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Dietary Inulin Supplementation Modulates Fecal Microbiota and Vaccine Response in Cats—A Double-Blind Randomized Controlled Pilot Study

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Abstract: Twenty-six cats were fed either a control or an inulin-supplemented diet (0.6%) for six weeks with measurements of fecal short-chain fatty acids (SCFAs) and microbiota. In the second phase, cats were fed their respective diets for another six weeks and were then primo-vaccinated against the feline leukemia virus (FeLV) at days 0 and 21. Serum anti-FeLV IgG responses were monitored. Compared to the control group, the inulin group exhibited higher Firmicutes (+14%) and lower Bacteroidetes (−56%) and Prevotellaceae (−51%) after six weeks of supplementation. Compared to T0, SCFAs initially decreased at week 3 but subsequently increased at week 6 with inulin supplementation, leading to higher butyrate compared to the control group at week 6. A significant diet-by-time interaction was also observed for propionate and total SCFAs. Compared to the control group, the inulin group tended to show a higher serum anti-FeLV IgG response after the first vaccination ($p = 0.09$), with significant differences at days 5 and 11 post-vaccination. These preliminary results suggest that a six-week dietary supplementation with a low dose of inulin in cats can modify the fecal microbiota and its functional metabolites, potentially influencing the early immune response to vaccination.

Keywords: inulin; fecal microbiota; prebiotic; immune response; fructans; short-chain fatty acids

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1. Introduction

A stable and balanced microbiota is critical for maintaining intestinal health and immunity. Thus, finding nutritional strategies to keep a balanced microbiota is of interest. Prebiotics are defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit [1]. Inulin, a naturally occurring β-(2-1)-fructans, is a well-known prebiotic in various species. Based on chain length, inulin-type fructans are usually rather arbitrarily divided into subcategories with a relatively small (2–4) or medium (5–10) (generally referred to as fructooligosaccharides) or a relatively large chain length (11–60 fructose units) [2]. Natural inulin from chicory root has a chain length between 2 and 60 fructose units, with 75% < 10 and 25% > 10 units. The effect of inulin and/or fructooligosaccharides supplementation (0.5–4% in the cat diet) in promoting the growth of beneficial hindgut microbiota (e.g., *Lactobacillus* or *Bifidobacterium* spp.) has been previously reported in cats by several authors. Sparkes et al. (1998 a,b) showed an increase in Lactobacilli and a decrease in *Clostridium perfringens* and in *E. coli* with 0.75% of fructooligosaccharides for eight weeks [3,4]. Barry et al. [5] observed an increase in *Bifidobacterium* and a decrease in *E. coli* with 4% inulin+fructooligosaccharides for a month. Kanakupt et al. [6] showed an increase in

Bifidobacterium with 0.5% of oligofructose for three weeks. Barry et al. [7] observed an increase in Actinobacteria (including *Bifidobacterium* spp.) with 4% inulin+fructooligosaccharides for a month. García Mazcorro et al. [8] showed an increase in Veillonellaceae and a decrease in Gammaproteobacteria with 0.4% inulin+fructooligosaccharides for two weeks. Whether variations in chain-length composition cause different physiological responses is currently unknown. In addition, studies testing the effects of low-dose inulin on cats' microbiota are missing.

Vaccination is a controlled way to expose the immune system to a specific amount and type of antigen. It has been proposed that the body's response to this antigenic exposure is the most relevant way to assess the functioning of the immune system in the absence of an infectious challenge [9]. A study in humans showed that long-chain (10–60 units), but not short-chain, fructans (2–25 units) boosted the immune response to a hepatitis B vaccine [10]. Another study demonstrated that a mix of long-chain inulin and short-chain oligofructose enhanced some aspects of the immune response to the seasonal flu vaccine [11]. To the authors' knowledge, so far, no studies have been performed using response to vaccination as a tool to examine the effect of β(2-1)-fructans inulin on adult cat immune function.

