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Research Article

Chain length-dependent effects of inulin-type fructan dietary fiber on human systemic immune responses against hepatitis-B

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Keywords: chain length, dietary fiber, hepatitis B, inulin-type fructans, vaccination

Received: 16/02/2017; Revised: 18/05/2017; Accepted: 29/05/2017

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201700171.

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Abbreviations: Anti-HBsAg, anti-Hepatitis B surface antigen; **CD,** cluster of differentiation; **CTL,** cytotoxic T lymphocyte; **DP,** degree of polymerization; **FoxP3,** forkhead box P3; **HepB,** hepatitis B; **ITF,** inulin-type fructan; **NK,** natural killer; **PBMC,** peripheral blood mononuclear cell; **TBET,** T-box protein expressed in T cells; **Th,** Thelper; **TLR,** Toll-like receptor.

Abstract

Scope: *In vivo* studies demonstrating that only specific dietary-fibers contribute to immunity are still inconclusive, as measuring immune effects in healthy humans remains difficult. We applied a relatively inefficacious vaccination-challenge to study chain length-dependent effects of inulin-type fructan (ITF) dietary fibers on human immunity.

Methods and results: ITFs with two different 'degree of polymerization-' (DP)-profiles were tested *in vitro* for effects on PBMC-cytokines and TLR2 activation. In a double-blind placebo-controlled trial, 40 healthy volunteers (18-29 y.) were divided into 3 groups and supplemented from day 1 to day 14 with DP10-60 ITF, DP2-25 ITF (both n=13), or fructose placebo (n=14), 8g/d. On day 7, all volunteers were vaccinated against hepatitis B. Anti-HbsAg-titer development and lymphocyte subsets were studied. *In vitro*, DP10-60 ITFs stimulated a Th1-like cytokine profile and stimulated TLR2 more strongly than DP2-25 ITFs. *In vivo*, DP10-60 increased anti-HBsAg titers, Th1-cells, and transitional B-cells. Both ITFs increased CD45RO^{hi} CTLs at day 35, and CD161^{+} cytokine producing NK-cells at day 21 and 35.

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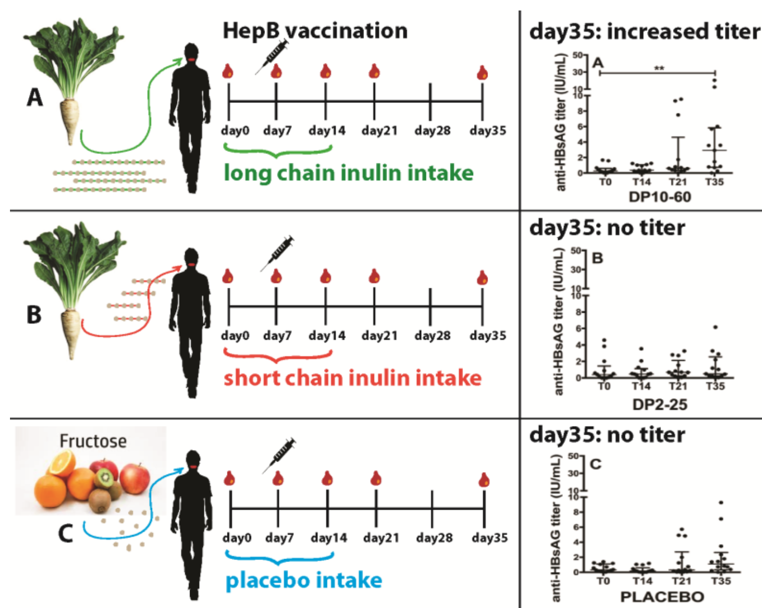
Conclusion: Support of immunity is determined by the chain length of ITFs. Only long-chain ITFs support immunity against pathogenic hepB-epitopes introduced by vaccination. Our findings demonstrate that specific dietary fibers need to be selected for immunity support.

Inulin-type dietary fibers of different chain lengths (short vs. long) were given to two healthy volunteer groups to study if they boost the immune response, and if chain length makes a difference in that result. Long chains but not short chains boosted the immune response to a hepatitis B vaccine. In the blood of these volunteers we observed a typical immune T cell profile that confirmed these results. When fibers were added to immune cells *in vitro*, these cells also released different signaling cytokines, depending on the fiber chain length.



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1 Introduction

High dietary fiber intake is recommended by health advisory groups as it can prevent several diseases [1–3] and regulate bowel habit [4–8]. Specifically for diseases with an immunological component, ameliorating effects have been demonstrated [9–12]. However, up to now no distinction is made in recommended types of fibers, while it has been shown *in vitro,* that only specific fibers contribute to immunity and that even within specific fiber types, clear chain length-dependent effects on immunity exist [13–16]. Whether this holds consequences for possible function-effector relationships in humans has not been reported up to now [17, 18].

Demonstrating chain length-dependent effects of dietary fibers on the immune system of healthy consumers has been cumbersome for several reasons. Often not healthy consumers, but immunocompromised populations such as infants or elderly with responses not representative for regular consumer immune responses were studied [19–28], or the dietary fibers were administered in mixtures [19–28]. Also, it has been difficult to apply an adequate challenge in healthy humans in which effects of oral intake of food ingredients on immunity can be measured. Vaccination protocols have been applied for testing supporting effects of food ingredients on mounting immune responses against common pathogens [24, 29–31]. Vaccinations such as measles [29], tetanus [30], influenza [24, 30], or pneumococcal vaccination [24] have been tested but effects of food ingredients could not easily be quantified, and high numbers of volunteers were required because of high standard efficacy of the vaccination protocols [24, 29–31]. We hypothesized that less efficacious vaccination protocols such as hepatitis B (hepB) vaccination may be more suitable for testing and comparing chain length-dependent fiber effects in humans, because of the broader range for improvement of immunity, and possible quantification of smaller effects than can be reached with the aforementioned protocols [32].

One family of fibers for which clear chain length-dependent effects are demonstrated *in vitro,* are inulin-type fructans (ITFs) [13, 33–36]. Previous studies have demonstrated that the degree of

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polymerization (DP, i.e. chain length) of ITFs determines the cytokine induction of human peripheral blood mononuclear cells (PBMCs) [13]. Long chain ITFs induced a pro-inflammatory cytokine profile, whereas short chain ITFs induced an increased IL-10/IL-12 ratio, indicating a more anti-inflammatory effect [13]. In addition, ITFs activate Toll-like receptor (TLR)2 in a DP-dependent manner [13]. Thus, ITFs exert chain length-dependent direct effects on immune cells, independent of effects on microbiota [35] .

The aim of this study was to determine the chain length-dependent effects on human immunity. We first selected and tested two ITF formulations with distinct DP profiles and immune effects. To this end, PBMCs of non-supplemented human subjects were challenged with two ITFs with different DP profiles, approved by the medical ethical committee for human application. Cytokine profiles were compared. Additionally, we studied TLR2 stimulation by these two ITFs, and they were subsequently applied in a human HepB vaccination trial. During the trial, anti-HBsAg titers were analyzed and peripheral blood lymphocyte subsets of the supplemented subjects were studied using flow cytometry, to identify possible underlying mechanisms of chain length-dependent effects of the dietary fibers.

2 Materials and Methods

2.1 Study Design

This study was approved by the ethical board of the University Medical Center Groningen, documented as METC_097, and registered in the Nederlands Trial Register (NTR41644). Written informed consent was obtained from all participants, and data are presented anonymously. All clinical investigations were conducted within the University Medical Center Groningen according to the principles of the Declaration of Helsinki.

