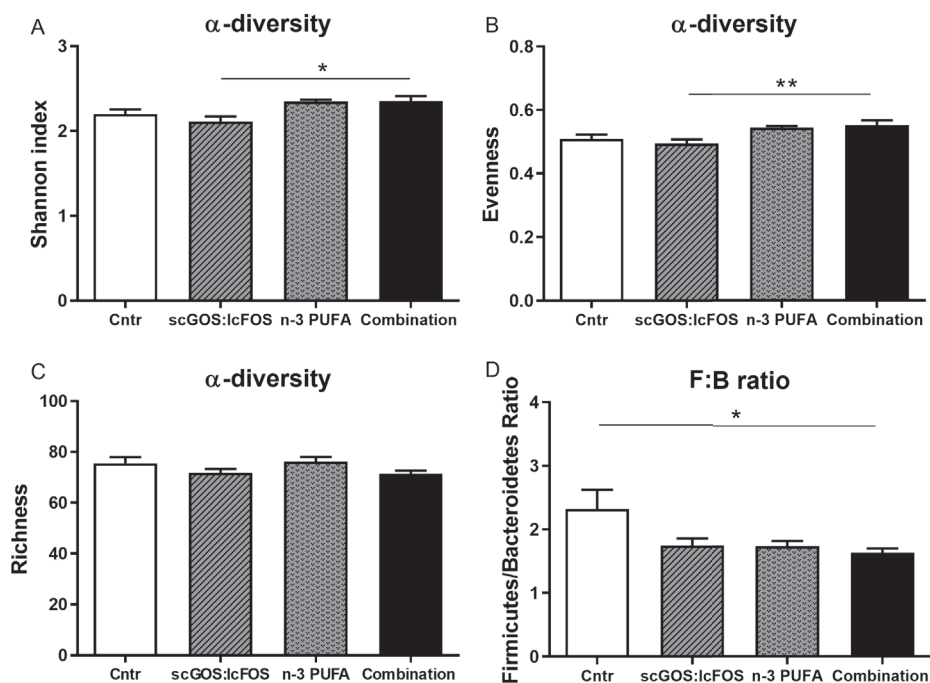
**Figure 2.** Caecum content α-diversity and Firmicutes-to-Bacteroidetes ratio for the control and dietary mice groups. α-diversity indices were examined at the taxonomic level of genus. α-diversity indices rarefied to 45,000 sequences per sample. Analysis of the (A) Shannon index and (B) evenness both indicated a significant dietary effect across all groups, with the combination diet diversity significantly higher than scGOS:lcFOS. (C) Richness diversity was not significantly different across groups. At the taxonomic level of phylum, the (D) Firmicutes-to-Bacteroidetes ratio significantly decreased in the combination diet mice compared to the control mice. Data were square root transformed for statistics. A-D: Data shown as the mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$. $n$ = 8-10 mice per group.

Significant differences in caecum content microbial community structure were observed between the control and each of the three dietary mice groups in β-diversity analyses conducted on bacteria genera (ANOSIM ($P$ = 0.001): **Table 1**). Additionally, the microbial communities differed between each dietary group (scGOS:lcFOS vs. combination, $P$ = 0.002; and n-3 PUFA vs. combination, $P$ = 0.001) (ANOSIM: **Table 1**). By incorporating Pearson's correlation, this analysis indicated individual genera that are strongly associated (R > 0.6) to either the microbial communities of the control group or the three dietary groups (nMDS: **Figure 3**). These results indicate that scGOS:lcFOS, n-3 PUFAs and the combination significantly altered the intestinal microbiota profiles.

At the taxonomic level of phylum, the Firmicutes-to-Bacteroidetes ratio was significantly decreased ($P$ < 0.05) in the combination diet group compared to the control group (**Figure 2D**). At the taxonomic level of genus, eight microbial features were significantly different (FDR-$P$ < 0.05) between groups, including *Allobaculum*, *S24-7* Unclassified,

*Oscillospira*, *Ruminococcaceae* Unclassified, *Turicibacter*, *Akkermansia*, *Lachnospiraceae* Unclassified and *Rikenellaceae* Unclassified (**Figure 4**). In comparison to the control mice, the relative abundances of *Allobaculum* and *S24-7* Unclassified were higher (**Figures 4A-B**), and butyrate-producing *Oscillospira* lower in the scGOS:lcFOS mice (**Figure 4C**). Both the relative abundances of propionate-producing genera *Turicibacter* (**Figure 4E**) and *Akkermansia* (**Figure 4F**) were increased in n-3 PUFA mice, compared to control mice. Furthermore, the microbial composition in the combination diet mice indicated similar significant relative abundance bacterial alterations as scGOS:lcFOS, in comparison to the control mice. However, the combination diet mice also had a significant decrease in the relative abundances of acetate-producing *Ruminococcaceae* Unclassified (**Figure 4D**) and butyrate-producing *Lachnospiraceae* Unclassified (**Figure 4G**), with a significant relative abundance increase in propionate-producing *Akkermansia* (**Figure 4F**) in comparison to the control mice.

Upon examining the microbial alterations between dietary groups, n-3 PUFA mice had significantly higher relative abundances of acetate-producing *Ruminococcaeae* Unclassified, propionate-producing *Turicibacter*, and *Rikenellaceae* Unclassified, but lower relative abundance of propionate-producing *Akkermansia*, when compared to the combination diet mice (**Figures 4D, E, F, H**). Lastly, the combination diet mice had a significantly higher relative abundance of propionate-producing *Akkermansia*, assessed to the scGOS:lcFOS mice (**Figure 4F**).

**Table 1.** Group analysis of similarity (ANOSIM) results for mouse caecum content microbiota compositions.

| Comparison – genus level | n | Global *R* | P-value[a] |
|---|---|---|---|
| Control vs scGOS:lcFOS | 8 | 0.600 | 0.001 |
| Control vs n-3 PUFA | 10 | 0.518 | 0.001 |
| Control vs Combination | 10 | 0.817 | 0.001 |
| scGOS:lcFOS vs Combination | 10 | 0.366 | 0.002 |
| n-3 PUFA vs Combination | 10 | 0.573 | 0.001 |

a = $P < 0.05$; Global *R* comparison was based on ANOSIM performed within the software package Primer7; P-values were calculated based on a permutational analysis, employing 999 permutations; square-root transformation.
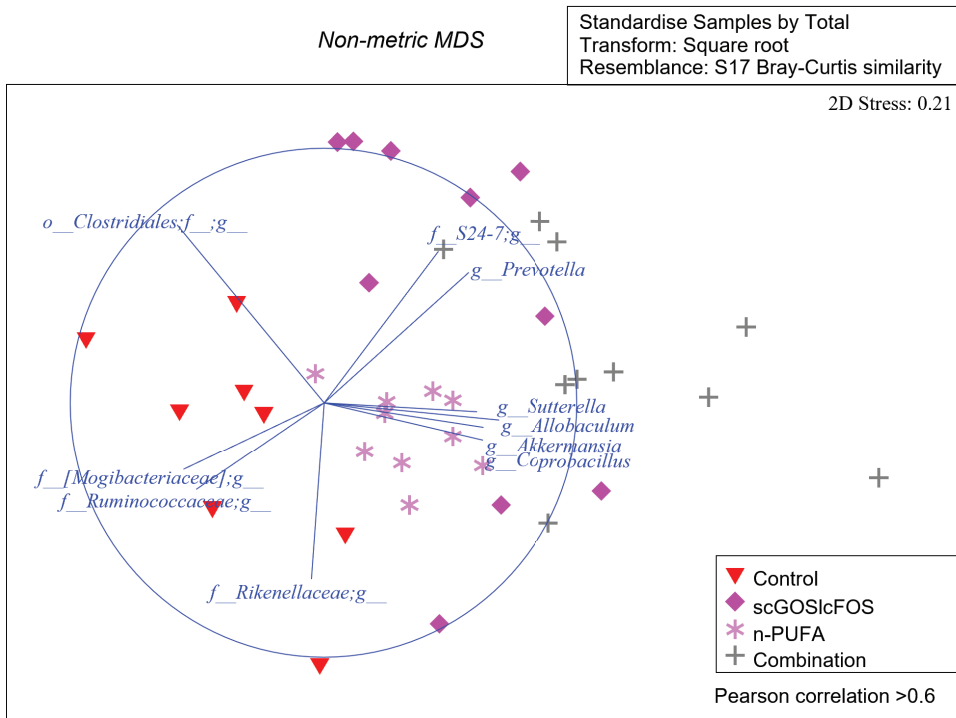
**Figure 3.** Visual of the non-multidimensional scaling (nMDS) plot depicting caecum content microbial community structures between control and dietary mice samples. A significant microbial community structure was observed between the control mice and the three dietary treatments. For statistical details, reference the analysis of similarity (ANOSIM) calculations in **Table 1**. Identified taxa with a Pearson's correlation (> 0.6) were strongly associated with either the control or dietary interventions.

Intestinal bacteria ferment carbohydrates and proteins into the different SCFAs and BCFAs. Using our inferred SCFA metabolite 16S rRNA bacterial taxa model, the percent relative abundances of putative bacteria that ferment carbohydrates and produce acetate, propionate and butyrate differed between the control and dietary mice groups. The genera examined as inferred SCFA-producing bacterial metabolites included acetate (*Ruminococcaceae* Unclassified, *Lactobacillus*, *Ruminococcus*, *Parabacteroidetes*, *Dorea*, *Streptococcus*, and *Bifidobacterium*); propionate (*Bacteroides*, *Akkermansia*, *Turicibacter*, and *Prevotella*); and butyrate (*Lachnospiraceae* Unclassified, [*Ruminococcus*], *Oscillospira*, *Lachnospiraceae* Other, *Coprococcus*, *Roseburia*, *Anaerofustis*, *Butyrivibrio*, *Anaerostipes*, and *Anaerotruncus*).
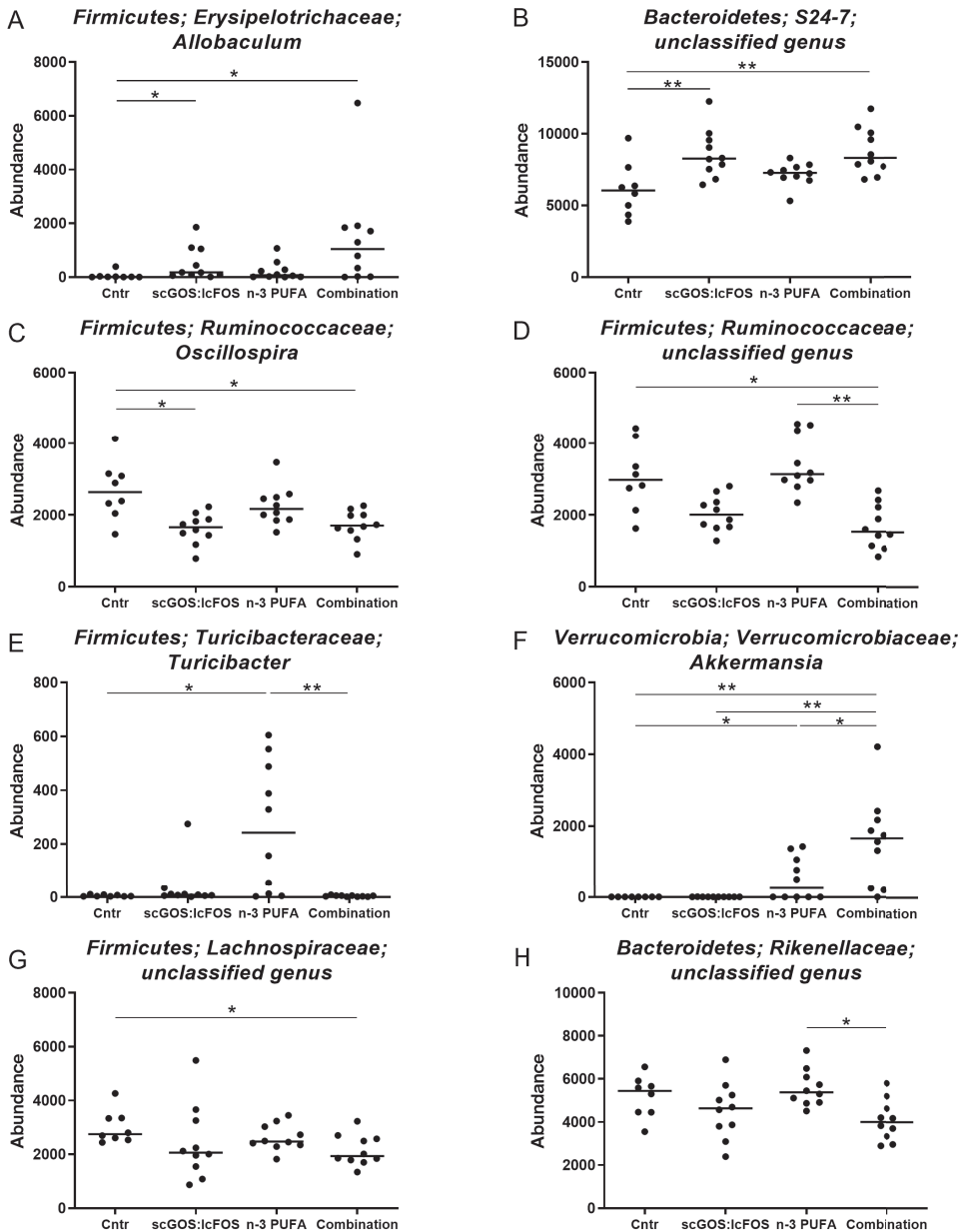
**Figure 4.** Significant genera taxa-specific relative abundances across mice groups (caecum content). The abundances of eight significant genera across mice groups are identified as: (A) *Allobaculum*, (B) *S24-7* Unclassified, (C) *Oscillospira*, (D) *Ruminococcaceae* Unclassified, (E) *Turicibacter*, (F) *Akkermansia*, (G) *Lachnospiraceae* Unclassified, and (H) *Rikenellaceae* Unclassified. A-H: Data shown as individual data points and median. Assessed for significance using Kruskal-Wallis test controlling for false-discovery rate (FDR): * FDR-$P$ < 0.05, ** FDR-$P$ < 0.01. $n$ = 8-10 mice per group.

The combination diet mice significantly lowered the percent relative abundance of putative genera acetate-producing bacteria, compared to the n-3 PUFA mice (**Figure 5A**). Additionally, the combination diet mice had a significantly higher relative abundance of putative genera propionate-producing bacteria than the control mice (**Figure 5B**). Finally, only the n-3 PUFA mice significantly lowered the percent relative abundance of putative genera butyrate-producing bacteria compared to the control mice (**Figure 5C**). Interestingly, these inferred bacterial genera metabolomics data were approximately mirrored by the actual targeted SCFA metabolomics concentrations levels in the caecum content.

Next, the targeted SCFA and BCFA metabolomics concentrations were examined using gas chromatography (**Figures 5D-J**). When compared to the control mice, the caecum content total SCFA and propionate concentration levels were significantly increased in the scGOS:lcFOS mice (**Figure 5D, F**). Additionally, the propionate concentration level was significantly increased in the combination diet mice, compared to the control mice (**Figure 5F**). Although not significantly different across all four mice groups, the scGOS:lcFOS mice concentration levels suggest that both acetate and butyrate are trending higher, compared to the other mice groups (**Figures 5E, G**). Finally, the concentration levels of the BCFAs for valerate, iso-butyrate and isovalerate were affected by the different diets, with a significant decrease in valerate, iso-butyrate, and iso-valerate in the combination mice, compared to the n-3 PUFA mice (**Figures 5H-J**).

## Intestinal serotonin-producing cells

Serotonin is a key neurotransmitter in the bidirectionally communication between the enteric and the central nervous system. To assess the effect of the diets on the intestinal serotonin-producing cells, the number of enterochromaffin cells was measured in the intestine. The number of these cells was significantly increased in the jejunum of the mice receiving a diet supplemented with scGOS:lcFOS, n-3 PUFAs or the combination compared to mice receiving the control diet (**Figure 6A**). The number of enterochromaffin cells in the ileum was unaffected by the different diets (**Figure 6B**). The cell number in the colon was significantly lower in the combination diet group than in the n-3 PUFA group. However, no differences were observed compared to the control (**Figure 6C**). **Figure 6** contains representative pictures of 5-HT + enterochromaffin cells in the jejunum (**D**), ileum (**E**) and colon (**F**) of control mice.

## Immune modulation

The n-3 PUFA diet significantly increased the percentage of total activated T cells (CD69^{+} of CD4^{+} cells) compared to the control group in the MLNs (**Figure 7A**). This n-3 PUFA-induced increase was not due to more activated Th1 or Th2 cells in the MLN (**Figures S2A-B**). In the MLN, the Th1 (CXCR3^{+}CD4^{+}) and the Th2 (T1ST2^{+}CD4^{+}) cell response tended towards an increase in, respectively, the scGOS:lcFOS group and the n-3 PUFA group compared to the combination diet (**Figures 7B-C**). Representative flowcytometry plots and histograms are shown in **Figures S1**, **S3, S4** and **S5**. These analyses were also performed in the spleen, but no significant differences were observed (**Figures S1C-G**).

In this study, the percentage of Tregs (CD25+Foxp3+CD4+) was unaltered by the dietary interventions in the MLN as well as in the spleen (**Figures S6A**, **C**). Additionally, the percentage Th17 cells (RORγt+CD4+) was unaffected by the different diets (**Figures S6B**, **D**).
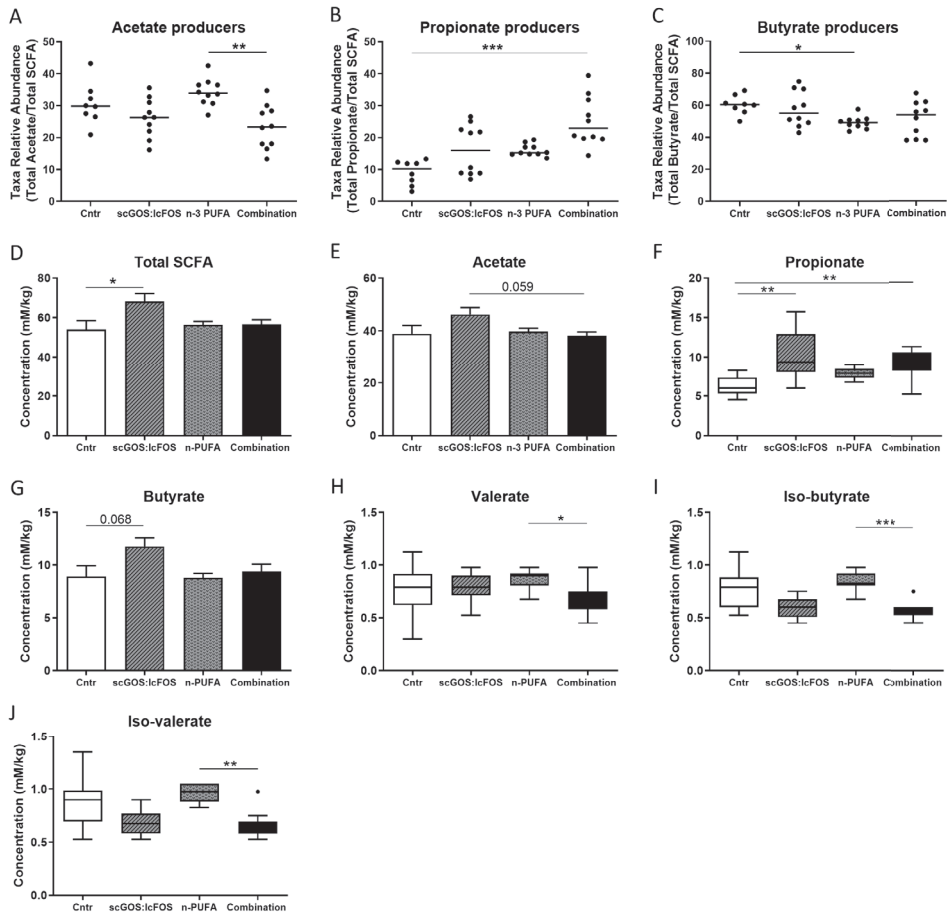


**Figure 5.** Caecum content predicted and targeted short-chain fatty acids metabolite concentrations between mice groups. Genera taxa metabolite predictive models depicting the relative abundances of (A) acetate- (B) propionate- and (C) butyrate-producing taxa were examined in the control and dietary mice groups. Targeted SCFA and BCFA graphs depict (D) total SCFAs (mM/kg); (E) acetate (mM/kg); (F) propionate (mM/kg); (G) butyrate (mM/kg); (H) valerate (mM/kg); (I) iso-butyrate (mM/kg) and (J) iso-valeric acid (mM/kg) in the mice groups. A-C: Data shown as individual data points and median. D,E,G: Data shown as the mean +/- SEM. F,H-J: Data shown as box-and-whiskers Tukey plots. A-C,F,H-J: Analysed by Kruskal-Wallis and Dunn's multiple comparisons post hoc test. D,E,F: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. A-J: $n$ = 8-10 mice per group. SCFA: short-chain fatty acids.
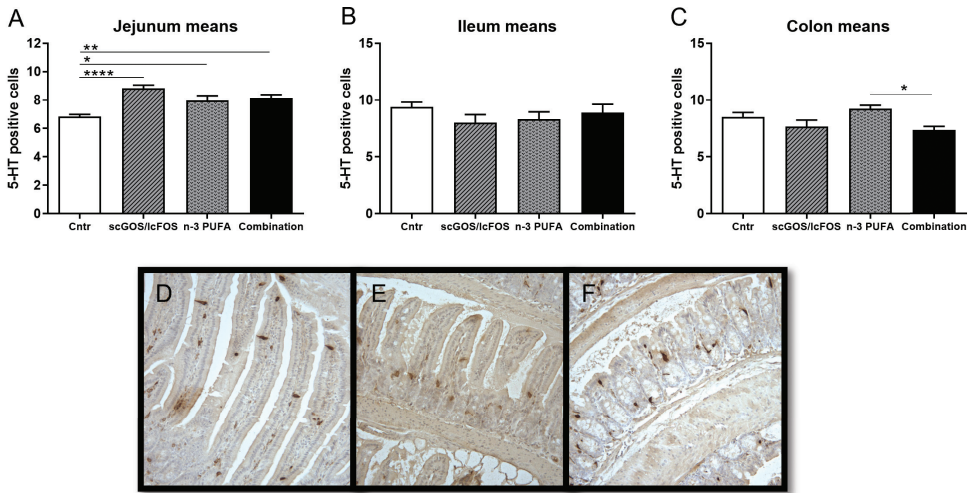
**Figure 6.** Serotonin-producing cells in the jejunum, ileum and colon. (A) In the jejunum, the number of serotonin-producing cells increased in the scGOS:lcFOS, n-3 PUFA and the combination diet groups compared to control. (B) In the ileum, the diets did not affect the serotonin-producing cells. (C) In the colon, the number of serotonin-producing cells was significantly decreased in the combination diet group compared to the n-3 PUFA group. (D-F) Representative pictures (control group) of the jejunum (D), ileum (E) and colon (F). A-C: Data shown as the mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. $n$ = 6 mice per group.
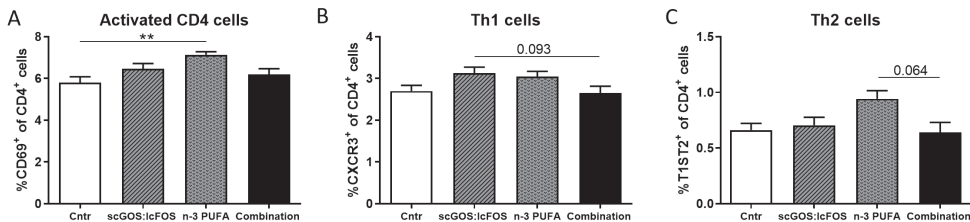


**Figure 7.** The dietary effect of scGOS:lcFOS, n-3 PUFAs and the combination of scGOS:lcFOS and n-3 PUFAs on activated CD4 cells, Th1 cells and Th2 cells in the MLN. (A) The percentage of activated CD4 (CD69+ CD4+) cells is significantly increased in the n-3 PUFA group compared to control. (B) The percentage of Th1 cells (CXCR3+ CD4+) tended to a decrease in the combination diet group compared to the scGOS:lcFOS group. (C) The percentage of Th2 cells (T1ST2+ CD4+) tended to a decrease in the combination diet group compared to the n-3 PUFA group. An outlier in the control group was excluded by use of ROUT analysis. A-C: Data shown as the mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. ** $P < 0.01$. $n$ = 5-10 mice per group, 2 samples in the control group, 3 samples in the n-3 PUFA group and 2 samples in the combination diet group were excluded due to low number of viable cells. Th1: T helper 1 cells. Th2: T helper 2 cells.

## Monoamines levels in brain regions

Several monoamines were measured in the amygdala, prefrontal cortex and hippocampus. In the amygdala, the 5-HT (serotonin) and the 5-HIAA (serotonin metabolite) levels were significantly increased in the combination diet group compared to the scGOS:lcFOS group (**Figures 8B-C**). However, tryptophan levels as well as serotonin turnover, were not significantly changed by the different diets compared to the control diet (**Figures 8A, D**). Except for the level of DOPAC, which tended towards an increase in the combination diet group compared to the n-3 PUFA group, the levels of noradrenaline, dopamine and the dopamine metabolites were unaltered as well as the turnover of dopamine (**Figure S7**). In the prefrontal cortex and dorsal hippocampus, no significant effects of the different diets were observed (**Figures S8** and **S9**, respectively).
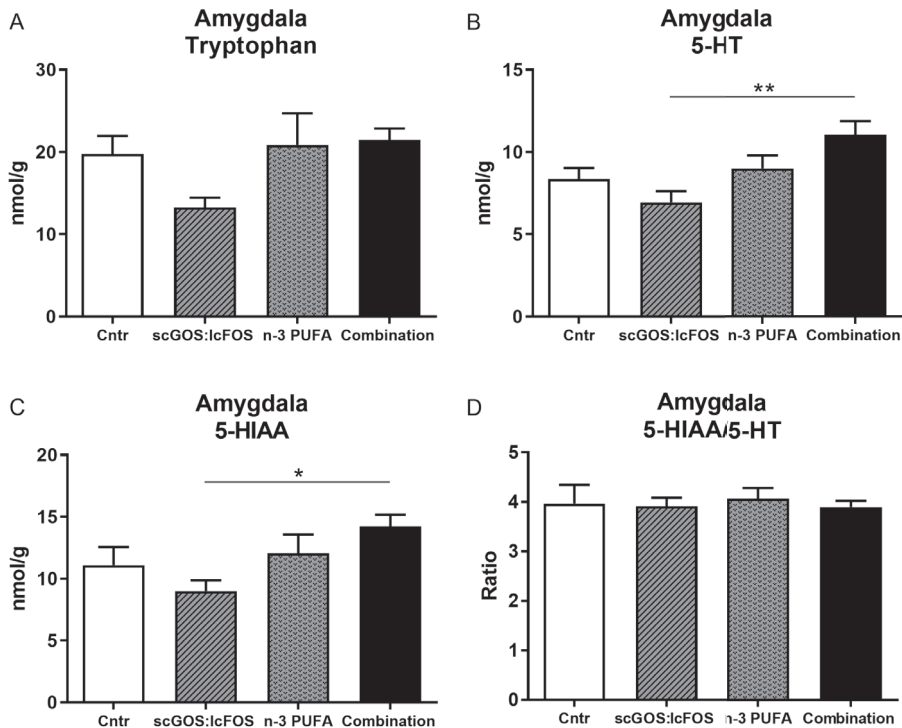


**Figure 8.** The amygdala levels of tryptophan, 5-HT, 5-HIAA and the serotonin turnover. (A) The tryptophan level did not differ between the dietary groups. (B, C) The 5-HT and 5-HIAA levels were significantly increased in the combination diet group compared to the scGOS:lcFOS group. (D) The serotonin turnover was unchanged in the dietary groups. A-D: Data shown as the mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$. $n$ = 4-5 samples per group, samples were pooled in pairs, in order to reach detection minimum, each sample contained two left brains. 5-HT: serotonin. 5-HIAA: 5-hydroxyindoleacetic acid (serotonin metabolite).

**Behavioural modulation**

During adolescence and early adulthood anxiety-like, self-grooming, explorative and social behaviour were assessed.

*Marble burying and self-grooming*

Overall, the number of marbles buried was unaffected by diet ($F_{(3, 10.224)}$ = 1.640, $P > 0.05$) but tended towards an effect by age ($F_{(1, 34)}$ = 3.323, $P = 0.077$), and the change in number of buried marbles over time was independent of the diet (interaction effect between diet and age ($F_{(3, 34)}$ = 2.198, $P > 0.05$)) (**Figure 9A**). The mice receiving the control diet buried significantly more marbles in early adulthood compared to adolescence ($P < 0.05$). The number of buried marbles in the dietary groups was unchanged over time. During adolescence the number of buried marbles was unaffected by the diets. However, in early adulthood the mice receiving the scGOS:lcFOS diet tended to bury less marbles compared to the control group ($P = 0.095$). One might be doubtful that the number of buried marbles is not significantly different between the control and scGOS:lcFOS group in early adulthood. These data are controlled for litter effect and can lead to the fact that visuals and statistics are not completely aligned.
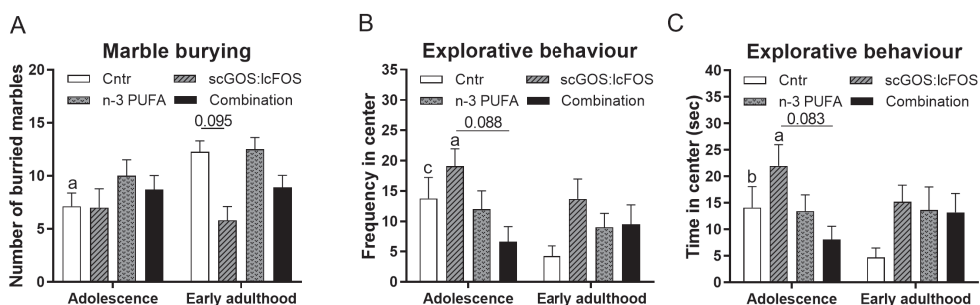


**Figure 9.** Anxiety-like behaviour assessed by marble burying and explorative behaviour in the open field. (A) Number of buried marbles by mice receiving the control, scGOS:lcFOS, n-3 PUFA or combination diet in adolescence and early adulthood (B) Explorative behaviour, the frequency the mice receiving the control, scGOS:lcFOS, n-3 PUFA or combination diet entered the centre of the open field in adolescence and early adulthood. (C) Explorative behaviour, the time the mice receiving the control, scGOS:lcFOS, n-3 PUFA or combination diet spent in the centre of the open field. A-C: Data shown as the mean +/- SEM. Analysed with mixed models, controlled for repeated measures, litter effect and Sidak's multiple comparisons post hoc test. a = * $P < 0.05$ compared with early adulthood within diet group, b = ** $P < 0.01$ compared with early adulthood within diet group, c = *** $P < 0.001$ compared with early adulthood within diet group. A-C: $n$ = 8-10 mice per group.

Grooming duration was overall affected by age ($F_{(1, 33.381)}$ = 6.502, $P < 0.05$) and diet ($F_{(3, 33.222)}$ = 3.387, $P < 0.05$). The change in grooming duration over time was independent of the diet ($F_{(3, 33.388)}$ = 0.629, $P > 0.05$) (**Figure S10B**). The grooming duration tended towards a decrease overtime in the control ($P = 0.079$) as well as in the scGOS:lcFOS group ($P = 0.058$). In early adulthood, grooming duration showed an increasing trend in the combination

group compared with the scGOS:lcFOS group ($P = 0.074$). The frequency of grooming was neither affected by age nor diet (**Figure S10C**).

*Open field*

The explorative behaviour was measured by frequency and time in the centre of the open field. The frequency in the centre was significantly affected by age ($F_{(1, 34)} = 12.558$, $P < 0.01$), but not by diet ($F_{(3, 10.935)} = 1.519$, $P > 0.05$) and the frequency in the centre over time was dependent on the diet (interaction effect between diet and age ($F_{(3, 34)} = 5.820$, $P < 0.01$)) (**Figure 9B**). Over time, the number of entries was significantly reduced in the control group and in the scGOS:lcFOS group ($P < 0.001$, $P < 0.05$, respectively). In adolescence, the number of entries tended towards a reduction in the combination diet group compared to the scGOS:lcFOS group ($P = 0.088$). The time spent in the centre of the open field tended towards an overall effect by age ($F_{(1, 34)} = 3.025$, $P = 0.0951$) but no overall effect by diet ($F_{(3, 11.042)} = 1.409$, $P > 0.05$). The time spent in the centre over time depended on the diet (interaction effect between diet and age ($F_{(3, 34)} = 4.414$, $P < 0.05$)). The statistical effects for time in centre were similar to frequency in centre (**Figure 9C**). Locomotor activity (total distance moved) also showed the same pattern (Age: $F_{(1, 34)} = 2.932$, $P = 0.096$. diet: $F_{(3, 11.762)} = 1.545$, $P > 0.05$, interaction: $F_{(3, 34)} = 3.828$, $P < 0.05$) (**Figure S10A**).

*Social interaction*

Social interaction, shown as relative time in zone (target/no target), was neither affected by age ($F_{(1, 32.933)} = 0.997$, $P > 0.05$) nor diet ($F_{(3, 8.300)} = 0.576$, $P > 0.05$) (interaction ($F_{(3, 32.827)} = 0.086$, $P > 0.05$)) (**Figure S10D**). However, overall the locomotor activity (distance moved) was significantly affected by age and unaffected by diet (Age: $F_{(1, 34)} = 7.425$, $P < 0.05$, diet: $F_{(3,34)} = 1.231$, $P > 0.05$, interaction: $F_{(3, 34)} = 2.343$, $P > 0.05$). Locomotor activity in the control and scGOS:lcFOS receiving mice was significantly decreased over time ($P < 0.05$ for both groups) (**Figure S10E**). In adolescence, locomotor activity tended towards a reduction in the combination diet group compared to the scGOS:lcFOS group ($P = 0.076$).

**Feature selection regarding repetitive and explorative behaviour**

Though the effects of the different early life dietary interventions on repetitive and explorative behaviour of healthy adolescent and early adult mice are not very clear, we have analysed the whole dataset regarding the marble burying (anxiety-like and repetitive behaviour) and open field (explorative behaviour) tests in early adulthood to achieve insight into which microbial, immune and monoamine features influence the two behavioural outcomes independent on the dietary intervention. After running the REFS algorithm 10 times, for marble burying behaviour the best signature is at 9 features, with an average global accuracy of all classifiers of 0.73 (**Figure 10A**). The optimal associated receiver-operating characteristic (ROC) curve for the best performing classifier Ridge is shown in **Figure 10B** that demonstrates an area under the curve (AUC) of 0.84 ± 0.17, which is considered good to outstanding (74, 75). The magnitudes of the 9 features separating the two groups are presented in **Figure 10C**. In **Table 2**, the direction of change

comparing the groups ≥ 10 (label 1) with <10 (label 0) number of buried marbles of the 9 features are presented. A reduction in marble burying (associated with reduced repetitive or anxiety-like behaviour) was associated with reductions in the caecal content relative abundance of the genus *Adlercreutzia*, the α-diversity Shannon index, and tryptophan levels in dorsal hippocampus; upregulation of the caecal content relative abundance of the genus *Dehalobacterium*, percentages of Th1 (CXCR3+ CD4+) and Th17 (RORγt+ CD4+) cells in MLN, and percentage of activated Th2 splenocytes (CD69+ T1ST2+ CD4+) (**Figure 10C**; **Table 2**). An overview of the involved features and their connection is depicted in **Figure 11A**.
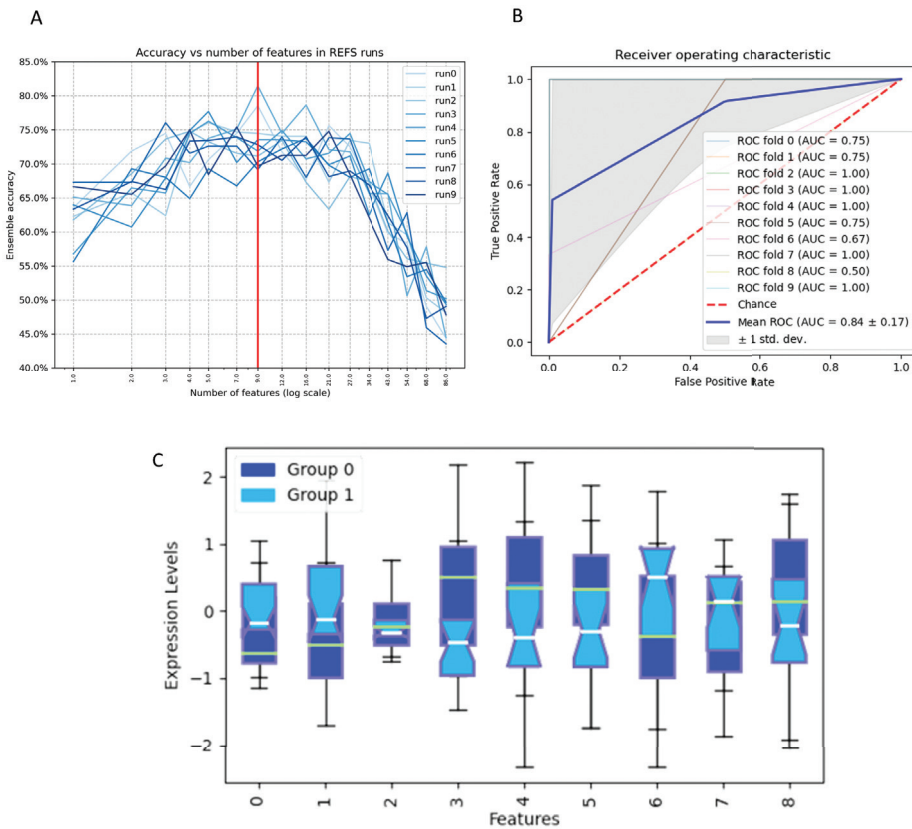


**Figure 10.** The datasheet of the entire study was analysed regarding marble burying (number of marbles buried) at 8 weeks of age (early adulthood). (A) After running the REFS algorithm 10 times, the best signature is at 9 features, with an average global accuracy of all classifiers of 0.73. (B) The optimal associated receiver-operating characteristic (ROC) curve for the best-performing classifier Ridge demonstrates an area under the curve (AUC) of 0.84 ± 0.17. (C) The 9 features separating the two labels: 0 = *Adlercreutzia*, 1 = DH: tryptophan (nmol/gr), 2 = *Lachnospiraceae* Other, 3 = *Dehalobacterium*, 4 = Th17 cells in MLN, 5 = Activated Th2 cells in spleen, 6 = α-diversity (Shannon index), 7 = PFC: noradrenaline (nmol/gr), 8 = PFC: noradrenaline (nmol/gr), and 9 = Th1 cells in MLN. Label 0: number of buried marbles <10. Label 1: number of buried marbles ≥10.

**Table 2.** Feature reduction marble burying test (repetitive/anxiety-like behaviour).

| Feature | Label 1 vs label 0 |
|---|:---:|
| *Coriobacteriaceae Adlercreutzia* | ↓ |
| DH: tryptophan (nmol/gr) | ↓ |
| *Lachnospiraceae* Other | - |
| *Dehalobacteriaceae Dehalobacterium* | ↑ |
| Th17 cells in MLN | ↑ |
| Activated Th2 cells in spleen | ↑ |
| α-diversity (Shannon index) | ↓ |
| PFC: noradrenaline (nmol/gr) | - |
| Th1 cells in MLN | ↑ |

Label 0: number of buried marbles < 10. Label 1: number of buried marbles ≥ 10. Arrow indicates if the feature is reduced or enhanced when the number of buried marbles is reduced.

For open field behaviour, after running the REFS algorithm 10 times the best signature is at 16 features with average accuracy of all classifiers of 0.77 (**Figure 12A**). The ROC curve for the best performing classifier Support Vector is shown in **Figure 12B** that demonstrates an area under the curve (AUC) of 0.82 ± 0.20. The magnitudes of 16 features separating the two groups are presented in **Figure 12C**. In **Table 3**, the direction of change comparing the groups ≥ 10 (label 0) with <10 (label 1) number of entries into the centre of the open field of 16 features is presented. Increased entry into the centre of the open field (more explorative behaviour) is associated with an enhancement in the caecal content relative abundance of the genera *Odoribacter* and *Turibacter*, dopamine and serotonin turnover in the amygdala and prefrontal cortex, respectively, and 5-HIAA levels in the prefrontal cortex. A reduction in the caecal content relative abundances of the phyla Cyanobacteria (class 4C0d; order YS2; unclassified family and genus), genera *Oscillospira*, *Ruminococcus*, *Lachnospiraceae* Other;Unclassified and *Adlercreutzia* as well as reduced levels of HVA in the prefrontal cortex are associated with enhanced explorative behaviour (**Figure 12C**; **Table 3**). An overview of the involved features and their connection is depicted in **Figure 11B**.
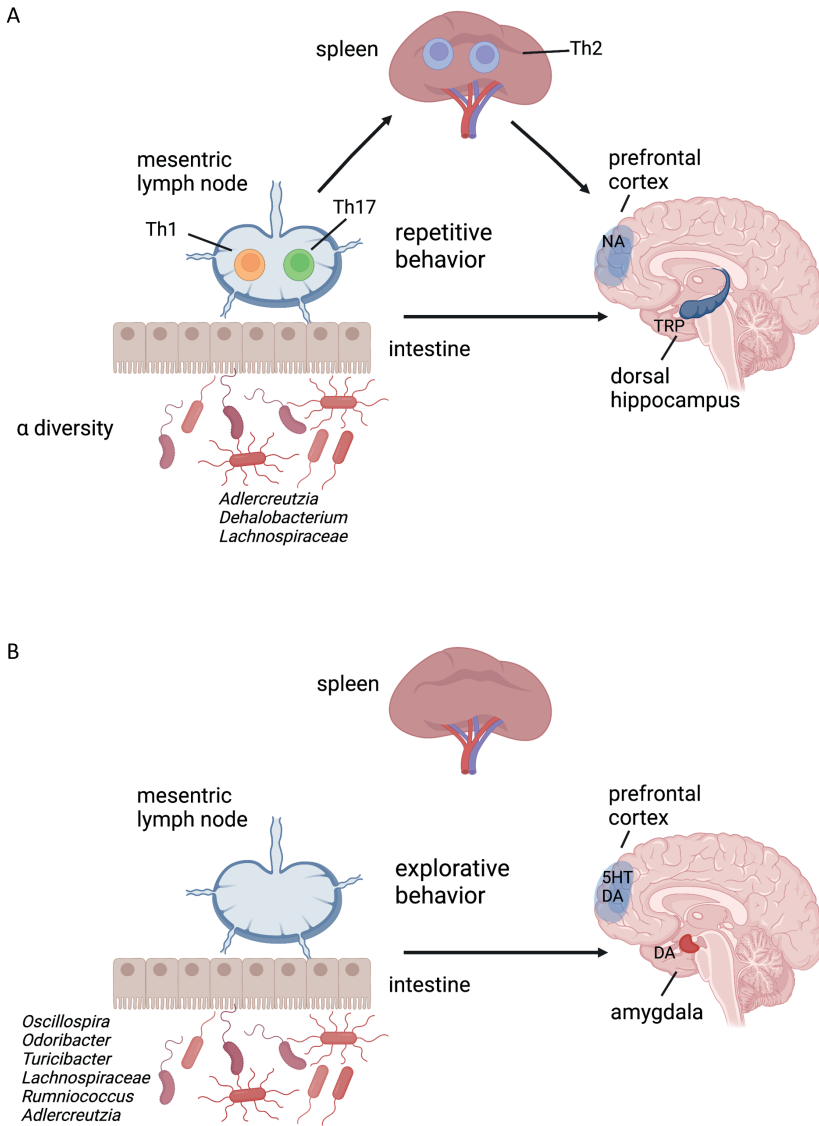
**Figure 11.** Indications of which feature combination significantly influence repetitive and anxiety-like behaviour and explorative behaviour evaluated by the marble burying (A) and open field behaviour (B) tests, respectively. (A) The α-diversity and relative abundances of the genera *Adlercreutzia* and *Dehalobacterium*, changes in Th1 and Th17 cells in MLN, changes in activated Th2 cells in spleen, and tryptophan levels in dorsal hippocampus significantly predict changes in repetitive behaviour. (B) The relative abundances of the genera *Oscillospira*, *Ruminococcus*, *Odoribacter*, *Turibacter*, *Lachnospiraceae* other/Unclassified and *Adlercreutzia* and changes in serotonin and dopamine metabolism in PFC and amygdala significantly predict changes in explorative behaviour. Th1: T helper 1 cell, NA = noradrenaline, TRP: tryptophan, 5-HT: serotonin, DA: dopamine.
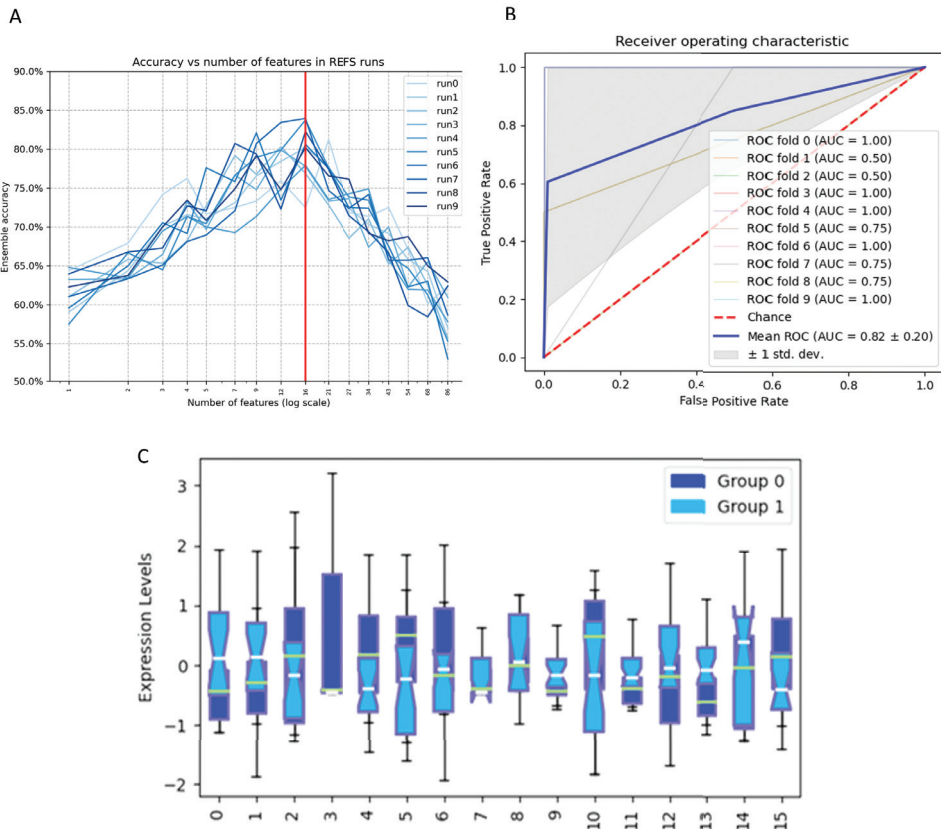
**Figure 12.** The datasheet of the entire study was analysed regarding the open field test (number of entries in the open field) at 8 weeks of age (early adulthood). (A) After running the REFS algorithm 10 times the best signature is at 16 features with average accuracy of all classifiers of 0.77. (B) The optimal associated ROC curve for the best performing classifier Ridge demonstrates an area under the curve (AUC) of 0.82 ± 0.20. (C) The 16 features separating the two labels (0 = *Cyanobacteria* Unclassified, 1 = *Oscillospira*, 2 = *Odoribacter*, 3 = *Turicibacter*, 4 = AM: (DOPAC+HVA)/DA, 5 = PFC: 5-HIAA/5-HT, 6 = Lachnospiraceae Unspecified, 7 = Allobaculum, 8 = DH: noradrenaline (nmol/gr), 9 = *Lactobacillus*, 10 = PFC: 5-HIAA (nmol/gr), 11 = *Lachnospiraceae* Other, 12 = *Ruminococcus*, 13 = *Adlercreutzia*, 14 = PFC: HVA (nmol/gr), and 15 = AM: (DOPAC + HVA + 3MT)/DA). Label 0: number of entries to open field ≥ 10. Label 1: number of entries to open field < 10.