The aim of this double-blind randomized controlled pilot study was, therefore, to evaluate the effect of three to six weeks of dietary inulin supplementation (0.6% chicory root inulin) on fecal short-chain fatty acids (SCFAs), fecal microbiota, and immune function in middle-aged cats.

This article is a revised and expanded version of a paper entitled Effects of dietary inulin supplementation on the fecal microbiota and its function and on vaccine response in cats, which was presented at the 27th Congress of the European Society of Veterinary and Comparative Nutrition, Vila Real, Portugal, 7–9 September 2023 [12].

2. Materials and Methods

2.1. Animals

Neutered male (n = 12) and neutered female (n = 14) cats, all four years old, were selected for this randomized, double blinded, placebo-controlled pilot study. All animals were considered healthy after a physical examination by a veterinarian, complete blood count, and serum biochemistry. They were all fully vaccinated against feline calicivirus (FCV), feline herpes virus (FHV), and feline panleukopenia virus (FPV), with the last vaccine administered 1 year and 3 months before the vaccine challenge in the second phase of the study (see Section 2.3). Cats tested negative for feline leukemia virus (FeLV) at their entrance to the center, and they had never been vaccinated against FeLV before the beginning of this study (see Section 2.3). The cats were housed in two groups of 13 animals in two indoor parks (40 m^2 each, with climatization to maintain the temperature at around 21 °C) at Affinity Nutrition Center (Affinity-Petcare SA, Barcelona, Spain), with free access to two outdoor parks (9 m^2 each). Indoor and outdoor parks were enriched with different elements to allow the expression of natural behavior and behavioral needs, such as scratching, hiding, and climbing. Cats interacted with their caretakers at least three times per day, and trained caretakers monitored the well-being of the cats daily through visual examination.

2.2. Study Part 1: Short-Chain Fatty Acids, CD4+, CD8+, Microbiota

In the first part of the study (microbiota composition and activity), the 26 cats were randomly divided into two groups of thirteen cats each (six males and seven females). All 26 cats in both groups were fed a complete and balanced extruded dry diet formulated for adult cats (control) for one month (T0) (Table 1). After this period, one group of cats (control group) remained on the control diet, while the second group (inulin group) received the same diet supplemented with 0.6% inulin for six weeks (0.7% Inulin SIPS, Beneo-Orafti, Tienen, Belgium, with a minimum of 86% purity, degree polymerization from 2 to 60 fructose units, with 75% < 10 and 25% > 10 units) (Table 1, Figure 1). The dose of inulin was arbitrarily determined from previously published studies showing effects

with doses as low as 0.4 to 0.75%. A specific ingredient (corn) was decreased by 0.7% and replaced by 0.7% of the inulin ingredient (0.6% pure inulin).

Table 1. Metabolizable energy, main analyzed [13] nutrients, and main ingredients of the control and inulin diets.

		Control	Inulin
Metabolizable energy *	Kcal/kg	3932	3914
Main nutrients			
Moisture	% as is	5	5
Protein	% as is	35	35
Fat	% as is	13	13
Ash	% as is	6.7	6.7
Crude fiber	% as is	1.5	1.9
Total dietary fiber	% as is	6.1	7.4
Methionine	% as is	0.9	0.9
Tryptophan	% as is	0.2	0.2
Lysine	% as is	1.6	1.7
Threonine	% as is	1.3	1.2
Main ingredients			
Chicken slurry	% as is	15	15
Whole wheat	% as is	14	14
Poultry meal	% as is	11	11
Corn gluten meal	% as is	11	11
Rice	% as is	11	11
Pork meal	% as is	12	12
Corn	% as is	8.9	8.2
Sugar beet pulp	% as is	2	2
Inulin (ingredient)	% as is	-	0.7

* Calculated with NRC equation (2006), as reported in Fediaf guidelines [13].