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A double-blind placebo-controlled (DB-PC) human dietary intervention trial was designed to study the effect of ITFs on vaccination efficacy and peripheral blood lymphocyte populations. To study the predefined hypothesis that ITF DP influences the type of immune reaction which is induced, we included healthy volunteers, who were supplemented from day 1 until day 14 with (i) ITFs of DP10-60, or (ii) with ITFs of DP2-25, or (iii) with fructose (Sigma-Aldrich, the Netherlands) as placebo (n=13, 13, and 14 per group respectively, 8g single dose/day). To this end, ITFs and placebo (in powder form) were weighed and aliquoted into 8 g portions, which the subjects then ingested daily at the same time, which was chosen by the subjects for their convenience as to improve adherence to the protocol. To mask the taste of the supplements, they were diluted into plain lemonade provided by the study coordinator (Jumbo fruits of the forest lemonade with no added sugars, and containing Stevia, diluted 1:7 in tap water by the subjects). Simple randomization was implemented by allocating the subjects to the experimental groups in series, according to the time of enrollment. Inclusion criteria for this study were defined as age 18-29 yr., healthy male and female Caucasian, Dutch speaking subjects who were enrolled to be vaccinated against hepatitis B, and who provided written informed consent. Exclusion criteria were defined as the presence of acute or chronic diseases, gastrointestinal disorders (e.g. inflammatory bowel disease, celiac disease), previous gastrointestinal surgery, treatment with antibiotics within 6 months of the start of the study, prior vaccination with hepatitis B, previous hepatitis B infection, immunodeficiencies, and the use of anti-coagulant drugs. All groups were vaccinated against hepatitis B (Engerix-B, GlaxoSmithKline Biologicals s.a., Belgium) on day 7. The volunteers consumed their habitual diet and filled out a nutrition diary for the duration of the study. Blood samples were collected at days 0, 7, 14, 21, and 35. Supporting information figure S1 depicts a schematic overview of experimental interventions.

The approach of measuring immunological parameters in the early period around hepB vaccination, especially the 35-day period directly after vaccination, has not been published before to the best of our knowledge. Due to this lack of preexisting literature, there was no possibility to do a power calculation to determine the exact group size required for reliable, robust results regarding the

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primary parameter, the anti-HbsAg titer in this early time frame. As a solution, the experiment was set up as a pilot study and our group size was based on the recommendations of Julious [37], rendering a required group size of 12 individuals. To account for expected drop out of participants, we started our experiments with group sizes of 13, 13, and 14 for the two experimental groups and the placebo group respectively, and group sizes were not altered during the study. Rules for stopping data collection were defined and documented in detail in advance, as required for the medical ethical board application, and they are reported in the accessible study protocol in the NTR trial register as well. The endpoint of the study was defined as the blood collection for HBsAg measurement on day 35 of the study. Blood samples were analyzed with flow cytometry per time point as much as possible, and plasma samples for anti-HBsAg titer measurements were frozen and stored during the course of the study, and were all analyzed at the same time at the completion of the study. The total experiment was performed once, and was limited to single flow cytometry measurements per subject per time point, to allow for measurements of the same study time point of different subjects to be as close together as possible. Results in time were substantiated by repeated measurement analysis.

2.2 Investigational compounds

Supplement A (Frutafit®TEX! Sensus, Roosendaal, the Netherlands), is a powdered food ingredient of DP10-60, based on native chicory inulin. Supplement B (Frutafit®CLR Sensus), is a highly soluble powdered fructo-oligosaccharide (FOS) of mainly DP2-25, produced by partial hydrolysis of chicory inulin. Supplement C is powdered fructose, used as placebo, as it consists of the monomer building blocks of fructans and does not have β(2-1) bonds. DP profiles of supplement A and B are depicted in Supporting information figure S2.

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2.3 Multiplex cytokine analysis of human peripheral blood mononuclear cells

Human PBMCs of 6 healthy volunteers were isolated and cultured as previously described [13]. 1x 10^6 cells were stimulated overnight with ITFs of DP10-60 or DP2-25 (100 μg/mL), or culture medium as control. Supernatant cytokine profiles were quantified with a human Bio-PlexTM 6-plex premixed cytokine assay (group I, IL-1Ra, IL-1β, IL-6, IL-10, IL-12p70, and TNF-α) according to the manufacturer's instructions (Bio-Rad Laboratories, Veenendaal, The Netherlands) [13].

2.4 TLR2 reporter cell culture and reporter assays

A HEK(293)-Blue cell line with constructs for human TLR2 and secreted embryonic alkaline phosphatase (SEAP), coupled to the NF-κB/AP-1 promotor (InvivoGen, Toulouse, France), was cultured as previously described [13]. 2.8 x 10^5 cells were stimulated overnight with a concentration series of DP10-60 and DP2-25 ITFs from 100 μg/mL to 1000 μg/mL, with culture medium as control, or with heat killed *Listeria monocytogenes* (1 x 10^8 cells/mL, InvivoGen). Analysis was performed according to the manufacturer's instructions, as previously described [13].

2.5 Whole blood immunostaining and multi-parameter flow cytometry

Percentages of B-cell, T-cell, NK-cell, and NKT-cell populations and subsets were analyzed with flow cytometry. Staining protocols and flow cytometry gating strategies are described in Supporting information figure S3.

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2.6 Anti-HBsAg titer analysis

Anti-HBsAg titers were analyzed at days 0, 14, 21, and 35. Per sample, 500 μL of plasma was aliquoted in Architect tubes (Abbott, Illinois, U.S.A.), and analyzed for anti-HBsAg titers using an Architect Immunoassay Analyzer (Abbott Diagnostics) following the manufacturer's instructions. In our analysis we specifically compared time of onset of titer development, titer response on the final measuring day, and/or the presence and number of 'responders', characterized as subjects with titers equal to or above 10 IU/mL [32].

2.7 Statistical analysis

GraphPad Prism 5.0 was used for statistical analysis of all data. The parametric distribution of data was confirmed using d'Agostino Pearson test. Values are expressed as mean ± standard deviation (SD) except where nonparametric, in which case median ± interquartile range was used. Statistical comparisons were performed using repeated measures ANOVA and Tukey's multiple comparison test for grouped analysis of parametrically distributed data. Where no parametric distribution could be demonstrated, we applied Friedman, Mann-Whitney U, or Kruskal-Wallis test, and Dunn's multiple comparison tests. $p<0.05$ was considered as statistically significant (*$p<0.05$, **$p<0.01$, and ***$p<0.001$), and $0.05 \leq p \leq 0.1$ was considered representative for a statistical trend.

3 Results

3.1 DP10-60 and DP2-25 ITFs induced dose-dependent TLR2 activation and DP determined the cytokine patterns induced in human PBMCs.

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DP10-60 and DP2-25 ITFs dose-dependently induced TLR2-mediated NF-κB/AP-1 activation (Figure 1). TLR2 was activated by both ITFs at a concentration of 200 µg/ml, but DP10-60 stimulated TLR2 consistently stronger than DP2-25 ITFs at the different doses tested. In human PBMCs (Figure 2), TNFα production was significantly increased by DP10-60 ITFs as compared to DP2-25 ITFs (1289.0 ± 835.0% of control vs. 350.5 ± 121.7% of control, $p<0.05$), indicating a more immune stimulating effect of long chain ITFs. IL-6 production was induced by DP2-25 but not by DP10-60 ITFs (685.0 ± 109.9% of control vs. 114.7 ± 21.3% of control, $p<0.05$), indicating a more regulatory effect of short chain ITFs. IL-12 production was induced by both formulations but did not significantly differ between ITFs. Because DP10-60 ITFs induced more immune stimulating cytokines, while DP2-25 ITFs only enhanced IL-6, we hypothesized that DP10-60 ITFs would enhance immune responses against hepB antigens, while DP2-25 ITFs would be ineffective.