**Table 3.** Feature reduction open field test (explorative behaviour).

| Feature | Label 0 vs label 1 |
|---|:---:|
| Cyanobacteria (c_4C0d;o_YS2) | ↓ |
| *Ruminococcaceae Oscillospira* | ↓ |
| *Odoribacteraceae Odoribacter* | ↑ |
| *Turicibacteraceae Turicibacter* | ↑ |
| AM: (DOPAC+HVA)/DA | ↑ |
| PFC: 5-HIAA/5-HT | ↑ |
| *Lachnospiraceae* Unspecified | ↓ |
| *Erysipelotrichaceae Allobaculum* | - |
| DH: noradrenaline (nmol/gr) | - |
| *Lactobacillaceae Lactobacillus* | - |
| PFC: 5-HIAA (nmol/gr) | ↑ |
| *Lachnospiraceae* Other | ↓ |
| *Ruminococcaceae Ruminococcus* | ↓ |
| *Coriobacteriaceae Adlercreutzia* | ↓ |
| PFC: HVA (nmol/gr) | ↓ |
| AM: (DOPAC+HVA+3MT)/DA | ↑ |

Label 0: number of entries to open field ≥ 10. Label 1: number of entries to open field < 10. Arrow indicates if the feature is reduced or enhanced when the number of entries to open field is enhanced.

## DISCUSSION

The present study demonstrates that dietary supplementation with the combination of scGOS:lcFOS and n-3 PUFAs leads to a distinct caecal content microbiota composition and indicates a balanced immune response compared to the individual food components. In our previous work (31), the scGOS:lcFOS effects on the in intestinal microbiota were associated with improved social behaviour and reduced anxiety-like and stereotypic behaviour assessed by marble burying and self-grooming behavioural tests. In this study, the effects of scGOS:lcFOS on these behaviours are less clear.

All three diets modulated caecal content microbial community structures. The distinct caecal content microbial profile alterations were significant comparing the control mice to the three dietary mice groups. Further, the caecal content bacterial composition in the combination group was different from both scGOS:lcFOS and n-3 PUFAs. These results indicate that the NDO diets modulated the caecal content microbial community structures uniquely, which is in line with other studies (23, 24, 31, 38). Based on the relative abundances of individual bacteria at the taxonomic level of genus, the combination diet induced a microbial profile similar to the profile induced by scGOS:lcFOS. Interestingly, scGOS:lcFOS and the combination diet interventions increased the relative abundances of genera *Allobaculum* and *S24-7* Unclassified, while reducing the abundance of the genera *Oscillospira* and *Ruminococcaceae* Unclassified. The genera *Allobaculum* and *S24-7* Unclassified have both been reported to be involved in the fermentation of fibres and

to be putative SCFA-producing bacteria (31, 76-78). The genus *Oscillospira* has been linked to slow faecal transit, which could lead to more water absorption from the stool and eventually result in constipation. scGOS:lcFOS has been reported to affect stool consistency, decrease transit time (79) and increase defecation frequency (80). Thus, these functions of scGOS:lcFOS could be due to the lower relative abundance of the genus *Oscillospira*. The relative abundance of the genus *Ruminococcaceae* Unclassified has been described to be positively correlated with plasma levels of serotonin (81). This might indicate that taxa associated with the family of Ruminococcaceae could play a role in the tryptophan metabolism converting tryptophan into serotonin (82).

To date, intestinal microbiota modulation by n-3 PUFAs has been less defined. In this study, the n-3 PUFA diet induced changes to the relative abundances of the genera *Turicibacter* and *Akkermansia*. Since *Turicibacter* has been shown to enhance the levels of PUFAs (83), this could be a positive feedback mechanism, where enhanced availability of lipids such as n-3 PUFAs increased the abundance of *Turibacter*. *Akkermansia* is induced by n-3 PUFAs as mentioned above, but *Akkermansia* is induced significantly more by the combination diet group, indicating an additive effect on the induction on *Akkermansia* when scGOS:lcFOS and n-3 PUFAs are combined. *Akkermansia* has been reported to be a propionate-producing bacteria (84) which could explain the increased caecal content propionate levels in the combination diet group. *Akkermansia* has been reported to communicate with the immune system of the host and i.e., induce Tregs (85) and reduce inflammation (86). With regard to the F:B ratio, an increase in this ratio has been associated with high-fat diets (Westernised diets) (87) and obesity (88). Moreover, the F:B ratio in mice receiving a n-3 PUFA-rich diet was decreased (89), which is in line with our findings, indicating a positive effect on the F:B ratio by scGOS:lcFOS and n-3 PUFAs.

As expected and observed before (31), the absolute SCFA levels in the scGOS:lcFOS group were increased indicating a saccharolytic fermentation profile. We expected the effects of scGOS:lcFOS on the SCFA levels to be visible in the combination group, however, that was not the case, except for propionate. Both caecal content inferred propionate producing genera and propionate metabolite measurements were significantly enhanced in the combination diet group, compared to the control group. Based on the acetate producers, one would expect less acetate in the combination diet group than in the n-3 PUFA group, but the acetate levels in these groups were similar. This could be due to overlapping function of other bacteria compensating for decreased relative abundance of acetate producing bacteria in the combination group. Concerning the SCFA levels, this might indicate that the n-3 PUFAs in some way influence the fermentation of scGOS:lcFOS, which leads to less pronounced effects in the SCFA levels in the combination diet group. The other possibility is the impact of n-3 PUFAs and scGOS:lcFOS on intestinal epithelial cells uptake of SCFAs. If n-3 PUFAs and scGOS:lcFOS improve intestinal epithelial function and increase their ability to uptake more SCFAs, then luminal SCFA levels would remain unchanged in spite of increased production by the SCFA-producing bacteria.

SCFAs and intestinal bacteria have the capacity to influence the immune system (90, 91). Therefore, modulation of intestinal microbiota composition and/or the activity might eventually lead to immune modulation. SCFAs can act both pro-and anti-inflammatory depending on the kind and state of the immune cell (90). They influence the immune system through inhibition of HDAC activity and the GPCRs GPR41, GPR43 and GPR109. Among others, SCFAs induce genes that maintain intestinal barrier function and induce differentiation and function of T cell subsets into Th1, Th17 and Tregs (92). These intestinal microbial and SCFA data indicate that as the individual components, scGOS:lcFOS and n-3 PUFAs influence the intestinal microbiota and it seems that the combination of scGOS:lcFOS and n-3 PUFAs balances intestinal microbiota composition – the best of both.

Previous studies have shown that scGOS:lcFOS is able to induce a Th1 response (93, 94) and stimulate the secretion of IL-10 from DCs, which eventually results in upregulation of the number of suppressive Tregs (34, 35). Although in this study the T cell subsets were unaffected by scGOS:lcFOS and n-3 PUFAs, Th1 and Th2 responses tended towards a decrease in the combination diet group compared with scGOS:lcFOS and n-3 PUFAs, respectively. Considering that this study takes place in healthy mice, one could argue that a pronounced immune response is undesired. Therefore, these data might indicate that the combination of scGOS:lcFOS and n-3 PUFAs leads to a balanced immune response. Both scGOS:lcFOS and n-3 PUFAs can induce Tregs. A reduced Treg response plays an essential role in preventing diseases such as food allergies (35, 44). It is most likely considered acceptable, that the percentages of Tregs and Th17 were unaffected by the dietary interventions as significant changes may be undesired in a healthy host. n-3 PUFAs are known to be health-promoting because of their anti-inflammatory potential. However, in this study, n-3 PUFAs induced the activated $CD4^+$ cells in the MLN, which was not in line with other studies (44, 95). Since the n-3 PUFA-induced increase in $CD69^+ CD4^+$ cells was not caused by enhanced activation of Th1 or Th2 cells, another explanation could be that n-3 PUFAs induce memory T cells, as CD69 can also be expressed by these cells (96). However, in the combination diet group, this increase in activated $CD4^+$ cells was no longer observed. This might indicate that the induction of activated $CD4^+$ cells is hampered by scGOS:lcFOS. All the immunological findings were only observed in the MLN. The diets had no pronounced effects in the spleen, suggesting that the diets might exert their modulatory effects locally in the intestine.

As mentioned earlier n-3 PUFAs and scGOS:lcFOS can modulate the intestinal microbiota, the immune system, and also brain development and behaviour (25, 31, 38, 40, 45, 46, 48). Overall, the change in behavioural parameters in this study mainly occurred over time: less repetitive and explorative behaviour and more anxiety-like behaviour. The scGOS:lcFOS behavioural effects are in line with previous studies where scGOS:lcFOS modulated repetitive and anxiety-like behaviour in healthy mice (31, 38). Additionally, in a mouse model of stress-induced anxiety, prebiotics improved or prevented the anxiety-like behaviour (assessed by light/dark preference and open field tests) (97). n-3 PUFAs have been shown to improve anxiety-like and social behaviour in healthy and allergic rodents, respectively (47, 48); this is not in line with our observations. However, Robertson *et al.*

reported no effects of n-3 PUFAs on repetitive and anxiety-related behaviour (assessed by marble burying, light/dark preference and elevated plus maze tests) in healthy mice (25) and this matches our data. Robertson *et al.* also reported the importance of n-3 PUFAs; n-3 PUFA deficiency in healthy mice led to impaired behaviour in adolescence and later in life (25). This indicates that n-3 PUFAs are an essential dietary component, but to observe behavioural improvements, maybe the window of opportunity is too small in a healthy host.

Behavioural changes are often accompanied by alterations in neurochemical mediators such as serotonin in the brain. Serotonin is a metabolite from tryptophan, and tryptophan is an essential amino acid that needs to be obtained from the diet. Serotonin is able to modulate anxiety and social behaviour (64, 98). Although not significantly lower, tryptophan levels in the brain regions investigated in this study show a similar pattern. The tryptophan levels seemed lower in the scGOS:lcFOS group and might indicate a lower availability of tryptophan. As tryptophan enters the body through the stomach and intestine, it could be that tryptophan-utilising intestinal bacteria use some of the tryptophan. Another option is that serotonin production by specific epithelial cells called enterochromaffin cells increases by scGOS:lcFOS stimulation. Indeed, the number of serotonin positive cells in the jejunum was significantly increased compared with the control group. For this boost of serotonin secretion, tryptophan is essential and consequently less tryptophan is available for the brain. Intriguingly, this phenomenon was only observed in the scGOS:lcFOS group. All three dietary interventions increased serotonin-positive cells in the jejunum, but no changes in tryptophan levels in the brain were observed in the combination and n-3 PUFA diet groups compared to the control group. The reason for this discrepancy remains unclear. Enterochromaffin cells are located throughout the intestinal tract (99), and the reason that the dietary effect is only observed in the jejunum and not in the ileum and colon is unknown. The analysis of goblet and Paneth cell counts would provide a more complete picture; however, these analyses are missing in this study. Intestinal bacteria associated with the family Ruminococcaceae possibly play an additional role. A recent study showed that the enhanced abundance of the family Ruminococcaceae correlated significantly with increased 5-HT$_{2A}$ receptor density in the PFC in a maternal activation (MIA) murine model (100). The fact that MIA offspring mice show impaired behaviour and that the abundance of faecal family Ruminococcaceae is increased in young children diagnosed with autism spectrum disorder, might explain why scGOS:lcFOS improves behaviour via a reduction in Ruminococcaceae in the intestines of healthy mice (31, 100-102). More studies are needed to elucidate a more detailed mechanism.

Serotonin and serotonin metabolite levels in the investigated brain regions approximately follow the same pattern in the dietary groups. The combination diet group increased serotonin and serotonin metabolite levels compared with the scGOS:lcFOS group in the amygdala brain region. In general, more serotonin is desirable according to the literature. However, in our previous paper, scGOS:lcFOS led to improved behaviour, but lower serotonin levels (31). Noticeable, in this study we observed no significant differences

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between the dietary groups in the other monoamines. The monoamines do follow the same pattern; the levels in the scGOS:lcFOS and n-3 PUFA groups are lower than in the control and the levels in the combination diet group approximated to the levels in control. This again indicates that the combination diet balances the effects of scGOS:lcFOS and n-3 PUFAs, which might be of valuable interest when combining these components in a healthy host or in models of disease. The main impact of n-3 PUFAs in this study was the modulated intestinal microbiota, which match findings of other studies (89, 103). To be able to explain the results, data on the n-3 PUFA metabolites in blood and/or brain tissues might have been useful. Overall, regarding the effects of scGOS:lcFOS with or without n-3 PUFAs on microbial composition and monoamine levels in different brain areas, it can be hypothesised that scGOS:lcFOS is influencing explorative behaviour either directly in the brain or indirectly through the microbe-brain axis. Future studies are essential to further elaborate on these hypotheses.

Finally, we implemented the REFS algorithm on all microbial, immune (MLN and spleen) and monoamine (brain) data and found 8 and 16 features, whose changes significantly seem to predict changes in repetitive and explorative behaviour with a global accuracy of 73% and 77%, respectively. Predictive changes for repetitive behaviour are mostly influenced by the composition of certain intestinal bacteria, which might in turn affect local and system immune balance, resulting in modified tryptophan levels in the dorsal hippocampus. Comparable to our findings, low hippocampal tryptophan levels are associated with an anxiolytic effect in BALB/c mice (104). A previous study has shown that enhanced intestinal abundance of *Adlercreutzia* is associated with inflammation-induced depressive-like behaviour in mice (105). Increased expression of the genus *Dehalobacterium* in mice is associated with ageing and food intervention-induced anti-inflammatory effects (106, 107). Moreover, reduced levels of intestinal *Dehalobacterium* observed in BTBR mice that have an autistic phenotype is associated with enhanced marble burying (108). Taken together, our finding that reduced marble burying is associated with reduced *Adlercreutzia* and increased *Dehalobacterium* abundance seems to be compatible with previous murine behavioural studies. Unlike repetitive behaviour, explorative behaviour was not associated with neither local nor systemic T lymphocytes, but was significantly associated with microbial alterations in the relative abundances of several bacterial genera plus changes in serotonin and dopamine metabolism in PFC and amygdala. No specific reports on the role of the genera *Oscillospira*, *Ruminococcus, Odoribacter*, *Turicibacter, Lachnospiraceae* Other/Unclassified and *Adlercreutzia* on explorative behaviour are published (109).

In conclusion, both early life dietary interventions with scGOS:lcFOS and/or n-3 PUFAs affected the caecal content microbial profiles, but had limited effects on behaviour and the immune system. No apparent additive effect was observed when scGOS:lcFOS and n-3 PUFAs were combined, as the data from the combination diet group show the same pattern as scGOS:lcFOS for some parameters and the n-3 PUFA pattern for other parameters. All parameters considered, the results suggest that these dietary components together create a balance – the best of both in a healthy host. The limited

effect on the immune system and behaviour is considered acceptable as this study was carried out in healthy mice. It may be concluded that improving intestinal microbiota composition by diet in a healthy host, where SCFA production by bacteria and intestinal epithelial cells is normal, has no functional impact. However, in a diseased situation (i.e., allergy, colitis or neurological disease), where the microbiota is abnormal, improving the microbiota by diet might have a positive impact.

## ACKNOWLEDGMENTS

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# SUPPLEMENTARY INFORMATION

**Table S1.** Diet composition of the experimental diets.

| | Control | scGOS:lcFOS | n-3 PUFA | Combination |
|---|---|---|---|---|
| | g/kg diet | g/kg diet | g/kg diet | g/kg diet |
| **Carbohydrates** | | | | |
| Cornstarch | 367.5 | 36.5 | 367.5 | 367.5 |
| Dextrinized cornstarch | 122.5 | 122.5 | 122.5 | 122.5 |
| Sucrose | 91.0 | 91.0 | 91.0 | 91.0 |
| Lactose monohydrate EF | 9.5 | 0 | 9.5 | 0 |
| Dextrose monohydrate | 9.9 | 0 | 9.9 | 0 |
| **Fiber** | | | | |
| Cellulose | 50.0 | 19.8 | 50.0 | 19.8 |
| GOS sirop Vivinal (45% GOS) | 0 | 60.0 | 0 | 60.0 |
| Inulin HP (lcFOS) (97% FOS) | 0 | 3.1 | 0 | 3.1 |
| **Protein** | | | | |
| Soy protein | 200.0 | 200.0 | 200.0 | 200.0 |
| DL-methionine | 2.0 | 2.0 | 2.0 | 2.0 |
| L-cystine | 1.0 | 1.0 | 1.0 | 1.0 |
| **Fat** | | | | |
| Soybean oil | 100.0 | 100.0 | 40.0 | 40.0 |
| BioPure DHA IF tuna oil | 0 | 0 | 60.0 | 60.0 |
| **Others** | | | | |
| Mineral mix AIN-93G | 35.0 | 35.0 | 35.0 | 35.0 |
| Vitamin mix AIN-93VX | 10.0 | 10.0 | 10.0 | 10.0 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 | 2.5 |
| Tert-butylhydroquinone | 0.014 | 0.014 | 0.014 | 0.014 |
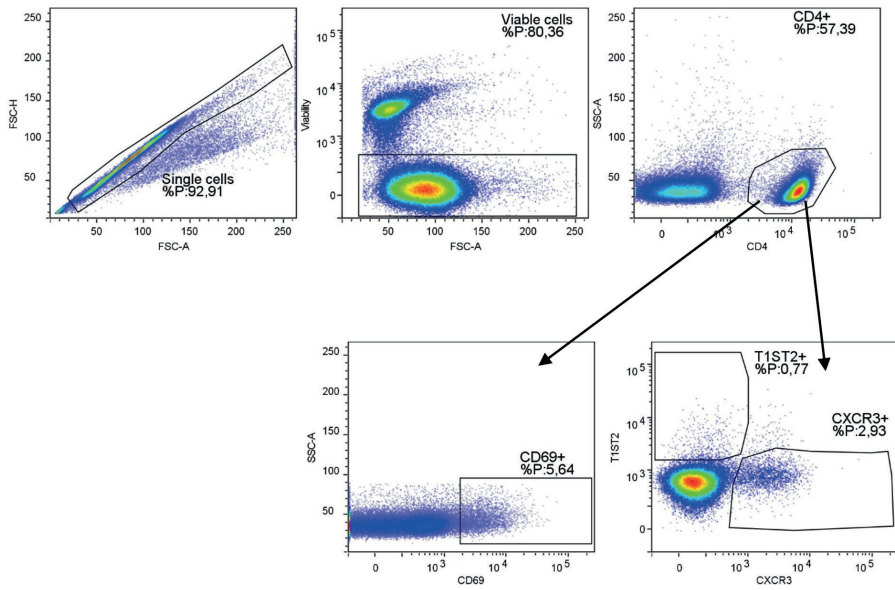
**Figure S1.** Gating strategy used in the flowcytometry analysis. First single cells were gated, then viable cells, followed by CD4+ cells. The activated (CD69+) cells as well as the Th1 (CXCR3+) and Th2 (T1ST2+) cells were gated from the CD4+ cells.
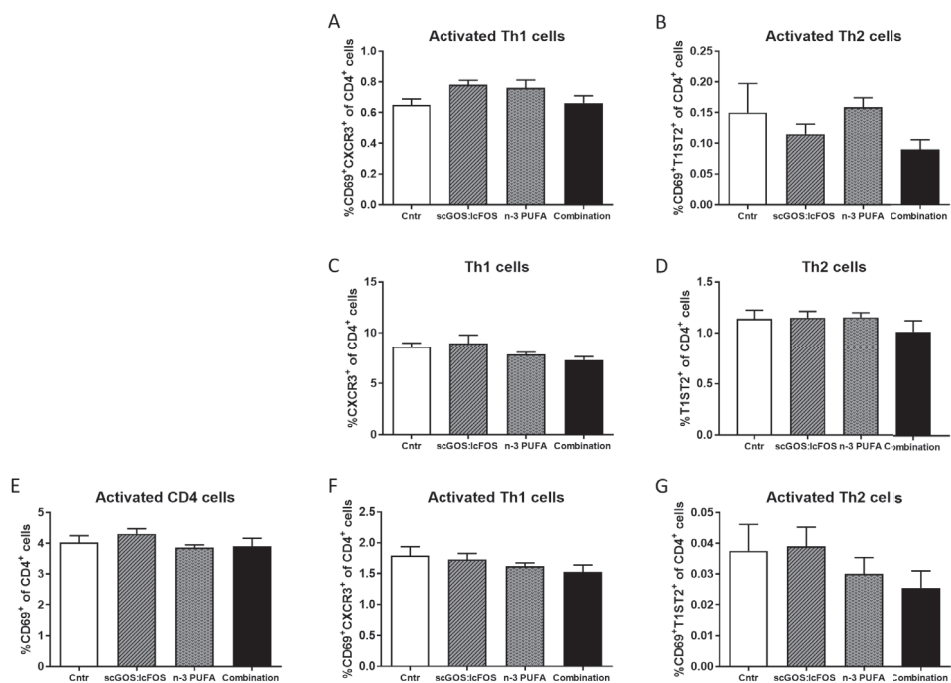
**Figure S2.** Th1 and Th2 response in MLN and spleen. (A,B) MLN: Activated Th1 and Th2 cells were not affected by the diets. (C-G) Spleen: Th1, Th2, activated T cells and activated Th1 and Th2 cells were unaffected by the diets. A-G: Data shown as mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. A-B: $n$ = 6-10 mice per group, 2 samples in the control group, 3 samples in the n-3 PUFA group and 2 samples in the combination diet group excluded due to low number of viable cells. C-G: $n$ = 8-10 mice per group, 1 sample in the combination diet group excluded due to low number of viable cells. Th1: T helper 1. Th2: T helper 2.
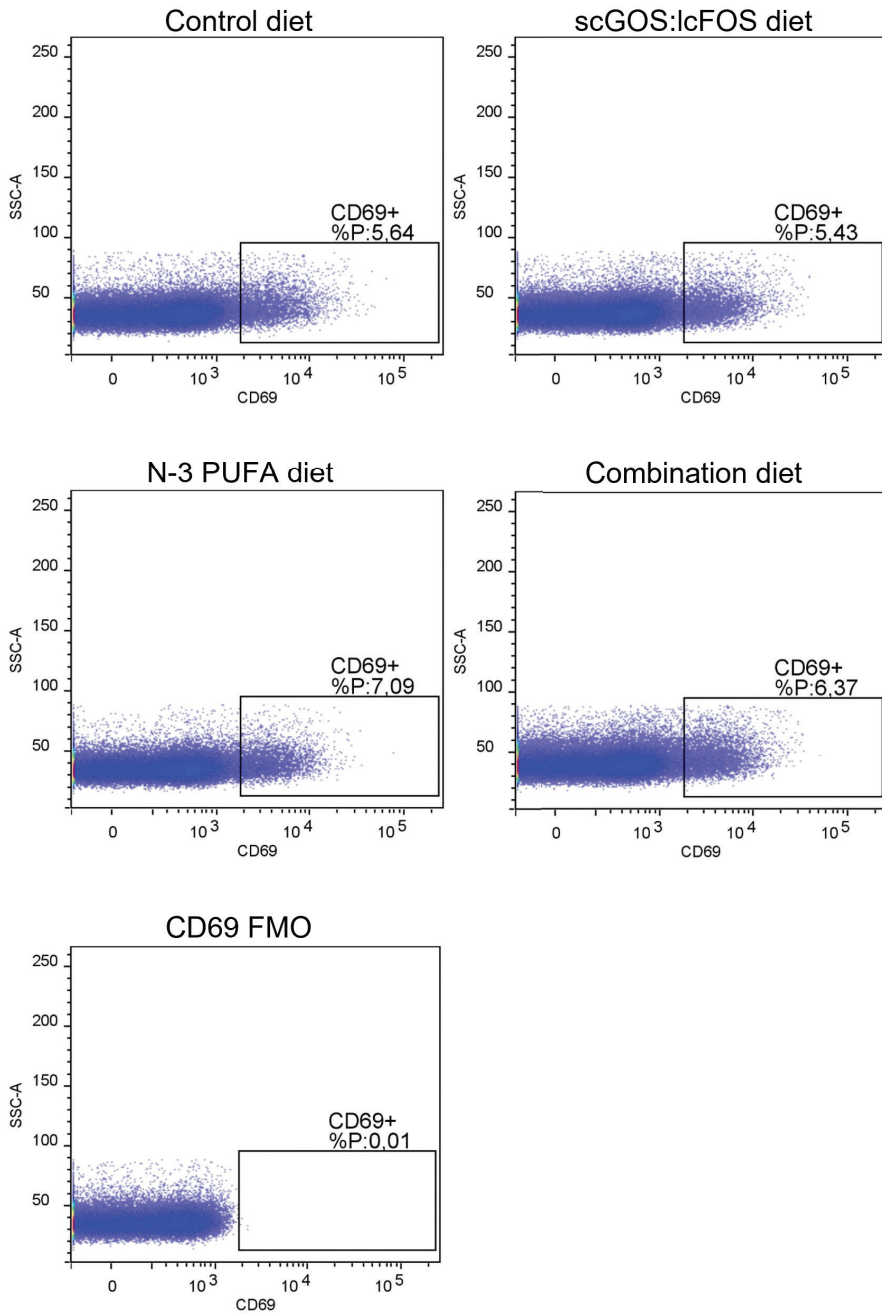
**Figure S3.** Representative dot plots of the CD69+ population from each diet group. The gate is based on the dot plot of the CD69 FMO.
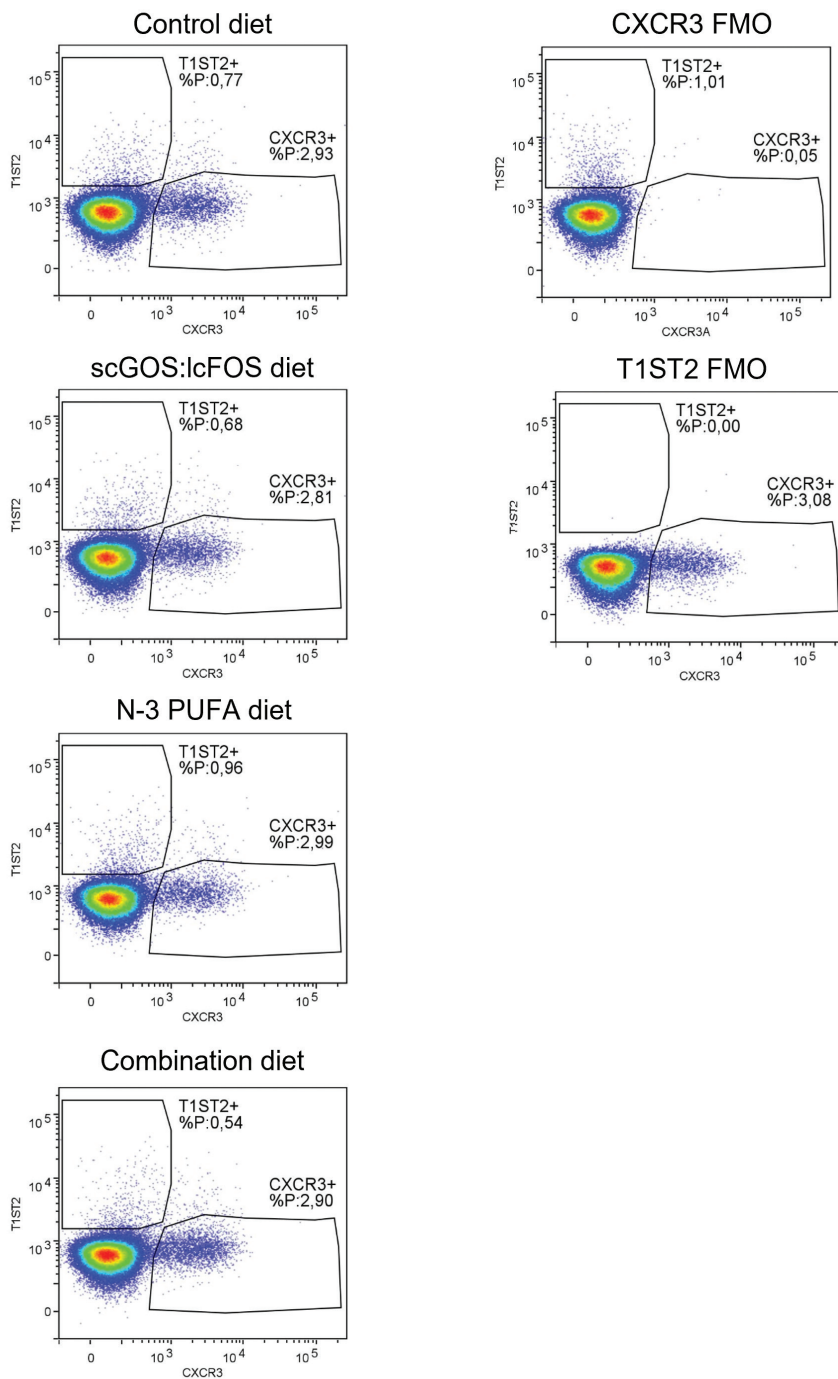
**Figure S4.** Representative dot plots of the Th1 (CXCR3$^+$) and Th2 (T1ST2$^+$) populations of each diet group. The gates were based on the CXCR3 FMO and T1ST2 FMO, respectively.
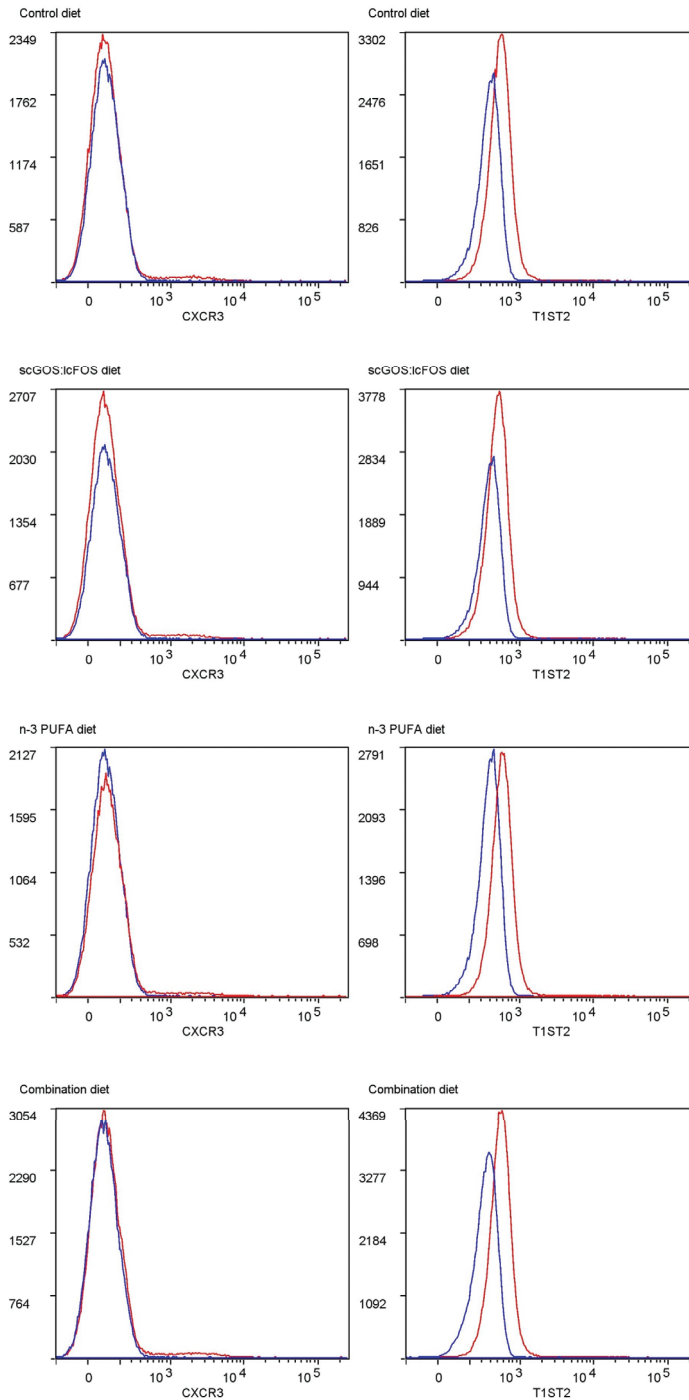
**Figure S5.** Representative histograms of CXCR3 and T1ST2 of each diet. The blue line indicates the FMO and the red line indicate the test sample.
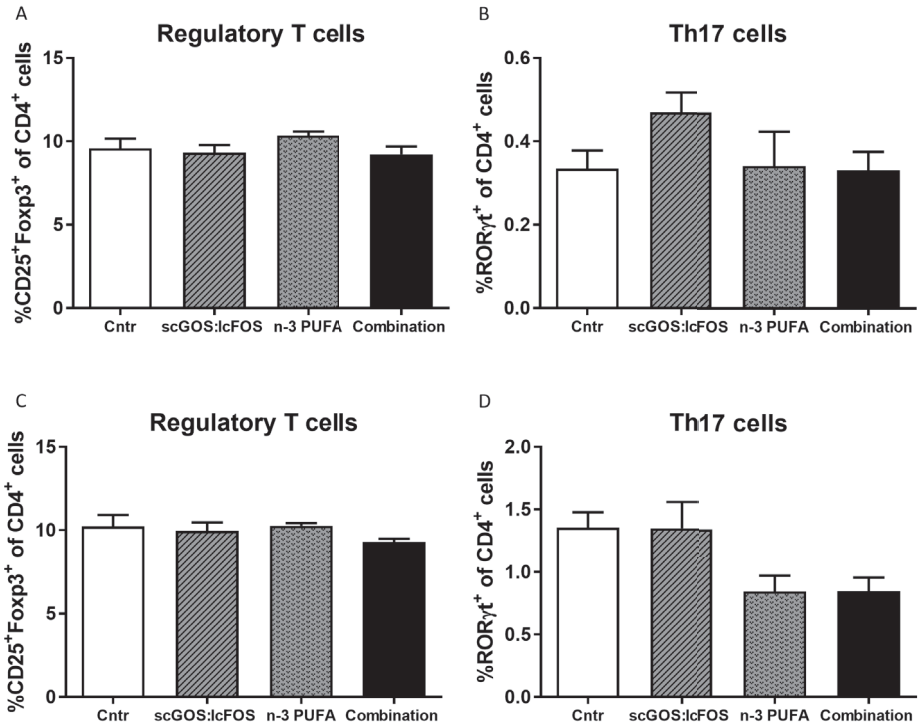
**Figure S6.** Treg and Th17 response in MLN and spleen. (A,B) No dietary effect on the Tregs and Th17 cells in the MLN. (C,D) No dietary effect on the Tregs and Th17 cells in the spleen. A-D: Data shown as mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. *n* = 7-10 mice per group, in the MLN 1 sample in the control, n-3 PUFA and combination diet excluded due to low number of viable cells. Th17: T helper 17.
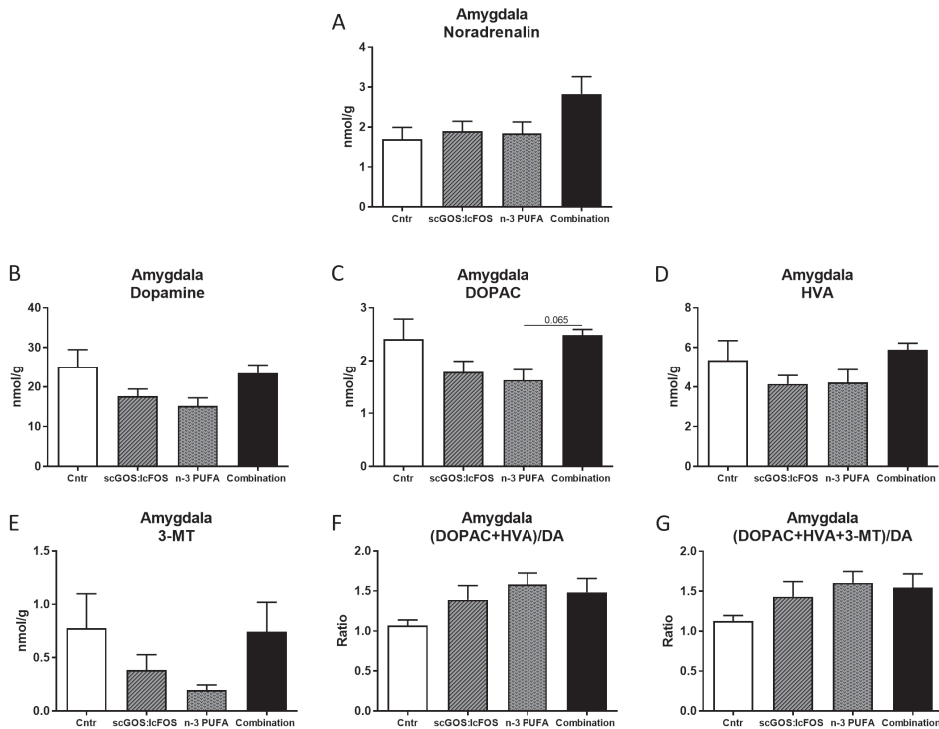
**Figure S7**. Monoamine levels in the amygdala. (A) Noradrenalin levels were unaffected by the diets. (B) Dopamine levels were unaffected by the diets. (C) The DOPAC level in the combination diet group tended to an increase compared to n-3 PUFA. (D,E) No dietary effect on HVA and 3-MT. (F,G) The turnover of dopamine was unaffected by the diets. A-G: Data shown as mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. $n$ = 4-5 samples per group, samples were pooled in pairs, in order to reach detection minimum, each sample contained two left brains. DOPAC: 3,4-dihydroxyphenylacetic acid. HVA: homovanillic acid. 3-MT: 3-methoxytyramine.
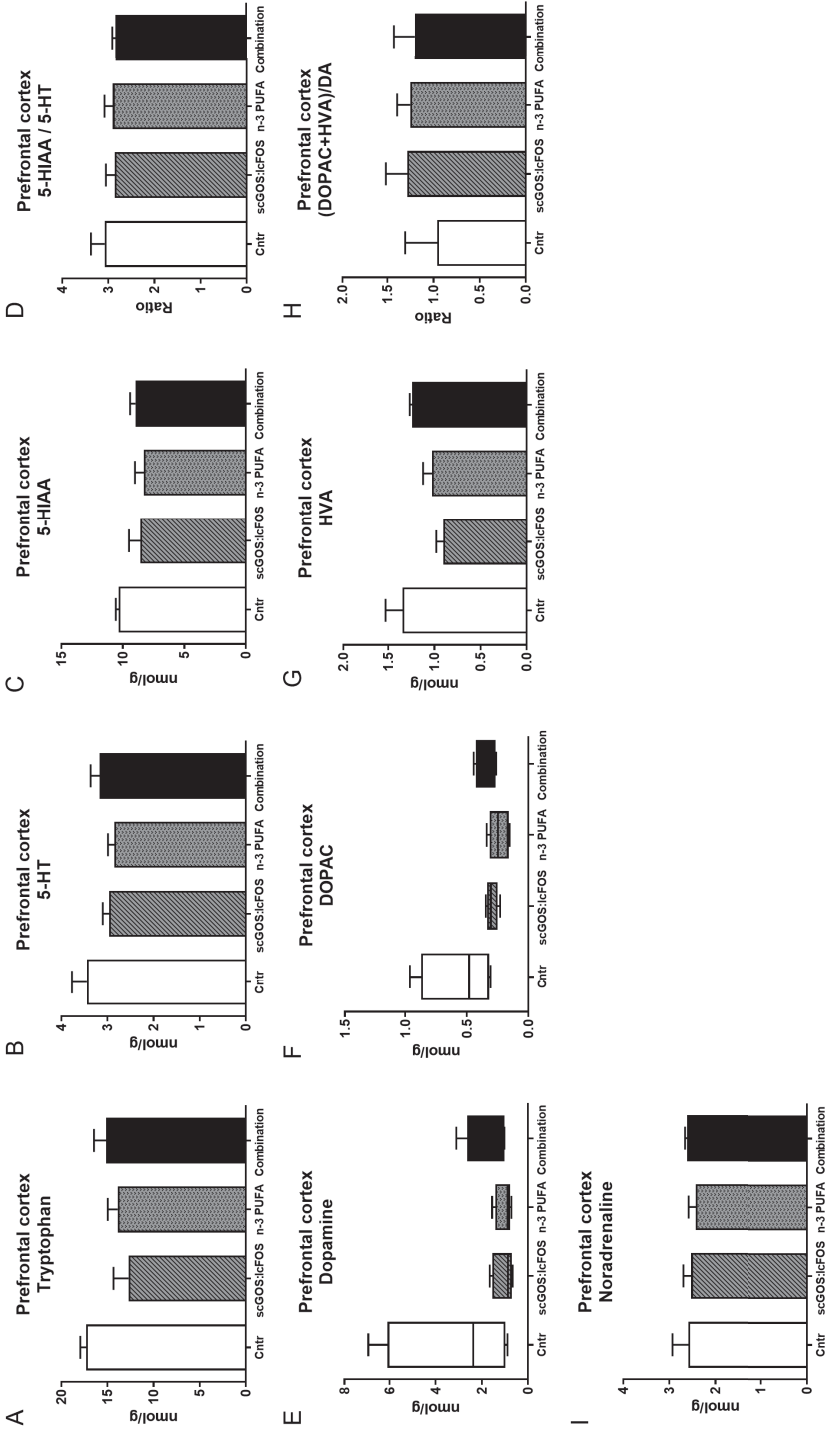
**Figure S8.** Monoamine levels and turnovers in the PFC. No differences were observed in any of the measured monoamines in the PFC. A-D,G-I: Data shown as mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. E,F: Data shown as box-and-whiskers Tukey plot. Analysed by Kruskal-Wallis and Dunn's multiple comparisons post hoc test. $n$ = 4-5 samples per group, samples were pooled in pairs, in order to reach detection minimum, each sample contained two left brains. 5-HT: serotonin. 5-HIAA: 5-hydroxyindoleacetic acid. DOPAC: 3,4-dihydroxyphenylacetic acid. HVA: homovanillic acid.
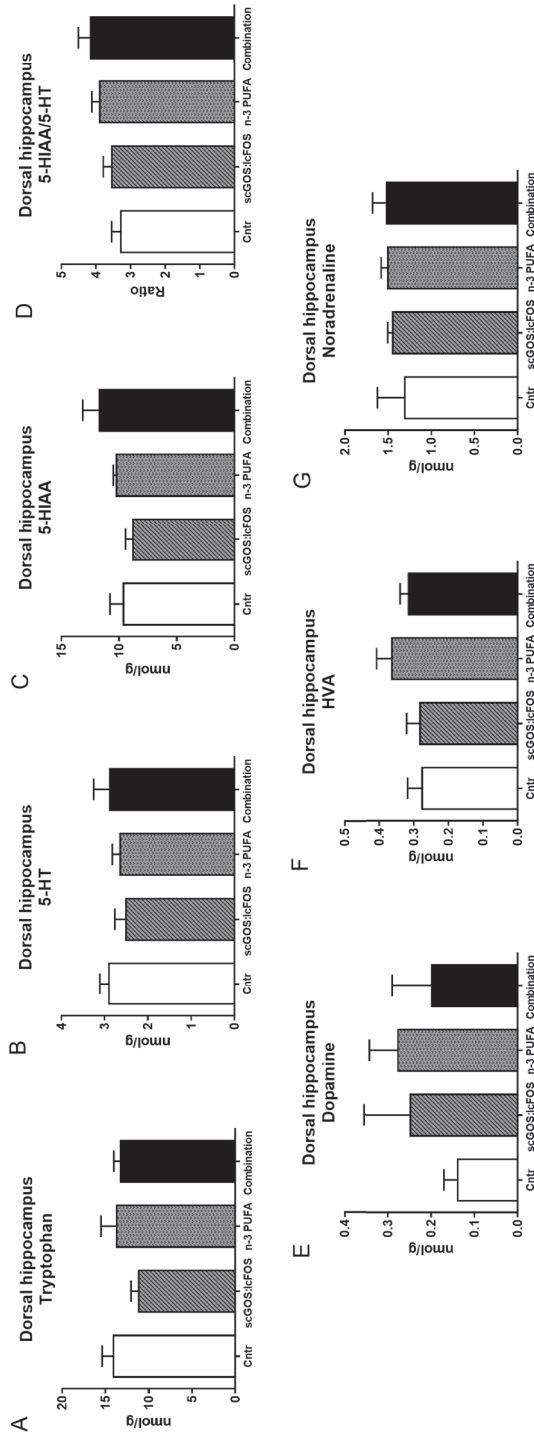
**Figure S9.** Monoamine levels and serotonin turnover in dorsal hippocampus. No differences were observed in any of the measured monoamines in the PFC. A-G: Data shown as mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. $n$ = 4-5 samples per group, samples were pooled in pairs, in order to reach detection minimum, each sample contained two left brains. 5-HT: serotonin. 5-HIAA: 5-hydroxyindoleacetic acid. HVA: homovanillic acid.
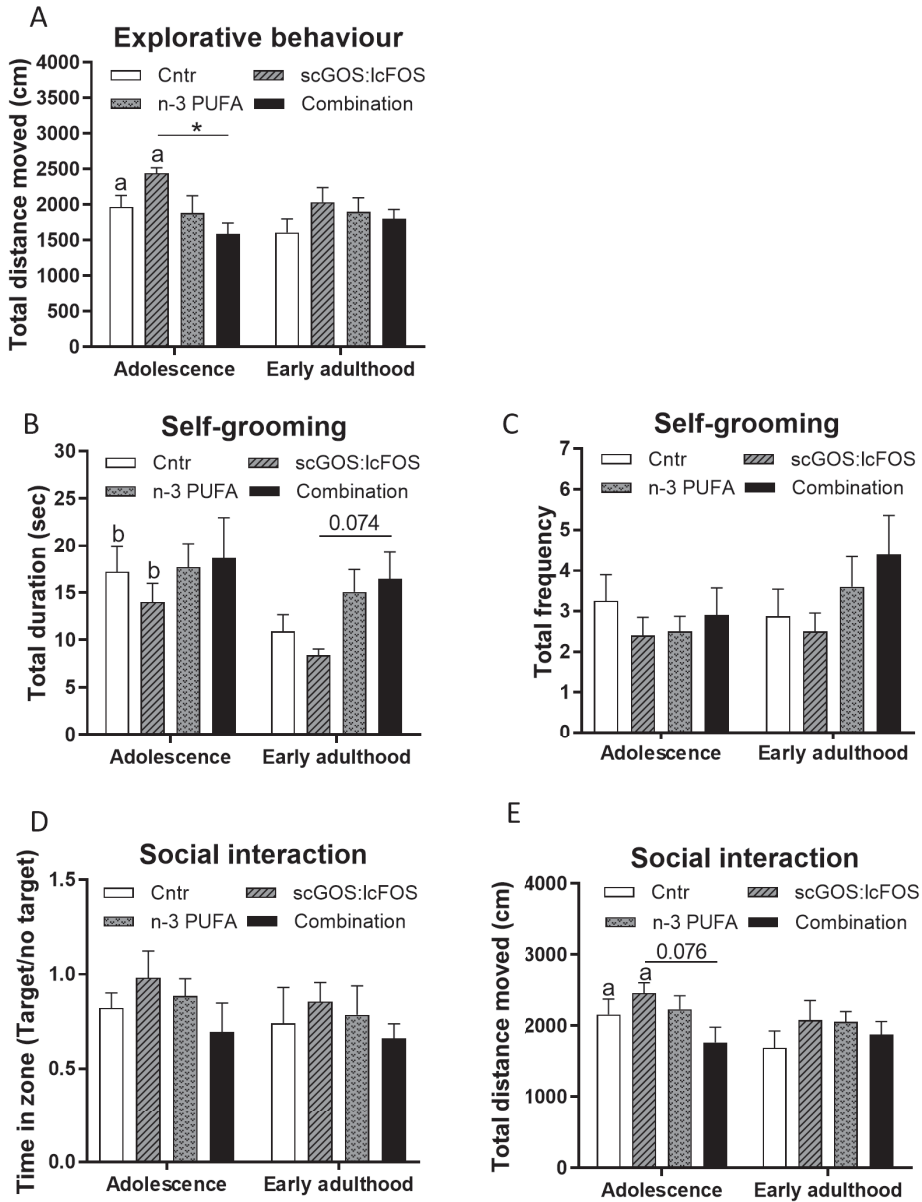
**Figure S10.** Behavioural data locomotion activity in the open field test, self-grooming and social interaction. (A) The mice receiving the control or the scGOS:lcFOS diet moved significantly less in early adulthood compared to adolescence. In adolescence, the locomotor activity was reduced in the combination diet group compared to the scGOS:lcFOS group. (B) The grooming duration tended towards a decrease overtime in the control as well as in the scGOS:lcFOS group. In early adulthood grooming duration showed an increasing trend in the combination group compared to the scGOS:lcFOS group. (C) The grooming frequency showed no significant differences. (D) The social interaction, shown as relative time in zone (target / no target), was neither affected by age nor diet. (E) The locomotor activity (distance moved) in the control and scGOS:lcFOS receiving mice

was significantly decreased over time. In adolescence, the locomotor activity tended towards a reduction in the combination diet group compared to the scGOS:lcFOS group. A-E: Data shown as mean +/- SEM. Analysed with mixed models, controlled for repeated measures, litter effect and Sidak's multiple comparisons post hoc test. * $P < 0.05$, a = * $P < 0.05$ compared with early adulthood within diet group, b = $P < 0.1$ trend compared with early adulthood within diet group. $n$ = 8-10 mice per group.