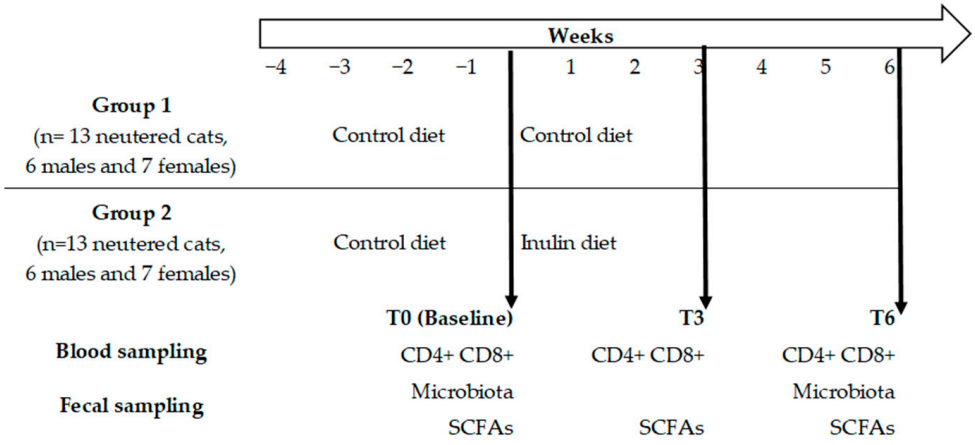

Figure 1. Experimental design of the first part of the study: Measurement of fecal short-chain fatty acids, fecal microbiota and blood CD4+, and CD8+ at baseline and after 3 and/or 6 weeks of feeding control or inulin diet.

Individual feed intake was adapted every week to maintain a stable body weight. Water was available *ad libitum*. Spontaneously voided fresh fecal samples were obtained from each cat at T0 and after three (T3) and six weeks (T6). Fecal short-chain fatty acids (SCFAs) (butyrate, acetate, propionate) were measured at T0, T3, and T6 by gas chromatography using an Agilent 6890 gas liquid chromatograph (Agilent Technologies España, S.L., Madrid, Spain) fitted with a capillary column (HP-FFAP polyethylene glycol TPA-treated, 30 m × 530 µm i.d. × 1 µm film thickness) and a flame ionization detector.

EDTA-anticoagulated blood samples were obtained at T0, T3, and T6 for CD4+ and CD8+ lymphocyte count determination by flow cytometry. Briefly, a blood volume of

500 µL was lysed with 1 mL of a red blood cell lysis buffer (ACK lysis buffer, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at room temperature (RT). After incubation, samples were washed in 1 mL of phosphate buffer saline (PBS) with 1% bovine serum albumin (BSA) washing solution (Sigma-Aldrich, St. Louis, MO, USA), centrifuged (350 g, 5 min), and lysed and washed a second time, and the resulting cell pellet was resuspended in 400 µL of a cryopreservation medium (CryoStor®, Merck, St. Louis, MO, USA) and frozen at −80 °C until analysis. All samples were analyzed on the same day. The cryopreserved samples were rapidly defrosted in a 37 °C bath, washed with washing solution, centrifuged, and resuspended in 100 µL of solution for antibody labeling (PBS/2% fetal bovine serum (FBS)/0.1% sodium azide). Cells were exposed to anti-feline-specific CD4 and CD8 monoclonal antibodies conjugated with fluorescein (FITC) and phycoerythrin (PE) (CD4-FITC MCA1346F), CD8-PE (MCA1347 PE), from Bio-Rad, Oxford, UK) for the characterization of T helper lymphocytes and T cytotoxic lymphocytes, respectively. The recommended antibody concentration from the manufacturer was added to the cells and incubated for 30 min at room temperature. After labeling, the cells were washed with 1 mL of the washing solution, centrifuged, and resuspended in 300 µL of solution (PBS/2% FBS/0.1% sodium azide) for flow cytometry analysis. Samples were analyzed using a Cytoflex LX flow cytometer (Beckman Coulter, Madrid, Spain) and CytExpert and FlowJo V10 software. Lymphocytes were initially identified by forward- and side-scattered properties, and the studied lymphocytes subsets were detected by gating the different positive or negative CDs using dot plots or histograms as appropriate.