3.2 DP10-60 ITF supplementation increased anti-HBsAg titers.

Figure 3 shows antibody titer development upon hepB vaccination of subjects treated with ITFs of placebo. Figures 3A-C show the titer development in time, plotted and analyzed per supplement. Significantly elevated antibody levels were reached at time point T35 compared to basal samples at T0 in the DP10-60 ITF group, but not in the DP2-25 ITF group or placebo group. In addition, upon comparing the effect of each treatment per time point (Figure 3D), DP10-60 ITFs induced a significant increase in antibody titer compared to treatment with DP2-25 ITFs at T35 ($p<0.5$), and an increased statistical trend compared to placebo treatment ($p=0.1$).

At T35, there were no responders (antibody titer above 10 IU/mL) [32], in the placebo group and the DP2-25 fructan group, however, in the DP10-60 fructan group, two responders were present. These results combined, demonstrate that supplementing young adults with a daily single dose of 8 g DP10-60 ITFs in the 14-day period around the first injection enhanced the efficacy of the anti-hepatitis B vaccination.

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3.3 Percentages of IgM^{hi} CD38^{hi} transitional B-cells were significantly increased at T14 and T21 in the DP10-60 and placebo group but not in the DP2-25 group.

As B-cells are essential in mounting antibody responses against vaccines, we will first describe the results of B-cell subsets in peripheral blood of subjects in the experimental groups. Results of B-cell flow cytometry are shown in Figure 4. The percentage of B-cells within the lymphocyte population was analyzed, and subsequently, the percentage of several populations within the lymphocyte population or within the B-cell population were analyzed and compared to basal samples at T0.

The percentages of B-cells in the total lymphocyte population did not change as compared to basal samples (Figure 4.I). Naïve non-class switched B-cells (CD19^{+} CD21^{+}, CD27^{-} IgD^{+}, data not shown), and class switched memory B-cell populations (CD19^{+} CD27^{+} IgD^{-}, data not shown) did not differ in time as compared with basal samples. Although non-class switched memory B-cells (CD19^{+} CD27^{+} IgD^{+} IgM^{+}, data not shown) also did not differ in time compared to basal samples, the percentage of IgM^{+} cells (CD19^{+} CD27^{+} IgD^{+} IgM^{+}) within this non-class switched memory B-cell population (Figure 4.II) was increased at T21 compared to basal samples ($p<0.05$) in the placebo group, but not in the DP10-60 group and DP2-25 group. Within the lymphocyte population and within the B-cell population, the percentage of transitional B-cells, and the percentages of plasmablasts or plasma cells did not differ in time for either ITF (data not shown). However, within the CD38^{hi} transitional B-cells, the percentages of IgM^{hi} cells were significantly increased at T14 and T21 in both the DP10-60 fructan group and the placebo group, as compared to basal samples ($p<0.05$). This effect was absent in the DP2-25 ITF group (Figure 4.III).

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3.4 DP10-60 ITFs showed a delayed increase in $CD161^+$ cytokine producing NK-cells.

Human NK-cells ($CD56^+$ $CD3^-$) are part of the first line of defense against viral pathogens and their activation can modulate the outcome of the adaptive immune response [38]. Similar to NK-cells, NKT-cells ($CD56^+$ $CD3^+$) are also implicated in the response against hepatitis B vaccine antigens [38]. No differences were observed for the percentage of NK-cells within the lymphocyte population (Figure 5.I). In the DP2-25 group and placebo group (Figure 5.II), an increase was observed for $CD161^+$ cytokine producing NK-cells at all time points compared to basal samples (T0) but in the DP10-60 group this effect was only observed on time points T21 and T35 ($p<0.05$). No changes were observed for $CD161^+$ cytotoxic NK-cells as compared to basal samples, irrespective of the treatment (data not shown). The percentage of $CD335^+$ cells did not differ in time within either NK-cell population (data not shown). In the DP10-60 group (Figure 5.III), NKT-cells tended to show a decrease on time point T14 compared to basal samples, expressed as percentage of the lymphocyte population ($p=0.1$), but no changes were observed in the DP2-25 and placebo group.

3.5 DP10-60 fructan supplement induced increased percentage of $TBET^+$ (Th1) cells on T35.

Results of T-lymphocyte flow cytometry are shown in Figure 6. T-cells are essential for stimulating the production of high-affinity antibodies as well as the induction of immune memory [39].

Percentages of T-cells (Figure 6.I), Th2-cells, $FoxP3^+$ Th-cells, and Th17-cells within the lymphocyte population (data not shown) did not differ at any of the time points from basal samples at T0. However, at T35, the percentage of $TBET^+$ (Th1) cells within the Th-cell population was significantly increased in the DP10-60 fructan group as compared to basal levels at T0 ($p<0.05$). This effect was not observed in the DP2-25 group or placebo group (Figure 6.II). The percentages of

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CD45RO$^+$ cells within the lymphocyte population did not differ in time for any of the treatment groups (data not shown).

It is generally believed that for the time frame following challenge with a virus or a viral vaccine, human CD8$^+$ memory cells are principally found within the CD45RO^{hi} population, which can be distinguished from the regular or intermediate CD45RO$^+$ cells based on population density and shape [40]. Therefore we also gated and analyzed these cells. Compared to T0, the percentage of CD45RO^{hi} CTLs expressed as percentage of CTL memory cells was increased at time point T14 and T35 in the DP10-60 group ($p<0.05$), and at time point T35 in the DP2-25 group ($p<0.05$), but no significant increases were observed in the placebo group (Figure 6.III). In addition to the CTLs, Th-cells also typically demonstrated changes in the CD45RO^{hi} population. However, only the placebo group demonstrated significant increases in CD45RO^{hi} Th-cells as percentages of the Th memory population (Figure 6.IV), at time point T14 and T35 as compared to basal samples at T0 ($p<0.05$).

4 Discussion

With this manuscript we provide scientific evidence of chain length-dependent effects of dietary fibers on immunity in humans. In our evaluation of the TLR2-activating and cytokine inducing potential of different ITFs, we found a more immune stimulating, Th1-inducing profile for DP10-60 ITFs compared to DP2-25 fructans, and a more immunosuppressive, Th1-inhibiting cytokine pattern for DP2-25 fructans. In parallel, oral intake of long chain DP10-60 ITFs significantly enhanced the antibody-titer response as compared to DP2-25 ITFs, and an increased trend was present as compared to placebo treatment. As the response to hepB vaccination is a Th1-dependent immune reaction, this confirmed our hypothesis that DP10-60 ITFs would stimulate vaccine responses *in vivo* while DP2-25 ITFs would be ineffective. Another observation that supports the immune stimulating effects, was the identification of two responders in the DP10-60 group vs. no responders in either the DP2-25 group or the placebo group. To the best of our knowledge, comparative chain length-dependent effects of ITFs

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in humans have not been reported up to now. In the majority of studies, ITFs were studied in combination with other oligosaccharides [19, 41–45]. In one study a mixture of short chain combined with long chain ITFs was studied [29], and enhanced blood IgG concentrations after measles vaccination were observed in 7-9 months old infants. The lack of effect of short chain ITFs is corroborated by Duggan *et al*., who found no effect on antibody response after vaccination with *H. influenza* type B vaccine in 6-12 month old infants supplemented with short chain ITFs (all fructan DP < 10, over 25% $\geq$ DP5, less than 75% DP $\leq$4; 0.7 g/d) [16]. Also in other studies, in infants supplemented with prebiotic mixtures with short-chain ITFs vaccine potentiating effects were not induced [30, 31]. Our data are in line with these findings, as short-chain ITFs are virtually ineffective in Th1 stimulation, which is required for adequate responses to these vaccines. Our data suggest that DP10-60 ITFs might be more effective.