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Dietary supplementation throughout life with non-digestible oligosaccharides and/or omega-3 polyunsaturated fatty acids in healthy mice modulates the gut  – immune system – brain axis

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# CHAPTER 5

No additional preventive effect of a combined dietary intervention with non-digestible oligosaccharides and omega-3 polyunsaturated fatty acids on cow's milk allergy development in mice

Kirsten Szklany[1], Mara A. P. Diks[1], Ruben de Vries[1], Johan Garssen[1,2], Aletta D. Kraneveld[1], Leon M. J. Knippels[1]

[1] Division of Pharmacology, Utrecht Institute of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[2] Danone Nutricia Research, Utrecht, The Netherlands

## ABSTRACT

Food allergy is a major global health burden. The earliest food allergy to occur is cow's milk allergy (CMA). Next to symptoms as skin rash and gastro-intestinal discomfort, food allergy has, in several cases, been associated with behavioural symptoms. Currently, no treatment for food allergy is available. Nutritional components such as short-chain galacto-oligosaccharides, long-chain fructo-oligosaccharides (scGOS:lcFOS) and omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been shown to possess preventive effects in the development of allergies like CMA. In this study, we investigated whether a combined dietary intervention with scGOS:lcFOS and n-3 PUFAs has additional capacity to prevent development of CMA and CMA-associated behaviour in a murine whey-induced food allergy model examining both sexes. Male and female C3H/HeOuJ mice received control or enriched diet with a combination of scGOS:lcFOS (9:1) and 6% tuna oil (n-3 PUFAs) or individually scGOS:lcFOS (9:1) or 6% tuna oil (n-3 PUFAs) and were orally sensitized with whey protein. The acute allergic skin response was evaluated after an intradermal challenge with whey. Behaviour, serum immunoglobulin levels, mast cell degranulation, *ex vivo* cytokine responses in splenocytes, caecal concentration of short-chain fatty acids and the intestinal mRNA expression of *Ppary* were assessed after oral challenge with whey protein. In allergic male mice, scGOS:lcFOS reduced the drop in body temperature during allergic reaction, reduced the *ex vivo* whey-induced cytokine secretion from splenocytes and increased the caecal butyrate concentration. In allergic female mice, the scGOS:lcFOS or n-3 PUFAs reduced the acute allergic response. ScGOS:lcFOS or the combination diet increased the caecal butyrate concentration. In both male and female mice, the ileal *Ppary* mRNA expression was affected by the dietary intervention. Both allergic and dietary effects on behaviour were limited in male and female mice. Combining scGOS:lcFOS and n-3 PUFAs led to no additional preventive effect on the measured allergic and behavioural parameters. The results of the combination diet group were either equal to the control diet or the other dietary interventions or even less beneficial. This outcome was unexpected, as both dietary components were able to prevent CMA development.

# INTRODUCTION

Food allergy is a major global health burden: worldwide 240-550 million people are estimated to suffer from food allergy (1). The earliest food allergy to occur is cow's milk allergy (CMA) with a prevalence of approximately 2-3% in westernized countries (2). Most children outgrow CMA during the first 5 years of life, however, these children are more prone to develop other (food) allergies later in life (3). During an allergic reaction, these children can experience symptoms like skin rash, gastro-intestinal discomfort (diarrhoea, vomiting) and in severe circumstances anaphylactic shock. In addition to these clinical symptoms, food allergy has, in several cases, been associated with behavioural symptoms, such as neurological manifestations like anxiety, depression and autism spectrum disorder-like behaviour (4-7). Currently, no treatment for food allergy is available; the only option is avoidance of the allergen. This and the increased risk of developing other allergies later in life as well as the possible neurological problems emphasize the importance of preventing the development of CMA.

During the first 6 months of life, human breast milk is the recommended nutrition. Although the evidence of the preventive effect of human breast milk on CMA is insufficient, the risk of developing other allergic disorders like asthma and eczema decreases when human breast milk is consumed during the first 3-4 months (8-10). Human breast milk contains components important in the development of the central nervous system, endocrine system and development and maturation of the gastro-intestinal tract and the immune system (11). These components include among others polyunsaturated fatty acids (PUFAs), human milk oligosaccharides (HMOs), immunoglobulins, proteins, vitamins and micronutrients (11). One of the most abundant components in human breast milk are HMOs. HMOs possess prebiotic activity promoting the growth and activity of beneficial bacteria like Bifidobacteria and Lactobacilli (12). Growth and activity of beneficial bacteria is important for a well-developed intestinal microbiota as the intestinal microbiota is an essential player in the development and maturation of the gastro-intestinal tract, immune system and the nervous system in early life (11). Unfortunately, human breast milk is not always available. Alternatively, infant milk formula containing non-digestible oligosaccharides (NDOs) that mimic the HMOs in human breast milk is provided. An example of such NDOs is the mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS). This specific mixture of scGOS and lcFOS (scGOS:lcFOS) also has prebiotic activity and can modulate the immune system directly and indirectly. Directly by interaction with dendritic cells (DCs) followed by induction of regulatory T cells (Tregs) to stimulate development of tolerance and by interaction with the intestinal epithelial cells leading to T helper 1 cells (Th1) and Treg responses also stimulating development of homeostasis (13, 14). And indirectly via fermentation by bacteria in the intestinal tract into short-chain fatty acids (SCFAs), which have anti-inflammatory properties and improve the intestinal barrier integrity (15-17). Although the mechanism is not completely unravelled, scGOS:lcFOS has preventive effects in the development of allergies like CMA (18, 19). Next to the immune modulatory capacity, prebiotics have been shown to reduce depression and anxiety-like behaviour

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in laboratory animals (20-22). Although the data are promising, more clinical trials are needed to support this evidence in humans (23).

Another immunomodulatory component present in human breast milk as well as in infant milk formula are PUFAs. PUFAs are incorporated into the cell membrane of immune and neuronal cells and influence the communication in the cell leading to immune and behaviour modulation (24, 25). The most important PUFAs are omega-3 (n-3) and n-6 PUFAs. The balance of n-6 and n-3 PUFAs is essential as n-6 PUFAs are converted into the pro-inflammatory mediators and n-3 PUFAs into anti-inflammatory mediators. Skewing of the n-6:n-3 PUFA ratio towards n-6 by consumption of meat and eggs at the expense of fatty fish is possibly playing a role in the rising prevalence of allergies (26). During the past decades this skewing towards n-6 PUFAs is observed in the western world and this might be linked with increased prevalence of allergic diseases. In populations with high fish intake and a n-6:n-3 ratio around 1:1, the prevalence of allergies is low indicating the importance of the balance of n-6 and n-3 PUFAs (27-29). Although the evidence is limited in clinical trials, n-3 PUFAs can induce tolerance and prevent development of allergies such as CMA in experimental models (29, 30). Finally, in rodents, n-3 PUFA deficiencies can lead to depression-like behaviour and n-3 PUFA supplementation reduces anxiety-like behaviour as well as ASD-like behaviour (31-33). However, in humans the beneficial properties of n-3 PUFAs in depression and anxiety is contradictory (34-37).

As both n-3 PUFAs and the mixture of scGOS:lcFOS have beneficial impact in prevention of CMA and might modulate disturbed behaviour, it is of interest to combine these components to evaluate a possible additional effect. In this study, we investigated whether a combined dietary intervention with n-3 PUFAs and scGOS:lcFOS has additional capacity to prevent development of CMA and CMA-associated behaviour in a murine whey-induced food allergy model examining both sexes. The sex difference was examined as sex difference has been reported in allergy and in behavioural disorders, both clinically and pre-clinically (38-45).

## MATERIALS AND METHODS

### Animals

Three week old male and female C3H/HeOuJ mice, purchased from Charles River Laboratories (Germany), were housed in Makrolon type III cages with *ad libitum* access to food and water on a 12 h light/dark cycle at the animal facility of Utrecht University (Utrecht, The Netherlands). The experimental procedures were executed during the light phase. The mice were randomly allocated to the cages upon arrival (n = 3 per cage). Following, the cages were randomly assigned to a treatment group. All animal experimental procedures were carried out in compliance with national legislation following the EU-Directive for the protection of animals used for scientific purposes and were approved by the Ethical Committee for Animal Research (Approval number AVD225002016521 and AVD1080020173506).

## Diets

Cow's milk protein free AIN-93G mice chow containing 10% fat (serving as control diet) were supplemented, in an iso-caloric manner, with 1% scGOS (degree of polymerization 2-8, Friesland Campina, The Netherlands) and lcFOS (degree of polymerization on average ≥ 23, Orafti, Wijchen, The Netherlands) in a 9:1 (w/w) ratio and/or 6% n-3 PUFA (6% of the soybean oil was substituted with tuna oil). The tuna oil was a kind gift from Bioriginal (Den Bommel, The Netherlands). The diets were manufactured by Ssniff Spezialdiäten gmbH (Soest, Germany).

## Animal procedures

The experimental design is schematic represented in **Figure 1**. The weight of the mice was recorded weekly (not shown in **Figure 1**) and area under the curve (AUC) for each experimental group was calculated to compare the mean bodyweight of the mice between treatment groups. The dietary interventions started at day -14 (day of arrival) and continued throughout the experiment (n = 11-15 per group). At day 0, 7, 14, 21 and 28 the mice were orally sensitised with 20 mg whey protein in 0.5 mL PBS containing 10 µl cholera toxin (CT, List Biological Laboratories, Campbell, CA, USA). Non-sensitised mice received CT only (10 µL/0.5 mL PBS). After the third sensitisation temperature transponders were injected subcutaneously. The day after the fourth sensitisation, the marble burying test was conducted. On day 35 mice were orally challenged with 50 mg whey protein in 0.5 mL PBS followed by blood sampling 1 h later to measure mast cell degranulation. The day after the oral challenge the social interaction and the open field test was conducted. On day 42 all mice were intradermally (i.d.) challenged in the ear pinnae (10 µg whey protein in 20 µl PBS per ear) to evaluate the acute allergic response. Subsequently, the mice were orally challenged (7 h after i.d. challenge) with 50 mg whey protein in 0.5 mL PBS. The next day (day 43) mice were euthanised, decapitated and blood and organs were collected.
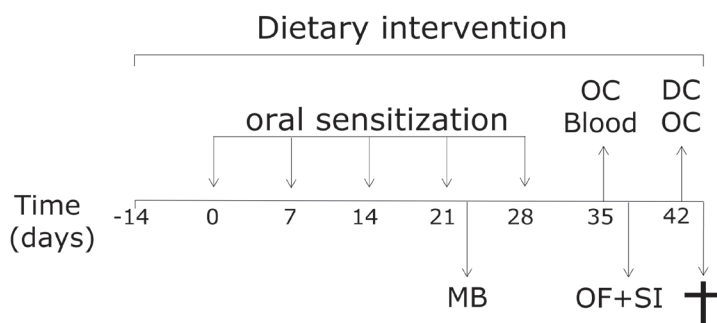
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**Figure 1**. Schematic overview of the experimental setup and group description. The mice received the allocated diet 2 weeks prior the first sensitization until the end of the experiment. On day 0, 7, 14, 21 and 28 of the experiment the mice were either sham- or whey protein sensitized. On day 22 the behavioural marble burying test was conducted. The open field and social interaction behavioural tests were conducted on day 36, one day after an oral whey protein challenge. Blood was sampled one hour after the oral challenge on day 35. On day 42 the mice were intradermally challenged in the ear and acute allergic symptoms were evaluated. Subsequently, the mice were orally challenged with whey protein. The following day the mice were euthanised and decapitated and organs were collected for further analysis. MB: marble burying behavioural test, OF: open field behavioural test, SI: social interaction behavioural test, OC: oral challenge, DC: intradermal challenge, CT: cholera toxin.

## Evaluation of the acute allergic response

To evaluate the severity of the allergic reaction and the symptoms, the acute allergic skin response, anaphylactic shock symptoms and body temperature were measured after i.d. challenge. Ear thickness of both ears was measured in duplicate before and 1 h after the i.d. challenge using a digital micrometre (Mitutoyo, Veenendaal, The Netherlands). The acute allergic response was calculated by subtracting the mean ear thickness at base line from the mean ear thickness 1 h after challenge, expressed as ear swelling ($\Delta$ µm). During the ear measurements and the i.d. challenge, mice were anesthetised twice using isoflurane inhalation. Symptoms of anaphylactic shock were scored at 30 min after i.d. challenge according to previous described and validated scoring table (46). To monitor the anaphylactic associated drop in body temperature, the temperature was recorded after 30 min by reading the transponders.

## mMCP-1 and whey specific immunoglobulins in serum

Blood collected 1 h after oral challenge (day 35) and after decapitation (day 43) were centrifuged at 10,000 rpm for 10 min, the serum was stored at -20 °C until further analysis. The concentrations of mouse mucosal mast cell protease-1 (mMCP-1) was detected using an mMCP-1 ELISA Ready-SET-Go (Fisher Scientific, Landsmeer, The Netherlands) according to manufacturer's protocol.

Whey specific immunoglobulin levels were determined as previously described (47), with few adjustments. Briefly, Costar 9018 high binding plates were coated overnight at 4 °C with 20 µg/mL whey in carbonate/bicarbonate buffer. After washing, the next day, the plates were blocked with 1% BSA in PBS for 1 h, followed by sample incubation for 2 h at room temperature (RT). After another wash, 1 µg/mL biotin-labelled rat anti-mouse IgE, IgG1 or IgG2a was added for 1.5 h at RT. Following, the plates were washed and incubated with streptavidin-horseradish for 45 min, washed again and developed with o-phenylendiamine. The reaction was stopped with 4M $H_2SO_4$ and using a Benchmark plate reader (Bio-Rad, Veenendaal, The Netherlands) the absorbance was measured at 490 nm. The immunoglobulin levels are shown as arbitrary units based on a titration of pooled sera serving as a standard.

## Evaluation of behaviour

### Social behaviour

Social behaviour was evaluated as previously described (20, 48). In brief, a perforated plexiglass cage allowing visual and olfactory interaction was placed against a side in the open field of L45 x W45 cm. The mice were habituated for 5 min in the open field, followed by 5 min with an age- and sex-matched unfamiliar mouse in the plexiglass cage. Time in interaction zone, frequency in the interaction zone and distance moved were analysed.

### Repetitive behaviour

Repetitive behaviour was evaluated as previously described (20, 49). Briefly, mice were placed individually in a cage (L35 x W20 x H15 cm) with twenty black marbles placed on the bedding. After 30 min the number of marbles buried for $^2/_3$ in the bedding was counted.

### Open field

Explorative behaviour was evaluated using the open field test as previously described (50). Individually the mice were placed in the centre of an open field (L45 x W45 cm), cleaned with 70% alcohol, and recorded for 5 min with a Sony Handycam DCR-SR72 video camera. The time spent in the centre and in the corners of the open field and the locomotor activity were blindly analysed by use of tracking software (Ethovision 3.1.16; Wageningen, The Netherlands).

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**Caecal short-chain and branched-chain fatty acid concentrations**

The caeca contents were stored at -80 °C until analysis of the SCFA concentrations: acetate, propionate, butyrate and valerate and the branched-chain fatty acid (BCFAs) concentrations: iso-valerate and iso-butyrate. The SCFA and the BCFA concentrations were quantified by a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) as described previously (51).

**Isolation of spleen cells**

Spleen tissue was crushed through a 70 µm cell strainer to single-cell suspensions and the red blood cells were lysed using a lysis buffer (8.3 g/L NH4Cl, 1 g/L KHCO3, and 37.2 mg/L EDTA). The cell suspensions were resuspended in RPMI 1640 medium (Lonza) with heat-inactivated foetal calf serum (FCS, 10%, Bodinco, Alkmaar, The Netherlands), penicillin (100 U/mL)/streptomycine (100 µg/mL) (Sigma-Aldrich) and β-mercapto-ethanol (20 µM, Thermo Fisher Scientific).

**Cytokine response – *ex vivo* splenocyte restimulation**

The splenic single-cell suspensions ($8 \times 10^5$ cells/well) were stimulated with medium, anti-CD3/CD28 polyclonal stimulation (10 µg/mL anti-CD3 and 1 µg/mL anti-CD28, eBioscience) or whey (50 µg/mL) and harvested after 48 or 96 h, respectively. The anti-CD3/CD28 stimulation was used to verify the functionality of the splenic cells (**Figures S2**, **S3**). The production of IL-5, IL-13, IL-10 and IFNɣ in the supernatants were quantified by ELISAs according to the protocol described for IgE above, the only adjustment: biotinylated antibodies were incubated for 1 h. Purified rat anti-mouse antibodies (IL-5 and IFNɣ 1 µg/mL, IL-10 2 µg/mL), recombinant mouse cytokines (IL-5, IFNɣ and IL-10) and biotinylated rat anti-mouse antibodies (IL-5, IFNɣ and IL-10 1 µg/mL) were purchased at BD Biosciences. Purified rat anti-mouse IL-13 (2 µg/mL), recombinant mouse IL-13 and biotinylated rat anti-mouse IL-13 (0.4 µg/mL) were purchased at R&D Systems).

**Intestinal *Pparɣ* expression**

Intestinal tissues (jejunum and ileum) were stored in RNAlater® (Sigma-Aldrich, Lithuania) at -20 ºC after 2 days at 4 °C. The intestinal tissue was precipitated in LPB lysis buffer and subsequently lysed and homogenized using a Precellys 24 (Bertin Technologies, France) and centrifuged at 14,000 rpm. The supernatant was transferred to a Nucleospin® gDNA removal column and centrifuged for 30 sec at 11,000 *g*. The flow-through was mixed with the binding solution (BS) and transferred to the Nucleospin® RNA plus column and centrifuged for 15 sec at 11,000 *g*. The samples were washed with Buffer WB1 and again centrifuged for 15 sec at 11,000 *g*. The samples were incubated for 15 min with RNase free DNase (Qiagen, Hilden, Germany). Buffer WB2 was added, and the samples were centrifuged for 15 sec at 11,000 *g*. For the final washing step WB2 was added and the samples were centrifuged for 2 min at 11,000 *g*. RNA was eluded from the column using RNase-free water and centrifuged for 1 min at 11,000 *g*. RNA was isolated using a Nucleospin® RNA plus kit (Machery-Nagel, Düren, Germany)**.** cDNA synthesis was

performed using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, United States) and a T100 thermal cycler (Bio-Rad, Hercules, United States). Hard-shell® PCR 96-well thin wall plates (Bio-Rad, Hercules, United States) were filled with cDNA samples, iQ− SYBR Green (Bio-Rad, Hercules, United States), and either *Pparγ* mouse primer (#PPM05108C-200, Qiagen, Hilden, Germany) or *Rps13* mouse primer (#PPM25809A-200, Qiagen, Hilden, Germany). Quantitative real-time PCR analysis was performed using a CFX96 Real-time system (Bio-Rad, Hercules, United States) and the mRNA levels were analysed using the Bio-Rad CFX Manager. mRNA expression was quantified as fold-change using qPCR Ct values from both *Pparγ* and *Rps13*.

### Statistical analysis

The statistical analysis was performed by comparing pre-selected comparisons using one-way ANOVA and Sidak's multiple comparison post hoc test. The data were transformed in case normality was not obtained or the variance was unequal. If transformation failed, the data were analysed by the Kruskal-Wallis test and Dunn's multiple comparisons post hoc test. GraphPad Prism software (version 8.4.3; GraphPad Software, San Diego, CA, USA) was used to statistically analyse the data. Data were considered statistically significant when $P < 0.05$.

## RESULTS

### Allergic and dietary effect on bodyweight

From three weeks of age to 10 weeks of age the mice were weighed weekly to follow the dietary effect on the bodyweight (**Figure 2**). In both sexes, the weight gain was unaffected by sensitization and challenge with whey. In males, the scGOS:lcFOS fed whey-sensitized mice tended to be heavier than the whey-sensitized mice receiving the control diet. The n-3 PUFA or the combination diet had no influence on the bodyweight in whey-sensitized and challenged male mice. The scGOS:lcFOS fed whey-sensitized mice were significantly heavier than whey-sensitized male mice fed the combination diet (**Figure 2A**, **Table 1**). In whey-sensitized and challenged female mice, the bodyweight was unaffected by the diets (**Figure 2B**, **Table 1**). The male mice were heavier than the female mice.

**Table 1.** AUC of weight curve of male and female mice from 3-10 weeks of age.

|  | Neg control | Pos control | scGOS:lcFOS | n-3 PUFA | Combination |
|---|---|---|---|---|---|
| Male | 162.9 ± 2.96 | 160.0 ± 3.21[#] | 172.0 ± 3.79[a,#] | 160.2 ± 1.91 | 158.7 ± 2.71[a] |
| Female | 130.0 ± 3.04 | 135.3 ± 2.61 | 133.5 ± 3.25 | 135.8 ± 2.82 | 132.0 ± 3.06 |

Data shown as AUC ± SEM AUCs with same superscript letter in each row are significantly different. a: AUCs of the scGOS:lcFOS group and the combination group differ significantly $P < 0.05$. #: AUCs of the positive control and the scGOS:lcFOS group tend to differ $P < 0.1$. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. $n = 11-15$ mice per group.
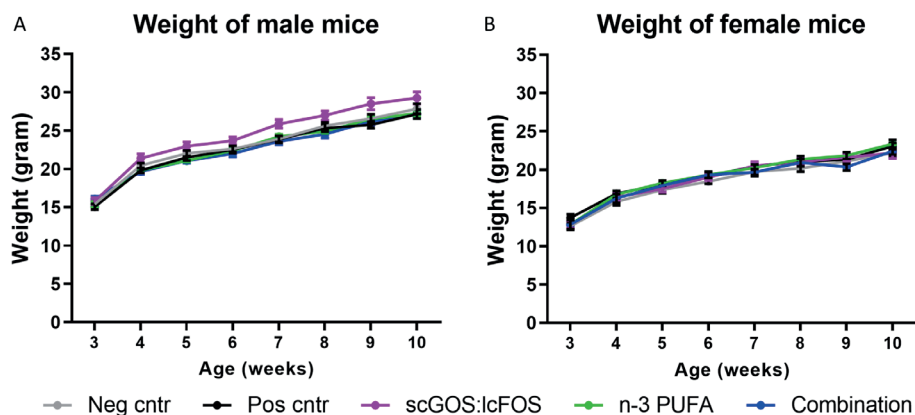
**Figure 2.** Weight of male (A) and female (B) mice over time throughout the experiment. A,B: Data shown as mean +/- SEM. Analysis based on AUC see **Table 1**. *n* = 11-15 mice per group.

### Dietary interventions affect the allergic symptoms in male and female mice differently

The acute allergic skin response was measured one hour after the i.d. challenge (day 42) in the ear to evaluate the capacity of the dietary interventions to prevent an allergic response to whey. The acute allergic skin response, in both male and female whey-sensitized control mice, was increased compared to sham-sensitized control mice (**Figure 3**). In male mice the allergic skin response was unaffected by any of the dietary interventions (**Figure 3A**). In the female mice, the allergic skin response was significantly reduced in mice receiving the scGOS:lcFOS and n-3 PUFA interventions separately compared to the whey-sensitized control mice. On the other hand, the allergic skin response in the female mice fed the combination diet tended to a decrease compared to the whey-sensitized control mice. This allergic response in mice receiving the combination diet group tended to be increased compared to the n-3 PUFA fed mice.

Other symptoms of an acute allergic reaction are anaphylactic shock and lowered body temperature. For both male and female allergic control diet fed mice the shock scores were significantly increased, and the body temperature was significantly decreased after i.d. challenge compared to the sham-sensitized control mice (**Figures 4A-D**). In both male and female mice, none of the dietary interventions led to significant effects on the allergic shock score. In males, the combination diet tended to increase the allergic shock score compared to the whey allergic control diet fed mice (**Figures 4A, B**). No significant correlation was observed between the acute allergic skin response data (ear swelling) and the shock score in male and female mice (**Figures S1A, B**).
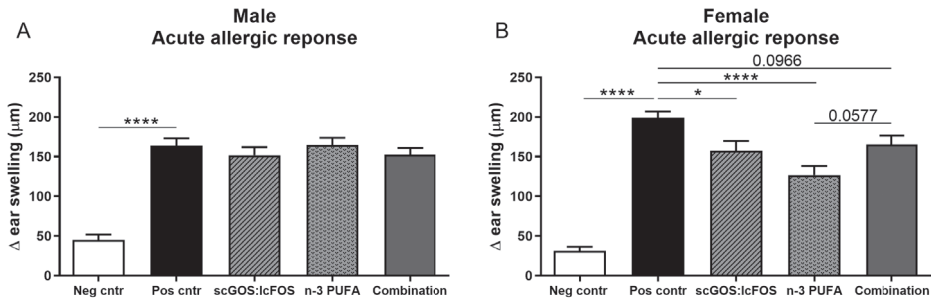
**Figure 3.** Effects of the dietary interventions on the acute allergic skin response one hour after intradermal challenge with whey protein. (A) Male mice. (B) Female mice. A,B: Data shown as mean +/- SEM. Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, **** $P < 0.0001$. $n$ = 11-15 samples per group.

After i.d. challenge whey-sensitized male and female mice showed a significant drop in body temperature. Only in male mice, the drop in temperature caused by the allergic reaction was reduced by the scGOS:lcFOS dietary intervention. The dietary intervention with n-3 PUFAs had no effect on the allergic reaction-induced decreased body temperature. Compared to the scGOS:lcFOS dietary intervention, the body temperature in the allergic mice fed the combination diet was significantly lower (**Figure 4C**). In the female mice, the allergy-induced drop in body temperature was unaffected by all dietary interventions (**Figure 4D**). In addition, no significant correlation was observed in neither male nor female mice between the acute allergic skin response data and changes in body temperature (**Figures S1C, D**).

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**Figure 4**. Effects of the dietary interventions on shock and body temperature; symptoms of an allergic reaction after exposure to whey protein. Shock score in male (A) and female (B) mice. Body temperature of male (C) and female (D) mice. A,B: Data shown as individual datapoints. C,D: Data shown as box-and-whiskers Tukey plots. A,B,D: Analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. C: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. $n$ = 11-15 samples per group.

**The whey allergic reaction-induced immunoglobulin serum levels and mast cell degranulation were unaffected by the dietary interventions**

Serum whey specific immunoglobulin (Ig) E, G1 and G2a levels, measured 18 h after oral challenge, were increased in whey allergic male and female mice compared to non-allergic mice (**Figure 5**). The levels of all measured classes of Ig's were unaffected by any of dietary interventions in both male and female allergic mice (**Figure 5**). The serum Ig levels in whey allergic males were lower than in whey allergic females, all receiving the control diet.
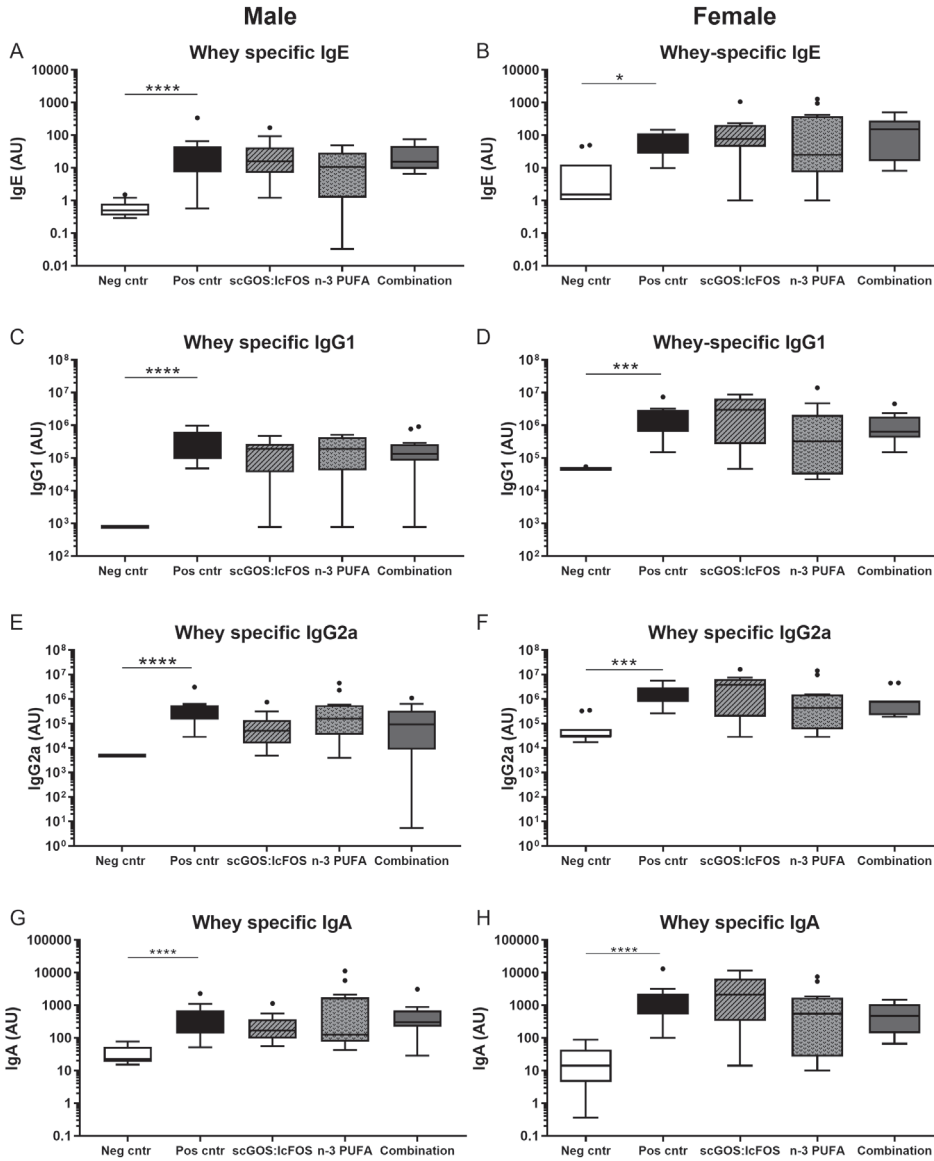
**Figure 5**. Dietary effects on allergen specific serum immunoglobulins. Whey specific IgE (A,B), IgG1 (C,D), IgG2a (E,F) and IgA (G,H) levels in male and female mice, respectively. A-H: Data shown as box-and-whiskers Tukey plots. A-H: Analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. $n$ = 11-15 samples per group.

The intestinal mucosal mast cell degranulation, assessed by serum levels of mMCP-1, one hour after oral whey challenge, was increased in whey-sensitized male and female mice when compared to sham-sensitized mice (**Figure 6**). Whey sensitization in male mice seems to lead to higher mMCP-1 concentrations after oral challenge compared to female mice. The allergic response-associated increases in serum mMCP-1 in both whey-sensitized sexes were unaffected by all dietary interventions (**Figure 6**).
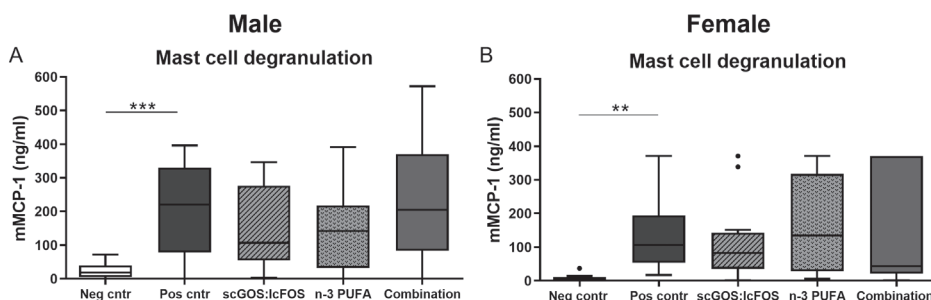


**Figure 6.** Influence of the dietary interventions on the mast cell degranulation measured in serum one hour after oral challenge with whey protein. (A) Male mice. (B) Female mice. A,B: Data shown as box-and-whiskers Tukey plots. A,B: Data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. ** $P < 0.01$, *** $P < 0.001$. $n$ = 9-15 samples per group. mMCP-1: mucosal mast cell protease-1.

## Effect of dietary interventions on the systemic immune response assessed by *ex vivo* whey-specific cytokine responses in splenocytes

To evaluate the functionality of the murine splenocytes, cytokine responses were measured after *ex vivo* stimulation with whey. The allergic clinical parameters in both male and female allergic mice were accompanied by a splenocyte whey specific cytokine response. The whey specific IL-13 response of splenocytes was significantly increased in the whey allergic control diet fed group, in both males and females, compared to the non-allergic control group. The whey-induced IL-13 response was unaffected by the dietary interventions in both males and females (**Figures 7A, B**). In the medium control, a whey allergic baseline effect of spontaneous IL-13 production by splenocytes was observed in both male and female mice (**Figures 7A, B**). This allergic effect was affected by scGOS:lcFOS in male mice (**Figure 7A**).

The whey specific IL-5 response was significantly increased in splenocytes obtained from the whey allergic male and female mice fed the control diet, compared to the non-allergic control. The whey induced IL-5 response was unaffected by any of the dietary interventions compared to the allergic control diet fed group, in both males and females (**Figures 7C, D**). In whey exposed males, compared to the non-allergic control, the combination diet led to a significant increased IL-5 *ex vivo* response. Intervention with scGOS:lcFOS or n-3 PUFAs in whey exposed male mice showed no significant increase in *ex vivo* IL-5 release, compared to non-allergic control mice. This indicates preventive effects of scGOS:lcFOS

or n-3 PUFAs on the IL-5 response in splenocytes after whey specific *ex vivo* stimulation. In the medium control, an allergic baseline effect was observed in splenocytes from allergic male mice, which was unaffected by any dietary intervention (**Figure 7C**). In female mice no allergic baseline effect was observed **(Figure 7D)**.
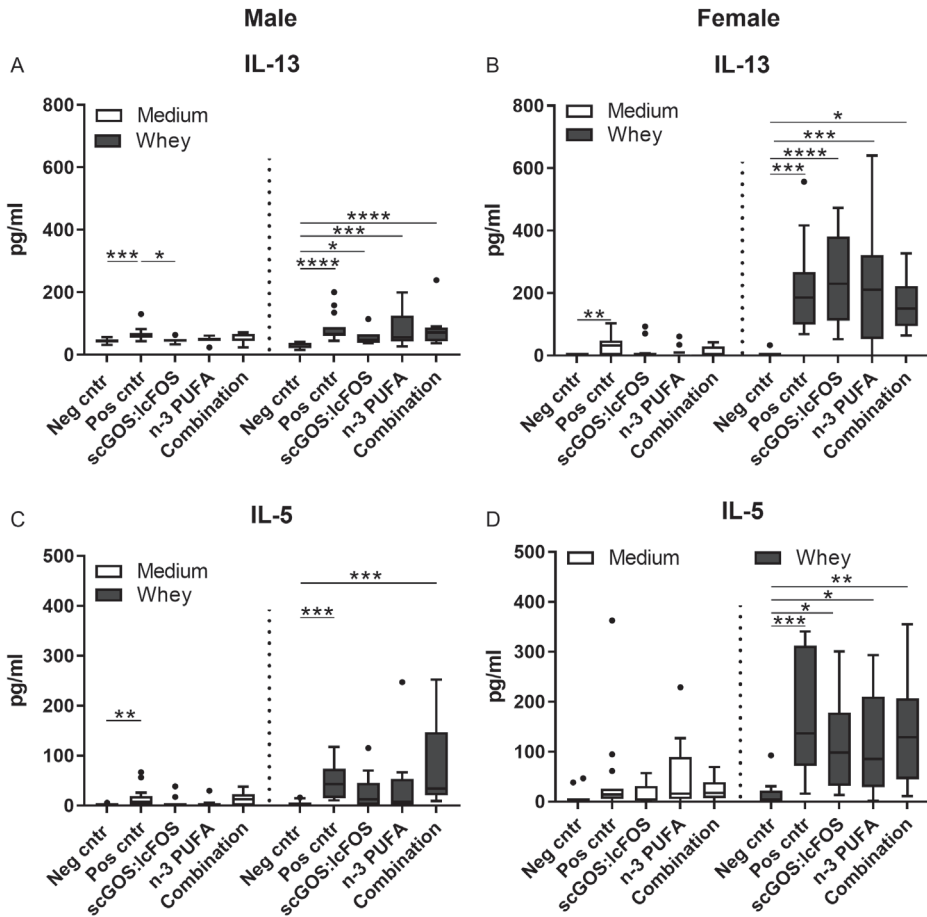


**Figure 7**. Effect of dietary interventions on the systemic IL-13 and IL-5 responses assessed by *ex vivo* whey-specific cytokine responses in splenocytes. Unstimulated and whey stimulated e*x vivo* IL-13 response (A,B) and IL-5 response (C,D) in splenocytes in male and female mice, respectively. A-D: Data shown as box-and-whiskers Tukey plots. A,D: Whey stimulated data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. B,C: Whey stimulated data analysed by Kruskal Wallis and Dunn's multiple comparison post hoc test. A-D: Medium control data analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. $n$ = 9-15 samples per group.

The whey specific IL-10 response was significantly increased in splenocytes of the whey allergic control in both males and females, compared to the non-allergic control (**Figures 8A, B**). The *ex vivo* IL-10 response, in allergic male mice, was unaffected by the n-3 PUFA or combination intervention (**Figure 8A**). The scGOS:lcFOS intervention in allergic male mice did not lead to a significant IL-10 release when compared to the release in the non-allergic control group. Next to that, in males, the IL-10 response tended to be lower in the scGOS:lcFOS group than in the combination diet group (**Figure 8A**). In the medium control, an allergic baseline effect was observed. This allergic effect was affected by scGOS:lcFOS (**Figure 8A**). In female mice, the whey IL-10 response was unaffected by the dietary interventions compared to the allergic control (**Figure 8B**).

The whey specific IFNɣ response was significantly increased in the whey allergic control in both males and females, compared to the non-allergic control. The whey-induced IFNɣ response was unaffected by the dietary interventions in both males and females (**Figures 8C, D**). Compared to the non-allergic control group, the IFNɣ response was unaffected by scGOS:lcFOS. Furthermore, the IFNɣ response tended to be lower in the scGOS:lcFOS group than in the combination diet group. In the medium control, an allergic baseline effect was observed of splenocytes obtained from allergic male mice, which was unaffected by the dietary interventions (**Figure 8C**). In females, no dietary effects on unstimulated and whey-stimulated splenocytes were observed compared to the allergic control (**Figure 8D**). Lastly, the whey specific cytokine responses, except the IFNɣ response, were higher in splenocytes from allergic females than from allergic males.
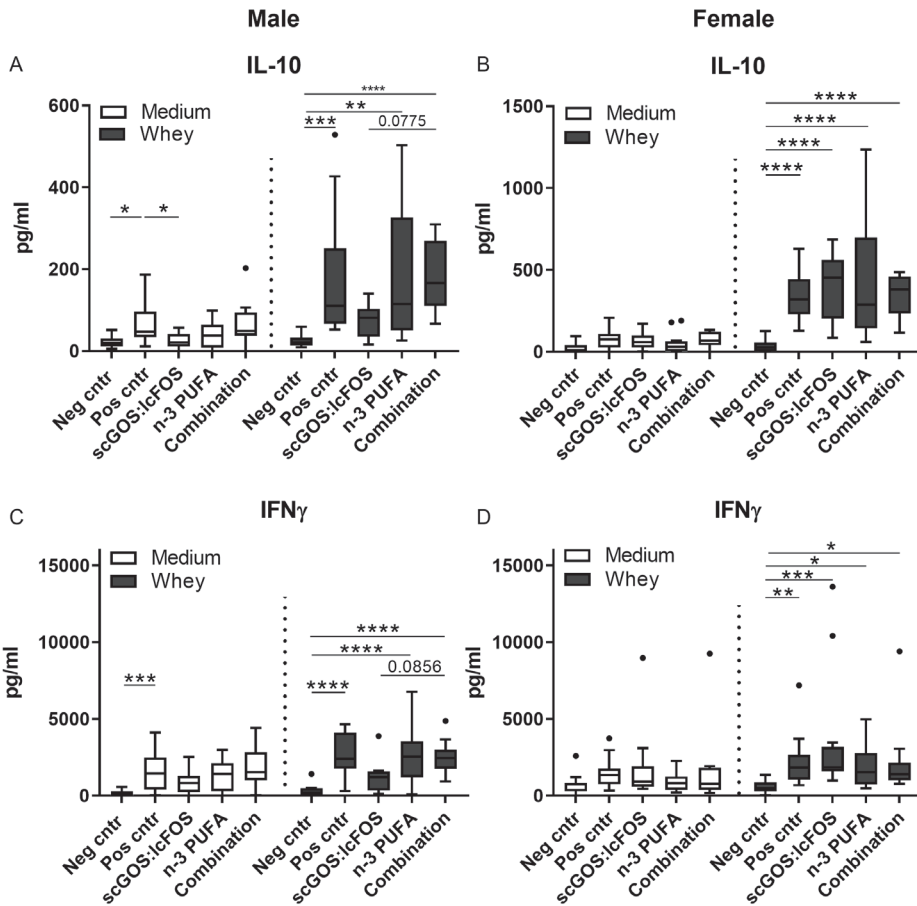
**Figure 8**. Effect of dietary interventions on the systemic IL-10 and IFNɣ responses assessed by *ex vivo* whey-specific cytokine responses in splenocytes. Unstimulated and whey stimulated e*x vivo* IL-10 response (A,B) and IFNɣ response (C,D) in splenocytes in male and female mice, respectively. A-D: Data shown as box-and-whiskers Tukey plots. A,D: Whey stimulated data analysed by Kruskal Wallis and Dunn's multiple comparison post hoc test. B,C: Whey stimulated data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. A,C,D: Medium control data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. B: Medium control data analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. * $P$ < 0.05, ** $P$ < 0.01, *** $P$ < 0.001, **** $P$ < 0.0001. $n$ = 11-15 samples per group.

## Limited effects on behavioural outcomes by the allergy as well as the dietary interventions

On the day following a sensitization or a challenge, behavioural parameters were recorded. The social behaviour, time in the interaction zone (**Figures 9A, B**) as well as frequency in the interaction zone (**Figures S4A, B**), was not impacted by CMA as well as by the dietary interventions in both male and female mice. The distance moved, a measure for locomotor activity, tended to be increased in the whey allergic control male mice

compared to the non-allergic mice. This increase in locomotor activity was significantly decreased in the n-3 PUFA treated male allergic mice (**Figure 9C**). The distance moved was unaffected by the scGOS:lcFOS and combination diets in allergic male mice (**Figure 9C**). In female mice neither the CMA nor the dietary interventions significantly changed the total distance moved (**Figure 9D**). The increased distanced moved by whey allergic male mice might affect the chance of these more active mice to enter the interaction zone. Indeed, it seems that CMA induced the frequency in zone, although not significantly (F**igure S4A**). Therefore, we normalized the time the mice spend in the interaction zone by the total distance moved. Although not significantly, whey allergic male mice seem to reduce normalized time in the interaction zone compared to non-sensitized mice (**Figure 9E**). This was not observed in female whey allergic mice (**Figure 9F**). Of the dietary interventions tested only the n-3 PUFA diet seems to prevent the CMA-induced reduction of normalized time in the interaction zone in male mice (**Figure 9E**).

Repetitive/anxiety-like and explorative behaviour were evaluated by means of marble burying and open field tests, respectively. These behavioural parameters were not impacted by CMA (**Figures S5** and **S6**). The only dietary effect observed, was in the female allergic mice, time in centre, was significantly enhanced in the n-3 PUFA group compared to the whey allergic control group (**Figure S6B**).