At T0 and T6, microbial DNA was extracted from 200 mg of a fecal sample using ZymoBIOMICS 96 DNA Kit (Zymo research, Irvine, CA, USA). To monitor for potential contamination of the laboratory or reagents, blank samples were processed without the initial material. To assess the performance of the DNA extraction and sequencing, a positive control (ZymoBIOMICS Microbial Community Standard, Zymo Research, Irvine, CA, USA) was also co-processed and analyzed. PCR amplification, library preparation, and massive sequencing of the V3–V4 hypervariable regions of the 16S rRNA gene were PCR-amplified, and the sequencing library was prepared using Quick-16S NGS Library Prep Kit for each included sample. No template control (NTC) samples were included in each PCR reaction to monitor for potential contamination in this step. Samples were sequenced on an Illumina MiSeq® using MiSeq® Reagent Kit v3 (600-cycle) under the manufacturer's conditions (Illumina, Eindhoven, The Netherlands). After sequencing, the reads were demultiplexed and separated by barcode. Then, the sequences were imported into the Quantitative Insight Into Microbial Ecology 2 (QIIME2) software, which was used to further analyze the data.

Within the QIIME2 pipeline, DADA2 was used as a quality filtering method in order to denoise and dereplicate paired-end sequences. This first step provided filtered and non-chimeric sequences without the primers and with a mean length of 410 bp. Sequences were classified into Amplicon Sequence Variant (ASV), which were used to classify and assign taxonomy to the sequences using SILVA 132 at 99% identity to reduce redundancy. The taxonomic analysis was performed for each sample or group of samples at all the taxonomic levels. Alpha- and beta-diversity analyzed the differences within and among samples, respectively, and were carried out using a sequencing depth of 40,500 sequences per sample. Alpha-diversity is represented by observed species (richness or the total number of features) and the Shannon index (evenness or the relative abundance of features in addition to the richness). Beta-diversity estimates how similar the microbiome profiles are among different samples, and we calculated them using the Bray–Curtis and unweighted and weighted Unifrac distance matrices. Bray–Curtis considers the microbiome composition with the relative abundance; weighted UniFrac considers the phylogeny, apart from the microbiome composition and the relative abundance; and finally, unweighted UniFrac is a qualitative metric that considers the microbiome composition and its phylogeny, but without considering the relative abundance.

2.3. Study Part 2: Vaccine Challenge and Specific Immune Response

In the second part of the study (specific immune response), after a six-month washout period with the control diet, the same cats were again divided into the two dietary groups as above (control vs. 0.6% inulin diet) (day −42). Six weeks after the change in diet (day 0), the cats were primo-vaccinated against FeLV with a recombinant *E. coli* FeLV p45 vaccine (Leucogen, Virbac, Carros, France), with a second vaccination after 21 days. Native blood (3 mL) was obtained from each cat from the cephalic vein and coagulated for 45 min before being centrifuged for 10 min at 2000× *g*. The serum was then separated and frozen at −80 °C until analysis. Anti-FeLV p45 antibodies (IgG) were measured in the serum by ELISA before vaccination (day −42, 0), after the first vaccination (days 5, 11, 14, 21), and after the second vaccination (day 25, 28, 34, 42, 56) as described [14] (Figure 2).