To study the effect of different DP of fructans on changes in peripheral lymphocytes, we analyzed B-cell, T-cell, NK-cell, and NKT-cell subsets of supplemented individuals for their percentage of the total lymphocyte population, percentage of relevant subpopulations, and for differences in activation marker expression. Class-switching is one of the hallmarks of activation and maturation of B cells. We, however, found no evidence of class-switching in our study: we observed no increase in class-switched B-cells, and no changes in IgG^+ or IgA^+ class switched memory B-cells. This may be a time dependent effect, as these cells may arise later or may already have migrated to the spleen and lymph nodes. A second vaccination might be required to specifically enhance this population, as was suggested by Siegrist [39]). Another explanation could be that the induction of memory cells or class switched cells is too low to measure with flow cytometry. This may be in line with the fact that there are very few antibody responders in our study. However, we found an increased percentage of maturing transitional B-cells ($CD38^{hi}$ IgM^{hi}), in the DP10-60 group and the placebo group for time points T14 and T21 compared to the basal samples, but not in the DP2-25 group. Increased percentages of this population indicate that B-cells are activated and stimulated to differentiate into antibody producing plasma cells, which is a functional objective of vaccination [39].

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The fact that the transitional B cell population was stimulated by DP10-60 fructan supplementation and not by DP2-25 fructan supplementation underscores the effective differences of these two types of dietary fibers.

Numbers of NK-, and NKT-cells did not demonstrate clear supplement dependent effects. However, CD161-expressing NK-cells were increased in every group during the vaccination protocol. CD161 is an NK-cell activating receptor [46], thus suggesting the NK-cells become activated following hepB vaccination. The lack of effects of supplements on NK-, and NKT-cells suggests that B-cells and T-cells could be more involved in boosting the vaccination response via dietary ITF supplementation. Supplementation with long chain DP10-60 ITFs indeed induced differences in T-cell populations in time. The increased titer response for the long chain group on T35 was associated with an increased percentage of (T-bet^{+}) Th1-cells compared to basal samples. Contrary to Th1-cells, (CD294^{+}) Th2-cells did not change in time for either treatment. These results suggest that in the long chain DP10-60 group, a shifted Th1/Th2 balance may have been induced, which was skewed towards Th1-cell responses. This stimulation of Th1-type reactions for DP10-60 fructans was corroborated by the *in vitro* results using PBMCs, and also by several reports [35, 36, 47–49], which describe that ITFs can shift the balance towards Th1-reactions rather than to Th2-mediated responses. The induction of memory T-cells is important in the efficacy of vaccinations, as these cells are responsible for fast recognition of a second encounter with the relevant antigen and mounting an adequate immune response [39]. Our results indicate that both fructan treatments stimulate the induction of memory CD45RO^{hi} CTLs, which could represent one of the effects of ITFs which are not related to DP differences, and could be considered as a shared linear fructan property.

The chain length-dependent effects of ITFs can be explained by several mechanisms. Upon consumption, ITFs of different DP may selectively stimulate different microbiota populations [50–52], and therefore have different effects on SCFA production [53]. However, SCFA only attenuate immune responses and cannot explain the Th1-effects of ITFs [53, 54]. We suggest the possibility that

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direct effects of ITFs on immune cells in the mucosa and not microbiota effects are mainly responsible for the immune-stimulating effect [13, 35, 36], which is in line with our in vitro data. Our working model is that ITFs of different composition prime immune cells in the mucosa in a chain length-dependent manner. We expect that PRRs are the main receptors that recognize ITF fibers. As we have shown *in vitro*, TLRs, and specifically TLR2, can recognize ITFs ([13], and current results), and this receptor could also be involved in ITF recognition *in vivo*, in the intestinal mucosa, either expressed by dendritic cells [55, 56] or intra-epithelial lymphocytes [57]. Since dendritic cells are likely the cell type that migrates farthest through the body, and are relevant in antigen presentation such as after vaccination, we expect these to be primed differently by long chain ITFs as compared to short chain ITFs. As we previously stated [55] this may be caused by mechanistic differences in receptor interactions at the cellular surface, where short chain ITFs activate fewer receptors at a time, at a relatively large distance from each other, whereas long chain ITFs might cluster TLR2 and possible signaling partners into a complex that enhances downstream signal transduction, in a similar mode as was reported for TLR4 [13, 58]. In addition, TLR2 is known to signal in complex with many different partners such as dectin-1, TLR1, or TLR6 [9, 59, 60], which can lead to different downstream pathway activation. Thus, we can also envision long chain ITFs signaling through a different TLR2 heterodimer or TLR2-complex than short chains, and in that way cause a different outcome in innate and even adaptive immune responses. Dendritic cells migrate to several draining lymph nodes including the nodes near the systemic intramuscular site where the pathogen-derived surface antigen is injected. There, the antigen is presented to B-, and T-cells, followed by a primary immune response [39].

The limitations of this study and the interpretation of results consist mainly of the relatively small group size, and the fact that it was set up as a pilot study. Keeping this in mind, our results can nevertheless be used to design a similar study with larger experimental group sizes, and different vaccines to confirm the long-chain ITF supplementation effects on human immunity in healthy subjects. As the applied supplements are highly pure and already approved as safe for human

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consumption, these prospective studies are likely to enable a general recommendation of DP10-60 ITF supplementation during vaccination programs.

This is the first *in vivo* study that demonstrates a structure-function relation of a dietary fiber on a human immune response against pathogenic surface antigens. We demonstrate that events in the gut that occur upon supplementing with a dietary fiber have consequences for systemically induced changes in the immune system. The *in vivo* immune stimulatory potential of long-chain enriched ITFs and absence of effect of shorter chain ITFs, underpins that not all dietary fibers exert beneficial health effects and that more specific recommendations for promoting health benefits may be necessary. Finally, we feel that the use of a low efficacy vaccination model such as hepB may be instrumental to demonstrate immunologically relevant effects of a nutritional supplements [15, 30, 32]. These human studies can be performed with relatively low numbers of subjects and still have a high statistical power.

Author contributions: L.M.V., M.E.E., M.M.F., and P.d.V. designed the research. L.M.V., T.B., and B.J.d.H. have carried out all experimental work, P.d.V. has supervised the study, L.M.V., M.M.F., and P.d.V. have written the manuscript. L.M.V. had primary responsibility for the final content.

We thank prof. Van Leer-Buter and coworkers for assistance in the high quality HBsAg titer measurements. This research was jointly financed by the European Union, European Regional Development Fund and the Ministry of Economic Affairs, Peaks in the Delta, the Municipality of Groningen, the Provinces of Groningen, Fryslan and Drenthe, as well as the Dutch Carbohydrate Competence Center (CCC WP2a), and by Royal Cosun. Within the framework of the Carbohydrate Competence Center (CCC WP2a) to which Royal Cosun contributed both financially and

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intellectually, the data obtained in the course of this study and reported in this article are considered proprietary data of the parties to CCC WP2a, patent pending.

The authors have declared no conflicts of interest.

5 References

[1] Aune, D., Chan, D.S., Lau, R., Vieira, R., et al., Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *BMJ* 2011, *343*, d6617.

[2] Schulze, M.B., Schulz, M., Heidemann, C., Schienkiewitz, A., et al., Fiber and magnesium intake and incidence of type 2 diabetes: a prospective study and meta-analysis. *Arch. Intern. Med.* 2007, *167*, 956–965.