**The production of caecal butyrate is induced by scGOS:lcFOS in allergic mice**
The total concentration of caecal SCFAs, acetate, propionate and butyrate together, was significantly decreased in whey allergic when compared to non-allergic male mice (**Figure 10A**). In contrast, in female mice, the total caecal SCFA concentration was unaffected in whey allergic mice when compared to non-allergic (**Figure 10B**). The total SCFA concentration was not influenced by any dietary intervention neither in allergic male nor female mice. The caecal concentration of butyrate was significantly decreased in the male whey allergic mice compared to the non-allergic mice (**Figure 10C**). This allergic effect was not observed on caecal butyrate concentration in the female mice (**Figure 10D**). The butyrate concentration, in both allergic male and female mice, were significantly enhanced by the scGOS:lcFOS dietary intervention (**Figures 10C, D**). The n-3 PUFA dietary intervention did not affect caecal butyrate concentration in male allergic mice. Additionally, in the male mice receiving the combination diet, the butyrate concentration tended to be decreased compared to the concentration in the mice receiving the scGOS:lcFOS diet (**Figure 10C**). In the female allergic mice, both the n-3 PUFA and the combination diet interventions (tended to) increased caecal concentration of butyrate compared to the whey allergic control diet fed mice (**Figure 10D**). The concentration of the other SCFAs (acetate, propionate and valerate) and branched chain SCFAs (BCFAs, iso-valerate and iso-butyrate) were unaffected by the allergic reaction and the dietary interventions, only acetate was significantly decreased in the male whey allergic mice compared to the non-allergic mice (**Figures S7** and **S8**). Generally, the SCFA concentrations were higher in female mice than in male mice.
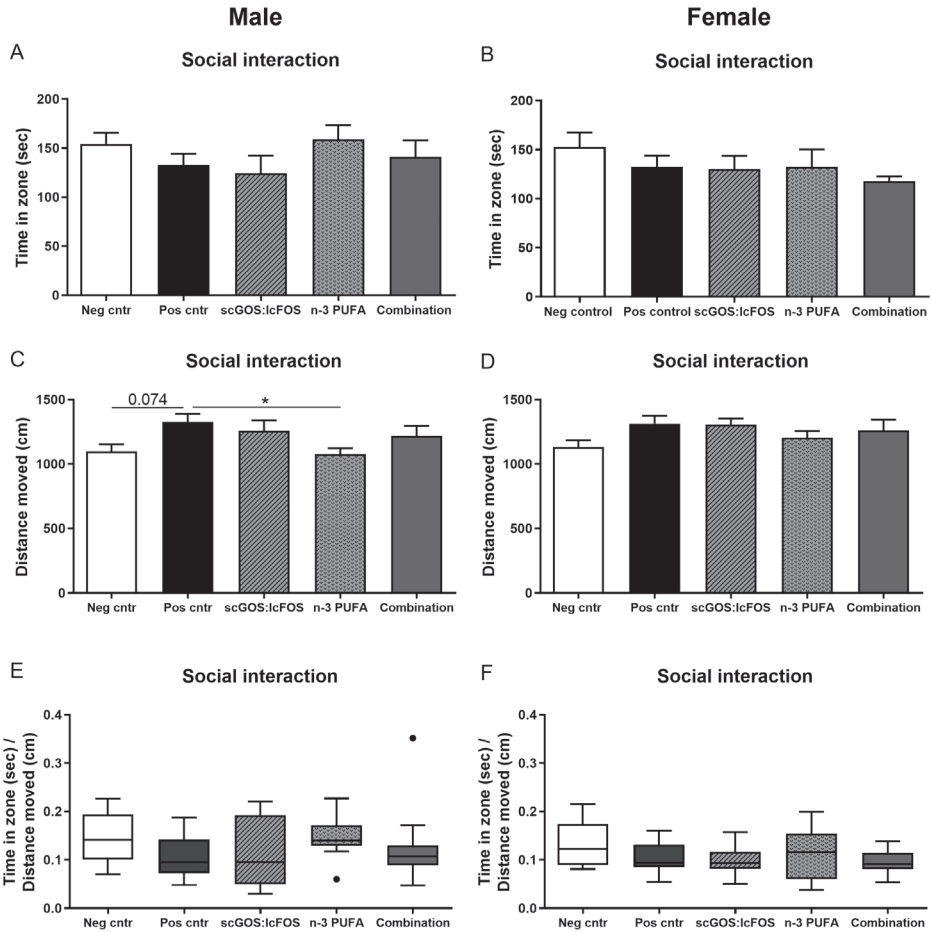
**Figure 9**. Allergic and dietary effects on social behaviour. (A,B) The time mice spend in the interaction zone in male and female mice, respectively. (C,D) Distance moved to measure locomotor activity in male and female mice, respectively. (E,F) Normalization of time spent in zone by distance moved in male and female mice, respectively. A-D: Data shown as mean +/- SEM. E,F: Data shown as box-and-whiskers Tukey plots. A-D,F: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. E: Data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$. $n$ = 11-15 samples per group.
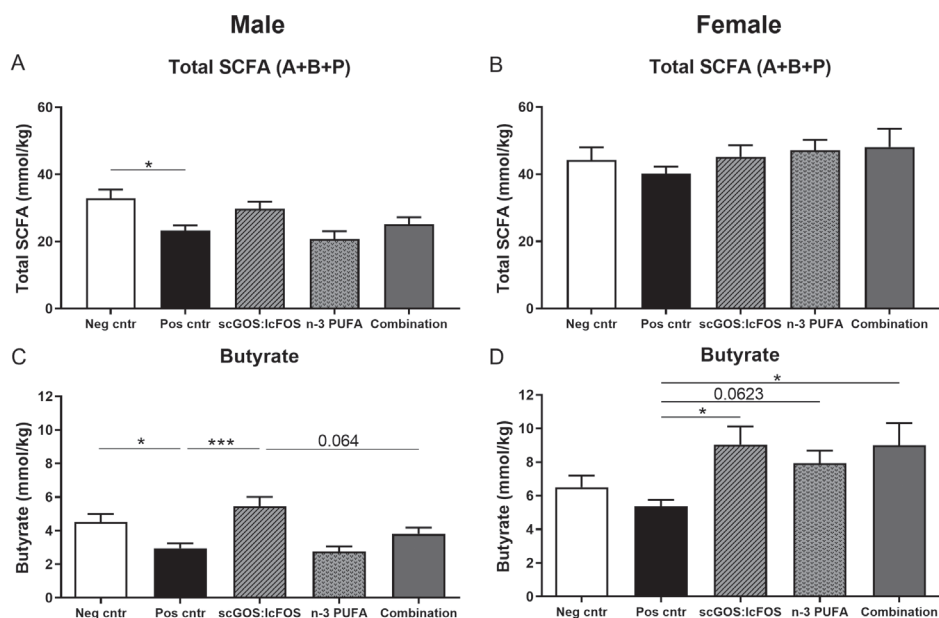
**Figure 10.** Caecum content concentration of total SCFAs and butyrate. (A,B) Total caecal SCFA concentration including acetate, propionate and butyrate in male and female mice, respectively. (C,D) Butyrate caecal concentrations in male and female mice, respectively. A-D: Data shown as mean +/- SEM. A-C: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. D: Data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. * $P < 0.05$, *** $P < 0.001$. $n$ = 9-15 samples per group.

## The *Ppary* expression is unaffected by allergy but affected by dietary interventions

The mRNA expression of *Ppary* in the ileum was unaffected by the whey allergy in both males and females. In allergic male mice, the ileal *Ppary* expression was significantly increased by the combination diet compared to the whey allergic control. Furthermore, the *Ppary* expression tended to be increased by the combination diet compared to the scGOS:lcFOS diet (**Figure 11A**). Similarly, in female allergic mice, the combination diet significantly induced the *Ppary* expression compared to the scGOS:lcFOS or n-3 PUFA diets (**Figure 11B**). As butyrate modulates the mRNA expression of *Ppary* (52), we correlated the ileal *Ppary* mRNA expression and the caecal butyrate concentration, but the parameters did not significantly correlate in both male and female mice (**Figures 11C, D**). The mRNA expression of *Ppary* in the jejunum showed no changes in neither male nor female allergic mice compared to non-allergic mice and in addition no dietary effects were observed (**Figure S9**).
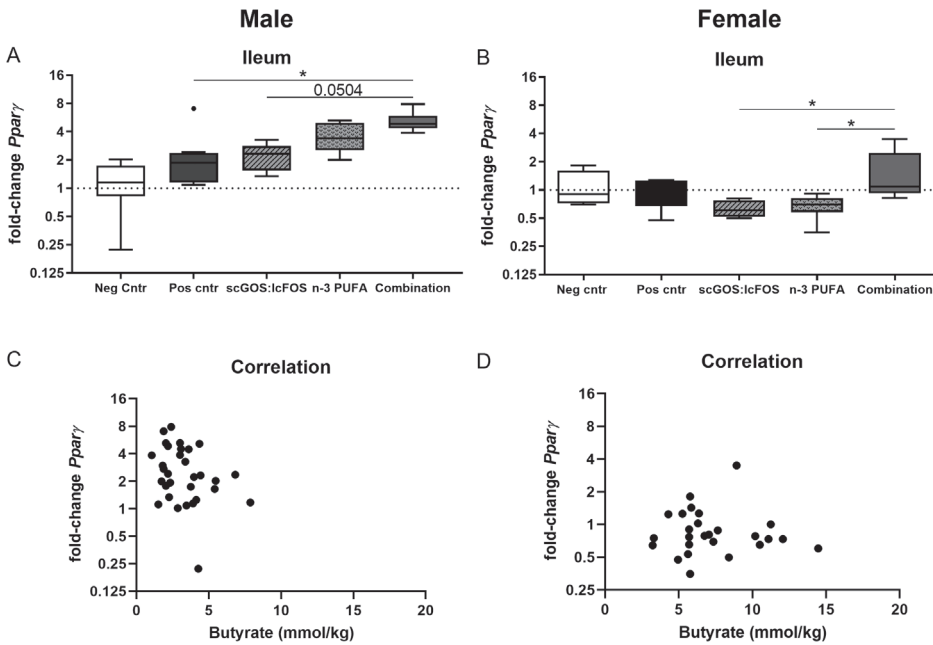
**Figure 11.** $Ppar\gamma$ mRNA expression in the ileum and correlation with butyrate concentration in caecum content. $Ppar\gamma$ mRNA expression in male (A) and female (B) mice. (C,D) Correlation between ileal $Ppar\gamma$ mRNA expression and caecal butyrate concentration in male and female mice, respectively. A,B: Data shown as box-and-whiskers Tukey plots. C,D: Data shown as individual data points. A,B: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. C,D: Analysed by Pearson correlation coefficient. * $P < 0.05$. $n$ = 4-8 samples per group.

## DISCUSSION

In these studies, the effects of the dietary interventions in prevention of allergy differed in male and female mice. In allergic male mice, scGOS:lcFOS seems to be the most effective dietary intervention in prevention of CMA based on the following results: reduced drop in body temperature during allergic reaction and reduced *ex vivo* whey-induced cytokine secretion from splenocytes. In allergic female mice, the scGOS:lcFOS or n-3 PUFAs reduced the acute allergic response (ear swelling). The preventive effects of scGOS:lcFOS or n-3 PUFAs were expected as this has been documented in several studies (18, 19, 29, 32) but the missing additional effect of the combination of these two dietary components was surprising.

The impact of the dietary interventions on the allergy development was actualised by means of allergy-induced drop in body temperature in male mice and by the acute allergic response (ear swelling) in female mice. In males, the development of allergy was reduced by scGOS:lcFOS and in females by scGOS:lcFOS and n-3 PUFAs individually. Surprisingly, in the combination of scGOS:lcFOS and n-3 PUFAs, the preventive effects of scGOS:lcFOS (in both males and females) and n-3 PUFAs (in females) seemed to be

reduced or even diminished. In males, the body temperature was significantly lower in the mice fed the combination diet than in the mice fed the scGOS:lcFOS diet and the shock score tended to be higher in the combination diet group than in the allergic control. In females, the acute allergic response in the combination diet group tended to be lower than in the allergic control and tended to be higher than in the n-3 PUFA group. Together, these data indicate that the combination dietary intervention negatively influenced the observed clinical allergy parameters indicating no additive or synergistic effect between scGOS:lcFOS and n-3 PUFAs in the prevention of CMA development in the used dosages in this model. This is surprising as both scGOS:lcFOS and n-3 PUFAs individually have shown the capacity to induce tolerance and prevent development of CMA (18, 19, 29, 32).

According to the clinical parameters both male and female mice develop CMA after sensitization and challenge. This is confirmed by the whey-specific immunoglobulin levels and the mast cell degranulation measured in serum. However, the dietary induced prevention of the clinical allergic symptoms is not accompanied by changes in the immunoglobulin and mast cell degranulation levels. This suggest that the humoral pathway is not involved in the allergy preventive capacities of the scGOS:lcFOS and n-3 PUFA diets. That the mast cell degranulation is unaffected by n-3 PUFAs and scGOS:lcFOS is in line with other studies, but the effect of n-3 PUFAs on the immunoglobulin levels is inconclusive (19, 29, 32).

Along the clinical allergic parameters, the systemic cytokine responses are important in the development of allergy and/or tolerance. Overall, the *ex vivo* splenic cytokine responses in males were prevented by the scGOS:lcFOS diet but in the combination diet group; this effect was lost. This is most evident in the IL-10 and IFNɣ responses. This indicates that scGOS:lcFOS have the ability to impact the allergy development. The modulated cytokine response can be facilitated directly by scGOS:lcFOS or indirectly, i.e., through butyrate immune modulation (13, 14, 53). However, the scGOS:lcFOS mediated cytokine response was impaired when scGOS:lcFOS and n-3 PUFAs were combined, which suggests a possible interaction between these components. The cytokine responses can be directly linked to affected immune cells Th1, Th2, and Tregs (54). These immune cells including DCs were analysed in splenocytes by flowcytometry, however, no allergic as well as dietary effects were observed (data not shown). That the numbers (percentages) of immune cells were unchanged but the cytokine responses were affected by the allergy and the dietary interventions might indicate that the functionality of the immune cells is improved (55, 56).

Next to immunological manifestations, CMA has also been associated with behavioural appearances such as depression, anxiety-like behaviour and reduced sociability. Even though the social behaviour seemed impaired in whey-sensitized male mice and the impairment seemed reduced by n-3 PUFAs which is in line with the findings of de Theije *et al.* (32), the allergy induced behavioural impact is limited in this study and this is in contrast to previous studies. By using more and/or different tests i.e. elevated zero maze test, that have been used previously to show CMA induced behavioural changes (38) and

a larger sample size, might give a better overview of the behavioural impairment in CMA in this mouse model.

Butyrate, a metabolite of scGOS:lcFOS after fermentation by the bacteria in the intestinal tract, possesses immune modulatory functions, supporting both Tregs and effector T cells (57-60). Butyrate facilitates tolerance in steady state by promoting Tregs and stimulates an active immune response during a pathogen invasion (60). The role of butyrate in the development of tolerance and the prevention of food allergy is supported by increasing evidence: In children, low faecal levels of butyrate was associated with food allergy (61). In preclinical murine models of egg, cow's milk and peanut allergy, the allergy development was reduced or even prevented by butyrate treatment prior to sensitization, indicated by reduced acute allergic symptoms like anaphylactic score, IgE levels as well as modulated cytokine responses (58, 62). Butyrate exhibits its function through several pathways by binding to GPCRs like GPR41, GPR43 and GPR109A, by inhibiting histone deacetylase (HDAC) activity leading to enhanced accessibility to gene transcription, and most likely binding to transcription factors like PPARɣ (63). Butyrate activates PPARɣ (64) and it has been hypothesised that the anti-inflammatory properties of butyrate might be mediated through upregulation of PPARɣ, among others (65). PPARɣ is expressed in many immune cells i.e. DCs and T cells and is shown to have immunomodulating properties (66). The immune modulatory properties most likely takes place by counteracting other pro-inflammatory transcription factors like NFκB. However, the exact role of PPARɣ in T cells appears to be inconclusive, it can be pro- as well as anti-inflammatory (66). PPARɣ is not only activated by butyrate but also by docosahexaenoic acid (DHA) and most likely by eicosapentaenoic acid (EPA) too, both n-3 PUFAs. Binding of DHA (and EPA) to the PPARɣ receptor in DCs leads to inhibition of NFκB signalling followed by reduction of pro-inflammatory cytokine production and induction of an anti-inflammatory mechanism (67). As the combination of scGOS:lcFOS and n-3 PUFAs showed no additive or synergistic preventive effects on the clinical allergic parameters, we hypothesised that the scGOS:lcFOS mediated enhanced butyrate concentration might influence the PPARɣ expression, because Lukovac *et al.* (52) reported butyrate stimulated murine intestinal organoids to express lower *Ppar*ɣ mRNA. A possible scGOS:lcFOS-reduced mRNA expression could then be followed by less functional capacity, whereas the preventive effects of n-3 PUFAs on CMA development would be impaired. To confirm or reject (part of) this hypothesis, we analysed the *Ppar*ɣ mRNA expression in intestinal tissues and expected lower expression of *Ppar*ɣ in the mice receiving the scGOS:lcFOS and the combination diet. However, the *Ppar*ɣ expression in the scGOS:lcFOS group was unchanged, whereas in the combination group the *Ppar*ɣ expression was significantly (and tending to be) increased compared to the allergic control male mice, the scGOS:lcFOS male and female mice and the n-3 PUFA female mice. This indicates that butyrate is not interfering with the immunomodulatory effects of n-3 PUFAs by reducing the mRNA expression of *Ppar*ɣ meaning that our hypothesis is invalid, at least in this model. Noteworthy, the expression of *Ppar*ɣ was measured in total intestinal tissue. Analysing Pparɣ mRNA and protein expression in different cell types in the intestinal tissue could

provide more information why the total mRNA expression is increased, and which cells are involved in the combination diet group.

Sex differences were examined and evaluation of the allergic parameters, mainly the cytokine responses, indicates a higher sensitivity in females than in males to this model. Moreover, the concentrations of all SCFAs seem to be higher in the female mice than in the male mice. It could be possible that the fermentation activity of intestinal bacteria is greater in females than in males intestines. Regarding behaviour, autism spectrum disorder, indicated by impaired social and repetitive behaviour is most prevalent in males (68) and CMA/food allergy is increasingly reported to be associated with autism spectrum disorder (43). Be aware, in girls, autism spectrum disorder is expressed differently. However, in the study reported here, we expected the males to be more susceptible to behavioural modulation but the modulation in general was limited.

Taking all results together, combining scGOS:lcFOS and n-3 PUFAs leads to no additional preventive effect on the measured allergic and behavioural parameters. The results of the combination diet group were either equal to the control diet or the other dietary interventions or even less beneficial. These results are contradictory to a dermatitis study in mice, where a diet consisting of the prebiotics GOS and polydextrose and the PUFAs arachidonic acid and DHA showed additional effect compared to the individual components (69). A possible hypothesis at the level of intestinal bacteria, can be that the fermentation of scGOS:lcFOS into butyrate is influenced by n-3 PUFAs. This might be the case in male allergic mice as the caecal butyrate concentration in the combination diet mice tended to be lower than in the scGOS:lcFOS diet group and was at the same level as in the n-3 PUFA diet mice. This indeed indicates that n-3 PUFAs might interfere with the fermentation into butyrate in male mice. This is not the case in female mice as the concentration of caecal butyrate levels is not disturbed by n-3 PUFAs, another unknown interaction might be occurring here.

In conclusion, the combination of scGOS:lcFOS and n-3 PUFAs has no additional effect on preventing CMA. This outcome was unexpected, as both dietary components were able to prevent CMA development. The possibility exist that these components interact with each other, either directly via physically or chemically interactions (**Chapter 6**) or indirectly by n-3 PUFA-mediated inhibition of intestinal microbial fermentation of scGOS:lcFOS (**Chapter 7**). However, both components individually are important in children's development. Both components are included in infant formula and our results do not mean that the formulas should only contain the one or the other. n-3 PUFAs are very important in the development of the brain and immune system and the oligosaccharides for the development of the gastro-intestinal tract and the immune system, and one cannot be left out. Both components have shown beneficial effects in prevention of allergy in pre-clinical models. Therefore, it might be a question of optimizing dosages. From this the importance of investigating combinations is clear.

## SUPPLEMENTARY INFORMATION
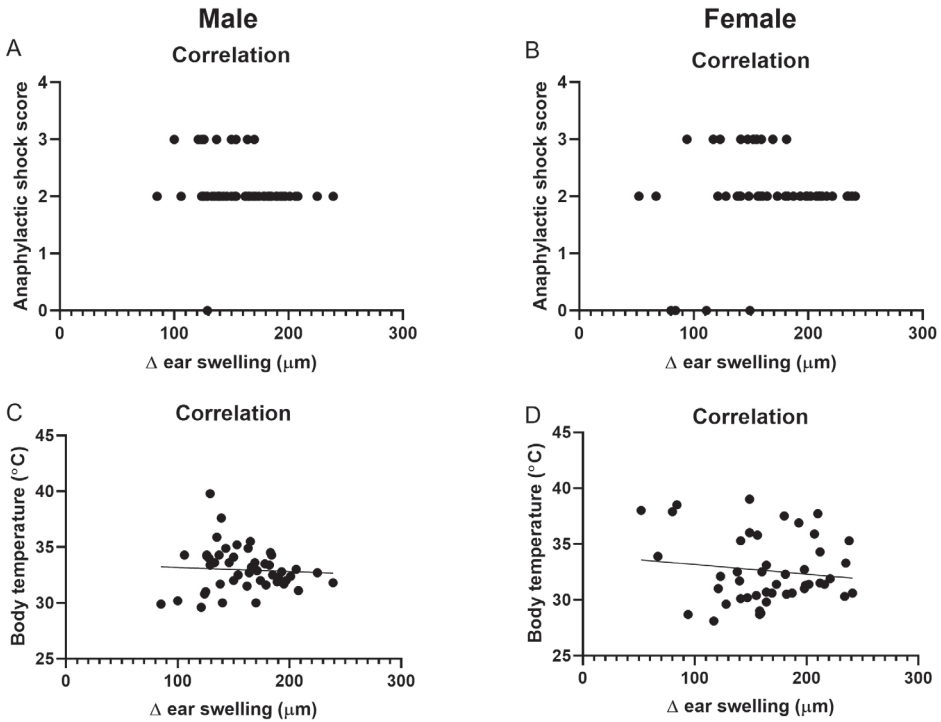


**Figure S1.** Correlations of clinical symptoms during an acute allergic reaction. (A,B) Correlation of shock score and acute allergic skin reaction (ear swelling) in male and female mice, respectively. Data included from whey allergic mice receiving the control, scGOS:lcFOS, n-3 PUFA and combination diet. (C,D) Correlation of body temperature and acute allergic skin reaction (ear swelling) in male and female mice, respectively. A-D: Data shown as individual data points. A,B: Analysed by Spearman correlation coefficient. C,D: Analysed by Pearson correlation coefficient. $n$ = 56-60 data points.

**Figure S2.** Stimulation with anti-CD3/CD28 to verify functionality of splenocytes. Unstimulated and anti-CD3/CD28 stimulated e*x vivo* IL-13 response (A,B) and IL-5 response (C,D) in splenocytes in male and female mice, respectively. A-D: Data shown as box-and-whiskers Tukey plots. A,C,D: Anti-CD3/CD28 stimulated data analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. B: Anti-CD3/CD28 stimulated data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. A-D: Medium control data analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. * $P < 0.05$. $n$ = 11-15 samples per group.
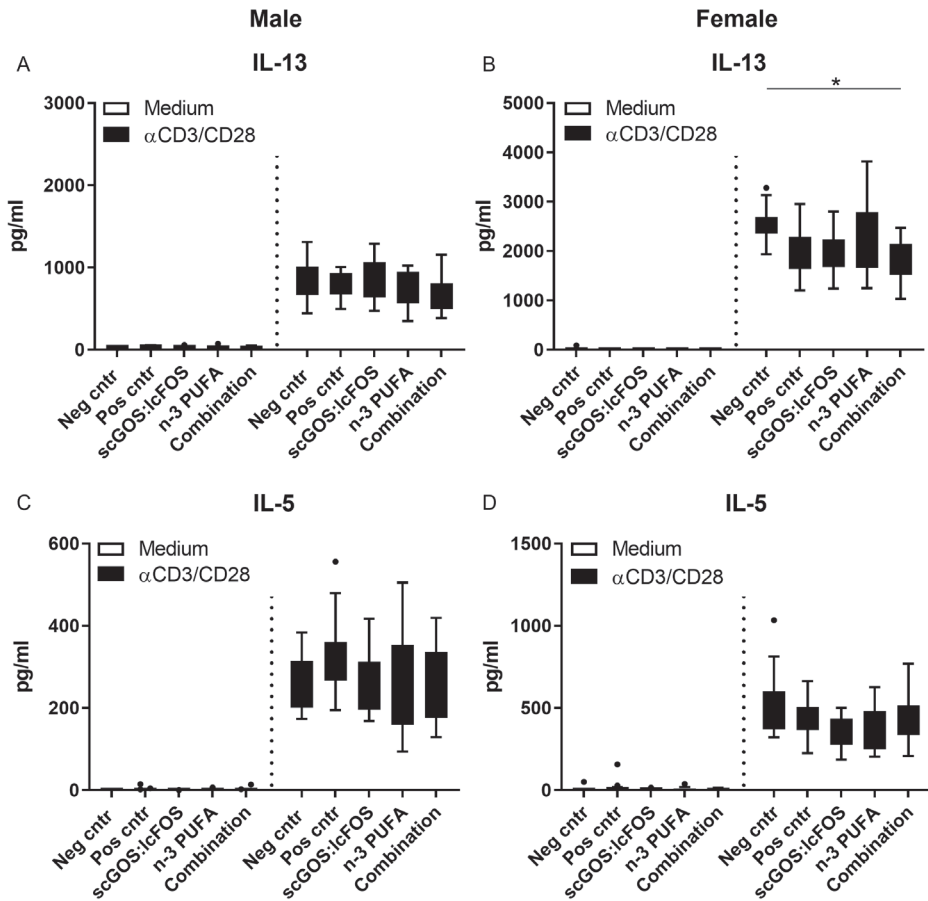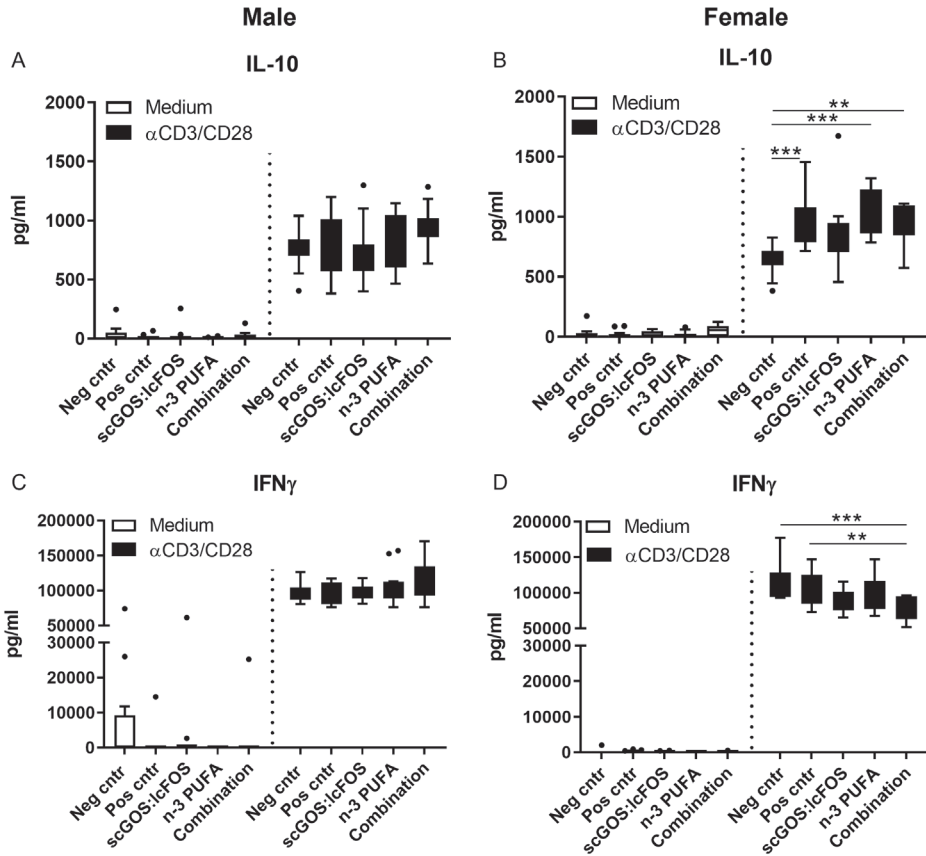
**Figure S3.** Stimulation with anti-CD3/CD28 to verify functionality of splenocytes. Unstimulated and anti-CD3/CD28 stimulated e*x vivo* IL-10 response (A,B) and IFNɣ response (C,D) in splenocytes in male and female mice, respectively. A-D: Data shown as box-and-whiskers Tukey plots. A: Anti-CD3/CD28 stimulated data analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. B-D: Anti-CD3/CD28 stimulated data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. A-D: Medium control data analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. ** $P < 0.01$, *** $P < 0.001$. $n$ = 11-15 samples per group.

## Male

### A  Social interaction



## Female

### B  Social interaction



**Figure S4.** Social behaviour, frequency in zone in male (A) and female (B) mice. A,B: Data shown as box-and-whiskers Tukey plots. A: Analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. B: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. *n* = 11-15 samples per group.

## Male

### A  Marble burying



## Female
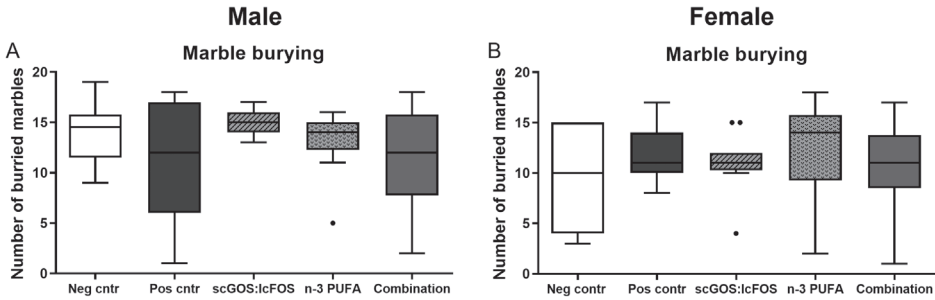
### B  Marble burying



**Figure S5.** Marble burying behaviour. Number of buried marbles in male (A) and female (B) mice. A,B: Data shown as box-and-whiskers Tukey plots. A,B: Analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. *n* = 11-15 samples per group.

## Male

## Female



**Figure S6.** Explorative behaviour. (A,B) Time in centre in male and female mice, respectively. (C,D) Frequency in centre in male and female mice, respectively. (E,F) Time in corners in male and female mice, respectively. (G,H) Distance moved in male and female mice, respectively. A,B: Data shown as mean +/- SEM. C-H: Data shown as box-and-whiskers Tukey plots. A-C,F,H: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. D,E,G: Analysed by Kruskal Wallis and Dunn's multiple comparisons post hoc test. * $P$ < 0.05. $n$ = 11-15 samples per group.

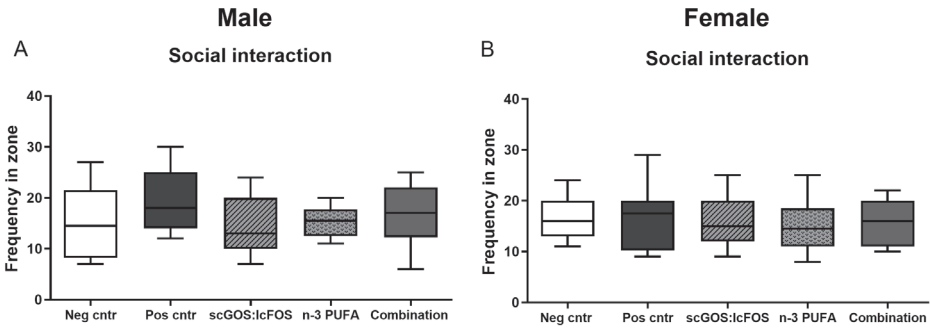**Figure S7**. Caecum content concentration of acetate and propionate. (A,B) Acetate concentration in male and female mice, respectively. (C,D) Propionate caecal concentrations in male and female mice, respectively. A-D: Data shown as mean +/- SEM. A-D: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. ** $P < 0.01$. $n$ = 9-15 samples per group.
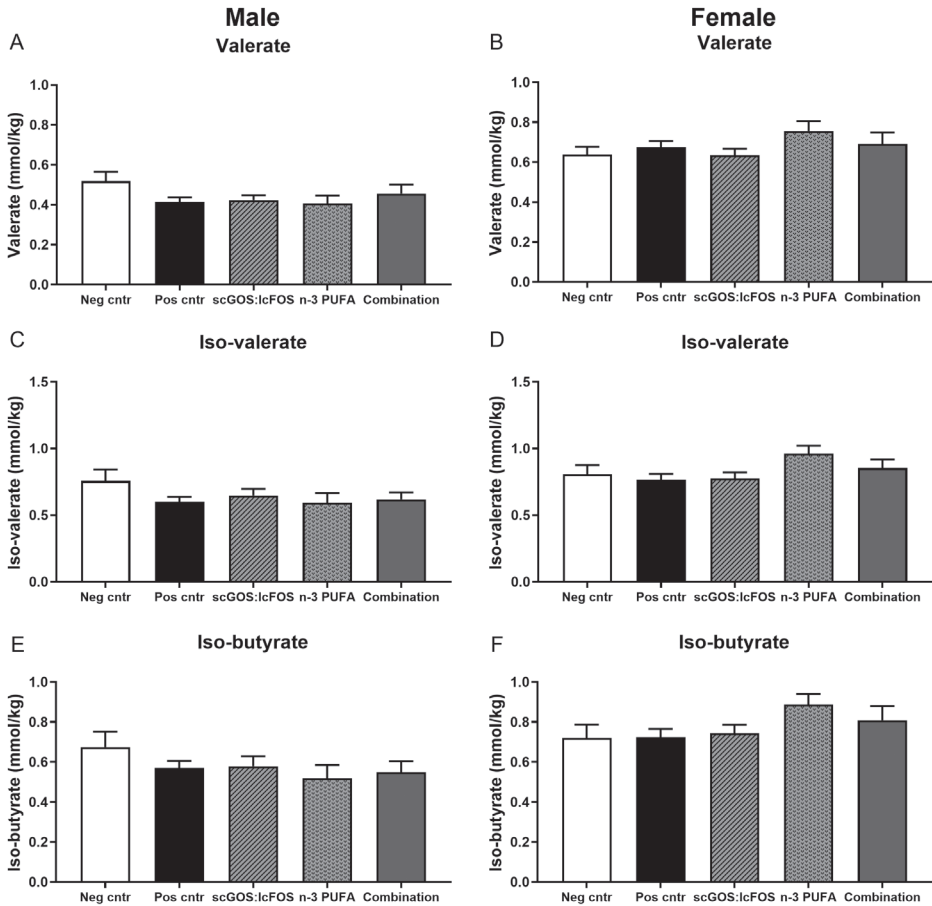
**Figure S8**. Caecum content concentration of branched chain fatty acids. (A,B) Valerate concentration in male and female mice, respectively. (C,D) Iso-valerate caecal concentrations in male and female mice, respectively. (E,F) Iso-butyrate caecal concentrations in male and female mice, respectively. A-F: Data shown as mean +/- SEM. A-E: Analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. F: Data transformed and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test. $n$ = 9-15 samples per group.

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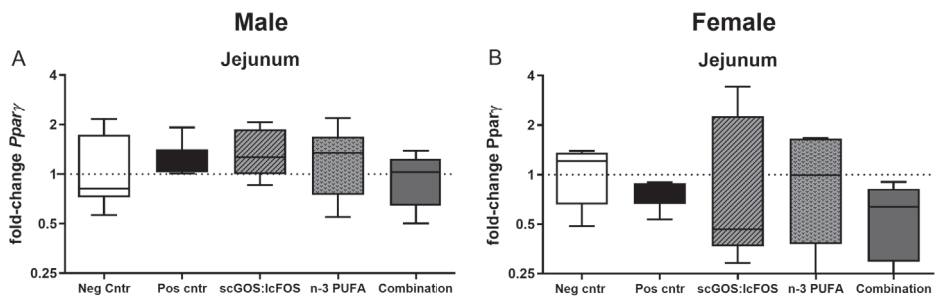
**Figure S9.** *Pparγ* mRNA expression in jejunum in male (A) and female mice (B). A,B: Data shown as box-and-whiskers Tukey plots. A,B: Analysed by one-way ANOVA. *n* = 4-9 samples per group.

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No additional preventive effect of a combined dietary intervention with non-digestible oligosaccharides and omega-3 polyunsaturated fatty acids on cow's milk allergy development in mice

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# CHAPTER 6

Molecular reactions and/or interactions between galacto-/fructo-oligosaccharides and omega-3 polyunsaturated fatty acids

Kirsten Szklany[1]*, Orsola Mocellin[1]*, Leon M. J. Knippels[1,2], Johan Garssen[1,2], Aletta D. Kraneveld[1], Reshmi Mukherjee[1]

[1]  Division of Pharmacology, Utrecht Institute of Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands
[2]  Global Centre of Excellence Immunology, Danone Nutricia Research, Utrecht, The Netherlands
*  These authors contributed equally

## ABSTRACT

Food allergy is one of the most common immune disorders in young children worldwide and an effective treatment is unavailable. Studies have indicated that several food supplementations like short-chain galacto-oligosaccharides (scGOS) and/or long-chain fructo-oligosaccharides (lcFOS), and omega-3 polyunsaturated fatty acids (n-3 PUFAs) can both have preventive effects on allergy development. However, we observed that the combined supplementation of both n-3 PUFAs and scGOS:lcFOS showed no synergy or additive effects on the prevention of cow's milk allergy development.

We hypothesized that the reduced effect of combining the n-3 PUFAs and scGOS:lcFOS compared to summing up the individual effects is due to chemical reaction and/or physical interactions between n-3 PUFAs and scGOS:lcFOS in the gastrointestinal tract due to the presence of lipases and/or the acidic environment of the stomach. This study aims to test the effects of digestive lipases and/or an acidic environment on the chemical reaction and physical interactions between scGOS:lcFOS and n-3 PUFAs in an *in vitro* setting.

We have employed a two-step *in vitro* model simulating *in vitro* digestion in the stomach and small intestine for testing the effect of lipases on n-3 PUFAs and scGOS:lcFOS. For testing the effect of a low pH stomach environment, we carried out an incubation of n-3 PUFAs and scGOS:lcFOS at pH 2. n-3 PUFAs were tested both in their triglyceride form (as tuna oil) and in their free form (as pure eicosapentaenoic acid, EPA). The products of the *in vitro* digestion and under low pH conditions were analysed using thin-layer chromatography (TLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Optical microscopy is used for the monitoring of changes in physical characteristics of n-3 PUFAs, scGOS:lcFOS, and EPA in the presence of digestive components like bile salts as well as at low pH. Possible formed complexes of scGOS:lcFOS and n-3 PUFAs were evaluated by computational molecular modelling.

TLC analysis showed the formation of new compounds after both the *in vitro* digestion and the low pH condition between EPA and scGOS:lcFOS. These results were confirmed by MALDI-MS analysis. The molecular mass of the new compounds indicates that they can be the result of a chemical reaction between n-3 PUFAs and scGOS or lcFOS, however, it was not possible to fully characterize them with only MALDI-MS. TLC analysis, however, showed that no new compounds were formed after *in vitro* digestion and pH 2 reaction between tuna oil and scGOS:lcFOS. *In vitro* digestion and a low pH condition of tuna oil and scGOS:lcFOS did not reveal the formation of any new chemical compounds. The optical microscopy showed that the tuna oil forms particulate micelle like structures in the presence of bile salts. When scGOS:lcFOS is added to tuna oil then the micellar structure is deformed to a droplet like structure. That scGOS:lcFOS and n-3 PUFAs can interact was also suggested by the molecular modelling data.

Although the used experimental procedures need optimisation, these preliminary data indicate possible chemical reactions and physical interactions between scGOS:lcFOS and n-3 PUFAs which might lead to less bioavailability and functionality. However the results of the clinical studies on scGOS:lcFOS with n-3 PUFAs clearly provide evidence that these combination positively impact the gut microbiota and immune system in early life.

## INTRODUCTION

Food allergies are one of the most common immune disorders in young children worldwide. The prevalence of food allergies is estimated to be 5-10% and increasing in children in westernised countries (1). Paediatric patients affected by food allergies experience a variety of symptoms such as pulmonary and gastric distress, atopic dermatitis, and anaphylaxis. Currently, no cure or treatments are available for food allergies; avoidance of the allergen is the only strategy so far to prevent the manifestation of symptoms. Nevertheless, the treatment and prevention strategy is changing rapidly nowadays in terms of early life exposures, immunotherapies and dietary interventions (2, 3).

Human breast milk is the recommended nutrition during the first 6 months of life (4). Consumption of human milk during the first 3-4 months decreases the risk of developing allergic disorders like wheeze, eczema and asthma. However, the evidence is insufficient to conclude a preventive effect of human milk on food allergy development (5-7). Human milk contains components like nutrients, growth factors, antigens, vitamins, bacteria, human milk oligosaccharides (HMOs) and polyunsaturated fatty acids (PUFAs). These components are derived from the maternal diet or immune system and are important in the development of the intestinal microbiota composition and the immune system to induce tolerance in the new-born (8, 9).

HMOs is one of the components in human milk and associations between profiles of these HMOs and food sensitisation have been indicated (10). As breast milk is not always available, the HMOs are mimicked by non-digestible oligosaccharides (NDOs) with prebiotic properties in infant formulas. A specific mixture of the prebiotics short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) also have preventive effects on allergy development (11-16). Although, the precise underlying mechanisms of prebiotics are not fully unravelled yet, several mechanisms of actions are reported. Prebiotics stimulate the growth of beneficial bacteria and the production of short-chain fatty acids (SCFAs), that have been shown to be important for a balanced mucosal immune system (17-20) . In addition, prebiotics have direct immunomodulatory activity in the intestinal tract resulting in the development of tolerance (21-26).

Another component in human milk is PUFAs, which are derived from the maternal diet. Omega-3 (n-3) and n-6 are the most important PUFAs, these fatty acids are incorporated into the cell membrane and possess immunomodulatory functions (27).
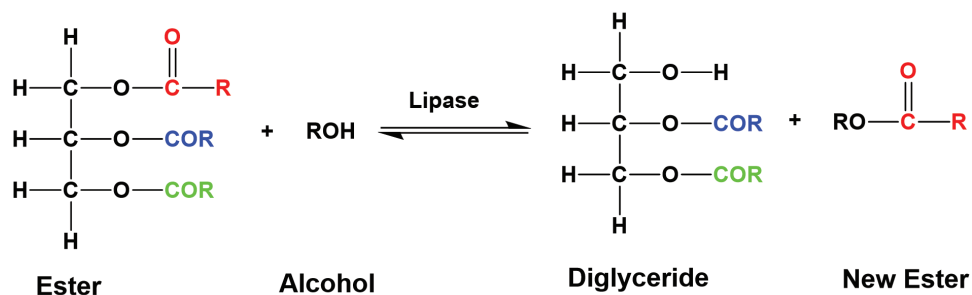
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**Figure 1.** A schematic representation of a lipase-catalysed alcoholysis reaction between an ester (e.g. triglyceride) and an alcohol. The red-coloured acyl group in the triglyceride (ester) detaches and forms a new ester (in which the red acyl group is attached with the black alkoxy group of the alcohol ROH) and diglyceride. This same reaction can occur with the blue and green coloured acyl groups present in the triglyceride (ester).

Lipid transport from the intestinal tract through the epithelial barrier across the plasma membranes towards the extracellular space and into the systemic circulation is a concerted process. PUFAs are triglycerides (TGs) and get hydrolysed by lipases in the stomach. TGs are esters composed of a glycerol backbone covalently bonded to three fatty acids. These molecules are the naturally occurring form of most fatty acids, including n-3 PUFAs. Lipases also catalyse alcoholysis reactions (28). An alcoholysis is a reaction between an ester and an alcohol where the acyl group of the ester is detached and conjugated with the alkoxy group of the alcohol forming a new ester. An example of alcoholysis is shown in **Figure 1** where the red coloured acyl group in a triglyceride (ester) detaches and forms a new ester (in which the red acyl group is attached with the black alkoxy group of the alcohol ROH) and diglyceride. This same reaction can occur with the blue and green coloured acyl groups present in the triglyceride (ester).

As a result of hydrolysis, free fatty acids and monoacylglycerol are produced from PUFAs. In the proximal part of the intestine, the free fatty acids interact with bile salts and form mixed micelles which are soluble in water (29). The mucus layer lining the microvilli of the enterocytes contains a pH gradient with a decreasing pH from the intestinal lumen towards the villi. At the physiological pH in the intestine, the free fatty acids are ionized and when the formed micelles diffuse through the mucus layer with a decreasing pH, dissociation of the micelles occurs due to protonation of the fatty acids. Protonation of the fatty acids facilitate the uptake of the free fatty acids into the enterocytes by passive and active diffusion although, the exact mechanism of suggested transporters is not well understood (30, 31). The lipid absorption and transport processes of lipids by enterocytes are shown in **Figure 2**.
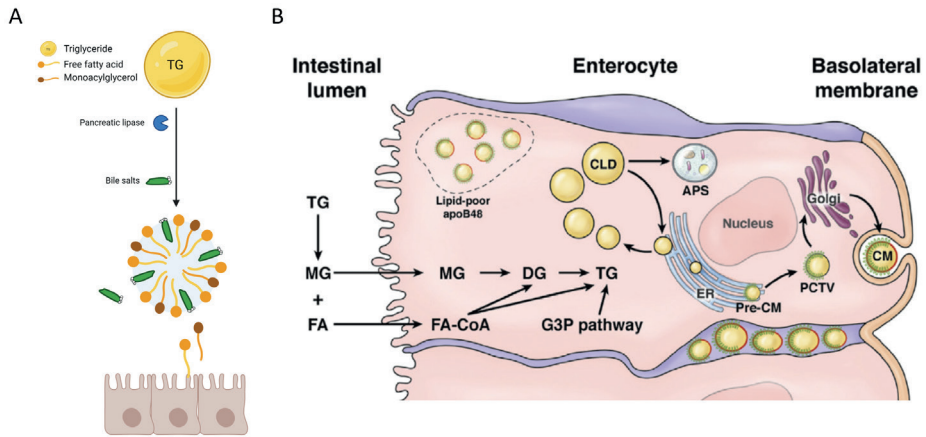
**Figure 2**. From triglyceride to chylomicron in the small intestine. (A) Triglycerides (TGs) are hydrolysed in the stomach and further in the duodenum by pancreatic lipase. As a result from the hydrolysis free fatty acids (FA) and monoacylglycerol (MG) are formed. Together with bile salts the FAs form mixed micelles. These micelles are dissociated when passing through mucus layer (figure created with BioRender.com). (B) FAs and MG are taken up by the enterocytes in the small intestine by passive and active diffusion. In the outer membrane of the endoplasmic reticulum (ER) TGs are reformed through the monoacylglycerol acyltransferase pathway, which is the main pathway for TGs resynthesis, or through the glycerol-3-phosphate (G3P) pathway. In the MGAT pathway MG and FAs combine to form diglycerides (DG) and TGs catalysed by acyl coenzyme A (CoA):monoacylglycerol acyltransferase and acyl-CoA:diacylglycerol acyltransferase, respectively. The G3P pathway uses glycerol-phosphate acyltransferase to resynthesise TGs. The resynthesized TGs are packed into pre-chylomicrons (Pre-CM) in the ER and further transported in pre-chylomicron transport vesicles (PCTV) to the Golgi. Here the maturation of the chylomicrons (CM) takes place. After maturation, the CMs leave the enterocytes via the basolateral membrane into the lamina propria. Lipid droplets formed in the ER might as well form cytoplasmic lipid droplets (CLDs) serving as a storage of TGs for later chylomicron formation in the ER or CLDs are degraded in autophagosome (APS). ApoB48 fuse with lipid droplets to form pre-CM, these apoB48 are stored in an apical pool. CoA, coenzyme A. Image adapted from Xiao *et al.* (32).

Once the PUFAs are inside the enterocytes, they are transported to the endoplasmic reticulum (ER) via transporter proteins like FATP4 to form again TGs. The reformation of the TGs occur through two pathways the monoacylglycerol acyltransferase (MGAT) pathway, which is the main pathway for TGs resynthesis, and the glycerol-3-phosphate (G3P) pathway. In the MGAT pathway MG and a FA combine to form diglycerides catalysed by acyl CoA:monoacylglycerol acyltransferase. Following another FA is added to form TGs and this is catalysed by acyl-CoA:diacylglycerol acyltransferase. The G3P pathway uses glycerol-phosphate acyltransferase to resynthesise TGs. The resynthesized TGs conjugate with apolipoproteins (ApoAI and apoB48) and are packed into pre-chylomicrons (Pre-CM) in the ER and further transported in pre-chylomicron transport vesicles (PCTV) to the Golgi. Here the prechylomicrons fuse and conjugate with other apolipoproteins (ApoCII and ApoCIII) and matures to large chylomicrons. These large chylomicrons leave the enterocytes from the basolateral sites into the lamina propria and further entering the lacteals from which they are actively transported through lymphatic vessels allowing them to be taken up by all organs in the body (31, 32).

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In several preclinical studies using among others a cow's milk allergy mouse model, dietary supplementation with scGOS:lcFOS (9:1; 1% w/w) prevented the development of an acute allergic response (**Figure 3 in chapter 5**, (14, 15)). The same was observed upon dietary supplementation with n-3 PUFAs (tuna oil added to the diet) (**Figure 3 in chapter 5**, (33, 34)). However, when administered together in the diet, scGOS:lcFOS combined with n-3 PUFAs showed no additive preventive effect on allergy development (**Figure 3 in chapter 5**). A proven reason for these results has not been elucidated thus far, and no studies investigating the possible chemical reaction and/or physical interactions between these components have yet been published. Knowledge about these interactions will help to decide on dosage regimens for use, thus it is important to shed light on this matter.