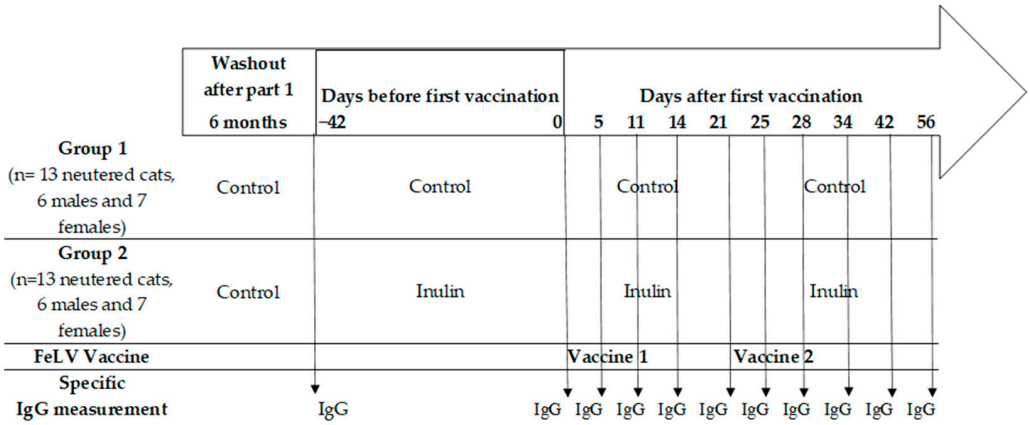

Figure 2. Experimental design of the second part of the study: specific IgG measurement in cats fed control or inulin-supplemented diet before first vaccination against feline leukemia virus (Day −42 and 0), after first vaccine (day 5 to 21), and after vaccine booster (day 25 to 56).

2.4. Statistics

SCFAs, CD4+, and CD8+ were log-transformed in the case of non-normal distribution and statistically analyzed using a mixed linear model for repeated measurements with diet, time, and their interaction as fixed effects, and with cat identity as a random effect. The simple effects of group*time least squares means were compared by time and by treatment, with Sidak adjustment.

The statistical significance of the alpha- and beta-diversity was analyzed using either independent or paired approaches. For independent analyses comparing both biologic groups at each time point, we used the QIIME2 diversity plugin. For paired analyses comparing the differences between paired individuals from each group at two different time points, we used the QIIME2 longitudinal plugin [15].

The statistical significance of the taxonomy was performed using Linear Discriminant Analysis (LDA) effect size (LEfSe). LEfSe is an algorithm to find significant differences in taxa among groups using the non-parametric factorial Kruskal–Wallis rank-sum test and Wilcoxon rank-sum test (if subclasses used). As a last step, LEfSe uses Linear Discriminant Analysis to estimate the effect size of each differentially abundant feature [16]. To assess taxonomical differences among the biological groups, we applied LEfSe in Galaxy URL (http://galaxy.biobakery.org/root?tool_id=LEfSe_for, accessed on 10 March 2020). We split the main data per time point and assessed whether there were differences among the control and inulin groups.

Anti-FeLV p45 antibodies, log-transformed in the case of non-normal distribution, were analyzed using a mixed generalized linear model for repeated measurements with diet, time, and their interaction as fixed effects and with cat identity as a random effect. Due to the specific pattern of the IgG response with different residual variability (before vaccination, after vaccine 1, and after booster), the data were not analyzed altogether, but were split into these three phases. No interaction was used in the pre-vaccination phase. No multiplicity

correction was applied, since the study was considered an exploratory pilot experiment and the analysis of each time or group of times aimed to answer different questions.

For all the analyses, a p-value ≤ 0.05 was considered as significant. A p-value ≤ 0.1 was considered as a trend. Values are presented as mean $\pm$ standard deviation.

2.5. Ethical Approval

The protocol was approved by the Government of Catalonia (Expedient ID: FUE-2018-00721761, reference number: CEA-OH/10002/1), following the European Union guidelines for the ethical care and handling of animals under experimental conditions, and by Affinity Petcare Ethical Committee (Study ID: RS000305).