[3] Pereira, M.A., O'Reilly, E., Augustsson, K., Fraser, G.E., et al., Dietary fiber and risk of coronary heart disease: a pooled analysis of cohort studies. *Arch. Intern. Med.* 2004, *164*, 370–376.

[4] Vuksan, V., Jenkins, A.L., Jenkins, D.J., Rogovik, A.L., et al., Using cereal to increase dietary fiber intake to the recommended level and the effect of fiber on bowel function in healthy persons consuming North American diets. *Am. J. Clin. Nutr.* 2008, *88*, 1256–1262.

[5] Weber, T.K., Toporovski, M.S., Tahan, S., Neufeld, C.B., et al., Dietary fiber mixture in pediatric patients with controlled chronic constipation. *J. Pediatr. Gastroenterol. Nutr.* 2014, *58*, 297–302.

[6] Kleessen, B., Sykura, B., Zunft, H.J., Blaut, M., Effects of inulin and lactose on fecal

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microflora, microbial activity, and bowel habit in elderly constipated persons. *Am. J. Clin. Nutr.* 1997, *65*, 1397–1402.

[7] Marteau, P., Jacobs, H., Cazaubiel, M., Signoret, C., et al., Effects of chicory inulin in constipated elderly people: a double-blind controlled trial. *Int. J. Food Sci. Nutr.* 2011, *62*, 164–170.

[8] Lopez Roman, J., Martinez Gonzalvez, A.B., Luque, A., Pons Minano, J.A., et al., The effect of a fibre enriched dietary milk product in chronic primary idiopatic constipation. *Nutr. Hosp.* 2008, *23*, 12–19.

[9] Oliveira-Nascimento, L., Massari, P., Wetzler, L.M., The Role of TLR2 in Infection and Immunity. *Front. Immunol.* 2012, *3*, eCollection 2012.

[10] Sonnenburg, E.D., Sonnenburg, J.L., Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. *Cell Metab.* 2014, *20*, 779–786.

[11] Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., et al., The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* 2013, *341*, 569–73.

[12] Breton, J., Plé, C., Guerin-Deremaux, L., Pot, B., et al., Intrinsic immunomodulatory effects of low-digestible carbohydrates selectively extend their anti-inflammatory prebiotic potentials. *Biomed Res. Int.* 2015, *2015*, 162398.

[13] Vogt, L., Ramasamy, U., Meyer, D., Pullens, G., et al., Immune Modulation by Different Types of Beta(2-1)-Fructans Is Toll-Like Receptor Dependent. *PLoS One* 2013, *8*.

[14] Popov, S. V, Ovodov, Y.S., Polypotency of the immunomodulatory effect of pectins. *Biochemistry.Biokhimiia* 2013, *78*, 823–835.

[15] Saavedra, J.M., Tschernia, A., Human studies with probiotics and prebiotics: clinical implications. *Br. J. Nutr.* 2002, *87 Suppl 2*, S241-6.

This article is protected by copyright. All rights reserved.

Accepted Article

[16] Duggan, C., Penny, M.E., Hibberd, P., Gil, A., et al., Oligofructose-supplemented infant cereal: 2 randomized, blinded, community-based trials in Peruvian infants. *Am. J. Clin. Nutr.* 2003, *77*, 937–942.

[17] Chuang, S.C., Norat, T., Murphy, N., Olsen, A., et al., Fiber intake and total and cause-specific mortality in the European Prospective Investigation into Cancer and Nutrition cohort. *Am. J. Clin. Nutr.* 2012, *96*, 164–174.

[18] Landberg, R., Dietary fiber and mortality: convincing observations that call for mechanistic investigations. *Am. J. Clin. Nutr.* 2012, *96*, 3–4.

[19] van Hoffen, E., Ruiter, B., Faber, J., M'Rabet, L., et al., A specific mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides induces a beneficial immunoglobulin profile in infants at high risk for allergy. *Allergy* 2009, *64*, 484–487.

[20] Raes, M., Scholtens, P.A., Alliet, P., Hensen, K., et al., Exploration of basal immune parameters in healthy infants receiving an infant milk formula supplemented with prebiotics. *Pediatr. Allergy Immunol.* 2010, *21*, e377-85.

[21] Perez, N., Iannicelli, J.C., Girard-Bosch, C., Gonzalez, S., et al., Effect of probiotic supplementation on immunoglobulins, isoagglutinins and antibody response in children of low socio-economic status. *Eur. J. Nutr.* 2010, *49*, 173–179.

[22] Scholtens, P.A., Alliet, P., Raes, M., Alles, M.S., et al., Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. *J. Nutr.* 2008, *138*, 1141–1147.

[23] Arslanoglu, S., Moro, G.E., Boehm, G., Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. *J. Nutr.* 2007, *137*, 2420–2424.

This article is protected by copyright. All rights reserved.

Accepted Article

[24] Bunout, D., Hirsch, S., Pia de la Maza, M., Munoz, C., et al., Effects of prebiotics on the immune response to vaccination in the elderly. *JPEN.Journal Parenter. Enter. Nutr.* 2002, *26*, 372–376.

[25] Guigoz, Y., Rochat, F., Perruisseau-Carrier, G., Effects of oligosaccharide on the faecal flora and nonspecific immunesystem in elderly people. *Nutr. Res.* 2002, *22*, 13–25.

[26] Schiffrin, E.J., Thomas, D.R., Kumar, V.B., Brown, C., et al., Systemic inflammatory markers in older persons: the effect of oral nutritional supplementation with prebiotics. *J. Nutr. Health Aging* 2007, *11*, 475–479.

[27] Vulevic, J., Drakoularakou, A., Yaqoob, P., Tzortzis, G., et al., Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *Am. J. Clin. Nutr.* 2008, *88*, 1438–1446.

[28] Amati, L., Marzulli, G., Martulli, M., Pugliese, V., et al., Administration of a synbiotic to free-living elderly and evaluation of serum cytokines. A pilot study. *Curr. Pharm. Des.* 2010, *16*, 854–858.

[29] Haschke, F., Firmansyah, A., Meng, M., Steenhout, P., et al., Functional food for infants and children. *Monatsschrift Kinderheilkd.* 2001, *149*, S66–S70.

[30] Stam, J., van Stuijvenberg, M., Garssen, J., Knipping, K., et al., A mixture of three prebiotics does not affect vaccine specific antibody responses in healthy term infants in the first year of life. *Vaccine* 2011, *29*, 7766–7772.

[31] van den Berg, J.P., Westerbeek, E.A., van der Klis, F.R., Berbers, G.A., et al., Neutral and acidic oligosaccharides supplementation does not increase the vaccine antibody response in preterm infants in a randomized clinical trial. *PLoS One* 2013, *8*, e70904.

[32] Hoebe, C.J., Vermeiren, A.P., Dukers-Muijrers, N.H., Revaccination with Fendrix(R) or

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HBVaxPro(R) results in better response rates than does revaccination with three doses of Engerix-B(R) in previous non-responders. *Vaccine* 2012, *30*, 6734–6737.

[33] Seifert, S., Watzl, B., Inulin and oligofructose: review of experimental data on immune modulation. *J. Nutr.* 2007, *137*, 2563S–2567S.

[34] Lomax, A.R., Calder, P.C., Prebiotics, immune function, infection and inflammation: a review of the evidence. *Br. J. Nutr.* 2009, *101*, 633–658.

[35] Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., et al., Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* 2010, *104 Suppl*, S1-63.

[36] Vogt, L.M., Meyer, D., Pullens, G., Faas, M.M., et al., Immunological properties of inulin-type fructans. *Crit. Rev. Food Sci. Nutr.* 2013.