Given that n-3 PUFAs mostly exist as esters (triglycerides) and scGOS and lcFOS contain hydroxyl groups like alcohols, an alcoholysis reaction catalysed by lipases may take place in the gastrointestinal tract. Moreover, in acid and basic environments esters can also form without lipases. If an alcoholysis reaction takes place between n-3 PUFAs triglycerides and scGOS:lcFOS, a branched molecule would form consisting of a scGOS or lcFOS "core" chemically bonded (covalently bonded) to several n-3 PUFAs "branches". As scGOS and lcFOS are mixtures of numerous oligosaccharides, the resulting molecules can be of many possible structures; one of the representative examples is shown in **Figure 3A**. These newly generated chemical structures resemble an already existing compound called olestra (**Figure 3B**).
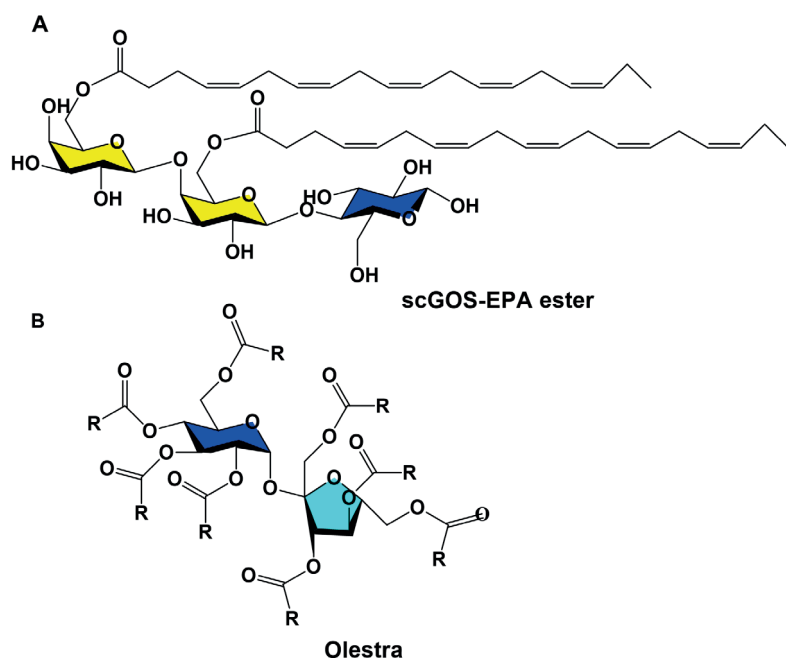


**Figure 3.** (A) Hypothetical molecular structure of an ester resulting from the chemical reaction between scGOS (degree of polymerization 3) and EPAs. (B) Molecular structure of olestra consisting of a sucrose core attached to eight fatty acids. R = acyl group of fatty acid. EPA: Eicosapentaenoic acid.

Olestra is an artificially created fat-substitute consisting of a sucrose molecule attached to six, seven or eight fatty acids. Because of its large and branched structure, olestra cannot be broken down in the body and passes through the gastrointestinal tract unchanged (35). We hypothesize that the branched molecules possibly generated from the chemical reaction between scGOS:lcFOS and n-3 PUFAs, will also not be absorbed by the host as well as scGOS:lcFOS and PUFAs are no longer available to exert effects on the host immune system and in the case of scGOS:lcFOS as well as to be digested by intestinal microbes into SCFAs. In this way, scGOS:lcFOS and n-3 PUFAs in combination can exert less beneficial effects, hence providing a possible explanation for the reduced effect of the combined n-3 PUFAs and scGOS:lcFOS dietary supplementation on the acute allergic response in cow's milk allergic mice (**Figure 3 in Chapter 5**).

We hypothesised that scGOS:lcFOS might chemically react and/or physically interact with n-3 PUFAs in the gastrointestinal tract. In the first part, we investigated the effects of digestive lipases and a pH 2 condition to observe the effect of lipases in the stomach and small intestine and to test the effects of gastric pH, respectively, on the chemical reaction between scGOS:lcFOS and n-3 PUFAs. The experimental products were analysed and characterized by thin-layer chromatography (TLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to evaluate ester formation between scGOS:lcFOS and n-3 PUFAs.

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In the second part, we investigated the physical interaction between scGOS:lcFOS and n-3 PUFAs in the form of tuna oil and the interaction of scGOS:lcFOS on the micelle formation of n-3 PUFAs. Our hypothesis is that, co-administration of n-3 PUFAs and scGOS:lcFOS in some way leads to encapsulation of the n-3 PUFAs within a core of scGOS:lcFOS. Consequently, the PUFAs are unavailable for protonation and for absorption into enterocytes.

## MATERIALS AND METHODS

### Materials

Pepsin from porcine gastric mucosa (P6887), lipase F-AP 15 from *Rhizopus oryzae* (80612), porcine bile extract (B8631), pancreatin from porcine pancreas (P7545), TLC plate (aluminium backed Silicagel 60 F 254 plates, 20 x 20 cm) were all purchased from Sigma-Aldrich. Tuna oil (BioPure DHA® IF oil) was used as a source of n-3 PUFAs in triglyceride form and was a kind gift from Bioriginal. Eicosapentaenoic acid (EPA) (GK4054) was purchased from Glentham Life Sciences. ScGOS (syrup) and lcFOS (10096752) were obtained from Friesland Campina and BENEO-Orafti, respectively. ScGOS:lcFOS (9:1) (w/w) standard was made by mixing scGOS syrup (22.2 mg/mL ) and lcFOS (1.1 mg/mL) in MilliQ water. ScGOS is a mixture of compounds containing galacto-oligosaccharides with a degree of polymerization (DP) between 2 and 8. It mainly consists of DP2 (42%) and DP3 (23%) (36), therefore lactose was chosen to represent DP2 and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p* (scGOS 1,4 β trimer) to represent DP3.

### *In vitro* digestion

To investigate the effect of digestive lipases, we used a two-step *in vitro* model (**Figure 4**) reproducing digestion conditions in the stomach and the upper part of the small intestine adapted from a model described by Larsson *et al.* (37).

Two different *in vitro* digestions were carried out: one employing tuna oil as a source of n-3 PUFAs in triglyceride form; the other using EPA in its free fatty acid form. The tuna oil *in vitro* digestion was performed as the following; first, a simulated gastric fluid (SGF) was prepared by mixing 3 g/L NaCl, 1.06 g/L KCl, 1.47 g/L $CaCl_2 \cdot 2H_2O$, 0.47 g/L $KH_2PO_4$, 0.74 g/L $MgCl_2 \cdot 6H_2O$, 2.25 g/L lipase (50 U/mg), and 2.53 g/L pepsin (3706 U/mg protein) in ultrapure water. Next, in a glass reaction vessel, 200 mg tuna oil was mixed with 66.7 mg scGOS syrup and 3.3 mg lcFOS and added to 3 mL of SGF (**Figure 4A**). A magnetic stir bar was added to the reaction vessel to enable mixing throughout the *in vitro* digestion. The pH was then lowered to 4 using 0.5 M HCl to simulate the first part of the stomach (**Figure 4B**). The sample was placed in a water bath at 37 °C with magnetic stirring at 600 rpm (**Figure 4C**). After 30 min, the pH was adjusted to 2 using 0.5 HCl to simulate the stomach (**Figure 4D**). The sample was returned in the water bath for additional 30 min (**Figure 4E**). Thereafter, to mimic the intestinal phase, the pH was raised to pH 7 using 1 M $NaHCO_3$ (**Figure 4F**) and 0.4 mL intestinal solution was added (ultrapure water containing 4.0 g/L pancreatin and 25 g/L bile extract) (**Figure 4G**). The sample was then placed in the water bath for the remaining of the *in vitro* digestion (2 h) (**Figure 4H**). To monitor the formation of new molecules at different stages of the *in vitro* digestion 0.5 mL samples were taken every 30 min throughout the entire *in vitro* digestion and kept frozen at -80 °C until extraction and analysis. To inhibit oxidation, all samples were flushed with $N_2$ for 15 s, vortexed for 15 s, and then flushed again with $N_2$ for 15 s.

The *in vitro* digestion was also conducted using EPA. The same protocol was used with lower amounts of each material, namely 66.7 mg EPA mixed with 22.2 mg scGOS syrup and 1.1 mg lcFOS in 1 mL of SGF. Because of the low volume, only two samples were taken, every 90 min.
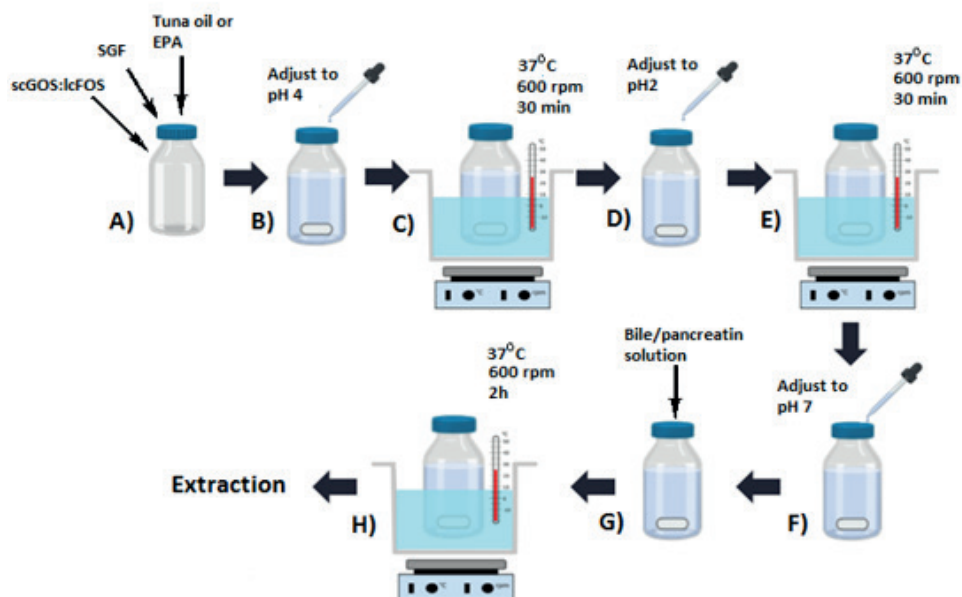
**Figure 4.** *In vitro* digestion process. (A) Tuna oil or EPA was mixed with scGOS syrup and lcFOS and added to the simulated gastric fluid (SGF). (B) A magnetic stir bar was added to the sample and the pH was lowered to 4. (C) The sample was placed in a water bath at 37 °C with magnetic stirring at 600 rpm for 30 min. (D) The pH was adjusted to 2 and (E) the sample was placed back in the water bath for 30 min. (F) To mimic the intestinal phase, the pH was raised to pH 7 and (G) bile/pancreatin solution was added. (H) The sample was then placed in the water bath for the remaining of the in vitro digestion (2 h), followed by extraction of the sample for analysis.

## Stomach simulation: pH 2 condition

As esters can also form without the presence of lipases in an acidic environment such as the stomach, we studied the interactions between scGOS:lcFOS and n-3 PUFAs at pH 2 (**Figure 5**). Two experiments were conducted, using either tuna oil or EPA. The tuna oil pH 2 condition experiment was conducted as following; in a glass reaction vessel, 200 mg tuna oil was mixed with 66.6 mg scGOS and 3.3 mg lcFOS and added to 3 mL MilliQ (**Figure 5A**). A magnetic stir bar was added to the reaction vessel to enable mixing throughout the experiment. The pH was then lowered to 2 using 0.5 M HCl (**Figure 5B**) and the sample was placed in a water bath at 37 °C with magnetic stirring at 600 rpm for 3 h (**Figure 5C**). Throughout the incubation, 0.5 mL samples were taken every 30 min and kept frozen at -80 °C until extraction and analysis. To inhibit oxidation, all samples were flushed with $N_2$ for 15 s, vortexed for 15 s, and then flushed again with $N_2$ for 15 s. The same protocol was used for the pH 2 condition experiment using EPA with lower amounts of each material, namely 66.7 mg EPA mixed with 22.2 mg scGOS syrup and 1.1 mg lcFOS in 1 mL ultrapure water. Because of the low volume, only two samples were taken, every 90 min.
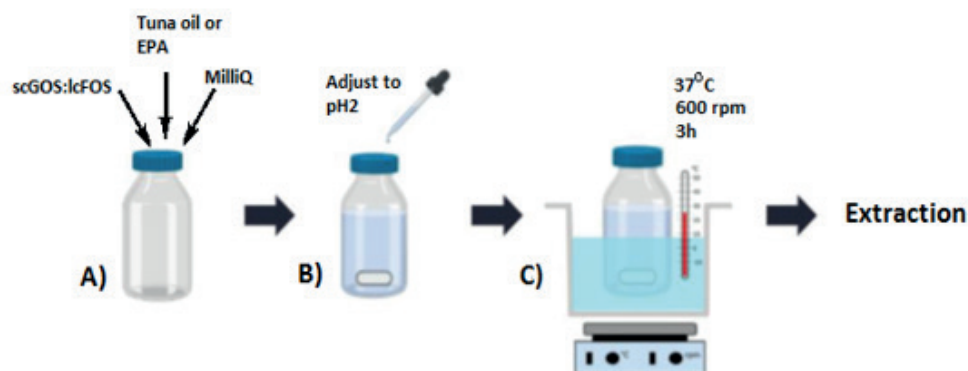
**Figure 5.** pH 2 reaction process. (A) Tuna oil or EPA was mixed with scGOS syrup and lcFOS and added to MilliQ. (B) A magnetic stir bar was added to the sample and the pH was lowered to 2. (C) The sample was placed in a water bath at 37 °C with magnetic stirring at 600 rpm for 3 h, followed by extraction of the sample for analysis.

## Extraction

An extraction in chloroform was carried out to separate the lipids from the water-soluble compounds. For the extraction, we modified the protocol described by Vinarov *et al.* (38) as follows; the *in vitro* digestion or pH 2 condition sample was thawed and 75 µL were transferred to a new vial. The remaining sample was flushed with $N_2$ and placed back at -80 °C until needed for additional analysis. Before the extraction of the *in vitro* digestion samples, the pH was lowered to 2 to decrease the solubility of the products in water. Then, 125 µL chloroform was added and the sample was sonicated for 15 min, agitating it by hand after every 5 min of sonication. The resulting dispersion was then centrifuged for 30 min at 4500 rpm and the organic phase was transferred to a new vial. Both the organic and the aqueous phase were placed at -80 °C until the analysis.

## Thin-layer chromatography (TLC)

TLC was used to analyse the products generated from the *in vitro* digestion or from pH 2 condition and to examine the formation of new products. A modified version of the protocol described by Vinarov *et al.* (38) was used; we employed aluminium backed Silica gel 60 F 254 plates, cut into 2 x 6.5 cm plates. An optimization of the carrier liquid was implemented to find the most suitable eluent to separate each compound present in the samples. A mixture of n-hexane:diethyl ether:acetic acid (ratio 73:25:2) was chosen as the carrier liquid for the separation of the products found in the organic phase, whereas a mixture of butanol:ethanol:water (ratio 5:3:2) was used for the separation of the products found in the aqueous phase (**Figure S1**). After running the samples, a phosphomolybdic acid solution (6% w/w phosphomolybdic acid dissolved in *i*-propanol) was used to dye the TLC plate and a hot plate at 150 °C was used to visualize the spots representing the different compounds.

**Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)**

MALDI-MS was employed for more sensitive analysis, to further characterize new molecules formed during the *in vitro* digestion or during pH 2 condition. The analysis was conducted using an Axima CFR system (Kratos Analytical) and all samples were analysed on reflectron mode. 2,5 dihydroxybenzoic acid (DHB) (10 mg/mL dissolved in acetonitrile) was used as the matrix. 4 μL DHB was added to 1 μL sample and vortexed for 15 s. The mixture was then transferred to a MALDI plate, air-dried, and placed in the MALDI-MS system for analysis. The peaks were analysed in MH$^+$ mode.

**Optical microscopy**

20 μL *in vitro* digested sample of different conditions from a single time point (3 h) was added to a 96 well plate (Greiner) and imaged under the microscope Nikon Eclipse TS2.

**Computational molecular modelling of the supramolecular complex of n-3 PUFAs (DHA) and scGOS**

The molecular modelling study was conducted using PCModel software developed by Serenasoft. scGOS is a mixture of compounds containing galacto-oligosaccharides with a degree of polymerization (DP) between 2 and 8. It mainly consists of DP2 (42%) and DP3 (23%) (36), therefore lactose was chosen to represent DP2 and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p* (scGOS 1,4 β trimer) to represent DP3. The components, lactose (α- and β-anomers), DHA, scGOS 1,4 β trimer (α- and β-anomers) have been energy minimized using MMFF94 force field. The 3D coordinates of α- and β-scGOS 1,4 β trimer and α- and β-lactose were generated by using GLYCAM forcefield (www.glycam.org). The 3D coordinates of DHA were generated by translating the SDF file to 3D coordinate file at the website https://cactus.nci.nih.gov/translate/. **Figure 6** shows the chemical structures of the components used in the modelling study.

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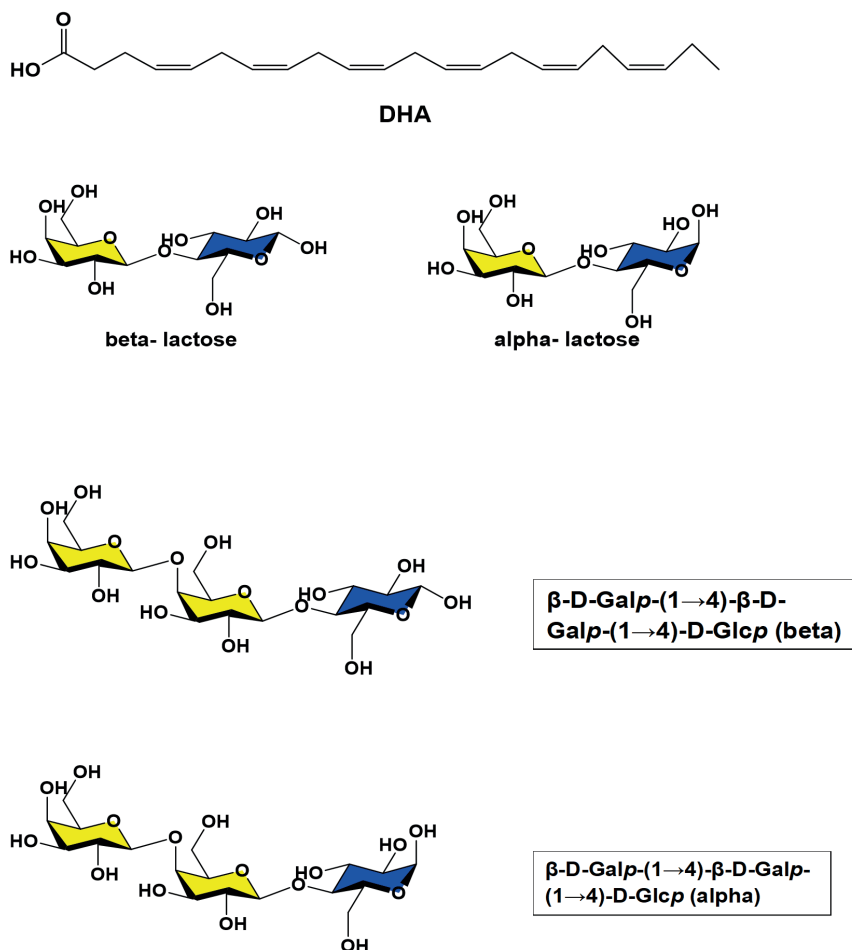
**Figure 6.** Molecular structures of DHA, α- and β-lactose and α- and β-scGOS 1,4 beta trimer.

We have chosen the ratio of components in 1:1:1 ratio; i.e. DHA:lactose:scGOS 1,4 β trimer as a possible representation of the complex in water. Energy minimization of both α-and β-anomers of lactose and of scGOS 1,4 β trimer was performed using generalised Born and surface area (GBSA) solvation model of PCmodel with water as a solvent. During energy minimization addition and deletion of hydrogens were used repeatedly until the lowest steric energy was achieved. Dynamics was calculated by raising temperature from 300 K (27 ºC) to 310 K (37 ºC) for 100 fs. Heat transfer time was 0.001 fs and equilibration time was 1 fs.

# RESULTS

### TLC analysis of the *in vitro* digestion

For the performance of the thin-layer chromatography, optimization of the carrier liquid was implemented to find the most suitable eluent to separate each component of the *in vitro* digestion and pH 2 condition samples. For the organic phase the combination of n-hexane:diethyl ether:acetic acid (ratio 73:25:2) and for the aqueous phase a mixture of butanol:ethanol:water (ratio 5:3:2) were used as carrier liquids. (**Tables S1** and **S2** and **Figure S1**)

After the extraction, the aqueous and organic phases of the *in vitro* digestion samples were analysed by TLC. For the aqueous phase, a TLC analysis was carried out comparing a scGOS:lcFOS standard in water, the aqueous phase of the tuna oil and scGOS:lcFOS *in vitro* digestion sample, and the aqueous phase of the EPA and scGOS:lcFOS *in vitro* digestion sample (**Figure 7A**). The scGOS:lcFOS standard only showed one band and a colouration at the bottom of the plate, corresponding to scGOS and lcFOS, respectively. The tuna oil and scGOS:lcFOS *in vitro* digestion sample did not present any differences compared to the scGOS:lcFOS standard, indicating that no new compounds were present in the sample. The EPA and scGOS:lcFOS *in vitro* digestion sample, however, showed a faint colouration in the upper part of the plate, suggesting the presence of one or more new compounds.

The TLC results for the organic phase compare a tuna oil standard in chloroform, an EPA standard in chloroform, the organic phase of the tuna oil *in vitro* digestion, and the organic phase of the tuna oil and scGOS:lcFOS *in vitro* digestion sample (**Figure 7B**). The tuna oil standard presented a band indicating the position of triglycerides. The EPA standard showed a band indicating the position of free fatty acids. The tuna oil *in vitro* digestion sample was analysed as a control to monitor the *in vitro* digestion of tuna oil without the influence of scGOS:lcFOS. In this sample, the main lipid components can be distinguished. The tuna oil and scGOS:lcFOS *in vitro* digestion sample showed the same lipid components and no notable differences compared to the tuna oil *in vitro* digestion, indicating that no other compounds were present in the sample.

The tuna oil standard and the tuna oil *in vitro* digestion sample were also compared to the EPA and scGOS:lcFOS *in vitro* digestion sample, next to an EPA standard from a different plate (**Figure 7C**). The EPA and scGOS:lcFOS *in vitro* digestion sample results showed an intense band corresponding to free EPA and some bands below it (circled in red) that were not visible in the EPA standard and that did not exactly correspond to the ones seen in the tuna oil *in vitro* digestion sample. Moreover, colouration can be seen in the lower part of the plate for the EPA and scGOS:lcFOS *in vitro* digestion sample, indicating that one or more new compounds could be present in this sample.
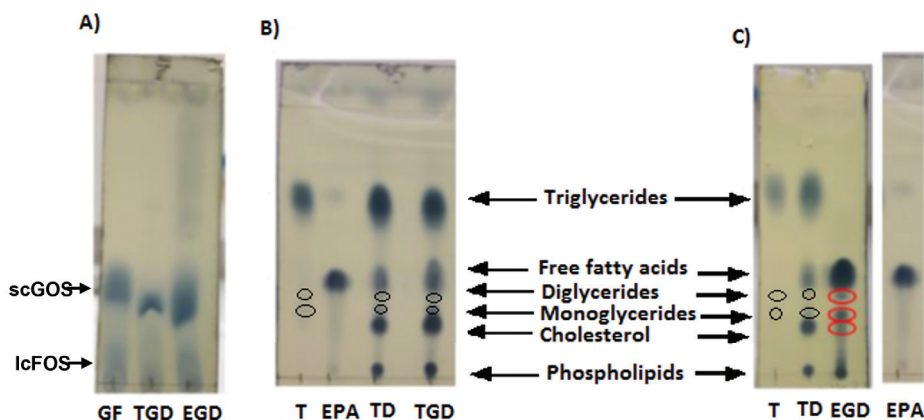
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**Figure 7.** TLC separation of the *in vitro* digestion samples. (A) Aqueous phase. The scGOS:lcFOS standard (GF) shows one band and a colouration at the bottom of the plate, corresponding to scGOS and lcFOS, respectively. The tuna oil and scGOS:lcFOS sample (TGD) displays no differences compared to the standard. The EPA and scGOS:lcFOS sample (EGD) shows a faint colouration in the upper part of the plate. (B) Organic phase. The tuna oil standard (T) presents a band indicating the position of triglycerides. The EPA standard (EPA) shows a band indicating the position of free fatty acids. The tuna oil digestion sample (TD) was used as control showing the main lipid components. The tuna oil and scGOS:lcFOS sample (TGD) shows the same lipid components and no differences compared to the tuna oil control. (C) Organic phase. The EPA and scGOS:lcFOS sample (EGD) results show an intense band corresponding to free EPA and some bands below it (circled in red) that are not visible in the EPA standard (EPA) and that does not exactly correspond to the ones seen in the tuna oil digestion sample (TD). Moreover, colouration can be seen in the lower part of the plate for the EPA and scGOS:lcFOS sample (EGD). EPA standard is from a different plate. Carrier liquid A: butanol:ethanol:water (5:3:2 ratio); B: n-hexane:diethyl ether:acetic acid (73:25:2 ratio); C: n-hexane:diethyl ether:acetic acid (73:25:2 ratio). Bands circled in red show newly observed compounds.

## TLC analysis of the stomach simulation: pH 2 condition

Analysis of the aqueous and the organic phase of the pH 2 condition samples was performed after extraction. The TLC analysis of the aqueous phase was carried out comparing a scGOS:lcFOS standard in water, the aqueous phase of the tuna oil and scGOS:lcFOS pH 2 condition sample, and the aqueous phase of the EPA and scGOS:lcFOS pH 2 condition sample (**Figure 8A**). The tuna oil and scGOS:lcFOS pH 2 condition sample did not present any differences compared to the scGOS:lcFOS standard, indicating that no new compounds were present in the sample. The results of the EPA and scGOS:lcFOS pH 2 condition sample showed a colouration on the upper part of the plate and a more defined band below the colouration, hence indicating that one or more new compounds were formed in this sample. The TLC results for the organic phase compare a tuna oil standard in chloroform, and EPA standard in chloroform, the organic phase of the tuna oil *in vitro* digestion (to compare the spots of free fatty acids, cholesterol etc.), and the organic phase of the tuna oil and scGOS:lcFOS pH 2 condition sample (**Figure 8B**). A band indicating the position of triglycerides can be seen in the tuna oil standard. The EPA standard allows the distinction of a band indicating the position of free fatty acids. The tuna oil *in vitro* digestion sample showed the results of the *in vitro* digestion of tuna oil

without the influence of scGOS:lcFOS, where the main lipid components can be identified. The tuna oil and scGOS:lcFOS pH 2 condition sample only showed one clearly visible band corresponding to the band for triglycerides and no new compounds are detected. The tuna oil standard and the tuna oil *in vitro* digestion sample were also compared to the EPA and scGOS:lcFOS pH 2 condition sample, next to an EPA standard from a different plate (**Figure 8C**). The EPA and scGOS:lcFOS pH 2 condition sample showed results similar to those of the EPA and scGOS:lcFOS *in vitro* digestion sample (**Figure 7C**), although fainter. A distinct band corresponding to free EPA can be distinguished and some bands below it (circled in red) that are not visible in the EPA standard and that do not seem to be the same ones seen in the tuna oil *in vitro* digestion sample. Again, a faint colouration can be seen in the lower part of the plate for the EPA and scGOS:lcFOS pH 2 condition sample, indicating that one or more new compounds could be present in this sample.

The TLCs of the EPA and scGOS:lcFOS *in vitro* digestion sample and the EPA and scGOS:lcFOS pH 2 condition sample have very similar patterns. To evaluate the role of EPA in more details, the EPA and scGOS:lcFOS *in vitro* digestion sample together with the EPA standard and an EPA pH 2 condition sample (EPA only incubated in the pH 2 condition setup) were analysed on a separate TLC (**Figure S2**). These results indicate that the EPA only pH 2 also showed similar bands on the TLC as the EPA and scGOS:lcFOS *in vitro* digestion sample.

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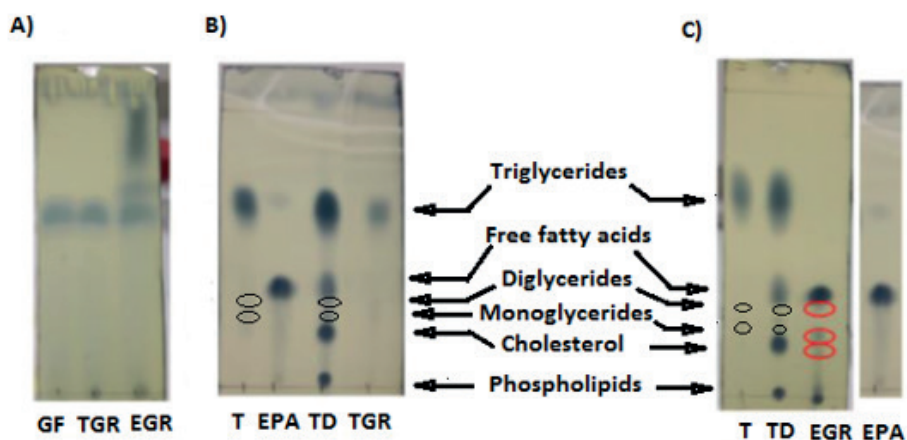


**Figure 8.** TLC separation of the pH 2 condition samples. (A) Aqueous phase: The tuna oil and scGOS:lcFOS sample (TGR) present no differences compared to the scGOS:lcFOS standard (GF). The EPA and scGOS:lcFOS sample (EGR) shows a colouration on the upper part of the plate and a more defined band below the colouration. (B) Organic phase: A band indicating the position of triglycerides can be seen in the tuna oil standard (T). The EPA standard (EPA) allows the distinction of a band indicating the position of free fatty acids. The tuna oil digestion sample (TD) shows the results of the *in vitro* digestion of tuna oil without the influence of scGOS:lcFOS, where the main lipid components can be identified. The tuna oil and scGOS:lcFOS sample (TGR) shows one clearly visible band corresponding to the band for triglycerides. (C) Organic phase: In the EGR sample a distinct band corresponding to free EPA can be distinguished and some bands below it (circled in red) that are not visible in the EPA standard (EPA) and that do not seem to be the same ones seen in the tuna oil in vitro digestion sample (TD). A faint colouration can be seen in the lower part of the plate

for the EPA and scGOS:lcFOS pH 2 condition sample (EGR). Carrier liquid: A: butanol:ethanol:water (5:3:2 ratio); B: n-hexane:diethyl ether:acetic acid (73:25:2 ratio); C: n-hexane:diethyl ether:acetic acid (73:25:2 ratio). Bands circled in red show newly observed compounds.

## MALDI-MS results for EPA and scGOS:lcFOS standards

The TLC analysis indicated that new compounds were formed during both the *in vitro* digestion and pH 2 condition between EPA and scGOS:lcFOS. MALDI-MS analysis was carried out for further characterization of the possible new compounds detected in these samples. EPA, scGOS and lcFOS standards were analysed to obtain reference spectra. All molecules were detected as monosodium adducts, therefore results appear as the mass of the original molecule with the addition of the mass of sodium $[M+Na]^+$. The spectrum for the EPA standard (**Figure 9A**) showed a peak at *m/z* 324.8924, corresponding to EPA, along with other peaks corresponding to unidentified contaminants. The peak at *m/z* 324.8924 is absent in the MALDI matrix (DHB spectrum) (**Figure S3**).

The spectra for the scGOS (**Figure 9B**) and lcFOS (**Figure 9C**) standards showed multiple peaks for each compound, corresponding to various degrees of DP. Results for scGOS showed seven clear peaks (*m/z* 365.3983 to *m/z* 1337.9685) corresponding to DP 2 to 8.
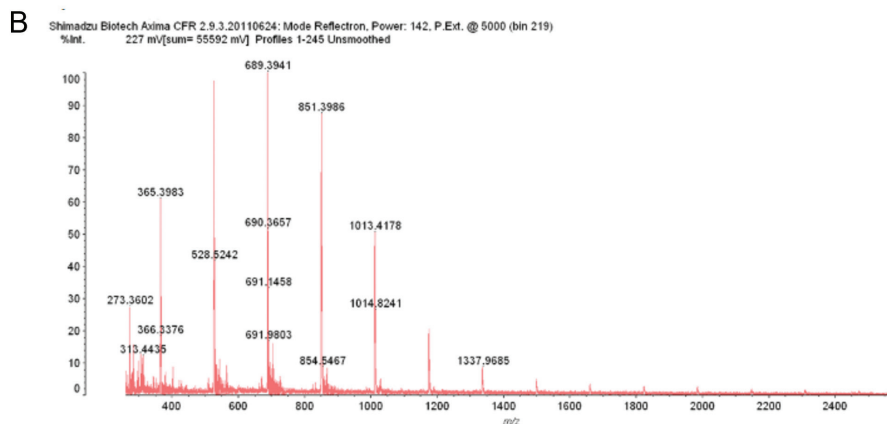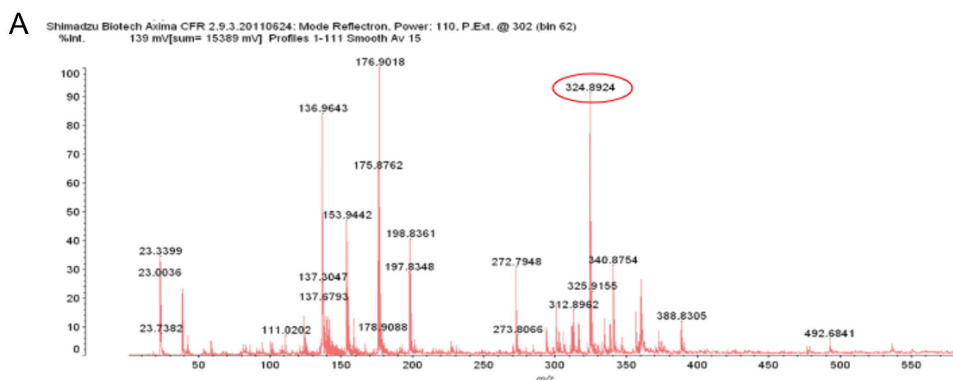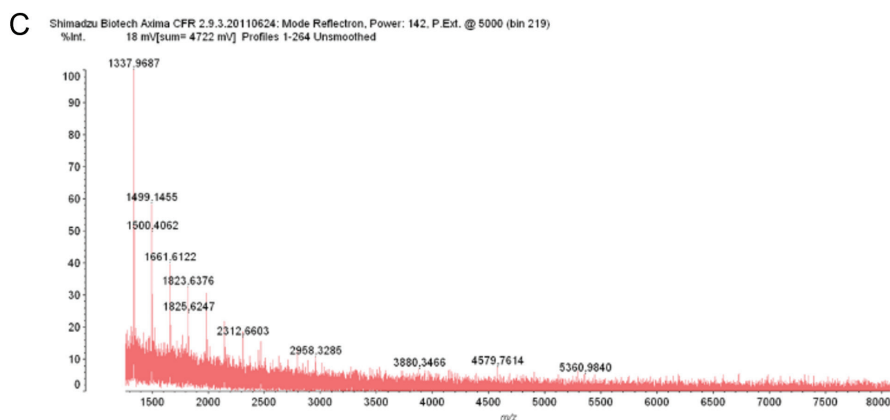
**Figure 9.** MALDI-MS spectra for the (A) EPA, (B) scGOS and (C) lcFOS standards. (A) The peak corresponding to EPA (circled in red) is found at a *m/z* value of 324.8924. (B) and (C) Multiple peaks for scGOS and lcFOS represent different degrees of polymerization.

**MALDI-MS results for the EPA and scGOS:lcFOS *in vitro* digestion sample**

The organic and aqueous phase of the EPA and scGOS:lcFOS *in vitro* digestion samples were analysed to characterize the indicated new compounds detected during the TLC analysis. The spectrum for the organic phase of the sample (**Figure 10A**) revealed multiple peaks corresponding to new molecules. These peaks were not found in the standards tested and the *m/z* values can be used to calculate the mass of these new compounds, thus allowing speculation on their identity. Based on their masses, it was calculated that some of these compounds could be the result of a chemical reaction between scGOS and several EPA molecules. However, most of the peaks has to be considered as less reliable signals due to the low height. On the other hand, the spectrum for the aqueous phase of the EPA and scGOS:lcFOS *in vitro* digestion sample (**Figure 10B**) did not show any new peaks. The peaks for scGOS and lcFOS were also not detected in this spectrum, even though these compounds were present in the sample and they were visible upon TLC analysis. The results for the aqueous phase of this sample were therefore inconclusive.

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**Figure 10.** MALDI-MS spectra of the (A) organic phase and (B) aqueous phase of the EPA and scGOS:lcFOS *in vitro* digestion sample. *m/z* values circled in red correspond to peaks for new compounds.

## MALDI-MS results for the EPA and scGOS:lcFOS pH 2 condition sample

The organic and the aqueous phase of the EPA and scGOS:lcFOS pH 2 condition samples were analysed as well. The spectrum for the organic phase of the EPA and scGOS:lcFOS pH 2 condition sample (**Figure 11A**) showed numerous peaks corresponding to new compounds. These peaks were not observed in any of the standards tested. Based on the *m/z* values of these peaks, we calculated the mass of the new molecules and found that some of them could correspond to compounds resulting from a chemical reaction between scGOS and several EPA molecules. However, most of the peaks has to be considered as less reliable signals due to the low height. As for the aqueous phase of the EPA and scGOS:lcFOS pH 2 condition sample (**Figure 11B**), the spectrum showed peaks

corresponding to scGOS but did not show any new peaks. The results thus indicate that no new compounds were present in the aqueous phase of the sample.
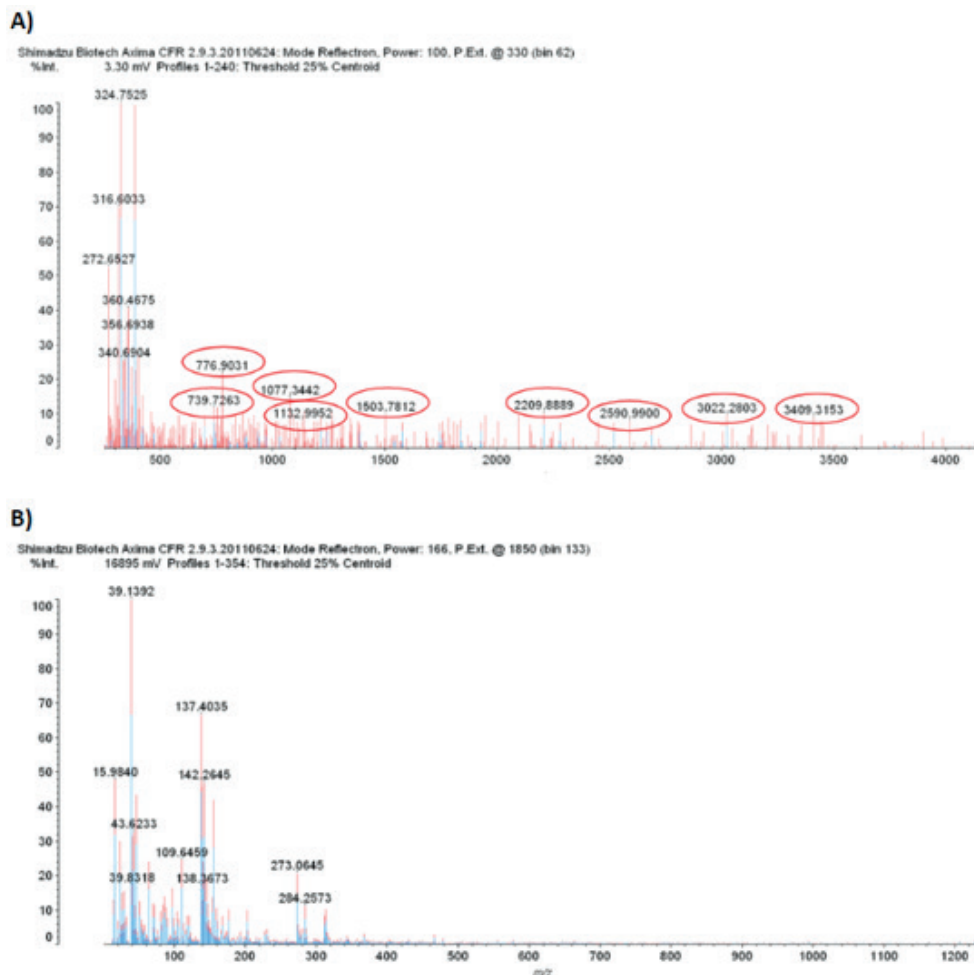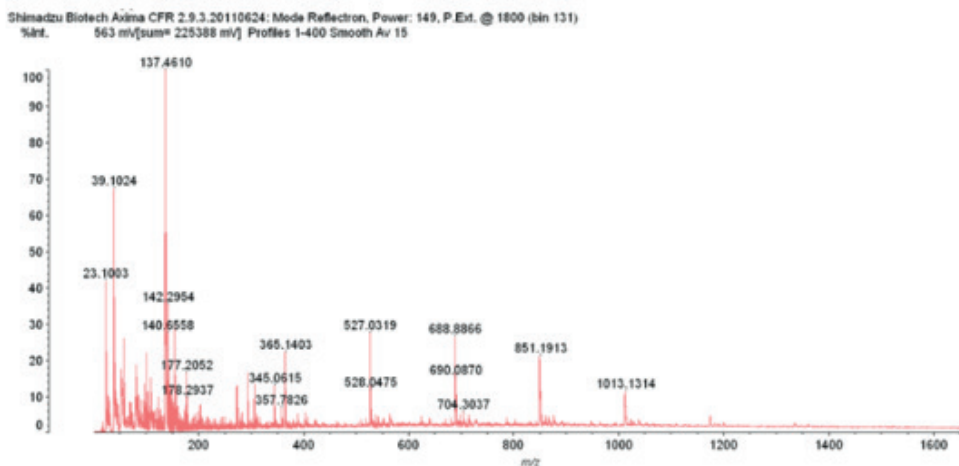


**Figure 11.** MALDI-MS spectra of (A) the organic phase and (B) aqueous phase of the EPA and scGOS:lcFOS pH 2 condition sample. *m/z* values circled in red correspond to peaks for new compounds.

## n-3 PUFAs mixed micelle formation influenced by scGOS:lcFOS

To evaluate the hypothesis that scGOS:lcFOS interact with the mixed micelle formation of n-3 PUFAs in the form of tuna oil samples from the *in vitro* digestion setup were subjectively analysed by microscope. ScGOS:lcFOS in water did not form any particulate structure (**Figure 12A**). Bile salts in water formed some random particulate structure (**Figure 12B**). We observed that pure tuna oil also had a particulate sphere-like structure (**Figure 12C**).

In water, the tuna oil formed oil droplets-structures (**Figure 12D**). In the presence of digestive mixtures (e.g. bile salts, pancreatin and lipases) tuna oil formed nanoparticle like structures, this could be a micelle formation (**Figures 12E**, **F**). These nanoparticulate structures, however, morphologically changed in the presence of scGOS:lcFOS (**Figures 12G, H**). In the organic phase, no particulate structures were observed (data not shown). The microscopic evaluation of the samples of tuna oil digestion and of tuna oil with scGOS:lcFOS digestion before the extraction resulted in pictures too dark and dense to investigate properly (**Figure S4**).
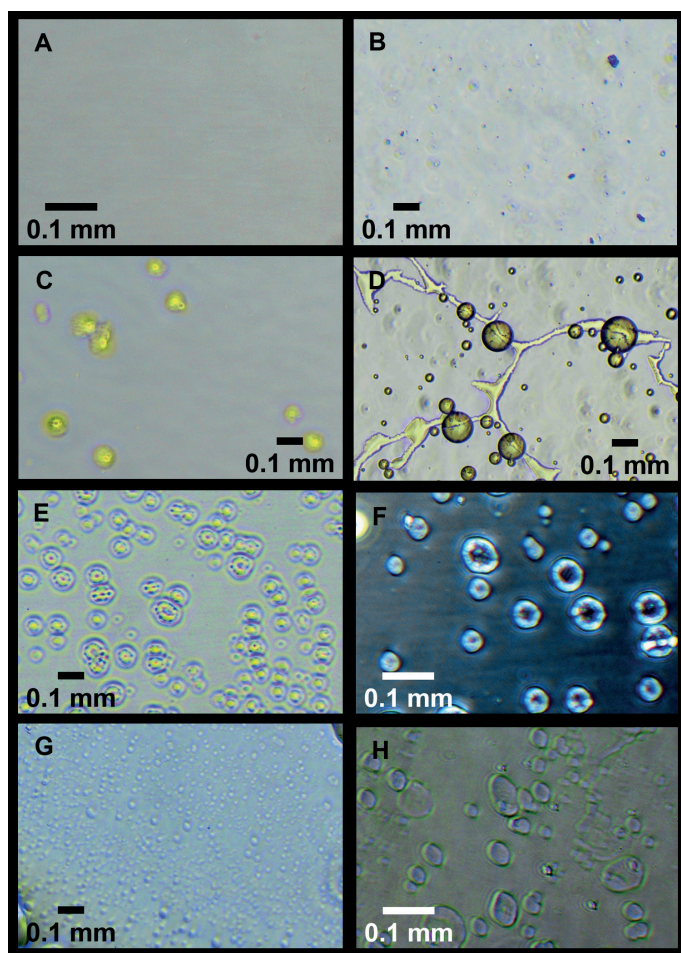


**Figure 12.** Optical microscopic images of components used in *in vitro* digestion. (A) scGOS:lcFOS in MilliQ water (20X), (B) bile in MilliQ water (10X), (C) tuna oil only (10X), (D) tuna oil in MilliQ water (10X), (E) sample of aqueous layer of tuna oil after digestion (10X), (F) sample of aqueous layer of tuna oil after digestion (20X), (G) sample of aqueous layer of tuna oil combined with scGOS:lcFOS after digestion (10X), (H) sample of aqueous layer of tuna oil combined with scGOS:lcFOS after digestion (20X).

## Computational molecular modelling of hydrogen binding between n-3 PUFAs and scGOS

The chemical structures of the components used in the modelling study are shown in **Figure 6**. The molecular modelling of the possible chemical complex of DHA and scGOS resulted in many possible structures. Some of the energy minimized structures of each component (**Figures 13A-E**) and of 1:1:1 combination of DHA:lactose:scGOS 1,4 β trimer are shown in **Figures 13F-I**. DHA is the most abundant n-3 PUFA of the tuna oil and therefore DHA was used in the modelling. ScGOS is a mixture of compounds containing galacto-oligosaccharides with a degree of polymerization (DP) between 2 and 8. It mainly consists of DP2 (42%) and DP3 (23%) (36), therefore lactose was chosen to represent DP2 and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-D-Glc*p* (scGOS 1,4 β trimer) to represent DP3. The combination of 1:1:1 of DHA:β-lactose:β-scGOS 1,4 β trimer seems unstable, that the components are separating (**Figure 13F**). The **Figures 13G** and **13H** show that the complexation between DHA:α-lactose:α-scGOS 1,4 β trimer is stable. The combination of DHA:β-lactose:α-scGOS 1,4 β trimer (**Figure 13I**) shows hydrogen bondings (yellow lines in the figure) between the three components DHA, β-lactose and α-scGOS 1,4 β trimer. This suggests that in water these components can form a supramolecular assembled structure at 37 °C. It is also worth noting that DHA has Xlogp = 10.82, which suggests that it is insoluble in water. Both the α- and β-lactose have Xlogp = -5.921 and both α- and β-scGOS 1,4 β trimer have Xlogp = -8.33. The negative Xlogp value suggests that they are soluble in water. The 1:1:1 complex of DHA:(α or β)-lactose:(α or β)-scGOS 1,4 β trimer has Xlogp = -3.428 suggesting that the complex is still soluble in water. The complexation is favoured as DHA is included within carbohydrates (lactose and scGOS 1,4 β trimer) and form interactions in such a way that it is soluble in water.