[37] Julious, S.A., Sample sizes for clinical trials with normal data. *Stat. Med.* 2004, *23*, 1921–1986.

[38] Albarran, B., Goncalves, L., Salmen, S., Borges, L., et al., Profiles of NK, NKT cell activation and cytokine production following vaccination against hepatitis B. *APMIS* 2005, *113*, 526–535.

[39] Siegrist, C.A., Vaccine Immunology, in: Plotkin, S., Orenstein, W., Offit, P. (Eds.), *Vaccines*, Elsevier, e-book. 2013, pp. 17–36.

[40] Wills, M.R., Carmichael, A.J., Weekes, M.P., Mynard, K., et al., Human virus-specific CD8+ CTL clones revert from CD45ROhigh to CD45RAhigh in vivo: CD45RAhighCD8+ T cells comprise both naive and memory cells. *J. Immunol. (Baltimore, Md. 1950)* 1999, *162*, 7080–7087.

[41] Vos, A.P., Haarman, M., Buco, A., Govers, M., et al., A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. *Int.*

This article is protected by copyright. All rights reserved.

Accepted Article

Immunopharmacol. 2006, *6*, 1277–1286.

[42] Vos, A.P., Haarman, M., van Ginkel, J.W., Knol, J., et al., Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. *Pediatr. Allergy Immunol.* 2007, *18*, 304–312.

[43] Bakker-Zierikzee, A.M., Tol, E.A., Kroes, H., Alles, M.S., et al., Faecal SIgA secretion in infants fed on pre- or probiotic infant formula. *Pediatr. Allergy Immunol.* 2006, *17*, 134–140.

[44] Macfarlane, G.T., Steed, H., Macfarlane, S., Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* 2008, *104*, 305–344.

[45] Shadid, R., Haarman, M., Knol, J., Theis, W., et al., Effects of galactooligosaccharide and long-chain fructooligosaccharide supplementation during pregnancy on maternal and neonatal microbiota and immunity--a randomized, double-blind, placebo-controlled study. *Am. J. Clin. Nutr.* 2007, *86*, 1426–1437.

[46] Pozo, D., Valés-Gómez, M., Mavaddat, N., Williamson, S.C., et al., CD161 (Human NKR-P1A) Signaling in NK Cells Involves the Activation of Acid Sphingomyelinase. *J. Immunol.* 2006, *176*, 2397–2406.

[47] Lomax, A.R., Calder, P.C., Probiotics, immune function, infection and inflammation: a review of the evidence from studies conducted in humans. *Curr. Pharm. Des.* 2009, *15*, 1428–1518.

[48] Kelly, G., Inulin-type prebiotics: a review. (Part 2). *Altern. Med. Rev.* 2009, *14*, 36–55.

[49] Rijnierse, A., Jeurink, P. V, Garssen, J., Knippels, L.M., Food-derived oligosaccharides exhibit pharmaceutical properties. *Eur. J. Pharmacol.* 2011.

[50] Alles, M.S., Hautvast, J.G., Nagengast, F.M., Hartemink, R., et al., Fate of fructo-oligosaccharides in the human intestine. *Br. J. Nutr.* 1996, *76*, 211–221.

This article is protected by copyright. All rights reserved.

Accepted Article

[51] Rumessen, J.J., Bode, S., Hamberg, O., Gudmand-Hoyer, E., Fructans of Jerusalem artichokes: intestinal transport, absorption, fermentation, and influence on blood glucose, insulin, and C-peptide responses in healthy subjects. *Am. J. Clin. Nutr.* 1990, *52*, 675–681.

[52] van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H., et al., Inulin-type fructans of longer degree of polymerization exert more pronounced in vitro prebiotic effects. *J. Appl. Microbiol.* 2007, *102*, 452–460.

[53] Meijer, K., de Vos, P., Priebe, M.G., Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? *Curr. Opin. Clin. Nutr. Metab. Care* 2010, *13*, 715–721.

[54] Smelt, M.J., de Haan, B.J., Bron, P.A., van Swam, I., et al., Probiotics can generate FoxP3 T-cell responses in the small intestine and simultaneously inducing CD4 and CD8 T cell activation in the large intestine. *PLoS One* 2013, *8*, e68952.

[55] Cambi, A., Figdor, C.G., Dual function of C-type lectin-like receptors in the immune system. *Curr. Opin. Cell Biol.* 2003, *15*, 539–546.

[56] Abreu, M.T., Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat. Rev.* 2010, *10*, 131–144.

[57] Abadie, V., Discepolo, V., Jabri, B., Intraepithelial lymphocytes in celiac disease immunopathology. *Semin. Immunopathol.* 2012, *34*, 551–566.

[58] Visintin, A., Latz, E., Monks, B.G., Espevik, T., et al., Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. *J. Biol. Chem.* 2003, *278*, 48313–48320.

[59] Wetzler, L.M., The role of Toll-like receptor 2 in microbial disease and immunity. *Vaccine* 2003, *21 Suppl 2*, S55-60.

This article is protected by copyright. All rights reserved.

[60] van Bergenhenegouwen, J., Plantinga, T.S., Joosten, L.A., Netea, M.G., et al., TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors. *J. Leukoc. Biol.* 2013, *94*, 885–902.

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Figure legends

Figure 1. Activation of TLR2 by DP10-60 and DP2-25 ITFs. HEK293-hTLR2 reporter cells were stimulated and activation of NF-κB was plotted as fold-induction of control (medium). Concentrations of ITFs are in μg/mL. Statistical differences as compared to control were determined with a Kruskal-Wallis test and Dunn's post test, *($p<0.05$), n=5. HKLM, heat killed *Listeria monocytogenes*, 10^8 cells/mL.

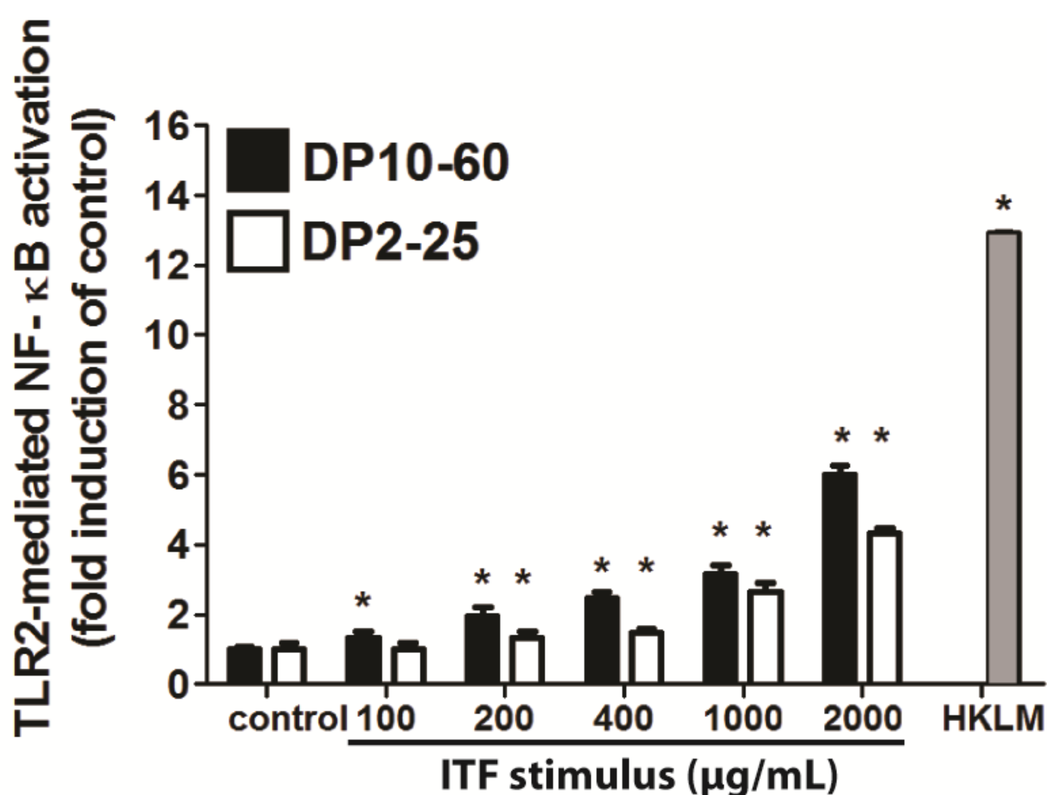

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Figure 2. Human PBMC cytokine profiles induced by *in vitro* stimulation with ITFs of DP10-60 and DP2-25 in isolated human PBMCs. Relative cytokine production as compared to unstimulated controls was plotted for treated PBMCs (DP10-60 in black bars, DP2-25 ITFs in white bars, both 100 μg/mL). Wilcoxon signed rank test was used to test for differences in cytokine induction between the different ITFs, * ($p < 0.05$), n=6.

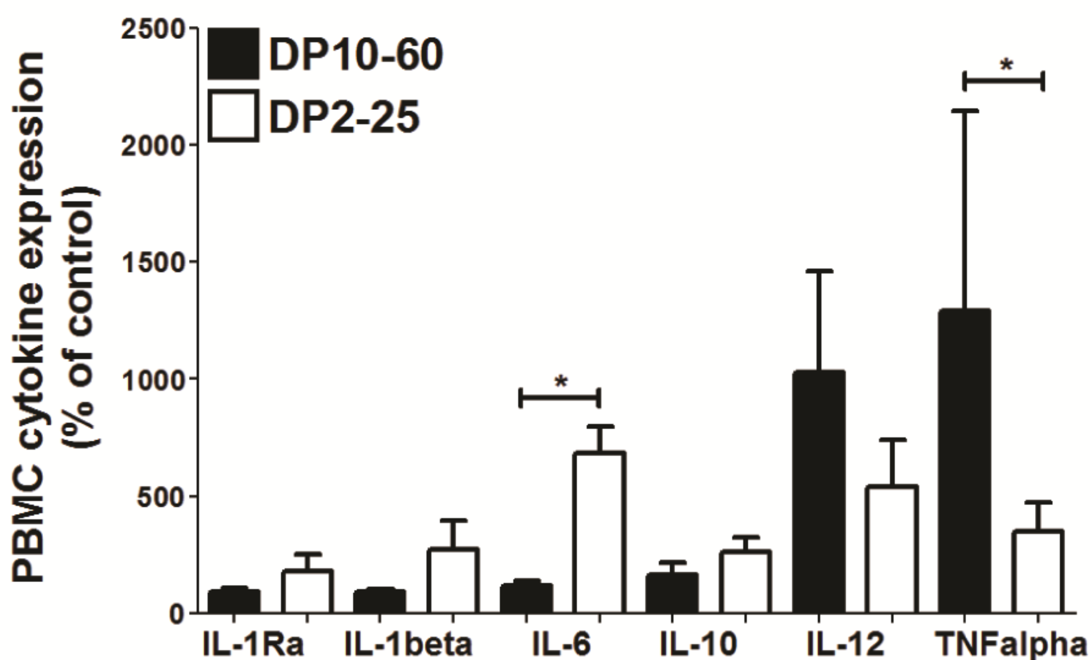

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Figure 3. Anti-HBsAg antibody titer development in ITF-, and placebo supplemented individuals in time. Statistical significance levels were determined with a Friedman test and Dunn's post-test for individual supplements to test for titer development compared to basal samples at T0 (A-C), and Mann Whitney for differences between supplements (D). Median and IQR of the anti-HBsAg titers are plotted in IU/mL. n=13, n=13, and n=14 respectively for the supplemental groups DP10-60, DP2-25, and placebo. p=0.1 indicates a statistical trend, * (p<0.05), ** (p<0.01).