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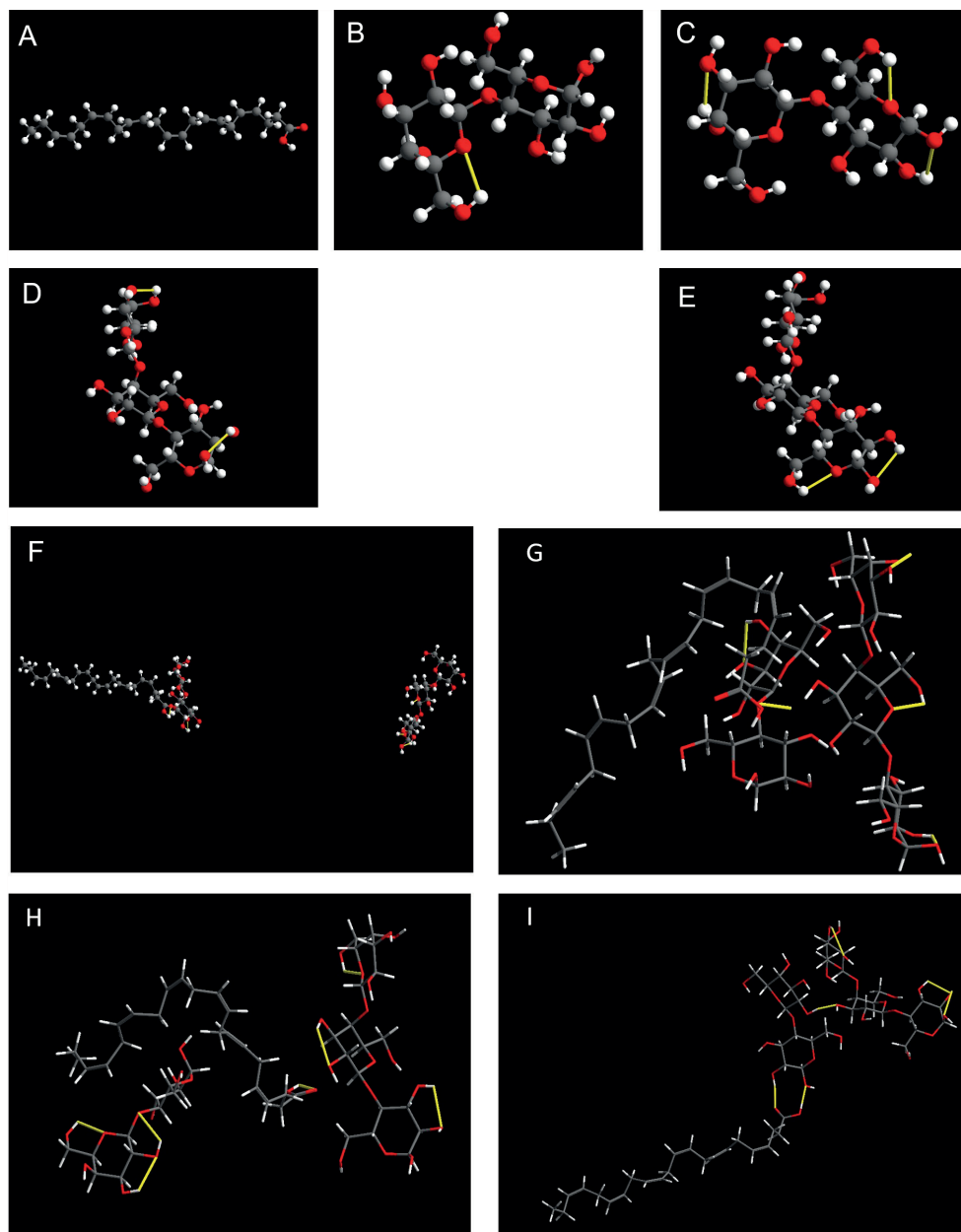
**Figure 13.** The 3D structures of single components and the complexation between DHA:lactose:scGOS-1,4 β trimer in the ratio 1:1:1. Hydrogen bonds are shown as yellow lines. (A) DHA, (B) α- lactose, (C) β- lactose, (D) α-scGOS 1,4 β trimer, (E) β-scGOS 1,4 β trimer, (F) DHA:β-lactose:β-scGOS 1,4 β-trimer (shows instability of the complex), (G) DHA:α-lactose:α-scGOS 1,4 β-trimer, (H) α-lactose:DHA:α-scGOS 1,4 β trimer, (I) β-lactose:DHA:α-scGOS 1,4-β trimer (shows hydrogen bonding between 3 components).

## DISCUSSION

Our observations indicate that both the *in vitro* digestion and pH 2 condition of EPA and scGOS:lcFOS result in the formation of numerous new compounds, while no new compounds were observed after the *in vitro* digestion and pH 2 condition of tuna oil with scGOS:lcFOS. Tuna oil contains fatty acids mostly in the triglyceride form and EPA was used in its free fatty acid form. The TLCs of the tuna oil *in vitro* digestion samples show an intense band for triglycerides and fainter bands for free fatty acids, monoglycerides and diglycerides, indicating that the lipase conversion of the triglycerides in the tuna oil into free fatty acids might be less effective than expected. This suggests that the *in vitro* digestion model we employed is not entirely effective. Since the model has been successfully used before (37), it is important to determine if and why the model was less effective in our study. The protocol applied in our experiments presents some slight modifications compared to the model described by Larsson *et al.* (37). A difference is that the *in vitro* digestion was carried out in a water bath instead of an incubator. This factor might have affected lipase efficiency, thus impacting our results related to a reduced lipase conversion of the triglycerides in the tuna oil into free fatty acids. Another possibility is that the used fungi lipases are not optimal and the use of human or mice lipases might lead to better conversion. We repeated the *in vitro* digestion of tuna oil and scGOS:lcFOS in a nitrogen rich environment to simulate the anaerobic condition of the gastrointestinal tract. This did not lead to newly formed compounds, indicating that the anaerobic environment is not affecting the chemical reaction between scGOS:lcFOS and n-3 PUFAs (data not shown). Given that the interaction between n-3 PUFAs and scGOS:lcFOS in the gastrointestinal tract has not been studied before, there might be important variables in *in vivo* conditions that we did not account for and that are not replicated by this model. In that case, alternative models with different concentrations of enzymes and simulated digestive fluids should be tested (39, 40). Finally, our method is a static *in vitro* digestion model that does not simulate peristaltic movements. This can also play a role in our study, the use of a more translatable model like the TNO gastro-intestinal model-1 (TIM-1) model (41) can provide some insight into the conditions required to carry out a study like this.

On its own (without scGOS:lcFOS) EPA is unable to be chemically modified at pH 2. However, the TLC of the EPA only pH 2 condition sample shows similar bands as the TLCs of pH 2 condition and *in vitro* digestion model of EPA with scGOS:lcFOS. The observation of higher molecular weight in MALDI-MS is surprising, because these compounds can be generated due to interactions between EPA and other molecules like scGOS or bile salts etc. This suggests that the new bands in the TLC might be associated with an impurity present in the EPA sample. However, based on the spectra of the MALDI-MS, the newly formed compounds cannot only be due to impurities of EPA. The molecular weight of the newly formed compounds is too large to be caused by EPA impurities. The peaks in the MALDI-MS of the newly formed compounds are different comparing the *in vitro* digestion sample and the pH 2 condition sample of EPA with scGOS:lcFOS. This indicates that the chemical composition of the newly formed compounds differs. The exact identity of the

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possible newly generated compounds is, however, still unclear and therefore it is difficult to formulate a precise molecular interpretation of these results based on what has been discovered so far. Additionally, the peak heights in the MALDI-MS spectra of the organic phase of both the *in vitro* digestion sample and the pH 2 condition sample were low and might lead to a less reliable signal to indicate presence or absence of new compounds. The MALDI-MS results for the aqueous phase of the scGOS and lcFOS standards were in line with previous literature (42). However, the MALDI-MS results of the samples from the *in vitro* digestion was inconclusive due to the non-appearance of the scGOS:lcFOS peaks. This could be due to an ionization problem in the MALDI-MS. The aqueous phase MALDI-MS peaks of pH 2 condition of EPA and scGOS:lcFOS, however, shows that there were no formation of new compounds residing in the aqueous phase.

Although the gastric pH in mice and infants is about 3 (43, 44), in this study we wanted to evaluate the effect of a more extreme pH on the possible chemical/physical reaction/interaction between scGOS:lcFOS and n-3 PUFAs and therefore these experiments were conducted at pH 2.

In conclusion, *in vitro* digestion and pH 2 condition of tuna oil and scGOS:lcFOS did not lead to the formation of any new compounds. However, both *in vitro* digestion of EPA and scGOS:lcFOS and the pH 2 condition of EPA and scGOS:lcFOS resulted in the possible formation of multiple compounds. Based on the molecular mass of these possible new compounds, despite the possible influence of the EPA impurities on the TLC results and the less reliability due to the peak height, it is possible to speculate that these compounds may be the result of a chemical reaction between scGOS and several EPA molecules. The large size of the newly formed molecules cannot be achieved by the impurities of EPA. The molecule resulting from the interaction between scGOS and several EPA molecules will be too large and branched to be digested by intestinal microbiota or metabolized by the host resulting in a reduced absorption by the body, as is the case for the similarly structured compound olestra (35).

After the chloroform extraction, most of the components move to the organic layer and less components were left in the aqueous layer. In the microscopic evaluation of structural changes, we did observe a change in the structure of tuna oil in the presence of scGOS:lcFOS in the aqueous layer of the samples. The structure was more droplet-like rather than particle-like, which might indicate that the n-3 PUFAs micelles were influenced by scGOS:lcFOS. Due to the deformation of the micelle formation of scGOS:lcFOS and n-3 PUFAs, n-3 PUFAs will be less available to be absorbed by the enterocytes. Consequently, less chylomicrons will be formed, resulting in less n-3 PUFAs transported through the lymphatic system to enter the blood circulation for delivery to storage and energy utilization in several tissues. When the bioavailability of n-3 PUFAs in the host is reduced, the preventive effect regarding allergy development might very well be reduced as well. This hypothesis needs further experimentation such as determining the systemic or tissue levels of n-3 PUFAs in the presence of scGOS:lcFOS. Due to the encapsulation by scGOS:lcFOS the n-3 PUFAs stay in the intestinal tract and will be excreted via the faeces. The determination of n-3 PUFA levels in the faeces can help to get more insight in this hypothesis.

Despite the limitations of the microscopy evaluation of the interaction of scGOS:lcFOS on the micelle formation of n-3 PUFAs, computational modelling data suggests that scGOS can interact with n-3 PUFAs in such a way that the complex has a negative Xlogp value and thus is soluble in water. This indicates that scGOS might encapsulate n-3 PUFAs and make them unavailable for uptake by enterocytes and further distribution through the body. Although optimisation is needed, with caution we suggest that with our preliminary data scGOS:lcFOS might influence the micelle formation of n-3 PUFAs.

## OVERALL CONCLUSION

From the *in vitro* digestion experiments we learned that EPA and scGOS:lcFOS might interact with each other. This complex might be unavailable to be digested by the microbiota and to be absorbed by the intestine leading to less availability of both scGOS:lcFOS and n-3 PUFAs. The computational modelling data do indeed indicate that scGOS:lcFOS is able to form supramolecular complexes with free fatty acids into a stable water-soluble complex. Although the used experimental procedures need optimisation, these preliminary data indicate possible interactions between scGOS:lcFOS and n-3 PUFAs which might lead to less bioavailability and functionality. However the results of the clinical studies on scGOS:lcFOS with n-3 PUFAs clearly provide evidence that these combination positively impact the gut microbiota and immune system in early life (45).

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# SUPPLEMENTARY INFORMATION

**Table S1.** Optimization of TLC separation of the organic phase.
An optimization of the carrier liquid was implemented to find the most suitable way to separate each compound in the digestion and pH 2 reaction samples. Multiple combinations of different solvents were tested and the results were observed and compared. This table presents all carrier liquids tested for the separation of the organic phase of the digestion and reaction samples. The goal was to clearly distinguish all different lipid components found in the samples. EPA: Eicosapentaenoic acid, GF: scGOS:lcFOS.



**Carrier liquid:**
Petroleum ether:diethyl ether:formic acid (80:20:1)
**Samples:**
T2 = Tuna oil standard
60 = Tuna oil digestion at timepoint (T) 60 min
120 = Tuna oil digestion



**Carrier liquid:**
N-hexane:diethyl ether:formic acid (80:20:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil digestion



**Carrier liquid:**
N-hexane
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil digestion



**Carrier liquid:**
N-hexane:formic acid (100:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion

**Carrier liquid:**
N-hexane:diethyl ether:formic acid (85:15:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion



**Carrier liquid:**
N-hexane:diethyl ether:formic acid (90:10:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion



**Carrier liquid:**
Diethyl ether:formic acid (100:1)
**Samples:**
T2 = Tuna oil standard
180 = Tuna oil digestion



**Carrier liquid:**
N-hexane:ethyl acetate:formic acid (80:20:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
150 = Tuna oil digestion



**Carrier liquid:**
Petroleum ether:ethyl acetate:formic acid (90:10:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil and GF digestion
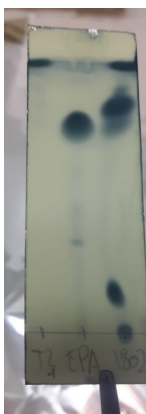


**Carrier liquid:**
Petroleum ether:ethyl acetate: (3:2)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil and GF digestion

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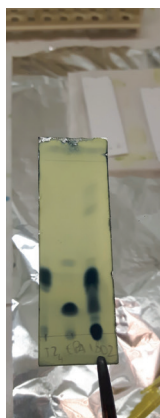
**Carrier liquid:**
Petroleum ether:ethyl acetate:formic acid (94:6:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil and GF digestion
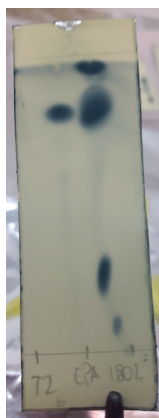


**Carrier liquid:**
Petroleum ether:ethyl acetate:formic acid (60:40:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil and GF digestion
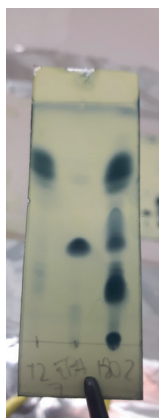


**Carrier liquid:**
Petroleum ether:ethyl acetate:formic acid (85:15:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
180 = Tuna oil and GF digestion
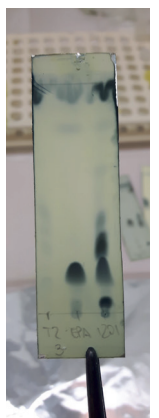


**Carrier liquid:**
Chloroform
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion



**Carrier liquid:**
Chloroform:methanol (2:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion
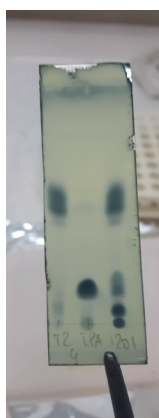


**Carrier liquid:**
N-hexane:ethyl acetate (9:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion
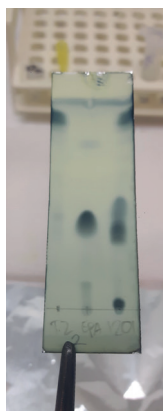
**Carrier liquid:**
N-hexane:ethyl acetate (4:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion
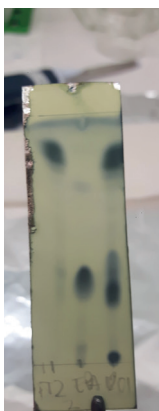


**Carrier liquid:**
N-hexane:ethyl acetate (8:1)
**Samples:**
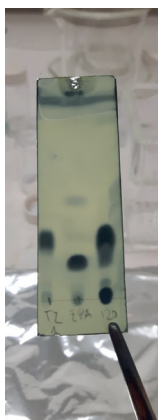T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion



**Carrier liquid:**
N-hexane:ethyl acetate (10:1)
**Samples:**
T2 = Tuna oil standard
EPA = EPA standard
120 = Tuna oil digestion



**Carrier liquid:**
Chloroform:methanol (99:1)
**Samples:**
GF = GF standard
180.1 = Tuna oil digestion
180.2 = Tuna oil and GF digestion



**Carrier liquid:**
Chloroform:methanol (98:2)
**Samples:**
T2 = Tuna oil standard
180.2 = Tuna oil and GF digestion
180.5 = EPA and GF reaction



**Carrier liquid:**
N-hexane:diethyl ether:acetic acid (73:25:2)
**Samples:**
T2 = Tuna oil standard
150.1 = Tuna oil digestion
150.2 = Tuna oil and GF digestion

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**Table S2.** Optimization of TLC separation of the aqueous phase.
The carrier liquids were tested for the separation of components of the aqueous phase. Two carrier liquids were tested and the goal was to visualize the components of the GF standard. GF: scGOS:lcFOS.



**Carrier liquid:**
Ethyl acetate:2-propanol:water (3:2:1)
**Samples:**
T2 = Tuna oil standard
GF = GF standard
180.2 = Tuna oil and GF digestion
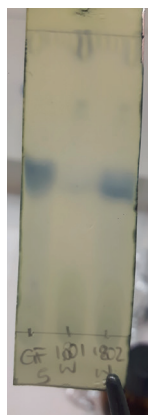


**Carrier liquid:**
Butanol:ethanol:water (5:3:2)
**Samples:**
GF = GF standard
180.1 = Tuna oil digestion at T 180 min
180.2 = Tuna oil and GF digestion
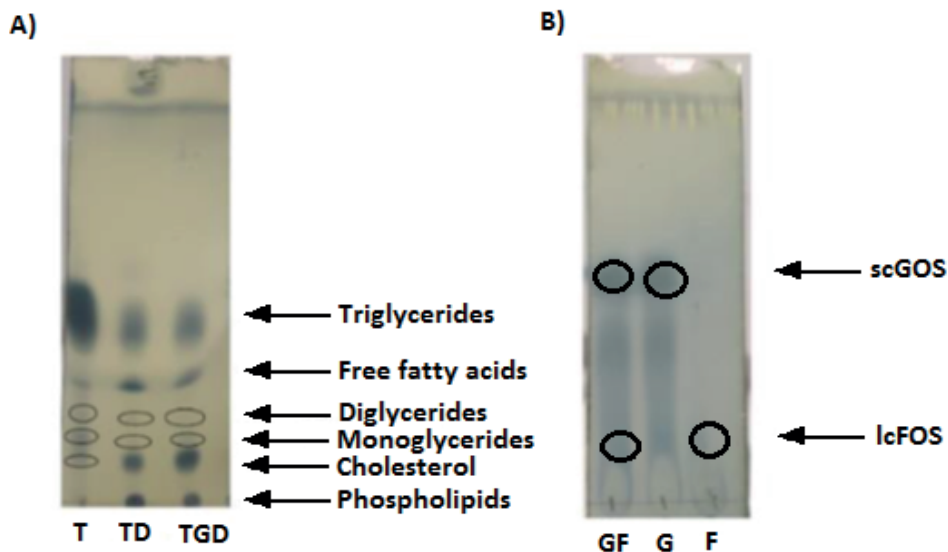


**Figure S1.** TLCs of the chosen carrier liquids after optimization. (A) TLC separation of lipids in the organic phase. T: tuna oil standard, TD: tuna oil *in vitro* digestion sample, TGD: tuna oil and scGOS:lcFOS *in vitro* digestion sample. Carrier liquid: n-hexane:diethyl ether:acetic acid (73:25:2 ratio). (B) TLC separation of scGOS and lcFOS standards in MilliQ. G: scGOS; F: lcFOS; GF: scGOS:lcFOS standard. Carrier liquid: butanol:ethanol:water (5:3:2 ratio).

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**Figure S2.** TLC of EPA standard, EGD (EPA and scGOS:lcFOS *in vitro* digestion sample) and EPA (pH2 condition). The TLC of the EPA only pH 2 condition sample shows similar bands as the *in vitro* digestion model of EPA with scGOS:lcFOS. EPA: Eicosapentaenoic acid.
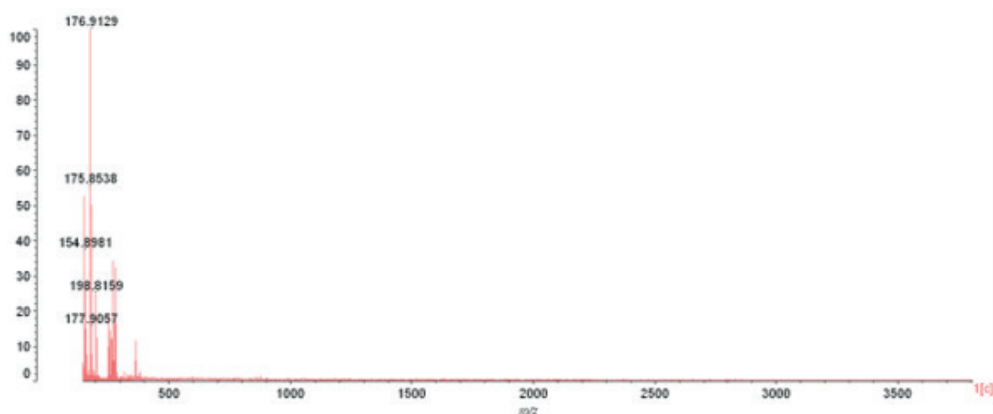


**Figure S3**. MALDI-MS spectrum of the 2,5 dihydroxybenzoic acid (DHB) blank (MALDI matrix).
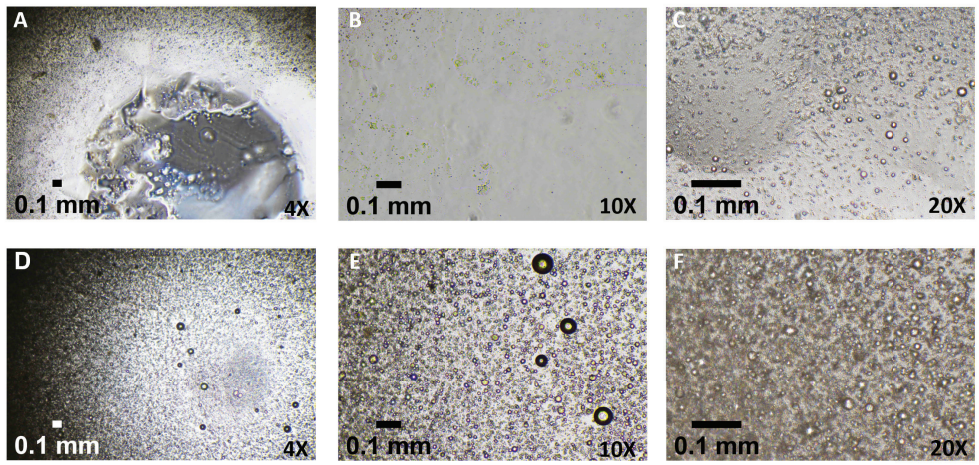
**Figure S4.** Microscopy pictures of freeze-thawed samples before extraction. A-C: Sample from tuna oil digestion in magnifications 4X (A), 10X (B) and 20X (C). (D-F) Sample from tuna oil with scGOS:lcFOS digestion in magnifications 4X (D), 10X (E) and 20X (F).

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# CHAPTER 7

*Ex vivo* caecal bacterial fermentation activity induced by non-digestible oligosaccharides is not inhibited by omega-3 polyunsaturated fatty acids – a preliminary study

Kirsten Szklany[1], Merel Vos[1], Marcel R. de Zoete[2], Johan Garssen[1,3], Aletta D. Kraneveld[1], Leon M. J. Knippels[1]

[1] Division of Pharmacology, Utrecht Institute of Pharmaceutical Sciences, Faculty of Science, Utrecht University, The Netherlands
[2] Department of Medical Microbiology, University Medical Center Utrecht, The Netherlands
[3] Danone Nutricia Research, Utrecht, The Netherlands

## ABSTRACT

The prevalence of cow's milk allergy (CMA) in westernized countries is approximately 2-3%. Currently, avoidance of the allergen is the only option to prevent allergic reactions as no cure or treatment for CMA is available. The prebiotic mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) as well as omega-3 polyunsaturated fatty acids (n-3 PUFAs) have promising effects in preventing CMA development in pre-clinical mouse models. A dietary combination of scGOS:lcFOS and n-3 PUFAs showed no additive preventive effect but suggested a possible interaction between scGOS:lcFOS and n-3 PUFAs. We tested the hypothesis that n-3 PUFAs might interfere with the intestinal bacterial fermentation of scGOS:lcFOS leading to reduced production of short-chain fatty acids (SCFAs). Less production of SCFAs might eventually lead to less immunomodulation and increased risk of CMA development. Caeca content from C3H/HeOuJ (healthy or CMA) mice were suspended in carbonate phosphate buffer in an anaerobic chamber. The suspensions were exposed to scGOS:lcFOS (0.5, 1 or 2% (w/v)), n-3 PUFA containing tuna oil (6, 12 of 18% (v/v)), to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (10, 50, 100 of 200 µM) or combinations of the components and incubated for 24 h at 37 ˚C. SCFAs were measured in the supernatants using gas chromatography. ScGOS:lcFOS increased the SCFA production following a dose-response curve. n-3 PUFA containing tuna oil as well as DHA and EPA without scGOS:lcFOS had no effect on the *ex vivo* SCFA production. The combination scGOS:lcFOS with tuna oil, DHA and/or EPA increased the SCFA production. The SCFA production was unaffected whether the donor mice were cow's milk allergic or healthy when unstimulated as well as after stimulation with scGOS:lcFOS, DHA, EPA or combinations of these components. The SCFA production was induced by scGOS:lcFOS and unaffected by DHA or EPA individually. Based on these preliminary data, scGOS:lcFOS is fermented into SCFAs *ex vivo*. The scGOS:lcFOS-induced SCFA production was neither affected by DHA nor EPA. This indicates that our hypothesis that n-3 PUFAs interfere with the bacterial fermentation of scGOS:lcFOS into SCFAs in the intestine leading to reduced SCFA production and less preventive efficiency of CMA development, is invalid.

## INTRODUCTION

In westernized countries the prevalence of children diagnosed with cow's milk allergy (CMA) is approximately 2-3% (1). Although the majority of these children outgrow CMA before the age of 5, they are at higher risk to develop other allergic disorders later in life (2). To date, no cure or treatment for CMA is available, avoidance of the allergen is the only option.

Human breast milk is the gold standard during the first six months of life. The documentation of the preventive effect of human milk on the development of CMA is limited, but the consumption of human milk in early life has been shown to reduce the risk of developing other allergic disorders such as eczema and asthma and is generally accepted (3-5). Human breast milk contains a wide range of components which serve all necessities of the infant (6). One of these components are the human milk oligosaccharides (HMOs) that have prebiotic capacities and drive the development of the intestinal microbiota, strengthen the function of the intestinal barrier and, support and modulate the maturation of the immune system and the nervous system in early life (6, 7). Although breastfeeding is recommended there are situations where breast milk is unavailable. Fortunately, infant formula is provided and most of these formulas contain non-digestible oligosaccharides (NDOs) which resemble HMOs. Examples of NDOs are short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS). Like HMOs the mixture of scGOS and lcFOS possess prebiotic activity and immune modulatory capabilities (8, 9). The immunomodulatory effects of scGOS:lcFOS may occur directly on intestinal epithelial or immune cells or indirectly through short-chain fatty acids (SCFAs) (10-13). SCFAs, mainly butyrate, are promising components in modulating the immune system to prevent the development of CMA (14). Another component possessing immunomodulatory properties in human breast milk as well as in infant formula is omega-3 polyunsaturated fatty acids (n-3 PUFAs). Two of the main n-3 PUFAs are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (15).

Although the clinical evidence is limited, in preclinical mouse models the effects of scGOS:lcFOS and n-3 PUFAs in preventing CMA are promising (16-19). Surprisingly, a combination of these dietary components did not lead to additional preventive effects on the development of CMA in mice. The effects of combining scGOS:lcFOS and n-3 PUFAs on the development of CMA were either absent or similar to the individual dietary interventions with scGOS:lcFOS or n-3 PUFAs (**Chapter 5**). These data suggested a possible interaction between scGOS:lcFOS and n-3 PUFAs. Such an interaction might be at the level of intestinal bacteria, where the fermentation of scGOS:lcFOS might be inhibited by n-3 PUFAs. Such inhibition of the intestinal bacterial fermentation activity will lead to reduced production of SCFAs. Inhibition of the bacterial fermentation of scGOS:lcFOS into SCFAs might eventually lead to less immunomodulation whereas the risk of CMA development increases.

Here we test the hypothesis that n-3 PUFAs interfere with the intestinal bacterial fermentation of scGOS:lcFOS by measuring SCFA levels as indication of microbial activity after exposing caecal content from healthy and cow's milk allergic mice *ex vivo* to scGOS:lcFOS and/or n-3 PUFAs.

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# MATERIALS AND METHODS

## Culture and stimulation of caecal content suspensions

Caeca from C3H/HeOuJ mice were isolated and in an anaerobic chamber the contents were removed and suspended in carbonate phosphate buffer (CPB, **Table S1**). Afterwards the suspension was filtered through a 4-layer sterile gauze. The caecal suspension was distributed into a 96 wells plate and exposed to a 9:1 mixture of scGOS (degree of polymerization 2-8, Friesland Campina, The Netherlands) and lcFOS (degree of polymerization on average ≥ 23, Orafti, Wijchen, The Netherlands) in final concentrations of 0.5, 1 or 2% (w/v), to n-3 PUFA (tuna oil, a kind gift from Bioriginal, Den Bommel, The Netherlands) in a final concentrations of 6, 12 or 18% (v/v), to DHA or EPA (both Merck) in final concentrations of 10, 50, 100 or 200 µM or combinations of the components. The EPA and DHA were resolved in ethanol, the ethanol had no effect on the fermentation assay (data not shown). The caecum content was diluted 30 times (w/v) in total. After incubation under anaerobic conditions for 24 h at 37 °C at an atmosphere of 10% $CO_2$, 5% $H_2$ and 85% $N_2$, the supernatants were collected and stored at -20 °C until further processing.

## SCFA measurement

The supernatants were thawed on ice, samples were diluted in PBS (Sigma-Aldrich). Afterwards the samples were centrifuged for 10 min at 13,000 rpm. The supernatant was stored at -20°C until SCFA analysis. Two methods were used to determine the SCFA concentrations.

Method 1: The levels of SCFAs were analysed as previously described (20). In short, the supernatant was heated for 10 min at 100 °C to inactivate all enzymes and centrifuged again. The SCFAs acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids were quantitatively determined by gas chromatography using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) as described previously.

Method 2: Esterification stimulate volatilization and was induced by adding 200 µl sample to an 800 µl mix consisting of MilliQ, sulfuric acid, and ethanol (6:5:1), and 50 µl of 1-butanol (10 mM). The samples were heated for 55 min at 110 °C. Afterwards the gas sample was injected in the gas chromatograph. The gas chromatograph consisted of a PorapakQ column of 1.1 m and an inner diameter of 3.2 mm and helium was used as carrier gas. The temperature was regulated and increased with 5 °C/min starting at 160 °C up to 200 °C. The temperature was maintained at 200 °C up till 20 min. The injection temperature was 200 °C. The concentrations of SCFAs were determined using the internal standard (1-butanol) and calibration curve standards for acetate, propionate and butyrate.

55 samples were measured using both methods to evaluate the similarity between the methods. The measured levels of SCFAs correlated significantly with a Pearson correlation coefficient of 0.9852 ($P < 0.0001$) (**Figure S1**). Method 1 was used to analyse one experiment evaluating the dose-response effect of scGOS:lcFOS and the experiment evaluating the effect of scGOS:lcFOS with or without DHA and/or EPA. Method 2 was used to analyse two of the experiments evaluating the dose-response effect of scGOS:lcFOS and the experiments with n-3 PUFAs (tuna oil).

## Cow's milk allergic mice

3 week old female C3H/HeOuJ mice were pre-treated daily on day 0-5 orally with 0.5 mL PBS and were weekly orally sensitized with 20 mg whey and 10 µg cholera toxin as adjuvant in 0.5 mL PBS from day 7-35. As a control, mice were orally treated with 10 µg cholera toxin in 0.5 mL PBS. On day 40 the acute allergic response was evaluated by ear swelling, shock score and temperature after challenge in the ear pinnae with 10 µg whey. On day 40, 18 h prior to euthanisation the mice were also exposed to an oral challenge with 50 mg whey. The experimental setup is shown in **Figure 1**. Further details about the experimental setup and data were recently published by Liu *et al.* (21). Whey-specific immunoglobulin serum levels were measured in the sera collected 18 h after oral challenge with whey using a previously reported method (22). After euthanisation and blood withdrawal, the caeca were isolated.
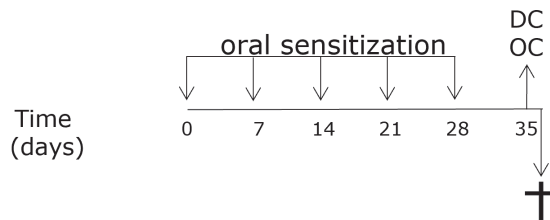


**Figure 1.** Experimental setup of the CMA model. The effect of scGOS:lcFOS with or without DHA and/or EPA on *ex vivo* caecal SCFA production was evaluated with caecum content from healthy mice and with mice included in a CMA model. On day 0, 7, 14, 21 and 28 of the experiment the mice were either sham- or whey protein sensitized. On day 35 the acute allergic symptoms were evaluated when mice were intradermally challenged in the ear. Afterwards, the mice were orally challenged with whey protein. The day after, the mice were euthanized and caeca were isolated. The mice received pre-treatments to induce tolerance prior to sensitization. This is irrelevant for the mice we used as they were pre-treated with PBS only, therefore this pre-treatment is not shown in this figure. DC: intradermal challenge, OC: oral challenge.

## Statistics

Data are expressed as mean ± SD and statistically analysed using one-way ANOVA and Tukey's multiple comparisons test. The correlation of the two methods used to measure the SCFA concentrations were analysed using Pearson correlation. Data were considered statistically significant at ($P$ < 0.05).

# RESULTS

**Dose response effect of scGOS:lcFOS on SCFA production in *ex vivo* caecal fermentation model**

ScGOS:lcFOS was added in three final dosages 0.5, 1 and 2% (w/v) to the caecal slurry (from healthy mice) to evaluate the effectiveness of the caecal fermentation *ex vivo* model. After 24 h, all three dosages of the scGOS:lcFOS mixture caused a significant increase in the total SCFA production compared with the control (**Figure 2A**). 1% and 2% scGOS:lcFOS led to significant higher total SCFA production compared with the 0.5% scGOS:lcFOS. No significant difference was observed in the total SCFA concentration between 1% and 2% scGOS:lcFOS. The effect of scGOS:lcFOS on the individual SCFAs was also determined. The higher the dosage of scGOS:lcFOS the higher the concentration of acetate (**Figure 2B**). 0.5% scGOS:lcFOS tended to increase the acetate production and the 1% and 2% scGOS:lcFOS significantly increased acetate production compared with the control. There were no significant differences in the induced acetate production between the three scGOS:lcFOS dosages. Also, the propionate production was significantly induced by all three dosages of scGOS:lcFOS compared to the control (**Figure 2C**). The 1% scGOS:lcFOS increased the propionate production significantly compared with the 0.5% and the 2%. The propionate production induced by 0.5% and 2% scGOS:lcFOS was similar. Butyrate production was significantly induced by all three dosages of scGOS:lcFOS (**Figure 2D**). 1% and 2% scGOS:lcFOS led to significantly higher butyrate concentrations than the 0.5%. Whereas the butyrate induced by 1% and 2% scGOS:lcFOS was similar.

**n-3 PUFAs (tuna oil) do not influence caecal SCFA production *ex vivo***

Based on the dose-response effect of scGOS:lcFOS on SCFA production, we continued the experiments with 1% scGOS:lcFOS. Three dosages of n-3 PUFAs 6, 12 and 18% (v/v, final concentration) were added to the caecal slurry to evaluate the capability of n-3 PUFAs to affect the production of SCFAs by caecal bacteria. The n-3 PUFA dosages did not affect the total SCFA as well as the individual SCFA production (**Figures 3A-D**). Next, the effect of the combination of scGOS:lcFOS and n-3 PUFAs on the SCFA production was assessed. Three dosages of n-3 PUFAs were combined with 1% scGOS:lcFOS. Compared with the control, the total SCFA production was significantly increased by scGOS:lcFOS and by the combinations of scGOS:lcFOS with 6, 12 or 18% n-3 PUFAs (**Figure 3A**). The total SCFA production was also significantly increased after stimulation with the combination of scGOS:lcFOS with 6%, 12% or 18% n-3 PUFAs compared with caecal slurry with 6%, 12% or 18% n-3 PUFAs only. Compared with the control, the acetate production tended towards an increase after stimulation with scGOS:lcFOS only. This scGOS:lcFOS induced acetate production was not affected by n-3 PUFAs (**Figure 3B**). In combination with scGOS:lcFOS, 6% or 18% n-3 PUFAs showed no effect on the acetate concentration compared with 6% or 18% n-3 PUFAs individually. 12% n-3 PUFAs in combination with scGOS:lcFOS tended to increase the acetate concentration compared with 12% n-3 PUFAs only. The production of propionate and butyrate showed the same pattern after *ex vivo* caecal stimulation with scGOS:lcFOS only and in combination with 6, 12 or 18% n-3 PUFAs as the total SCFA

concentration (**Figures 3C, D**). ScGOS:lcFOS alone and in combination with 6, 12 or 18% n-3 PUFAs led to significantly increased propionate and butyrate levels. Also, 6, 12 or 18% n-3 PUFAs in combination with scGOS:lcFOS increased the propionate and butyrate production significantly compared with 6%, 12% or 18% n-3 PUFAs only, respectively.



**Figure 2**. Effect of 0.5, 1 or 2% (w/v) scGOS:lcFOS on the SCFA production in *ex vivo* caecal fermentation model. (A) Total SCFAs. (B) Acetate. (C) Propionate. (D) Butyrate. A-D: Data shown as mean +/- SD based on three independent experiments. Each condition within each experiment was tested in duplicate or triplicate. Analysed by one-way ANOVA and Tukey's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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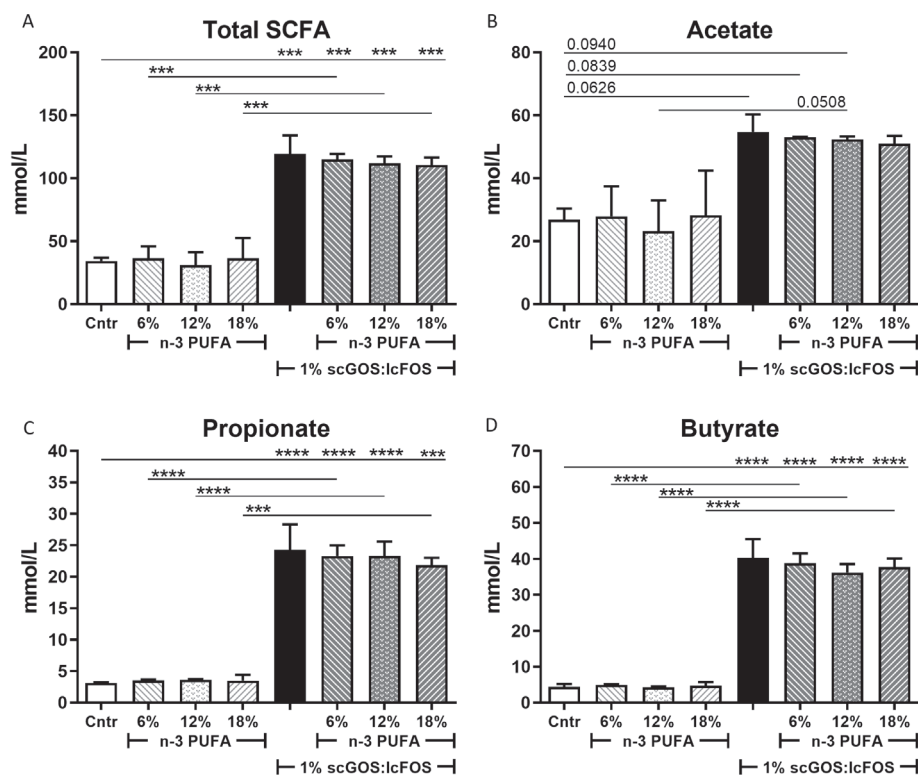
**Figure 3.** Effect of 6, 12 or 18% (v/v) n-3 PUFAs in the form of tuna oil with or without scGOS:lcFOS on the SCFA production in *ex vivo* caecal fermentation model. (A) Total SCFAs. (B) Acetate. (C) Propionate. (D) Butyrate. A-D: Data shown as mean +/- SD based on two independent experiments. Each condition within each experiment was tested once, in duplicate or triplicate. Analysed by one-way ANOVA and Tukey's multiple comparisons post hoc test. *** $P < 0.001$, **** $P < 0.0001$.

## DHA and EPA have no effect on caecal SCFA production *ex vivo*

The tuna oil containing n-3 PUFAs had no influence on the caecal SCFA production *ex vivo*. As this is a mixture of several fatty acids, the ability of the specific n-3 PUFAs DHA and EPA to affect intestinal bacterial activity by means of SCFA production was assessed. DHA was added to the caecal slurry in four dosages 10, 50, 100 or 200 µM. None of these DHA dosages affected the total SCFA or the individual SCFA production compared with the control (**Figures 4A-D**). The combination of scGOS:lcFOS with the dosages of DHA increased the total SCFA production significantly compared with the control (**Figure 4A**). Compared with the individually DHA dosages, the combination of scGOS:lcFOS and 10, 50, 100 or 200 µM DHA significantly induced the total SCFA production.

The acetate production significantly increased in presence of scGOS:lcFOS and 10 µM DHA and tended to increase in presence of scGOS:lcFOS and 200 µM DHA compared with the control (**Figure 4B**). In the presence of scGOS:lcFOS in combination with 50 or

100 µM DHA, the acetate production was unaffected compared with the control. The combinations with scGOS:lcFOS and 10 or 200 µM DHA increased the acetate production compared with the respectively DHA dosages only. The concentrations of propionate and butyrate were affected by the combinations of scGOS:lcFOS and DHA dosages in the same manner as the total SCFA concentrations (**Figures 4C, D**). The combinations of scGOS:lcFOS and the four dosages of DHA enhanced the propionate and butyrate production significantly compared with the control and compared with the respectively DHA dosages without scGOS:lcFOS.
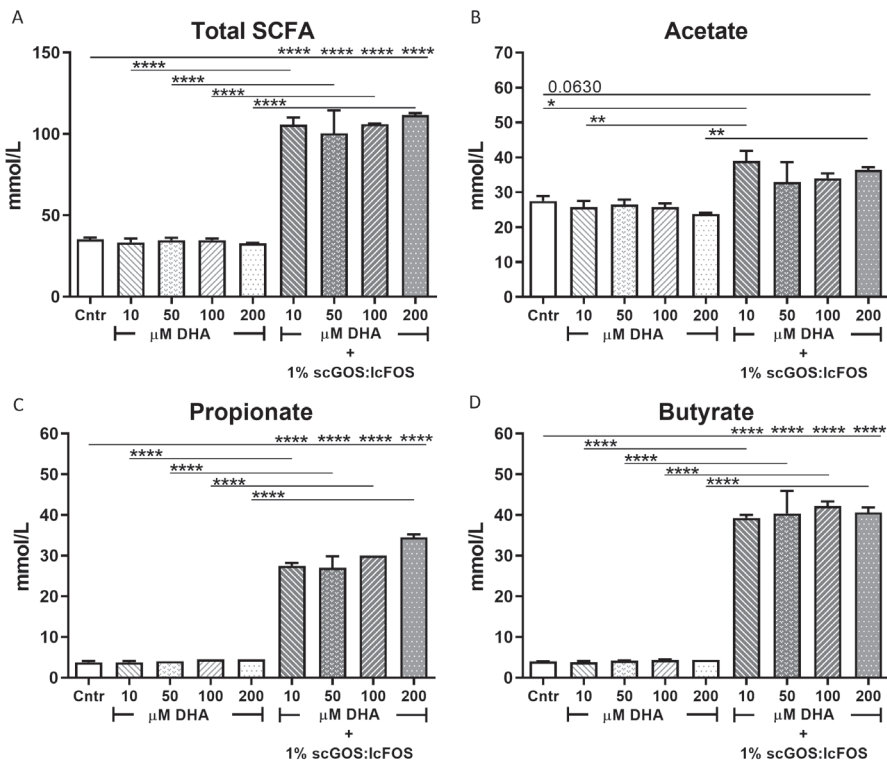


**Figure 4**. Effect of 10, 50, 100 or 200 µM of DHA with or without scGOS:lcFOS on the SCFA production in *ex vivo* caecal fermentation model. (A) Total SCFAs. (B) Acetate. (C) Propionate. (D) Butyrate. A-D: Data shown as mean +/- SD based on one experiment with each condition tested in duplicate. Analysed by one-way ANOVA and Tukey's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

EPA was also added in four dosages to the caecal slurry 10, 50, 100 and 200 µM. The effect of the EPA dosages alone or in combination with scGOS:lcFOS showed the same pattern in the production of total SCFAs and all three SCFAs individually (**Figures 5A-D**). The EPA dosages without scGOS:lcFOS had no effect on the SCFA production compared with the control. The combination of scGOS:lcFOS and the EPA dosages led to enhanced SCFA production compared with the control and compared with the EPA dosages without scGOS:lcFOS.

The increased SCFA production of scGOS:lcFOS in combination with either DHA or EPA is most probably due to scGOS:lcFOS. However, this statement cannot be made as the condition with only scGOS:lcFOS is missing in this data set. Based on the results from the experiments described above (dose-response of scGOS:lcFOS and scGOS:lcFOS in combination with n-3 PUFAs (tuna oil)) the expected levels of SCFAs induced by scGOS:lcFOS only are at the same levels as induced by scGOS:lcFOS in combination with DHA or EPA. Due to the missing condition of scGOS:lcFOS only and that some conditions were measured only once, these DHA and EPA data are preliminary.
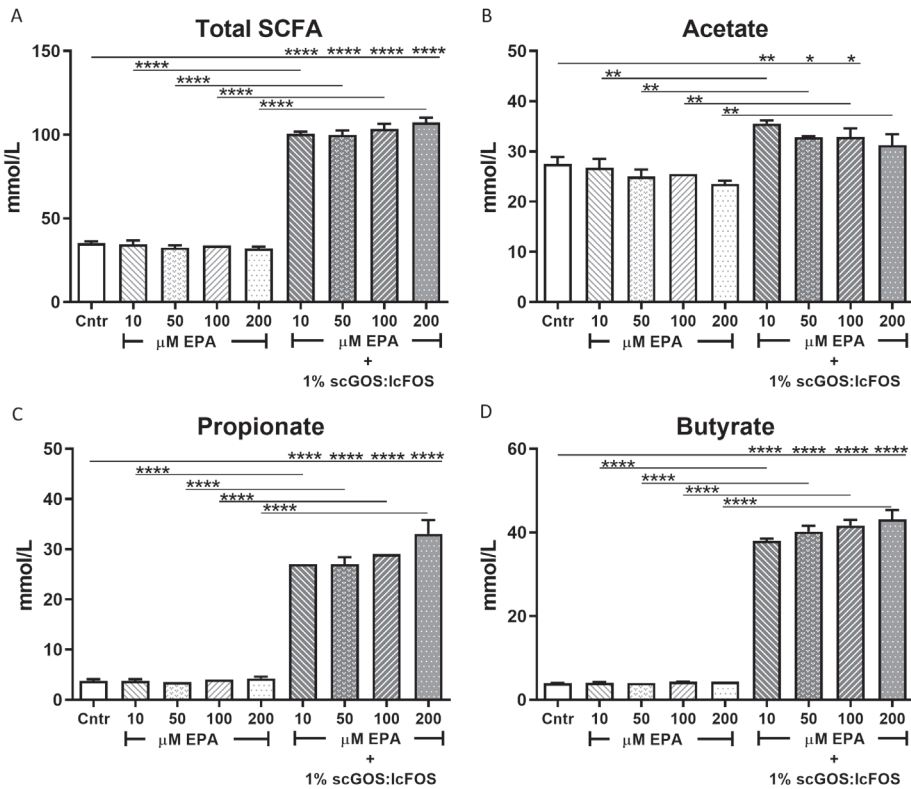


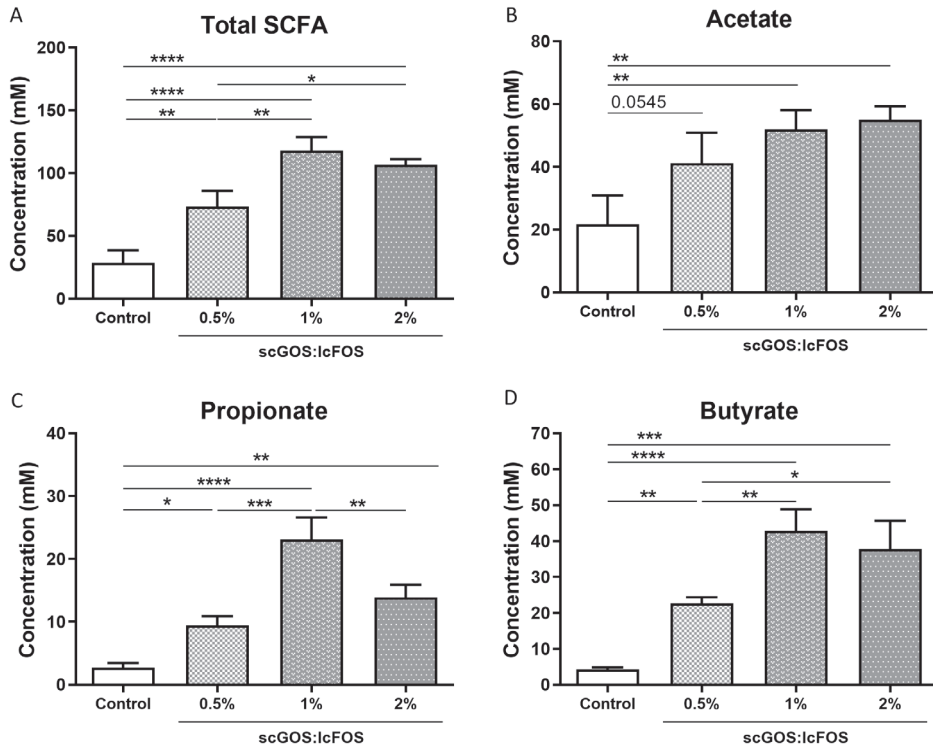**Figure 5.** Effect of 10, 50, 100 or 200 µM of EPA with or without scGOS:lcFOS on the SCFA production in *ex vivo* caecal fermentation model. (A) Total SCFAs. (B) Acetate. (C) Propionate. (D) Butyrate. A-D: Data shown as mean +/- SD based on one experiment with each condition tested in duplicate. Analysed by one-way ANOVA and Tukey's multiple comparisons post hoc test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

**The effect of scGOS:lcFOS with or without DHA and/or EPA on *ex vivo* caecal SCFA production from cow's milk allergic mice**

So far in this study the effect of n-3 PUFAs on the fermentation activity of intestinal bacteria was evaluated using caeca from healthy mice with the result that n-3 PUFAs don't intervene with the basal and scGOS:lcFOS-induced intestinal bacteria activity measured by SCFA production. To investigate the effect of n-3 PUFAs on the scGOS:lcFOS-induced intestinal bacterial fermentation activity in disease, caecum content from non-allergic and cow's milk allergic mice were stimulated *ex vivo* with scGOS:lcFOS with or without DHA and/or EPA.

The CMA mice from which the caeca were isolated, showed allergic symptoms such as increased ear swelling, increased shock score, decreased body temperature after intradermal challenge in the ear and increased IgE serum levels (**Table 1**).

**Table 1**. Allergic parameters from caecum donor mice (21)

|  | Ear swelling | Temperature | Shock score | Whey-IgE serum level |
|---|---|---|---|---|
|  | Δ um | °C |  | AU |
| Non-allergic | 26.5 ± 6.5 | 37.0 ± 0.2 | 0 | 0 ± 0 |
| Allergic | 91.5 ± 8.2 | 36.3 ± 1.0 | 0.5 | 37332 ± 22985.8 |

Ear swelling, temperature and whey-IgE shown as mean ± SEM Shock score shown as median. AU = arbitrary units. Non-allergic n = 2, allergic n = 6.

Total and individually SCFA production in caecal slurry were unaffected whether the donor mice were cow's milk allergic or not when unstimulated as well as after stimulation with scGOS:lcFOS, DHA, EPA or combinations of these components (**Figures 6A-D**). The total SCFA, acetate, propionate and butyrate production in caecum slurry all showed the same pattern after 24 h *ex vivo* stimulation with scGOS:lcFOS, DHA, EPA or combinations of these components (**Figures 6A-D**). The SCFA production in caecal slurry was induced by scGOS:lcFOS and unaffected by DHA or EPA individually. The scGOS:lcFOS-induced SCFA production was neither affected by DHA nor EPA.

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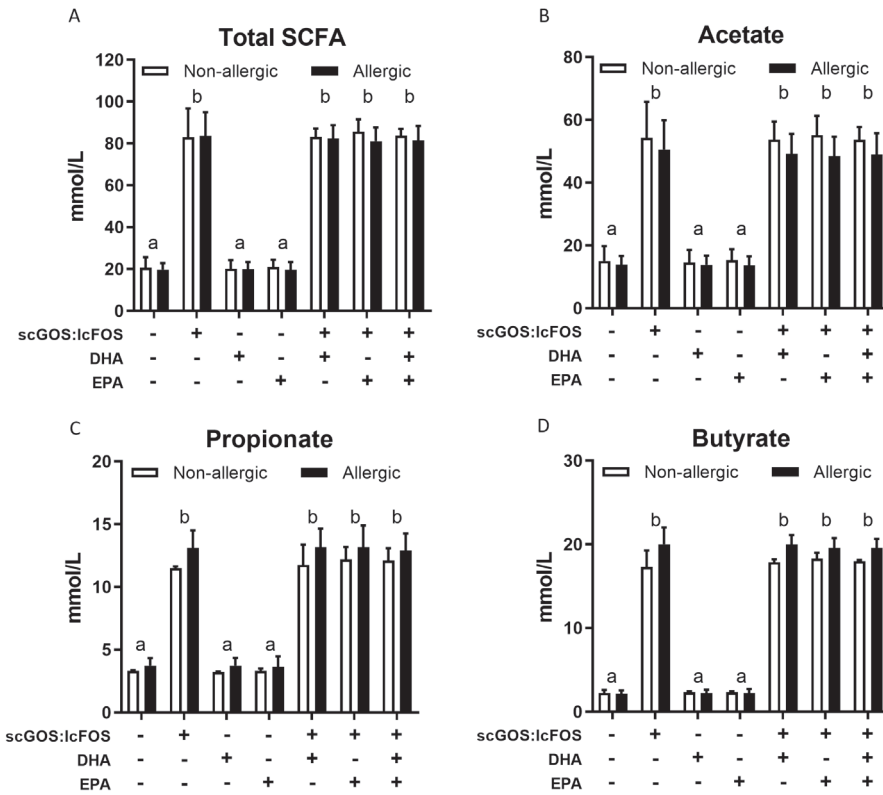
**Figure 6.** The *ex vivo* SCFA production in caeca from non-allergic and allergic mice with and without the effect of 1% (w/v) scGOS:lcFOS, 50 µM DHA, 10 µM EPA or combinations hereof. (A) Total SCFAs. (B) Acetate. (C) Propionate. (D) Butyrate. A-D: Data shown as mean +/- SD. Analysed by one-way ANOVA and Tukey's multiple comparisons post hoc test. Different letter means significant difference between conditions ($P < 0.0001$) in SCFA production between the stimulations, this is valid for caeca from both non-allergic as well as allergic mice. Non-allergic $n = 2$ mice, allergic $n = 6$ mice.

## DISCUSSION

This preliminary study demonstrates that the bacterial fermentation process of scGOS:lcFOS into SCFAs is not diminished by n-3 PUFAs from tuna oil or by individual n-3 PUFAs, DHA and EPA, in caecum content from healthy mice as well as from CMA mice. Adding scGOS:lcFOS - with or without tuna oil, with or without DHA and/or EPA - to the caecum content increases the SCFA production *ex vivo.* Whereas tuna oil, DHA and/or EPA without scGOS:lcFOS had no effect on SCFAs production. The *ex vivo* SCFA production was not significantly affected by the fact that the donor mice were cow's milk allergic. There was no difference observed comparing scGOS:lcFOS-induced SCFA production by caecal content from non-allergic and CMA mice.

Based on our previous observations where a dietary intervention with a combination of scGOS:lcFOS and n-3 PUFAs in a murine CMA model showed no additive preventive effect on the development of CMA, an interaction between scGOS:lcFOS and n-3 PUFAs was hypothesised (**Chapter 5**). A possible interaction can be that n-3 PUFAs interact with intestinal bacteria and thereby influence the fermentation of scGOS:lcFOS resulting in lower SCFAs production. SCFAs have been shown to be involved in prevention of allergy (14, 23). Thus, lower levels of SCFAs leads to less immunomodulation and consequently a potential higher risk of CMA development.

NDOs are classified as carbohydrates and carbohydrates can be fermented into SCFAs in an *in vitro* fermentation model using human faeces (24). This is in line with our data. With increasing dosages of scGOS:lcFOS the functionality of the *ex vivo* murine caecal fermentation model was successfully evaluated. In the presence of 0.5% and 1% scGOS:lcFOS, the SCFA production followed a dose-response curve. However, after stimulation with 2% scGOS:lcFOS, the SCFA production seemed to have reached a plateau. Based on these results, the 1% scGOS:lcFOS dosage was determined to be the optimal dosage to be used in the following experiments. Noticeable, the *ex vivo* bacterial fermentation of scGOS:lcFOS into acetate was less pronounced compared with the propionate and the butyrate levels. This can be explained by cross-feeding, a complex process where bacteria use acetate to produce propionate or butyrate (25).

The effect of tuna oil on the bacterial fermentation of scGOS:lcFOS was assessed, because tuna oil as a source of n-3 PUFAs was used in the dietary interventions in the murine CMA model (**Chapter 5**). Tuna oil had no effect on the *ex vivo* SCFA production indicating that tuna oil does not inhibit the microbial fermentation of scGOS:lcFOS. However, the hydrophobic tuna oil was not homogenously mixing with the CPB because of its hydrophilic properties. Possibly, the tuna oil n-3 PUFAs in this solution were unable to reach the bacteria in the buffer and thereby not affecting the fermentation properties. To overcome this hurdle, single DHA and EPA, that are both water soluble after dissolved in ethanol, were evaluated in the *ex vivo* fermentation model. DHA or EPA alone did not influence the SCFA production. The SCFA production increased significantly after *ex vivo* caecal feeding with scGOS:lcFOS in combination with DHA or EPA. Most likely, this increase is due to scGOS:lcFOS, but as a control with only scGOS:lcFOS is missing in the

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dataset due to experimental failure and the sample size for some conditions is only one, this cannot be verified.

The results described so far in this chapter are generated using caecum content from healthy mice. As the tested hypothesis is based on results from CMA mice, we next used caecum content from CMA mice. CMA had no significant influence on the *ex vivo* SCFA production and no differences in SCFA concentrations were observed after feeding scGOS:lcFOS to the caecal microbes with or without DHA and/or EPA. Although not significantly different, it seems that the acetate production is lower in caecum content obtained from CMA mice than in caecum content from non-allergic control mice after *ex vivo* stimulation with scGOS:lcFOS. Additionally, the levels of propionate and butyrate are higher in CMA than in non-allergic caecum content after scGOS:lcFOS stimulation. This might have to do with cross-feeding. It might be that the composition of caecal bacteria in CMA mice are more prone to ferment scGOS:lcFOS into propionate and butyrate either directly or by cross-feeding through acetate.

Throughout the murine gastro-intestinal tract, structural differences exist in the bacterial composition (26, 27). The most distinct differences are observed between the small and large intestine. The bacterial community in the caecum content, colon and faeces is shown to be similar at family and genus level (26, 27). This indicates that the use of caecal content is representable of the colon bacterial content to evaluate *ex vivo* bacterial fermentation processes.

The data sets presented here can be improved by increasing the sample size of each condition and the number of experiments including tuna oil, DHA and EPA. In addition, two different SCFA analysis methods were used, even though the data analysed by the two different methods did not substantially differ (**Figure S1**), one SCFA method of analysis is preferred. Another factor to consider is the brief exposure to oxygen. In the caecum, the environment is anaerobic and at the moment of caecum isolation (some of) the bacteria were exposed to oxygen. Consequently, anaerobic bacteria might be lost affecting the bacterial composition and maybe the functionality of the caecal bacteria.

In conclusion, based on the preliminary data, scGOS:lcFOS is *ex vivo* fermented into SCFAs. The bacterial fermentation of scGOS:lcFOS is not disturbed by DHA or EPA, indicating that the hypothesis, that n-3 PUFAs interfere with the fermentation of scGOS:lcFOS in the intestine, leading to less SCFA production and eventually less preventive efficiency of CMA development is invalid.

# SUPPLEMENTARY INFORMATION

**Table S1**. Composition of trace element solution and carbonate-phosphate buffer

| Trace element solution | | Carbonate-phosphate buffer | | | |
|---|---|---|---|---|---|
| | mg/L | | g/L | g/250 mL | g/500 mL |
| $FeSO_4 \cdot 7H_2O$ | 3680 | $NaHCO_3$ | 9.24 | 2.31 | 4.62 |
| $MnSO_4 \cdot 1H_2O$ | 1159 | $Na_2HPO_4$ (anhydrous) | 2.824 | 0.706 | 1.412 |
| $ZnSO_4 \cdot 7H_2O$ | 440 | NaCl | 0.47 | 0.12 | 0.24 |
| $CoCl_2 \cdot 6H_2O$ | 120 | KCl | 0.45 | 0.11 | 0.22 |
| $NiCl_2$ | 100 | Urea | 0.4 | 0.1 | 0.2 |
| $CuSO_4 \cdot 5H_2O$ | 98 | $CaCl_2 \cdot 2H_2O$ | 0.0728 | 0.018 | 0.036 |
| $Mo_7(NH_4)_6O_{24} \cdot 4H_2O$ | 17 | $Na_2SO_4$ (anhydrous) | 0.1 | 0.025 | 0.05 |
| $MgCl_2 \cdot 6H_2O$ | 0.1 | Trace element solution | 10 | 2.5 | 5 |
| | | Resazurin (make a 1 mg/mL solution) | 1 | 0.25 | 0.5 |
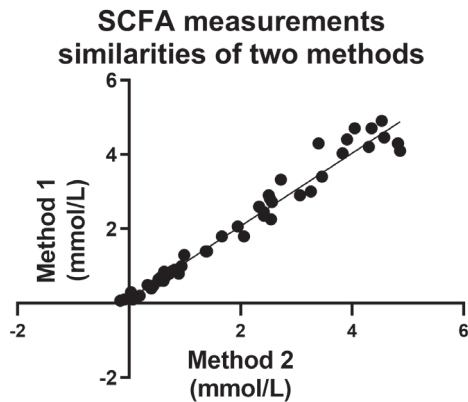


**Figure S1.** Correlation of the two methods used to measure the SCFA concentrations. Analysed by Pearson correlation with a coefficient of 0.9852 ($P < 0.0001$). $n$ = 55 samples.

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# CHAPTER 8

Summarising discussion

Three pillars play an important role in early life development: the intestinal microbiota, the immune system and the brain. Each of these three compartments undergo a tremendous maturation during the first years of life. The development of the immune system and maturation of the brain go hand in hand with the development of a stable intestinal microbiome. The last decades, the role of the intestinal colonising microbiota in the maturation of the immune system and the brain is becoming more evident (1). Several external factors such as antibiotic use, caesarean section and infant formula can influence colonisation of the intestine and as a consequent affect maturation of the brain and immune system (2-4). Disturbances in maturation of the intestinal microbiota have been associated with negative effects on brain, immune and metabolic health on the long-term (5, 6). The composition of the intestinal microbiota can be affected by among others dietary habits and/or food components (5). Thus, using specific food to maintain a healthy development in early life is of great importance.

Regarding nutrition, human breast milk is the golden standard during the first six months of life (7). Human milk contains all nutritional components necessary for the infant to develop during early life (1). Unfortunately, human breast milk is not always available. Infant formula is an alternative containing components which mimic the function of specific nutritional components in human breast milk. Some of the nutritional components present in formula milk that play a role in the maturation of the intestinal microbiome, immune system and/or the brain are prebiotics like short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) and omega-3 polyunsaturated fatty acids (n-3 PUFAs) (8-15).

This thesis presents preclinical studies on the effects of scGOS:lcFOS, n-3 PUFAs or a combination thereof in early life. The modulatory effects of these dietary components separately or combined on intestinal bacterial composition, immune system, brain and behaviour were studied in healthy mice. ScGOS:lcFOS and n-3 PUFAs are reported to possess cow's milk allergy (CMA) preventive capacities (16-19). The possibility that a combination of scGOS:lcFOS and n-3 PUFAs has additional preventive effects in the development of CMA was investigated as well. Since CMA has been associated with anxiety-like behaviour and impaired social behaviour in mice, these behaviours were also evaluated in this thesis. This was accompanied by testing several hypotheses on underlying mechanisms regarding possible interactions between scGOS:lcFOS and n-3 PUFAs affecting modulation of the immune system as well as brain and behaviour.

## Importance of scGOS:lcFOS and n-3 PUFAs during early life

One of the major components in human breast milk are human milk oligosaccharides (HMOs). HMOs are prebiotic substances, stimulating the growth of beneficial bacteria and therefore play an important role in the maturation of the intestinal microbiota (20, 21). A well-developed intestinal microbiota composition is essential in the development of the gastro-intestinal tract, immune system and nervous system early in life. Beyond the intestinal tract, HMOs also influence the maturation of the immune system by

regulating the T helper 1 cell (Th1)/Th2 lymphocyte balance after birth as well as inducing the interleukin 10 (IL-10) production by dendritic cells (DCs) promoting the differentiation of naïve T cells into regulatory T cells (Treg) (22-24). As mentioned above human milk is not always available and an alternative is infant milk formula. Infant formula contains prebiotics like scGOS:lcFOS which mimic the properties of some HMOs in human milk. The prebiotics in infant formula show immunomodulatory effects as well. These effects are exerted both directly (independent of intestinal microbiota changes) as well as indirectly via intestinal microbiota modulation, among others. Directly through communication with intestinal epithelial cells and DCs inducing Treg to develop tolerance and balance the Th1/Th2 response after birth (10, 11). And indirectly by induction of the growth of beneficial bacteria and by bacterial fermentation in the intestine into short-chain fatty acids (SCFAs) like acetate, propionate and butyrate (25, 26). SCFAs, in particular butyrate, are an energy source for colonocytes (27), promote the intestinal epithelial integrity (28) and are immunomodulatory (29-33). Butyrate supports the activity of several immune cells by stimulating the generation of Th1, Th17 cells and Tregs (31). Additionally, butyrate is reported to play a role in the prevention of CMA (32, 33).

Next to immunomodulatory capacities, SCFAs have neuroactive properties and are thought to play a role in neurodevelopment also. SCFAs are able to cross the blood brain barrier and have been reported to maintain the integrity of this barrier (34). In the central nervous system, SCFAs are capable of stimulating neuronal survival, growth and differentiation through modulation of neurotrophic factors like brain-derived neurotrophic factor (34). In microglia cells, SCFAs, in particular butyrate, regulate the homeostasis by promoting the expression of anti-inflammatory mediators and decreasing the activation of microglia cells and the secretion of pro-inflammatory cytokines (35-37). SCFAs have been suggested as treatments for neurodevelopmental and neurodegenerative diseases (34).

Other components present in human breast milk are the PUFAs. PUFAs include n-3 and n-6 PUFAs and are accumulating and incorporated into cell membranes of immune cells and brain cells (13, 15). PUFAs regulate the function of several immune cells like DCs, T cells and B cells through activation of transcription factors and subsequent changes in gene expression (13). During brain development in early life, the incorporation of n-3 and n-6 PUFAs into membranes of brain cells like microglia cells, astrocytes and neurons influence synaptogenesis, neurogenesis and neuronal migration and differentiation (14, 15). In the western world, the ratio of n-6:n-3 is increased from 4:1 to 20:1 during the last decades. This skewing is probably realised by an increased consumption of meat, corn and safflower oil containing n-6 PUFAs and a decreased consumption of fatty fish containing n-3 PUFAs (15). During pregnancy and lactation, n-3 and n-6 PUFAs are provided to the foetus and to the new-born (via breast milk) through the maternal diet. A maternal diet containing low amounts of n-3 PUFAs and high amounts of n-6 PUFAs might lead to insufficient n-3 PUFA levels in the foetus and/or new-born (14). Insufficient intake of n-3 PUFAs in children might lead to increased risk of neurodevelopmental diseases such as autism spectrum disorder (ASD) as n-3 PUFA plasma levels are low in children diagnosed with ASD (38). Next to that, increased consumption of n-6 PUFAs has been

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associated with enhanced risk of development of anxiety disorders (15). The skewing of the n-6:n-3 ratio in the western world is also associated with increased prevalence of allergic diseases (39, 40). Together this indicates the importance of a balanced n-6:n-3 ratio during brain and immune development in early life.

## scGOS:lcFOS and n-3 PUFA supplementation in healthy mice

### Microbiota and SCFAs

Next to the known prebiotic activity of scGOS:lcFOS, n-3 PUFAs have been regarded as prebiotic candidates as well (8, 12). During the last years, available literature is reporting the ability of n-3 PUFAs to modulate the intestinal microbiota composition, characterised by increased relative abundance of *Bifidobacterium* and *Lactobacillus* in both preclinical and human trials (41-44). Very recently, it was reported that a change in the n-6:n-3 ratio in a mouse model leads to compositional changes in the intestinal microbiota (43). The intestinal bacterial modulation by n-3 PUFAs is suggested to occur indirectly i.e. through altered tissue fatty acid composition in the host and/or immunological changes (41, 42). Whether PUFAs are able to directly mediate changes in bacterial composition is to my knowledge still unknown. Indeed, dietary supplementation of scGOS:lcFOS (**Chapter 3** (45) and **4** (46)) or n-3 PUFAs (**Chapter 4** (46)) were able to change the intestinal bacterial composition. The dietary combination of scGOS:lcFOS and n-3 PUFAs altered the intestinal bacterial community structures in a distinct manner compared to scGOS:lcFOS or n-3 PUFAs individually (**Chapter 4** (46)). These data show a clear modulation of the bacterial composition by the dietary components in the intestine. However, whether the compositional differences lead to functional changes in the bacterial community structures still needs to be investigated.

As mentioned above, intestinal bacteria ferment scGOS:lcFOS into SCFAs, which possess immunomodulatory as well as neuroactive capacities (29, 31, 32, 34). SCFAs are able to act pro- and anti-inflammatory. They exert their immunological function through several mechanisms being histone deacetylase HDAC inhibition and binding to the G protein-coupled receptors (GPCRs) GPR41, GPR43 and GPR109 resulting in altered gene expression of tight junctions and mediators such as Zonula occludens-1, interferon-ɣ (IFNɣ), IL-10, IL-17, improving the intestinal cell wall function and inducing T cell subset differentiation and function into Th1, Treg and Th17 (32, 47, 48). ScGOS:lcFOS supplemented diets resulted, as expected, in increased faecal (**Chapter 3** (45)) and caecal (**Chapter 4** (46)) SCFA levels, a saccharolytic profile. The scGOS:lcFOS-induced acetate and butyrate levels were diminished when scGOS:lcFOS was combined with n-3 PUFAs (**Chapter 4** (46)). This might indicate that n-3 PUFAs interfere directly or indirectly with the intestinal bacterial digestion of scGOS:lcFOS. Another option is that the uptake of SCFAs by intestinal epithelial cells might be improved by enhanced epithelial expression of SCFA transporters induced by scGOS:lcFOS and n-3 PUFAs whereas the SCFA levels in the lumen remain unchanged.

**Immune system**

ScGOS:lcFOS and n-3 PUFAs have been shown to balance the Th1/Th2 mediated immune response and to induce Treg function in order to possibly decrease the risk of developing inflammatory diseases like allergies (10, 11, 18, 49-51). In the healthy situation evaluated in **Chapter 4**, the changes in immune parameters induced by the dietary interventions were subtle (46). The scGOS:lcFOS and n-3 PUFA interventions did not affect the T cell subsets in the mesenteric lymph nodes (MLN), however in the combined dietary intervention a tendency to a decreased Th1 response and increased Th2 response was observed compared with the scGOS:lcFOS and n-3 PUFAs diet groups, respectively. The few observed immune modulations occurred in the MLN and not in the spleen, indicating a local immune response in the intestine only. As this represents a healthy situation one expects a resilient immune system, and distinct effects on the immune system are undesired as the immune response is already balanced.

**Behaviour and serotonin**

Nowadays it is well known that diet influences the intestinal microbiota and it is generally accepted that the intestinal microbiota have a pivotal role in brain development and behaviour in early life (1). In other words, the nutrition given to new-borns is a very important influencing factor in the development of the brain. Although the evidence of beneficial neurodevelopmental effects of prebiotics is limited (52-55), prebiotics modulate the intestinal microbiota, which influences neurodevelopment, indicating that prebiotics might be able to modulate behaviour. A dietary intervention with scGOS:lcFOS starting in early life led to intestinal microbiota compositional changes associated with improved social behaviour (**Chapter 3** (45)), reduced anxiety-like and repetitive behaviour (**Chapters 3** and **4** (45, 46)) and a tendency to increased explorative (although not significantly) (**Chapter 4** (46)) behaviour later in life.

Whereas the importance of prebiotics during brain development still needs to be elucidated, it is clear that n-3 PUFAs play an essential role. n-3 PUFA deficiencies during early life has been associated with impaired psychomotor development and enhanced risk of development of neurodevelopmental diseases and additionally associated with behavioural deficits later in life (41). Previously, n-3 PUFA supplementation has been shown to reduce ASD and anxiety-like behaviours in mice (19, 41, 56). In contrast, in healthy mice we observed no improved behavioural effects of n-3 PUFAs (**Chapter 4** (46)), which matches findings by Robertson *et al.* (41) who demonstrated no effects of n-3 PUFAs on anxiety-related and repetitive behaviour in healthy mice also. The combined dietary intervention of scGOS:lcFOS and n-3 PUFAs had limited to no effect on the evaluated behaviours. In a healthy situation, the processes in the host are balanced and resilient, thus changes in behaviour might be difficult. In a healthy host, the dietary combination of scGOS:lcFOS and n-3 PUFAs might support robust behavioural resilience.

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Behavioural changes often pair with affected monoamine levels. It is generally expected that higher serotonin levels lead to reduced anxiety-like and improved social behaviour (57). Intriguingly, the improved behaviour mediated by scGOS:lcFOS was accompanied by (significantly) reduced levels of serotonin in the brain (**Chapters 3** and **4** (45, 46)) and increased number of serotonin secreting enterochromaffin cells in the intestine (**Chapter 4** (46)). Serotonin is a metabolite of tryptophan, which is an essential amino acid needed to be acquired from the diet (58). Serotonin is unable to pass the blood brain barrier meaning that intestinal produced serotonin has no direct effect in the brain. The generation of serotonin in the brain acquires tryptophan to enter the brain (59). Although not significantly, the brain tryptophan levels in the scGOS:lcFOS receiving mice seemed lower (**Chapters 3** and **4** (45, 46)). This might be indicative of less availability of tryptophan in the brain as a result of higher activity of tryptophan utilising intestinal bacteria or increased serotonin production by enterochromaffin cells. The number of enterochromaffin cells (**Chapter 4** (46)) was also increased by the n-3 PUFAs and the combined dietary interventions, but the tryptophan and serotonin levels in the brain were unaffected compared to the control diet. Other monoamines such as dopamine and noradrenaline might be involved in behavioural changes as well (60). However, in our studies the levels of dopamine and noradrenalin were unaffected by the dietary interventions. Noteworthy, the level of monoamines is the total amount measured in brain homogenates leaving the available amount of the monoamines in the synaptic clefts unknown.

**Parameters influencing explorative and repetitive behaviour**
To achieve more insight in parameters influencing repetitive and explorative behaviour in mice independent on the dietary interventions, we used a prediction machine learning model including all microbial, brain and immune data: Recursive Ensemble Feature Selection (REFS, **Chapter 4** (46)). This model indicated that changes in repetitive behaviour is mostly predicted by the caecal content's relative abundance of the *Adlercreutzia* and *Dehalobacterium* genera, which in turn might influence local and systemic T cell responses eventually leading to changes in tryptophan levels in the brain. This is in line with a study reporting that reduced intestinal relative abundance of the *Dehalobacterium* genera is associated with increased repetitive behaviour (61). Changes in explorative behaviour are also predicted by the caecal content's relative abundance of the *Oscillospira, Odoribacter, Turicibacter, Lachnospiraceae Other;Unclassified, Ruminococcus* and *Adlercreutzia* genera, serotonin and dopamine metabolism in the brain, but not by immune responses. However, the role of the predictive bacteria in explorative behaviour is so far unknown and need to be verified in future studies.

## scGOS:lcFOS and n-3 PUFA supplementation in cow's milk allergic mice

Next to evaluation of the combination of scGOS:lcFOS and n-3 PUFAs in healthy mice, we also used a murine CMA model to assess the capacity of the dietary combination in the prevention of CMA development and related disease symptoms (**Chapter 5**).

**Allergy and behavioural clinical parameters**

CMA is one of the most prevalent food allergies occurring during the first year of life with a prevalence of approximately 2-3% in westernised countries (62). The symptoms of CMA are skin rash, gastro-intestinal discomfort such as diarrhoea and anaphylactic shock can occur in severe cases. Currently, no treatment or cure is available, only avoidance of the allergen can prevent allergic reactions. Most CMA diagnosed children outgrow the CMA before the age of three, however, these children are more prone to develop other allergies later in life emphasising the necessity to prevent the development of CMA (63). The individual dietary components scGOS:lcFOS and n-3 PUFAs have previously been shown to possess preventive capacity in CMA development (16-19). This was mainly shown by reduced acute allergic responses, measured by allergic swelling of the ear skin after intradermal challenge in the ear pinnae of the mouse. The impact of the dietary interventions scGOS:lcFOS or n-3 PUFAs in the prevention of the CMA development was manifested in a reduced drop in body temperature in male mice (scGOS:lcFOS) and in reduced acute allergic reaction, less swelling of the ear, in female mice (scGOS:lcFOS or n-3 PUFAs)(**Chapter 5**). Unexpectedly, the dietary combination of scGOS:lcFOS and n-3 PUFAs had no additional preventive effect on the CMA development in the dosages used in this model. In fact, the individual preventive effects of scGOS:lcFOS or n-3 PUFAs seemed to be negatively impacted when combined. This observation suggested a possible interaction between the investigated dietary components.

In several cases food allergy has been accompanied by neurological manifestations like depression, anxiety and ASD-like behaviour (64-69). ASD associated behaviour such as impaired social behaviour and induced repetitive behaviour was observed only in male mice allergic to whey (19, 70, 71). The CMA-induced behaviour was subtle (**Chapter 5**). Male mice seemed to show impaired social behaviour and n-3 PUFAs seemed to reduce this impairment which is conform the reported results of De Theije *et al.* (19). As expected, we observed no CMA-associated behavioural changes in the female mice. ASD is more prevalent in boys than in girls - with the side-note that ASD-associated behaviour in girls presents in a different way (72).

**Mechanism, role of cytokines and SCFAs**

At birth the immune response is Th2 prone. Thereafter, this response is regulated to a balanced and a resilient Th1/Th2 response to minimize the risk of development of Th2-mediated disorders such as food allergy (73). The maturation of the immune system is dependent on among others the exposure to environmental factors like food antigens. Exposure to food antigens promotes the development of oral tolerance. The mechanism of immunological oral tolerance development is not exactly known, but differentiation of naïve T cells into Treg instructed by DCs plays an essential role (74). In our study, the immune T cell subsets in MLNs and spleens were unaffected by the CMA as well as by the dietary interventions (**Chapter 5**). On the other hand, the *ex vivo* whey-specific cytokine response of splenocytes from cow's milk allergic male and female mice were evident, indicating that the splenocytes were sensitive to exposure to whey,

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despite the missing effects on T cell subsets in splenocytes measured by flowcytometry. Splenocytes obtained from scGOS:lcFOS treated CMA male mice demonstrated reduced cytokine responses upon *ex vivo* exposure to whey. However, this effect was gone when scGOS:lcFOS was combined with n-3 PUFAs, suggesting the occurrence of an interaction between scGOS:lcFOS and n-3 PUFAs in mice.

The altered splenocyte cytokine responses by scGOS:lcFOS in CMA mice can either be the results of direct effects by scGOS:lcFOS or indirectly i.e. through intestinal bacterial fermentation into SCFAs. The intestinal bacterial composition was not assessed in the CMA mice, but the caecal bacterial activity was analysed by means of measurement of SCFA levels (**Chapter 5**). The caecal butyrate level was increased by scGOS:lcFOS in male as well as female mice. In females the scGOS:lcFOS-induced butyrate level was present as well in the combination diet group. However, in males the scGOS:lcFOS induced butyrate level tended to be impaired by n-3 PUFAs in the combination diet group. Butyrate has been shown to exert effects on cytokine responses in splenocytes (32). Butyrate reduced the CMA-induced IL-4 and IL-13 cytokine levels and induced the IFNɣ and IL-10 cytokine levels in splenocytes stimulated *ex vivo* with β-lactoglobulin (specific allergen for CMA) (32). Also, in human peripheral mononuclear cells (PBMCs) from CMA children co-incubated with butyrate and β-lactoglobulin, butyrate exerted anti-allergic effects. Butyrate reduced the β-lactoglobulin-induced IL-4 and IL-13 release and increased IFNɣ and IL-10 cytokine production by CMA-PBMCs (32). These results indicate that the role of butyrate in prevention of CMA might be essential.

## Hypotheses of interaction between dietary scGOS:lcFOS and n-3 PUFAs

From the data presented in **Chapters 4** and **5** we conclude that the dietary combination of scGOS:lcFOS and n-3 PUFAs neither leads to additional CMA preventive effect nor behaviour improving effects. In a healthy situation (**Chapter 4**), the combination of the dietary components scGOS:lcFOS and n-3 PUFAs has no additional effects on behaviour, but might support behavioural resilience. In the CMA (**Chapter 5**) model the dietary components in some way seem to interfere with the preventive effect of each other. This can be the result of an interaction between the components or an interference in the mechanism of action. In this thesis, we evaluated three possible hypotheses:

### Butyrate interferes with the signalling pathway of n-3 PUFAs through lowering the expression of epithelial PPARɣ.

Peroxisome proliferator activated receptor ɣ (PPARɣ) is a transcription factor expressed in epithelial cells and different types of immune cells like DCs and T cells (75-77). PPARɣ regulates inflammatory gene expression directly but also counteract with pro-inflammatory factors like NFκB. In the presence of a PPARɣ agonist, the activation of NFκB can be suppressed and results in inhibition of inflammatory responses (78). PPARɣ might play a role in alleviation of food allergies (79). Activation of PPARɣ in ovalbumin allergic rats resulted in reduced clinical symptoms, ovalbumin-specific IgE and IgG1 serum

levels, inhibited mast cell degranulation and altered cytokine responses. In the presence of the PPARɣ agonist the level of IL-4 was decreased and the level of IFNɣ in serum of ovalbumin allergic rats was increased (79). Docosahexaenoic acid (DHA) and (most likely) eicosapentaenoic acid (EPA) are PPARɣ agonists suggesting that the preventive effect of n-3 PUFAs on CMA development might (partly) occur through activation of PPARɣ. Another PPARɣ agonist is butyrate, indicating that the anti-inflammatory effects of butyrate might appear through PPARɣ signalling (80). On the other hand, in murine intestinal organoids (81), butyrate was reported to decrease epithelial *Pparɣ* mRNA expression. This might lead to less available PPARɣ, resulting in less binding sites for n-3 PUFAs. We hypothesised that the scGOS:lcFOS-induced butyrate level might reduce the *Pparɣ* mRNA expression in the intestine of mice. This might result in less functionality of PPARɣ and consequently impairing the effect of n-3 PUFAs in the prevention of CMA development. In jejunal and ileal intestinal tissue of CMA mice the mRNA expression of *Pparɣ* was unaffected by the individual dietary components (**Chapter 5**). However, the *Pparɣ* mRNA expression was increased by the combined diet. Based on these results, butyrate is not interfering with the preventive effect of n-3 PUFAs through intestinal PPARɣ downregulation, meaning our hypothesis is invalid. Notable, the mRNA analysed was in total intestinal tissue; it would be interesting to analyse the effect on *Pparɣ* mRNA in different intestinal cell types as well as the protein expression to acquire more insight in the specific cell types that may be involved.

**Chemical reaction or physical interaction between scGOS:lcFOS and n-3 PUFAs reducing the availability of these components to the host**

The dietary components scGOS:lcFOS and n-3 PUFAs are consumed together and a reaction between these dietary components might take place in the gastro-intestinal tract of mice. n-3 PUFAs consist of triglycerides which can react with compounds containing hydroxyl groups like scGOS:lcFOS in the presence of lipases. Lipases are present in the gastro-intestinal tract where they are essential in the breakdown of triglycerides making them available for uptake by the intestinal cells. If a chemical reaction takes place between scGOS:lcFOS and n-3 PUFAs, generation of a covalently bonded branched molecule might occur. This molecule will consist of a scGOS or lcFOS "core" with n-3 PUFA free fatty acid "branches". This molecule seems to be similarly structured as olestra. Olestra consists of a sucrose molecule attached to six to eight fatty acids. It is developed by the food industry as a fat substitute that adds no calories to products because it passes through the gastro-intestinal tract in an unchanged structure. Olestra cannot be broken down in the body due to its size and branches. We hypothesized that due to the large size of the molecule consisting of scGOS or lcFOS with n-3 PUFA free fatty acids, this branched molecule will be unable to be either fermented by intestinal bacteria or absorbed by the intestinal epithelial cells. Consequently, the dietary components scGOS:lcFOS as well as n-3 PUFAs will become less available to exert their immunomodulatory functions in the host to prevent the development of CMA. This hypothesis was evaluated using an *in vitro* digestion model leading to preliminary results indicating that scGOS and/or

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lcFOS indeed might form a branched molecule with several EPA molecules (**Chapter 6**). More optimization of the method used in our study is needed and confirmation that the branched molecule consisting of scGOS, lcFOS and EPA is leaving the intestine unchanged. ScGOS:lcFOS and n-3 PUFAs might also interact in a physical manner. Before being taken up by the enterocytes, the n-3 PUFA free fatty acids can form micelles (accumulation of amphiphilic molecules with a hydrophilic head and a hydrophobic tail) in the intestinal tract. Our hypothesis is that scGOS:lcFOS encapsulate the n-3 PUFAs in a core, whereas the n-3 PUFAs are unavailable to be taken up by the intestinal cells in the host. This was evaluated by microscopy and computational modelling and although the data are very preliminary and further optimisation is needed, we suggest, with caution, that scGOS:lcFOS might interact with the micelle forming of n-3 PUFAs in the intestine, reducing the immunomodulatory effects of both scGOS:lcFOS and n-3 PUFAs.

### Bacterial activity inhibited by n-3 PUFAs leading to less fermentation of scGOS:lcFOS

Another possible interaction between scGOS:lcFOS and n-3 PUFAs is at the level of intestinal fermentation. We hypothesised that n-3 PUFAs might interfere with the fermentation of scGOS:lcFOS in the intestine, which consequently leads to less immunomodulation by scGOS:lcFOS due to less produced SCFAs. We evaluated this hypothesis using an *ex vivo* caecal fermentation assay, where isolated caeca were stimulated with several concentrations of scGOS:lcFOS and/or n-3 PUFAs in an anaerobic chamber simulating the condition for intestinal bacteria (**Chapter 7**). First, using caeca from healthy mice, the functionality of the assay was successfully assessed. ScGOS:lcFOS showed a dose-response curve: increasing concentration of scGOS:lcFOS resulted in increased concentration of SCFAs. Tuna oil consisting of DHA and EPA was not suitable in this assay due to difficulties in homogenisation with the cultivating buffer. Later, DHA and EPA were used and showed no effect on the scGOS:lcFOS-induced production of SCFAs by caecal bacteria.

The intestinal bacterial composition is reported to be different among several taxa in food allergic individuals (82). To test our hypothesis in a CMA setting, caeca from cow's milk allergic mice were stimulated with and without scGOS:lcFOS and/or n-3 PUFAs and evaluated by SCFA analysis. The basal SCFA production was similar in the caeca from CMA mice and non-allergic mice. After scGOS:lcFOS stimulation, similar increased SCFA production was measured in caeca of both CMA and non-allergic mice. The scGOS:lcFOS-induced SCFA levels were unaffected by DHA and/or EPA (**Chapter 7**). Although these results are preliminary and optimisation is necessary, the first indication is that our hypothesis is invalid.

## Future perspectives

HMOs and n-3 PUFAs are present in human breast milk and are both necessary in early life development. However, human breast milk is not always available, and the alternative is infant formula. To mimic the properties of HMOs, non-digestible oligosaccharides like scGOS:lcFOS in combination with 2'-fucosyllactose, an HMO, are added to the infant formula. A combination of scGOS:lcFOS and n-3 PUFAs is expected to support the development of intestinal microbiota, immune system and brain, delivering additional effects during early life. However, according to the findings in this thesis, no added effects occurred when these dietary components were combined, indicating that their functionality might interact with each other. Preliminary data suggest a number of possible interactions. These interactions need more research and should be investigated in more detail. Future studies need to evaluate the hypotheses using more advanced methods, which are presented below.

An optimal way to examine the hypothesis that n-3 PUFAs interfere with the bacterial fermentation of scGOS:lcFOS, is the SHIME model. The SHIME® (Simulator of the Human Intestinal Microbial Ecosystem) model is an *in vitro* technique simulating the gastro-intestinal tract (83, 84). In this model the physiological, chemical and microbiological properties of the human intestine are combined. Using this model has several advantages; similar physiological stomach and intestinal environment as in humans and no risk of anaerobic bacteria exposure to oxygen (84).

To retrieve more insight in the chemical reactions and physical interactions between scGOS:lcFOS and n-3 PUFAs, using the TNO gastro-intestinal model 1 (TIM-1) is preferred. In contrast to the SHIME model, where the stomach and small intestinal parts consist of two compartments, the TIM-1 model is an exact simulation of the stomach and three compartments of the small intestine representing duodenum, jejunum and ileum (83-85). The TIM-1 model mimics the physiological processes including peristaltic contractions, whereas in the SHIME model magnetic stirrers are used for mixing (83-85). Sampling at different points during digestion in the TIM-1 model, makes it possible to evaluate at where in the intestine the interaction/reaction takes place and in which order (85).

The *in vitro* SHIME and TIM-1 models are important models to investigate behaviour of oral administrations like nutritional components or drugs in the gastro-intestinal tract and can lead to reduction in the number of animals used in research. However, the use of laboratory animals cannot be completely avoided. Like in this case, the preventive effect of the dietary component in the development of CMA and behavioural deficits, needs to be investigated in a model with multiple compartments as the intestine, systemic immune system and brain and behaviour detection. Yet, it is still impossible to perform research involving multiple compartments like interactions between intestinal microbiota, immune system and behaviour without using animals.

The PPARɣ hypothesis can be investigated in more detail by evaluating the role of PPARɣ in the n-3 PUFA-induced prevention of CMA development by using a PPARɣ antagonist

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and n-3 PUFAs dietary intervention in the CMA model. If the n-3 PUFA preventive effect on CMA development is dependent on PPARɣ, this preventive effect will decline when the activation of PPARɣ is blocked. If this leads to the result that PPARɣ has a role in the n-3 PUFA prevention of CMA, it might be interesting to investigate if the CMA preventive properties of scGOS:lcFOS occur at least in part through PPARɣ signalling mediated by butyrate. This can be tested by combining treatment with a PPARɣ antagonist and a scGOS:lcFOS dietary intervention in the CMA model evaluating the preventive effect of scGOS:lcFOS. This might provide more information whether both the dietary interventions n-3 PUFAs and scGOS:lcFOS (partly mediated through butyrate) prevent the CMA development through PPARɣ. If n-3 PUFAs and scGOS:lcFOS (partly) mediate the preventive effect of CMA development through PPARɣ, this might lead to competition whereas the preventive effect of a dietary combination of scGOS:lcFOS and n-3 PUFAs might be less effective.

Next to testing the possible interactions, it is important to take different dosages of the supplementations into account. Are the used dosages of scGOS:lcFOS and n-3 PUFAs the optimal dosages? The dosages used in the *in vivo* studies are based on previous results in murine models investigating scGOS:lcFOS and n-3 PUFAs individually. It might be that the optimal dosages to acquire the best possible effect are different when combining scGOS:lcFOS and n-3 PUFAs.

## OVERALL CONCLUSION

In the sense of one plus one is three, the aim of this thesis was to evaluate the additional or even synergistic effect of the dietary supplements scGOS:lcFOS and n-3 PUFAs to improve health as well as to prevent the development of CMA and related disease symptoms. Surprisingly, in both the healthy and cow's milk allergic mice no additional effect of the combination of scGOS:lcFOS and n-3 PUFAs were present. The combination of scGOS:lcFOS and n-3 PUFAs might be less effective than scGOS:lcFOS and n-3 PUFAs individually. Possible interactions in the intestinal tract between the dietary components might take place, leading to reduced availability and efficacy for the host. This might be caused by several interactions at different points in the intestinal tract and/or at different points in the signalling pathway in the mechanism of action of the components such as interference with PPARɣ. The used dosages might also be of importance; adapted dosages dependent on individual or combined use. HMOs and n-3 PUFAs are both very important in the development of the intestinal bacterial environment, immune system and brain and behaviour in early life, meaning that it is no option to leave scGOS:lcFOS or n-3 PUFAs out of the infant formula. One thing is clear, it is essential to evaluate individual components but also combinations as they might act differently than expected.

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# ANNEX

# NEDERLANDSE SAMENVATTING

Tijdens de eerste levensjaren vinden er enorme veranderingen plaats in de samenstelling van bacteriën in de darmen en de ontwikkeling van het immuunsysteem en de hersenen. De ontwikkeling van het immuunsysteem (ook afweersysteem genoemd) en de hersenen gaan hand in hand met de ontwikkeling van een stabiele samenstelling van bacteriën in de darmen. De laatste decennia wordt de rol van de darmbacteriën in de ontwikkeling van het immuunsysteem en de hersenen steeds duidelijker. Verschillende externe factoren, zoals gebruik van antibiotica, een keizersnede en het type zuigelingenvoeding, kunnen de kolonisatie van de darm beïnvloeden en dit kan weer van invloed zijn op de ontwikkeling van de hersenen en het immuunsysteem. Verstoringen in de kolonisatie van de darmbacteriën zijn in verband gebracht met negatieve effecten op de ontwikkeling van de hersenen, het immuunsysteem en de metabole gezondheid op de lange termijn. De samenstelling van de darmbacteriën wordt met name beïnvloed door het type voeding. Daarom is het gebruik van optimale voeding voor een gezonde ontwikkeling in het vroege leven van groot belang.