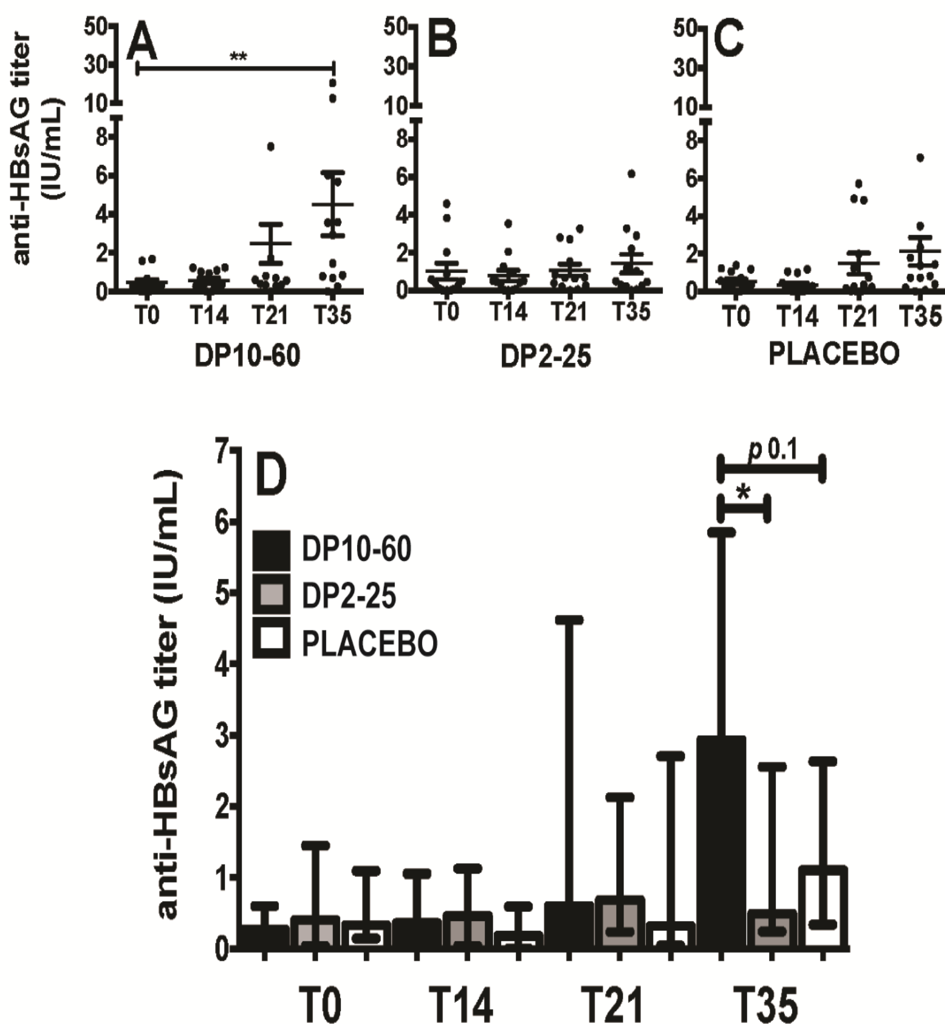

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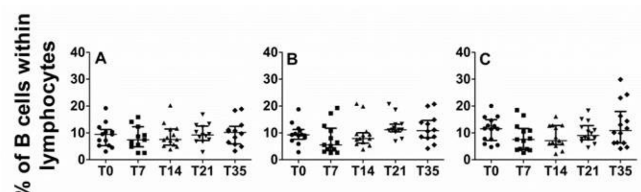
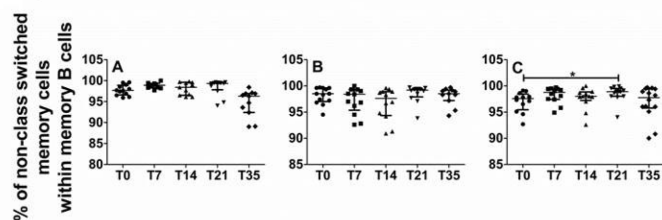
Figure 4. B-cells and B-cell subsets as percentages of lymphocytes, or within subsets in time. I) Percentage of B-cells within lymphocyte population. II) Percentage of non-class-switched IgM^+ memory cells ($CD19^+$ $CD27^+$ IgD^+ IgM^+), within memory B-cell population. III) Percentage of IgM^{hi} transitional B-cells within $CD38^{hi}$ B-cell parent population. A) DP10-60 fructans, B) DP2-25 fructans, C) placebo. A Friedman test and a Dunn's post-test were used to analyze time effect per supplement. Median and IQR are plotted as percentage of the indicated cell populations, n=13, 13, and 14 for supplement DP10-60, DP2-25, and placebo respectively. * ($p<0.05$), ** ($p<0.01$).

I) % of B cells within lymphocyte population



II) % of non-class-switched IgM^+ memory cells within memory B cell population



III) % of IgM^{hi} transitional B cells within $CD38^{hi}$ B cell parent population

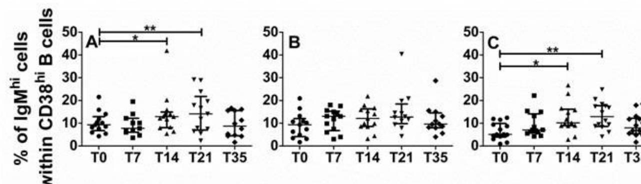


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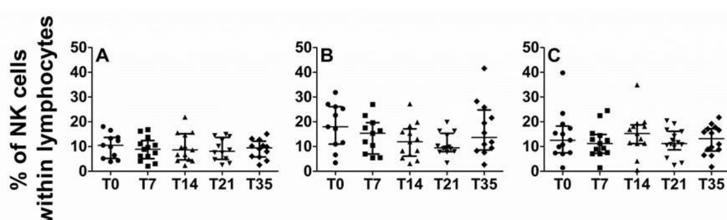
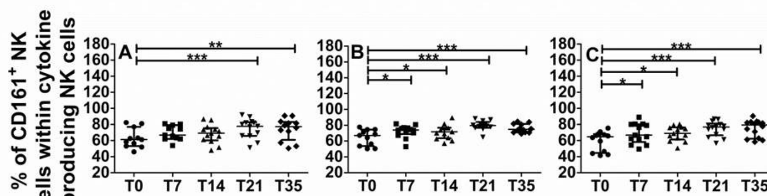
Figure 5. NK-cells, CD161^{+} NK-cell subset, and NKT-cells as percentage within lymphocytes or parent NK-cell population. I) Percentage of NK-cells within lymphocyte population. II) Percentage of CD161^{+} cells within cytokine producing NK-cell population. III) Percentage of NKT-cells within lymphocyte population. A) DP10-60 fructans, B) DP2-25 fructans C) placebo. Repeated measures ANOVA and Tukey's post-test or a Friedman test and a Dunn's post-test were used to analyze time effect per supplement. Average and SEM, or median and IQR are plotted as percentage of the indicated cell populations, n=13, 13, and 14 for supplement DP10-60, DP2-25, and placebo respectively. p=0.1 indicates a statistical trend, * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

I) % of NK cells within lymphocyte population



II) % of CD161^{+} cells Within cytokine producing NK cell population



III) % of NKT cells within lymphocyte population

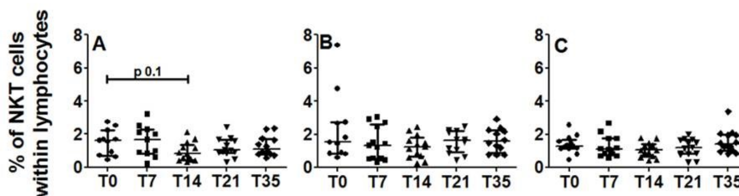


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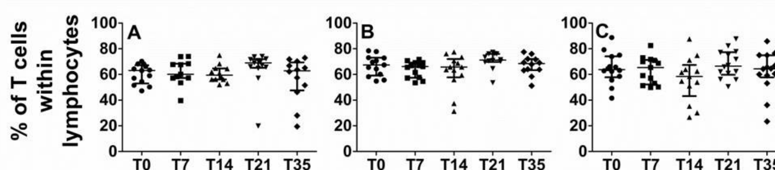
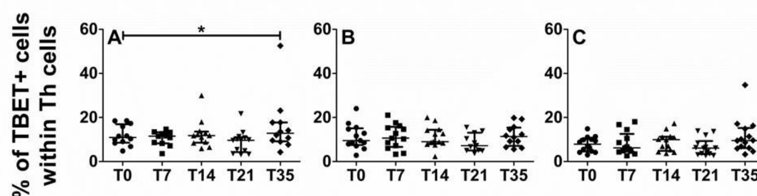
Figure 6. T-cells as percentage of lymphocytes, and T-cell subsets expressed as percentages of Th-cells or T-memory cells in time, plotted per supplement. I) Percentage of T-cells within lymphocyte population. II) Percentage of Th1-cells (TBET^{+}) within Th-cell population. III) Percentage of CD45RO^{hi} CTLs within CTL population. IV) Percentage of CD45RO^{hi} Th-cells within Th-cell population. A) DP10-60 fructans, B) DP2-25 fructans C) placebo. A Friedman test and a Dunn's post-test were used to analyze time effect per supplement. Median and IQR are plotted as percentage of the indicated cell populations, n=13, 13, and 14 for supplement DP10-60, DP2-25, and placebo respectively. * (p < 0.05).

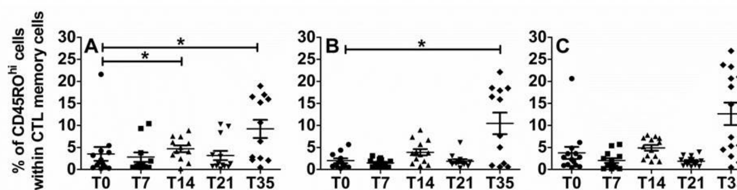
I) % of T cells within lymphocyte population



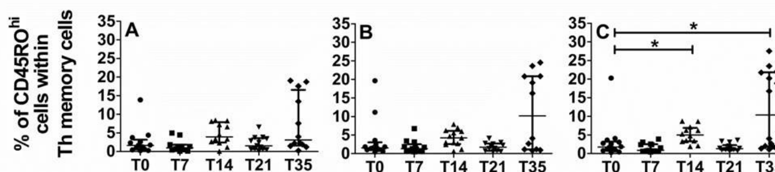
II) % of Th1 cells within Th population



III) % of CD45RO^{hi} CTLs within CTL memory cell population



IV) % of CD45RO^{hi} Th cells within Th memory cell population



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Tables

Table 1. Background of participants in experimental groups.

	DP10-60 group	DP2-25 group	Placebo group
Total number	13	13	14
Gender, females : males	8 : 5	7 : 6	8 : 6
Age (yrs.), mean, min-max.	20, 19-26	20, 18-29	20, 18-25
Dietary fiber intake (g/day), mean, min-max)	19.29, 13.23-28.1	17.3, 12.91-23.08	16.72, 10.87-22.22

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