Wat voeding betreft, is moedermelk de gouden standaard tijdens de eerste zes levensmaanden. Moedermelk bevat alle voedingscomponenten die nodig zijn voor de ontwikkeling van het kind tijdens het vroege leven. Helaas is moedermelk niet altijd beschikbaar. Zuigelingenvoeding is een goed alternatief en bevat componenten die de functie van voedingscomponenten in moedermelk nabootsen. Voorbeelden van voedingscomponenten in zuigelingenvoeding die een rol spelen bij de ontwikkeling van de darmbacteriëncompositie, het immuunsysteem en/of de hersenen zijn prebiotica, zoals korte keten galacto-oligosachariden (scGOS) en lange keten fructo-oligosachariden (lcFOS) en omega-3 meervoudig onverzadigde vetzuren (n-3 PUFAs).

Dit proefschrift beschrijft de effecten van scGOS:lcFOS, n-3 PUFAs of een combinatie daarvan in het vroege leven door middel van preklinische studies. Ten eerste werden de effecten van deze voedingscomponenten (afzonderlijk of gecombineerd) op de samenstelling van de darmbacteriën, het immuunsysteem, de hersenen en gedrag bestudeerd in gezonde muizen. Van scGOS:lcFOS en n-3 PUFAs is bekend dat ze de ontwikkeling van koemelkallergie (KMA) (deels) kunnen voorkomen. Daarom werd het effect van een combinatie van scGOS:lcFOS en n-3 PUFAs op de ontwikkeling van KMA ook onderzocht. Aangezien KMA in verband is gebracht met angstig gedrag en verminderd sociaal gedrag bij muizen, werden deze gedragingen ook in dit proefschrift geëvalueerd. Tevens is er geprobeerd om de onderliggende mechanismen met betrekking tot mogelijke interacties tussen scGOS:lcFOS en n-3 PUFAs, die het effect op het immuunsysteem evenals de hersenen en gedrag beïnvloeden, te ontrafelen.

## Belang van scGOS:lcFOS en n-3 PUFAs tijdens het vroege leven

Een van de belangrijkste componenten in moedermelk zijn humane oligosachariden (HMOs). HMOs zijn prebiotische stoffen die de groei van goede bacteriën stimuleren en spelen daarom een belangrijke rol bij de rijping van de darmbacteriën. Een juist ontwikkelde samenstelling van de darmbacteriën tijdens de eerste levensjaren is

essentieel voor de ontwikkeling van het maagdarmkanaal, het immuunsysteem en het zenuwstelsel. HMOs stimuleren de rijping van het immuunsysteem door o.a. de T helper 2 cel (Th2)-gemedieerde immuunrespons bij geboorte te reguleren. Zo ontstaat er een gebalanceerde en veerkrachtige Th1/Th2-respons, die het risico op de ontwikkeling van Th2-gemedieerde aandoeningen, zoals voedselallergie, minimaliseert. Ook bevorderen HMOs de differentiatie van naïeve T-cellen in regulerende T-cellen (Tregs). De differentiatie naar Tregs samen met blootstelling aan voedselantigenen speelt een essentiële rol in de ontwikkeling van immunologische tolerantie. Zoals eerder vermeld, is moedermelk niet altijd beschikbaar en een alternatief daarvoor is zuigelingenvoeding. Zuigelingenvoeding bevat prebiotische vezels zoals scGOS:lcFOS die de eigenschappen van bepaalde HMOs in moedermelk nabootsen. De prebiotische vezels in zuigelingenvoeding vertonen ook immunomodulerende effecten. Ze beïnvloeden rechtstreeks darmepitheelcellen en dendritische cellen (DCs), die Treg induceren om tolerantie te ontwikkelen en de Th1/Th2-respons na de geboorte in evenwicht te brengen. Indirect induceren prebiotische vezels de groei van goede bacteriën waardoor ze worden gefermenteerd tot korte keten vetzuren (SCFAs) zoals acetaat, propionaat en butyraat. SCFAs, voornamelijk butyraat, zijn een belangrijke energiebron voor colonocyten, bevorderen de integriteit van het darmepitheel en hebben een immunomodulerende werking. Butyraat ondersteunt de activiteit van verschillende immuuncellen door de differentiatie naar Th1-, Th17-cellen en Tregs te stimuleren. Bovendien speelt butyraat een rol bij de preventie van KMA.

Naast immunomodulerende eigenschappen bezitten SCFAs neuroactieve eigenschappen en het wordt gesuggereerd dat ze ook een rol spelen bij de neurologische ontwikkeling. SCFAs kunnen de bloed-hersenbarrière passeren en de integriteit van deze barrière onderhouden. In het centrale zenuwstelsel zijn SCFAs in staat neuronale overleving, groei en differentiatie te stimuleren door modulatie van neurotrofische factoren, zoals "brain-derived neurotrophic factor" (BDNF).

Andere componenten aanwezig in moedermelk zijn PUFAs. Voorbeelden van PUFAs zijn n-3 en n-6 onverzadigde vetzuren, die worden opgenomen in de celmembraan van onder andere immuun- en hersencellen. PUFAs reguleren de functie van verschillende immuuncellen zoals DCs, T-cellen en B-cellen. Tijdens de hersenontwikkeling in het vroege leven worden n-3 en n-6 PUFAs opgenomen in de celmembranen van hersencellen zoals microgliacellen, astrocyten en neuronen waardoor synaptogenese, neurogenese en neuronale migratie en differentiatie worden beïnvloed. In de westerse wereld is de verhouding van n-6:n-3 bij mensen de laatste decennia verhoogd van 4:1 naar 20:1. Deze verschuiving wordt waarschijnlijk veroorzaakt door een verhoogde consumptie van vlees en plantaardige oliën die n-6 PUFAs bevatten en een verminderde consumptie van vette vis die n-3 PUFAs bevat. Tijdens zwangerschap en het geven van borstvoeding krijgen de foetus en de pasgeborene n-3 en n-6 PUFAs binnen via het dieet van de moeder. Een dieet met lage hoeveelheden n-3 PUFAs en grote hoeveelheden n-6 PUFAs bij de moeder, kan leiden tot onvoldoende n-3 PUFAs bij de foetus en/of pasgeborene. Onvoldoende inname van n-3 PUFAs bij kinderen kan leiden tot een verhoogd risico op neurologische aandoeningen, zoals autismespectrumstoornis (ASS), aangezien n-3 PUFA-plasmaspiegels laag zijn bij

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kinderen met de diagnose ASS. Daarnaast is een verhoogde consumptie van n-6 PUFAs in verband gebracht met een verhoogd risico op het ontwikkelen van angststoornissen. De veranderde verhouding van de n-6:n-3-ratio in de westerse wereld wordt ook in verband gebracht met een verhoogde prevalentie van allergische aandoeningen. Samen geeft dit het belang aan van een evenwichtige n-6:n-3-verhouding tijdens de ontwikkeling van de hersenen en het immuunsysteem in het vroege leven.

## Het effect van scGOS: lcFOS en n-3 PUFAs in gezonde muizen

### Darmbacteriën, SCFAs en immuun parameters

Naast de bekende prebiotische activiteit van scGOS:lcFOS, wordt er gedacht dat ook n-3 PUFAs prebiotische activiteit hebben. Toevoeging van scGOS:lcFOS (**hoofdstuk 3** en **4**) of n-3 PUFAs (**hoofdstuk 4**) in het dieet was inderdaad in staat de samenstelling van darmbacteriën te veranderen in gezonde muizen. De combinatie van scGOS:lcFOS en n-3 PUFAs veranderde de samenstelling van de darmbacteriën op een andere manier in vergelijking met scGOS:lcFOS of n-3 PUFAs afzonderlijk (**hoofdstuk 4**). Of de verschillen in de bacteriële samenstelling in de darm leiden tot functionele veranderingen moet echter nog worden onderzocht.

Tevens werd de invloed van de voedingscomponenten op de activiteit van de darmbacteriën en de fermentatie naar SCFAs, geëvalueerd. Wanneer scGOS:lcFOS toegevoegd was aan de diëten resulteerde dit, zoals verwacht, in verhoogde SCFA-waarden. De door scGOS:lcFOS geïnduceerde acetaat- en butyraatspiegels waren verlaagd wanneer scGOS:lcFOS werd gecombineerd met n-3 PUFAs (**hoofdstuk 4**). Dit zou erop kunnen wijzen dat n-3 PUFAs direct of indirect interfereren met de bacteriële fermentatie van scGOS:lcFOS in de darmen.

De veranderingen in de bacteriële samenstelling waren niet geassocieerd met veranderingen in het immuunsysteem. De veranderingen in immuunparameters veroorzaakt door de dieetinterventies waren gering. Aangezien dit een gezonde situatie betreft, kan men een veerkrachtig immuunsysteem verwachten waar duidelijke effecten op het immuunsysteem ongewenst zijn.

### Gedrag en serotonine

Tegenwoordig is het algemeen bekend dat voeding de darmbacteriën beïnvloedt en zoals hierboven vermeld spelen de darmbacteriën een centrale rol bij de ontwikkeling van de hersenen en het gedrag in het vroege leven. Met andere woorden, de voeding van pasgeborenen is een zeer belangrijke factor in de ontwikkeling van de hersenen. In **hoofdstuk 3** en **4** laten we zien dat scGOS:lcFOS-geïnduceerde veranderingen in de samenstelling van de darmbacteriën geassocieerd waren met verbeterd sociaal gedrag, verminderd angstig en repetitief gedrag en een neiging tot meer verkennend gedrag op latere leeftijd.

Alhoewel het duidelijk is dat n-3 PUFAs een essentiële rol spelen in de ontwikkeling van de hersenen, werden er bij gezonde muizen geen veranderde gedragseffecten door

n-3 PUFAs waargenomen (**hoofdstuk 4**) en de gecombineerde voedingsinterventie van scGOS:lcFOS en n-3 PUFAs had beperkt tot geen effect op het geëvalueerde gedrag. In een gezonde situatie zijn de processen in de gastheer evenwichtig en veerkrachtig, waardoor gedragsveranderingen moeilijk kunnen worden gemeten. Bij een gezonde gastheer zou de voedingscombinatie van scGOS:lcFOS en n-3 PUFAs robuuste gedragsveerkracht kunnen ondersteunen.

Gedragsveranderingen gaan vaak gepaard met veranderende monoamine neurotransmitterwaarden. Over het algemeen wordt verwacht dat hogere serotoninespiegels leiden tot minder angstig en beter sociaal gedrag. Het is intrigerend dat het verbeterde gedrag dat wordt gemedieerd door scGOS:lcFOS in **hoofdstuk 3** en **4** gepaard ging met (significant) verlaagde niveaus van serotonine in de hersenen en een toename van het aantal serotonine-uitscheidende enterochromaffienecellen in de darm (**hoofdstuk 4**). Dit zou eventueel gerelateerd kunnen zijn aan de beschikbaarheid van tryptofaan voor de hersenen, want perifeer aangemaakt serotonine kan de bloed-hersenbarrière niet passeren. Tryptofaan wordt gebruikt door enterochromaffienecellen om serotonine te produceren in de darm met het gevolg dat er een verlaagde (alhoewel niet significant vergeleken met de controle) hoeveelheid tryptofaan beschikbaar is voor serotonine aanmaak in de hersenen. Het aantal enterochromaffienecellen (**hoofdstuk 4**) was ook verhoogd door de n-3 PUFAs en de gecombineerde voedingsinterventies, maar de tryptofaan- en serotoninespiegels in de hersenen werden niet beïnvloed in vergelijking met het controledieet. Hierbij moet vermeld worden dat de totale hoeveelheid serotonine en tryptofaan is gemeten in hersenhomogenaten, waardoor de daadwerkelijke hoeveelheid serotonine in de synaptische spleten onbekend blijft.

## Het effect van scGOS:lcFOS en n-3 PUFAs in muizen die allergisch zijn voor koemelk

Naast evaluatie van de combinatie van scGOS:lcFOS en n-3 PUFAs in gezonde muizen, is ook een muizen KMA-model gebruikt om de preventieve werking van de voedingscombinatie in de ontwikkeling van KMA en gerelateerde ziektesymptomen te beoordelen (**hoofdstuk 5**).

KMA is een van de meest voorkomende voedselallergieën tijdens het eerste levensjaar. De symptomen van KMA zijn huiduitslag, gastro-intestinale problemen, zoals diarree, en in ernstige gevallen kan een anafylactische shock optreden. Momenteel is er geen behandeling of genezing beschikbaar, alleen het vermijden van het allergeen kan allergische reacties voorkomen. De meeste kinderen met de diagnose KMA ontgroeien de KMA vóór de leeftijd van 3 jaar, maar deze kinderen hebben een hogere kans om later in hun leven andere allergieën en gedragsproblemen te ontwikkelen. Dit benadrukt de noodzaak om de ontwikkeling van KMA te voorkomen. In **hoofdstuk 2** beschrijven wij de potentie van voedingscomponenten die de ontwikkeling van het immuunsysteem vroeg in het leven ondersteunen om de ontwikkeling van koemelkallergie te voorkomen. Van de afzonderlijke voedingscomponenten scGOS:lcFOS en n-3 PUFAs is eerder aangetoond

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dat ze een preventieve werking hebben bij de ontwikkeling van KMA. In **hoofdstuk 5** beschrijven wij de impact van de voedingsinterventies scGOS:lcFOS en/of n-3 PUFAs op het voorkómen van de ontwikkeling van wei-geïnduceerde KMA. Dit kwam tot uiting in een verminderde daling van de lichaamstemperatuur na blootstelling aan het wei-allergeen bij mannelijke muizen (scGOS:lcFOS) en in een verminderde acute allergische reactie (minder zwelling van het oor) bij vrouwelijke muizen (scGOS:lcFOS of n-3 PUFAs). De voedingscombinatie van scGOS:lcFOS en n-3 PUFAs had geen extra preventief effect op de KMA-ontwikkeling. De individuele preventieve effecten van scGOS:lcFOS of n-3 PUFAs leken negatief te worden beïnvloed als de voedingscomponenten werden gecombineerd. Deze waarneming suggereert een mogelijke interactie tussen de onderzochte voedingscomponenten.

De KMA en de dieetinterventies hadden geen effect op de immuun-T-celsubsets in mesenteriale lymfe klieren en in de milt. De *ex vivo* wei-specifieke cytokinerespons van splenocyten van koemelkallergische mannelijke en vrouwelijke muizen was duidelijk. Splenocyten verkregen uit met scGOS:lcFOS behandelde mannelijke KMA-muizen vertoonden een verminderde cytokinerespons bij *ex vivo* blootstelling aan wei. Dit effect verdween echter wanneer scGOS:lcFOS werd gecombineerd met n-3 PUFAs, wat suggereert dat er een interactie is tussen scGOS:lcFOS en n-3 PUFAs. Aangezien butyraat een fermentatie product is van scGOS:lcFOS en mogelijk een essentiële rol speelt bij de preventie van KMA is de hoeveelheid butyraat in de blindedarm van de muizen gemeten. De hoeveelheid butyraat werd verhoogd door scGOS:lcFOS in het dieet bij zowel mannelijke als vrouwelijke muizen. Bij de vrouwelijke muizen was dit verhoogde butyraatgehalte ook aanwezig in de combinatiedieetgroep. Bij de mannelijke muizen leek dit verhoogde butyraatgehalte te worden beïnvloed door n-3 PUFAs in de groep met een combinatiedieet.

In een aantal gevallen ging voedselallergie gepaard met neurologische manifestaties zoals depressie, angst en ASS-achtig gedrag. In dit proefschrift was het KMA-geïnduceerde gedragsveranderingen subtiel (**hoofdstuk 5**). Mannetjesmuizen leken verminderd sociaal gedrag te vertonen en n-3 PUFAs leken deze stoornis te verminderen, wat in overeenstemming is met eerdere onderzoeken. Zoals verwacht, zagen we geen KMA-geassocieerde gedragsveranderingen bij de vrouwelijke muizen.

## Hypothesen van interactie tussen scGOS:lcFOS en n-3 PUFAs in de voeding

Uit de gegevens gepresenteerd in **hoofdstuk 4** en **5** kan geconcludeerd worden dat de voedingscombinatie van scGOS:lcFOS en n-3 PUFAs noch leidt tot extra KMA-preventieve effecten, noch tot positieve effecten op het gedrag. Dit kan het gevolg zijn van een interactie tussen de componenten of een storing in het werkingsmechanisme. In dit proefschrift hebben we drie mogelijke hypothesen geëvalueerd:

**Butyraat verstoort de signaalroute van n-3 PUFAs door de expressie van epitheliale PPARɣ te verlagen**

Aangezien de n-3 PUFAs, docosahexaeenzuur (DHA) en (hoogstwaarschijnlijk) eicosapenta-eenzuur (EPA), "peroxisome proliferator-activated receptor ɣ" (PPARɣ)-agonisten zijn, zou het (gedeeltelijk) preventieve effect van deze n-3 PUFAs op de ontwikkeling van KMA kunnen optreden via activering van PPARɣ, die ontstekingsremmende eigenschappen heeft. Een andere PPARɣ-agonist is butyraat, wat aangeeft dat de ontstekingsremmende effecten van butyraat zouden kunnen optreden via PPARɣ-signalering. Opvallend, in darmorganoïden van de muis, werd in de literatuur gerapporteerd dat butyraat de darmepitheliale *Pparɣ* mRNA-expressie verminderde. Dit kan leiden tot minder beschikbare PPARɣ, wat resulteert in minder bindingsplaatsen voor n-3 PUFAs. Onze hypothese was dat de door scGOS:lcFOS geïnduceerde butyraat concentratie, de *Pparɣ*-mRNA-expressie in de darm van muizen zou kunnen verminderen. Dit zou kunnen resulteren in minder functionaliteit van PPARɣ, waardoor het effect van n-3 PUFAs op het voorkomen van KMA-ontwikkeling wordt verminderd. In darmweefsel van KMA-muizen werd de mRNA-expressie van *Pparɣ* niet beïnvloed door de individuele voedingscomponenten (**hoofdstuk 5**). De *Pparɣ* mRNA-expressie werd echter verhoogd door het gecombineerde dieet. Op basis van deze resultaten interfereert butyraat niet met het preventieve effect van n-3 PUFAs door intestinale PPARɣ-downregulatie, wat betekent dat deze hypothese niet klopt.

**Chemische reactie of fysieke interactie tussen scGOS:lcFOS en n-3 PUFAs**

De voedingscomponenten scGOS:lcFOS en n-3 PUFAs worden samen geconsumeerd waardoor een reactie tussen deze voedingscomponenten kan plaatsvinden in het maag-darmkanaal. Als er een chemische reactie plaatsvindt tussen scGOS:lcFOS en n-3 PUFAs, kan er een covalent gebonden vertakt molecuul ontstaan. Dit molecuul zou kunnen bestaan uit een scGOS of lcFOS "kern" met n-3 PUFA vrije vetzuur "takken". Onze hypothese was dat het vertakte molecuul bestaande uit scGOS of lcFOS met n-3 PUFA vrije vetzuren, niet in staat zal zijn om te worden gefermenteerd door darmbacteriën of te worden geabsorbeerd door de darmepitheelcellen. Daardoor zullen de voedingscomponenten scGOS:lcFOS en n-3 PUFAs minder beschikbaar zijn om hun immunomodulerende functies in de gastheer uit te oefenen om de ontwikkeling van KMA te voorkomen. Deze hypothese werd geëvalueerd met behulp van een *in vitro* verteringsmodel dat leidde tot voorlopige resultaten die aangeven dat scGOS en/of lcFOS inderdaad een vertakt molecuul zouden kunnen vormen met verschillende EPA-moleculen (**hoofdstuk 6**). Meer optimalisatie van de methode die in onze studie werd gebruikt, is nodig, evenals bevestiging dat het vertakte molecuul bestaande uit scGOS, lcFOS en EPA de darm ongewijzigd verlaat.

ScGOS:lcFOS en n-3 PUFAs kunnen ook op een fysieke manier interageren. Voordat ze door de enterocyten worden opgenomen, kunnen de n-3 PUFA-vrije vetzuren micellen vormen (accumulatie van amfifiele moleculen met een hydrofiele kop en een hydrofobe staart) in de darm. Onze hypothese was dat scGOS:lcFOS de n-3 PUFAs inkapselt in een

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kern, waardoor de n-3 PUFAs niet beschikbaar zijn voor opname door de darmcellen in de gastheer. Dit werd geëvalueerd door microscopie en computationele modellering en hoewel de gegevens zeer voorlopig zijn en verdere optimalisatie nodig is, suggereren we met de nodige voorzichtigheid, dat scGOS:lcFOS mogelijk interageert met de micelvorming van n-3 PUFAs in de darm, waardoor de immunomodulerende effecten van zowel scGOS:lcFOS als n-3 PUFAs zijn gereduceerd.

**Bacteriële activiteit geremd door n-3 PUFAs leidt tot minder fermentatie van scGOS:lcFOS**

Een derde mogelijke interactie tussen scGOS:lcFOS en n-3 PUFAs is op het niveau van darmfermentatie. Onze hypothese was dat n-3 PUFAs zouden kunnen interfereren met de fermentatie van scGOS:lcFOS in de darm, wat leidt tot minder immunomodulatie door scGOS:lcFOS als gevolg van minder geproduceerde SCFA. Deze hypothese is geëvalueerd met behulp van een *ex vivo* caecale fermentatietest, waarbij geïsoleerde blindedarminhoud werd geïncubeerd met verschillende concentraties scGOS:lcFOS en/of n-3 PUFAs in een anaerobe kamer, die de omgeving van darmbacteriën nabootst (**hoofdstuk 7**). Een toenemende concentratie van scGOS:lcFOS resulteerde in verhoogde concentratie van SCFA. De n-3 PUFAs, DHA en EPA, vertoonden geen effect op de door scGOS:lcFOS geïnduceerde productie van SCFA door darmbacteriën. Om de hypothese in een KMA-setting te testen, werd de inhoud van de blindedarm van koemelkallergische muizen gestimuleerd met en zonder scGOS:lcFOS en/of n-3 PUFAs. De door scGOS:lcFOS geïnduceerde SCFA-niveaus werden niet beïnvloed door DHA en/of EPA. Hoewel deze resultaten voorlopig zijn en optimalisatie noodzakelijk is, is dit een eerste indicatie dat deze hypothese niet juist is.

## Algemene conclusie

Het doel van dit proefschrift was om het aanvullende of zelfs synergetische effect van de voedingscomponenten, scGOS:lcFOS en n-3 PUFAs, te evalueren op het verbeteren van de gezondheid en het voorkomen van de ontwikkeling van KMA. Verrassend genoeg was er bij zowel de gezonde als koemelkallergische muizen geen additioneel effect van de combinatie van scGOS:lcFOS en n-3 PUFAs aanwezig. De combinatie van scGOS:lcFOS en n-3 PUFAs is mogelijk minder effectief dan scGOS:lcFOS en n-3 PUFAs afzonderlijk. Mogelijke interacties tussen de voedingscomponenten kunnen plaatsvinden in de darmen, wat leidt tot verminderde beschikbaarheid en werkzaamheid in de gastheer. Dit kan worden veroorzaakt door verschillende interacties op verschillende locaties in de darm en/of op verschillende punten in de signaalroutes van deze componenten, zoals interferentie met PPARɣ. HMOs en n-3 PUFAs zijn beide erg belangrijk bij de ontwikkeling van de darmbacteriën, het immuunsysteem en de hersenen en het gedrag in het vroege leven, wat betekent dat het geen optie is om scGOS:lcFOS of n-3 PUFAs uit de zuigelingenvoeding te laten. Eén ding is duidelijk, het is essentieel om individuele componenten te evalueren, maar ook combinaties, aangezien deze anders kunnen werken dan verwacht.

# DANSK RESUMÉ

I løbet af de første leveår sker der en enorm udvikling af tarmfloraen, immunforsvaret og hjernen. Udviklingen af immunforsvaret og hjernen går hånd i hånd med udviklingen af en stabil sammensætning af bakterier i tarmen. I de seneste årtier er tarmbakteriernes rolle i udviklingen af immunforsvaret og hjernen blevet mere og mere tydelig. Flere eksterne faktorer, såsom brug af antibiotika, fødsel ved kejsersnit og typen af modermælkserstatning, kan påvirke kolonisering af bakterier i tarmen, som igen kan påvirke hjernens og immunforsvarets udvikling. Forstyrrelser i koloniseringen af tarmbakterier er blevet forbundet med negative effekter på hjernens udvikling, immunforsvaret og langsigtet metabolisk sundhed. Sammensætningen af tarmbakterierne er påvirket af typen af kost. Derfor er brugen af den rigtige ernæring til sund udvikling tidligt i livet af stor betydning.

Ernæringsmæssigt er modermælk guldstandarden i de første seks måneder af livet. Modermælk indeholder alle de ernæringsmæssige komponenter, der er nødvendige for barnets udvikling tidligt i livet. Desværre er modermælk ikke altid tilgængelig. Modermælkserstatning er et godt alternativ og indeholder komponenter, der efterligner funktionen af ernæringskomponenter i modermælken. Eksempler på ernæringskomponenter i modermælkserstatning, der spiller en rolle i udviklingen af tarmfloraen, immunforsvaret og/eller hjernen er præbiotika, såsom kortkædede galacto-oligosaccharider (scGOS) og langkædede fructo-oligosaccharider (lcFOS) og omega-3 flerumættede fedtsyrer (n-3 PUFA'er).

Denne afhandling beskriver virkningerne af scGOS:lcFOS, n-3 PUFA'er eller en kombination heraf i det tidlige liv gennem prækliniske studier. Først blev virkningerne af disse kostkomponenter (separat eller kombineret) på sammensætningen af tarmbakterier, immunforsvaret, hjerne og adfærd undersøgt hos raske mus. ScGOS:lcFOS og n-3 PUFA'er er kendt for (delvist) at forebygge udviklingen af komælksallergi (KMA). Derfor blev effekten af en kombination af scGOS:lcFOS og n-3 PUFA'er på udviklingen af KMA også undersøgt. Da KMA har været forbundet med ængstelig adfærd og hæmmet social adfærd hos mus, blev denne adfærd også evalueret i denne afhandling. Vi forsøgte også at finde de underliggende mekanismer vedrørende mulige interaktioner mellem scGOS:lcFOS og n-3 PUFA'er, som påvirker effekten på immunforsvaret, hjernen og adfærd.

## Betydningen af scGOS:lcFOS og n-3 PUFA'er i det tidlige liv

En af hovedkomponenterne i modermælk er humane oligosaccharider (HMO'er). HMO'er er præbiotiske stoffer, der stimulerer væksten af gavnlige bakterier og spiller derfor en vigtig rolle i modningen af tarmbakterierne. En veludviklet sammensætning af tarmfloraen i de første leveår er afgørende for udviklingen af mave-tarmkanalen, immunforsvaret og nervesystemet. HMO'er stimulerer immunforsvarets modning ved blandt andet at regulere den T-hjælper 2-celle (Th2)-medierede immunrespons ved fødslen. Dette skaber et afbalanceret og modstandsdygtigt Th1/Th2-respons, som minimerer risikoen for at udvikle Th2-medierede lidelser, såsom fødevareallergi. HMO'er fremmer også differentieringen af naive T-celler til regulatoriske T-celler (Tregs). Differentieringen

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til Tregs sammen med eksponering for fødevareantigener spiller en væsentlig rolle i udviklingen af immunologisk tolerance. Som nævnt ovenfor er modermælk ikke altid tilgængelig, og et alternativ til det er modermælkserstatning. Modermælkserstatning indeholder præbiotiske fibre såsom scGOS:lcFOS, der efterligner egenskaberne af visse HMO'er i modermælk. De præbiotiske fibre i modermælkserstatning viser også immunmodulerende virkninger. De interagerer direkte med tarm epitelceller og dendritiske celler (DC'er), som inducerer Treg til at udvikle tolerance og balancere Th1/Th2-responset efter fødslen. Indirekte inducerer præbiotiske fibre væksten af gavnlige bakterier som fermenterer de præbiotiske fibre til kortkædede fedtsyrer (SCFA'er) såsom acetat, propionat og butyrat. SCFA'er, hovedsageligt butyrat, er en vigtig energikilde for tarmceller, fremmer integriteten af tarmepitel og har en immunmodulerende effekt. Butyrat understøtter aktiviteten af forskellige immunceller ved at stimulere differentiering til Th1, Th17 celler og Tregs. Derudover spiller butyrat en rolle i forebyggelsen af KMA.

Ud over immunmodulerende egenskaber besidder SCFA'er neuroaktive egenskaber og foreslås også at spille en rolle i neuroudvikling. SCFA'er kan krydse blod-hjerne-barrieren og opretholde integriteten af denne barriere. I centralnervesystemet er SCFA'er i stand til at stimulere neuronal overlevelse, vækst og differentiering gennem modulering af neurotrofiske faktorer, såsom "brain-derived neurotrophic factor" (BDNF).

Andre komponenter til stede i modermælk er PUFA'er. Eksempler på PUFA'er er n-3 og n-6 umættede fedtsyrer, som blandt andet optages i immun- og hjernecellers membran. PUFA'er regulerer funktionen af forskellige immunceller såsom DC'er, T-celler og B-celler. Under hjerneudvikling i det tidlige liv inkorporeres n-3 og n-6 PUFA'er i cellemembranerne i hjerneceller, såsom mikrogliaceller, astrocytter og neuroner, og påvirker derved synaptogenese, neurogenese og neuronal migration og differentiering. I den vestlige verden er forholdet mellem n-6:n-3 steget fra 4:1 til 20:1 i de seneste årtier. Dette skift er sandsynligvis forårsaget af et øget forbrug af kød og vegetabilske olier indeholdende n-6 PUFA'er og et reduceret forbrug af fed fisk indeholdende n-3 PUFA'er. Under graviditet og amning modtager fosteret og den nyfødte n-3 og n-6 PUFA'er gennem moderens kost. En diæt med lave mængder af n-3 PUFA'er og høje mængder af n-6 PUFA'er hos moderen kan tænkes at føre til utilstrækkelige n-3 PUFA'er hos fosteret og/eller nyfødte. Utilstrækkeligt indtag af n-3 PUFA'er hos børn kan tænkes at føre til en øget risiko for neurologiske lidelser, såsom autismespektrumforstyrrelse (ASD), da n-3 PUFA-plasmaniveauer er lave hos børn diagnosticeret med ASD. Derudover er øget indtag af n-6 PUFA'er blevet forbundet med en øget risiko for at udvikle angstlidelser. Det ændrede n-6:n-3-forhold i den vestlige verden er også forbundet med en øget forekomst af allergier. Tilsammen indikerer dette vigtigheden af et afbalanceret n-6:n-3-forhold under udvikling af hjernen og immunforsvaret tidligt i livet.

## Effekten af scGOS:lcFOS og n-3 PUFA'er i raske mus

### Tarmbakterier, SCFA'er og immunparametre

Ud over den kendte præbiotiske aktivitet af scGOS:lcFOS menes n-3 PUFA'er også at have præbiotisk aktivitet. Faktisk kunne tilføjelse af scGOS:lcFOS (**kapitel 3** og **4**) eller n-3 PUFA'er (**kapitel 4**) til kosten ændre sammensætningen af tarmbakterier i raske mus. Kombinationen af scGOS:lcFOS og n-3 PUFA'er ændrede tarmbakteriers sammensætning på en anden måde sammenlignet med scGOS:lcFOS eller n-3 PUFA'er alene (**kapitel 4**). Hvorvidt forskellene i bakteriesammensætningen i tarmen fører til funktionelle ændringer, mangler dog at blive undersøgt.

Ernæringskomponenternes indflydelse på tarmbakteriernes aktivitet og fermenteringen til SCFA'er blev også evalueret. Tilføjelse af scGOS:lcFOS til diæterne resulterede som forventet i forhøjede SCFA-niveauer. Acetat- og butyratniveauer induceret af scGOS:lcFOS blev reduceret, når scGOS:lcFOS blev kombineret med n-3 PUFA'er (**kapitel 4**). Dette kunne indikere, at n-3 PUFA'er direkte eller indirekte interfererer med den bakterielle fermentering af scGOS:lcFOS i tarmen.

Ændringerne i bakteriesammensætningen var ikke forbundet med ændringer i immunforsvaret. Ændringerne i immunparametre forårsaget af diætinterventionerne var få. Da dette er en sund situation, kan man forvente et modstandsdygtigt immunforsvar, hvor tydelige effekter på immunforsvaret er uønskede.

### Adfærd og serotonin

I dag er det velkendt, at kosten påvirker tarmfloraen, og som nævnt ovenfor spiller tarmbakterierne en central rolle i hjernens udvikling og adfærd i det tidlige liv. Nyfødtes ernæring er med andre ord en meget vigtig faktor i hjernens udvikling. I **kapitel 3** og **4** viser vi, at scGOS:lcFOS-inducerede ændringer i tarmbakteriesammensætning var forbundet med forbedret social adfærd, reduceret angst og stereotyp adfærd og en tendens til mere udforskende adfærd senere i livet.

Selvom det er klart, at n-3 PUFA'er spiller en væsentlig rolle i hjernens udvikling, observerede vi ingen ændrede adfærdseffekter hos raske mus ved indtag af n-3 PUFA'er. Den kombinerede ernæringsmæssige intervention af scGOS:lcFOS og n-3 PUFA'er havde begrænset til ingen effekt på den evaluerede adfærd (**kapitel 4**). I en sund situation er processerne i værten afbalancerede og modstandsdygtige, hvilket gør adfærdsændringer svære at måle. I en sund vært kunne den ernæringsmæssige kombination af scGOS: lcFOS og n-3 PUFA'er understøtte robust adfærdsmæssig modstandskraft.

Adfærdsændringer er ofte ledsaget af justerede monoamin-neurotransmitterniveauer. Generelt forventes højere serotoninniveauer at føre til mindre angst og bedre social adfærd. Interessant nok var den forbedrede adfærd medieret af scGOS:lcFOS **i kapitel 3** og **4** ledsaget af (signifikant) nedsatte niveauer af serotonin i hjernen og en stigning i antallet af serotonin-udskillende enterochromaffinceller i tarmen (**kapitel 4**). Dette kan muligvis være relateret til tilgængeligheden af tryptofan til hjernen, fordi perifert

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produceret serotonin ikke kan krydse blod-hjerne-barrieren. Tryptofan bruges af enterochromaffinceller til at producere serotonin i tarmen med det resultat, at der er en reduceret (men ikke signifikant sammenlignet med kontrol) mængde tryptofan tilgængelig til serotoninproduktion i hjernen. Antallet af enterochromaffinceller blev også øget af n-3 PUFA ernæringsintervention og den kombinerede ernæringsintervention, men tryptophan- og serotoninniveauer i hjernen blev ikke påvirket sammenlignet med kontroldiæten (**kapitel 4**). Det skal bemærkes, at niveauet af serotonin og tryptofan er den samlede mængde målt i hjernehomogenater, hvilket efterlader mængden af serotonin tilgængelig i de synaptiske kløfter ukendt.

## Effekten af scGOS:lcFOS og n-3 PUFA'er i forebyggelse af komælksallergi hos mus

Udover at evaluere kombinationen af scGOS:lcFOS og n-3 PUFA'er i raske mus, brugte vi også en muse-KMA-model til at vurdere den forebyggende effekt af diætkombinationen i udviklingen af KMA og relaterede sygdomssymptomer (**kapitel 5**).

KMA er en af de mest almindelige fødevareallergier i det første leveår. Symptomerne på KMA omfatter hududslæt, mave-tarmproblemer, såsom diarré, og i alvorlige tilfælde kan anafylaktisk shock forekomme. Der er i øjeblikket ingen behandling eller kur tilgængelig, kun undgåelse af allergenet kan forhindre allergiske reaktioner. De fleste børn diagnosticeret med KMA vokser fra KMA før 3 års alderen, men disse børn er mere tilbøjelige til at udvikle andre allergier og adfærdsproblemer senere i livet. Dette understreger behovet for at forhindre udviklingen af KMA. I **kapitel 2** beskriver vi det potentiale som ernæringskomponenter har, til at understøtte immunudvikling tidligt i livet for at forhindre udvikling af komælksallergi. De enkelte ernæringskomponenter scGOS:lcFOS og n-3 PUFA'er har tidligere vist sig at have en forebyggende effekt i udviklingen af KMA. I **kapitel 5** beskriver vi virkningen af ernæringsinterventionerne scGOS:lcFOS og/eller n-3 PUFA'er på at forebygge udviklingen komælksprotein-induceret KMA. Dette afspejledes i et reduceret fald i kropstemperaturen efter eksponering for komælksallergenet hos hanmus (scGOS:lcFOS) og i en reduceret akut allergisk reaktion (mindre hævelse af øret) hos hunmus (scGOS:lcFOS eller n-3 PUFA'er). Den ernæringsmæssige kombination af scGOS:lcFOS og n-3 PUFA'er havde ingen yderligere forebyggende effekt på KMA-udvikling. De individuelle forebyggende virkninger af scGOS:lcFOS eller n-3 PUFA'er syntes at blive negativt påvirket, når de ernæringsmæssige komponenter blev kombineret. Denne observation antyder en mulig interaktion mellem de undersøgte ernæringskomponenter.

KMA og diætinterventioner havde ingen effekt på T hjælper celler i mesenteriske lymfeknuder og milt. Den *ex vivo* komælksprotein-specifikke cytokinrespons fra splenocytter fra komælksallergiske han- og hunmus var tydelig. Splenocytter fra scGOS:lcFOS-behandlede KMA-hanmus viste et reduceret cytokinrespons ved *ex vivo* eksponering for komælksprotein. Denne effekt forsvandt da scGOS:lcFOS blev kombineret med n-3 PUFA'er, hvilket tyder på en interaktion mellem scGOS:lcFOS og n-3 PUFA'er. Da butyrat er et fermenteringsprodukt

af scGOS:lcFOS og kan spille en væsentlig rolle i forebyggelsen af KMA, blev mængden af butyrat i musenes blindtarm målt. Mængden af butyrat blev øget ved diætisk scGOS:lcFOS i både han- og hunmus. Hos hunmus var dette forhøjede butyratniveau også til stede i kombinationsdiætgruppen. Hos hanmus så dette forhøjede butyratindhold ud til at være påvirket af n-3 PUFA'er i den kombinerede diætgruppe.

I flere tilfælde var fødevareallergi ledsaget af neurologiske manifestationer som depression, angst og ASD-lignende adfærd. I denne afhandling var KMA-inducerede adfærdsændringer subtile (**kapitel 5**). Hanmus så ud til at vise reduceret social adfærd, og n-3 PUFA'er syntes at reducere denne svækkelse, hvilket er i overensstemmelse med tidligere undersøgelser. Som forventet så vi ingen KMA-associerede adfærdsændringer hos hunmusene.

## Hypoteser om interaktion mellem scGOS:lcFOS og diæt n-3 PUFA'er

Ud fra de data, der præsenteres i **kapitel 4** og **5**, konkluderer vi, at diætkombinationen af scGOS:lcFOS og n-3 PUFA'er hverken fører til yderligere KMA-forebyggende virkninger eller til positive virkninger på adfærd. Dette kan skyldes en interaktion mellem komponenterne eller en interferens i virkningsmekanismen. I denne afhandling har vi vurderet tre mulige hypoteser:

### Butyrat forstyrrer signalvejen for n-3 PUFA'er ved at reducere epitelial PPARɣ-ekspression

Da n-3 PUFA'erne, docosahexaensyre (DHA) og (mest sandsynligt) eicosapentensyre (EPA) er "peroxisome proliferator-activated receptor ɣ" (PPARɣ)-agonister, kan den (delvise) forebyggende effekt af disse n-3 PUFA'er på udviklingen af KMA forekomme via aktivering af PPARɣ, som har anti-inflammatoriske egenskaber. En anden PPARɣ-agonist er butyrat, hvilket indikerer, at de anti-inflammatoriske virkninger af butyrat kan opstå via PPARɣ-signalering. Påfaldende er at det er blevet rapporteret i litteraturen, at butyrat i museintestinale organoider reducerer intestinal epitelial *Pparɣ*-mRNA-ekspression. Dette kan føre til mindre tilgængelig PPARɣ, hvilket resulterer i færre bindingssteder for n-3 PUFA'er. Vi antog, at den scGOS:lcFOS-inducerede butyratkoncentration kunne reducere *Pparɣ* mRNA-ekspression i tarmen hos mus. Dette kan resultere i mindre funktionalitet af PPARɣ, hvilket reducerer effekten af n-3 PUFA'er på at forhindre KMA-udvikling. I tarmvæv fra KMA-mus blev *Pparɣ* mRNA-ekspression ikke påvirket af de individuelle kostkomponenter (**kapitel 5**). Imidlertid blev *Pparɣ* mRNA-ekspression øget af den kombinerede diæt. Baseret på disse resultater interfererer butyrat ikke med den forebyggende effekt af n-3 PUFA'er gennem tarm PPARɣ nedregulering, hvilket betyder, at vores hypotese ikke er sand.

### Kemisk reaktion eller fysisk interaktion mellem scGOS:lcFOS og n-3 PUFA'er

Ernæringskomponenterne scGOS:lcFOS og n-3 PUFA'er forbruges sammen, hvilket tillader en reaktion mellem disse ernæringskomponenter at finde sted i mave-

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tarmkanalen. Når en kemisk reaktion finder sted mellem scGOS:lcFOS og n-3 PUFA'er, kan der dannes et kovalent bundet forgrenet molekyle. Dette molekyle kunne bestå af en scGOS eller lcFOS "kerne" med n-3 PUFA fri fedtsyre "grene". Vi antog, at det forgrenede molekyle bestående af scGOS eller lcFOS med n-3 PUFA-frie fedtsyrer ikke vil være i stand til at blive fermenteret af tarmbakterier eller absorberet af tarmepitelcellerne. Derfor vil ernæringskomponenterne scGOS:lcFOS og n-3 PUFA'er være mindre tilgængelige til at udøve deres immunmodulerende funktioner i værtsorganismen for at forhindre udviklingen af KMA. Denne hypotese blev evalueret ved hjælp af en *in vitro*-fordøjelsesmodel, der førte til foreløbige resultater, der indikerer, at scGOS og/eller lcFOS faktisk kunne danne et forgrenet molekyle med forskellige EPA-molekyler (**kapitel 6**). Der er behov for optimering af metoden brugt i vores undersøgelse, samt bekræftelse af, at det forgrenede molekyle bestående af scGOS, lcFOS og EPA forlader tarmen uændret.

ScGOS:lcFOS og n-3 PUFA'er kan også interagere på en fysisk måde. Inden de optages af tarmcellerne, kan de n-3 PUFA-frie fedtsyrer danne miceller (akkumulering af amfifile molekyler med et hydrofilt hoved og en hydrofob hale) i tarmen. Vi antog, at scGOS:lcFOS indkapsler n-3 PUFA'erne i en kerne, hvilket gør n-3 PUFA'erne utilgængelige for optagelse af tarmcellerne i værtsorganismen. Dette blev evalueret ved mikroskopi og beregningsmodellering. Selvom dataene er foreløbige, og der er behov for optimering, foreslår vi, med forsigtighed, at scGOS:lcFOS kan interagere med den micellære dannelse af n-3 PUFA'er i tarmen og derved mindske de immunmodulerende virkninger af både scGOS:lcFOS og n-3 PUFA'er.

**Bakteriel aktivitet hæmmes af n-3 PUFA'er, hvilket fører til reduceret fermentering af scGOS:lcFOS**

En tredje mulig interaktion mellem scGOS:lcFOS og n-3 PUFA'er er på tarmfermenterings-niveau. Vi antog, at n-3 PUFA'er kunne interferere med fermenteringen af scGOS:lcFOS i tarmen, hvilket fører til reduceret immunmodulering af scGOS:lcFOS på grund af mindre SCFA-produktion. Vi evaluerede denne hypotese ved hjælp af en *ex vivo* caecal fermentationsmodel, hvor indholdet af blindtarmen fra mus blev stimuleret med forskellige koncentrationer af scGOS:lcFOS og/eller n-3 PUFA'er i et anaerobt kammer, hvilket efterligner miljøet i tarmen hvor tarmbakterierne befinder sig (**kapitel 7**). En stigende koncentration af scGOS:lcFOS resulterede i øget koncentration af SCFA'er. n-3 PUFA'erne, DHA og EPA, viste ingen effekt på scGOS:lcFOS-induceret produktion af SCFA'er af tarmbakterier. For at teste vores hypotese i en KMA-situation blev blindtarmsindholdet fra komælksallergiske mus stimuleret med og uden scGOS:lcFOS og/eller n-3 PUFA'er. SCFA-niveauer induceret af scGOS:lcFOS blev ikke påvirket af DHA og/eller EPA. Selvom disse resultater er foreløbige, og optimering er nødvendig, er den første indikation, at denne hypotese ikke er sand.

## Generel konklusion

Formålet med denne afhandling var at evaluere den komplementære eller endda synergistiske effekt af ernæringskomponenterne, scGOS:lcFOS og n-3 PUFA'er, med henblik på om denne effekt kunne forbedre sundheden og forhindre udviklingen af KMA. Overraskende fandtes der ingen yderligere effekt af kombinationen af scGOS:lcFOS og n-3 PUFA'er i hverken raske eller komælksallergiske mus. Kombinationen af scGOS:lcFOS og n-3 PUFA'er kan være mindre effektiv end scGOS:lcFOS og n-3 PUFA'er alene. Potentielle interaktioner mellem de ernæringsmæssige komponenter kan forekomme i tarmen, hvilket fører til reduceret tilgængelighed og effektivitet i værten. Dette kan skyldes forskellige interaktioner på forskellige steder i tarmen og/eller på forskellige punkter i disse komponenters signalveje, såsom interferens med PPARɣ. HMO'er og n-3 PUFA'er er begge meget vigtige i udviklingen af tarmbakteriesammensætning, immunforsvar og hjerne og adfærd i det tidlige liv, hvilket betyder, at udelukkelse af scGOS:lcFOS eller n-3 PUFA'er fra modermælkserstatning ikke er en mulighed. En ting er klart, det er essentielt at vurdere individuelle komponenter, men også kombinationer, da de sammen kan fungere anderledes end forventet.

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## ABOUT THE AUTHOR

Kirsten Szklany was born on 21st of April 1983 in Skørping, Denmark. After graduating from high school (Støvring Gymnasium, Støvring, Denmark) she moved to The Netherlands. She spent the first year in The Netherlands to learn Dutch whereafter she started her bachelor studies (in Dutch) at Utrecht University. In 2009 she received her Bachelor's degree in Pharmacy and continued with the Drug Innovation Master's program at the same university. As a part of her Master's, she conducted a nine-month internship at Danone Research Centre for Specialised Nutrition in Wageningen, The Netherlands under the supervision of Belinda van 't Land. During this internship she assessed the immunomodulatory effects of dietary non-digestible oligosaccharides on T cell subsets. She did a second internship at the Brain-Body Institute at McMaster University in Hamilton, Canada supervised by Khalil Karimi and John Bienenstock. During the six-month internship she investigated the interaction between superior cervical ganglia neurons and naïve T cells. After obtaining her Master's degree in 2013, Kirsten worked as Assistant Scientist at Crucell (currently Janssen) in Leiden, The Netherlands. In 2014, she joined the division of Pharmacology within Utrecht Institute of Pharmaceutical Sciences at Utrecht University as a PhD candidate under the supervision of prof. dr. Johan Garssen, prof. dr. Aletta Kraneveld and dr. Léon Knippels. Kirsten was trained in the Drug Innovation PhD program of the Graduate School of Life Sciences. She actively participated in the PhD committee of the Drug Innovation program. During her PhD trajectory Kirsten focussed on the potential of a dietary combination of non-digestible oligosaccharides and omega-3 polyunsaturated fatty acids to modulate the gut-immune-brain axis in health and allergic disease. The results of this work is described in this thesis. In 2020 Kirsten started as Research Coordinator at BioAg Europe in Harderwijk, The Netherlands. Here she conducts and supervises research projects to investigate the importance of humic and fulvic acid in intestinal health in humans as well as in farm animals.

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