3. Results

3.1. SCFAs, CD4+, CD8+, and Fecal Microbiota (Part 1)

- A time effect was observed for all the fecal SCFAs and their sum, with the exception of acetate, which showed only a trend. After a numerical or significant decrease was observed at T3 compared to T0, all the SCFAs were significantly increased at T6 with inulin supplementation compared to T3. A diet-by-time interaction was or tended to be observed for acetate (p = 0.06), propionate (p = 0.04), and the sum of SCFAs (p = 0.04), with a numerical increase in these SCFAs at T6 with inulin supplementation and a numerical decrease with the control compared to the respective T0 (Table 2). A diet effect tended to be observed for fecal butyrate (p = 0.07), with a significantly higher butyrate concentration at T6 with inulin compared to the control (p = 0.02). When looking at the preplanned simple comparison, compared to the control group, the inulin group also tended to have a higher sum of SCFAs (p = 0.08) after six weeks of supplementation (Table 2).

Table 2. Simple effect comparisons of group*time least squares means by time and by treatment of the fecal short-chain fatty acid concentration (µmol/g dry matter, mean $\pm$ SD), as well as main p-values of the model.

		Acetate	Propionate	Butyrate	Sum
T0	Control	421 $\pm$ 159	240 $\pm$ 97 ^{b}	114 $\pm$ 51	775 $\pm$ 182
	Inulin	395 $\pm$ 120 ^{ab}	205 $\pm$ 74 ^{ab}	142 $\pm$ 64 ^{b}	742 $\pm$ 99 ^{b}
3 weeks	Control	391 $\pm$ 129	199 $\pm$ 58 ^{a}	93 $\pm$ 33	683 $\pm$ 199
	Inulin	364 $\pm$ 109 ^{a}	177 $\pm$ 59 ^{a}	106 $\pm$ 58 ^{a}	648 $\pm$ 205 ^{a}
6 weeks	Control	392 $\pm$ 166	189 $\pm$ 84 ^{a}	95 $\pm$ 40	676 $\pm$ 216
	Inulin	468 $\pm$ 179 ^{b}	224 $\pm$ 101 ^{b}	136 $\pm$ 60 ^{*b}	827 $\pm$ 233 $^{(*)b}$
p-values effects	Time	0.06	0.009	0.004	0.016
	Diet	0.94	0.61	0.07	0.74
	Diet*time	0.06	0.04	0.17	0.04

^{a,b} Different subscripts indicate significant differences ($p \leq 0.05$) between times (T0, 3 weeks, 6 weeks) in each treatment group (control or inulin). * indicates a significant difference ($p \leq 0.05$) and (*) indicates a trend ($p \leq 0.010$) that is different between control and inulin groups at each time point (T0, 3 weeks, or 6 weeks).

- A time effect was observed on CD4+ or CD8+, with a decrease over time compared to T0 with both diets. However, at T6, the percentage of CD4+ lymphocytes tended to be higher in the inulin group compared to the control group (p = 0.06) and numerically increased with inulin at T6 compared to T3 (Tables 3 and 4).

Table 3. Simple effect comparisons of group*time least squares means by time and by treatment of CD4 and CD8 (%, mean ± SD), as well as main *p*-values of the model.

		%CD4+	%CD8+
T0	Control	32 ± 5^{b}	18 ± 7^{b}
	Inulin	34 ± 7^{b}	19 ± 6^{b}
3 weeks	Control	28 ± 9^{a}	11 ± 6^{a}
	Inulin	27 ± 6^{a}	11 ± 6^{a}
6 weeks	Control	24 ± 7^{a}	13 ± 5^{a}
	Inulin	$31 \pm 7^{(*)a}$	12 ± 6^{a}
p-values effects	Time	<0.001	<0.001
	Diet	0.35	0.508
	Diet*time	0.144	0.7

[a,b] Different subscripts indicate significant differences ($p \leq 0.05$) between times (T0, 3 weeks, 6 weeks) in each treatment group (control or inulin). (*) indicates a trend ($p \leq 0.010$) that is different between control and inulin groups at each different time point (T0, 3 weeks, or 6 weeks).

Table 4. Amplicon Sequence Variant (ASV) alpha-diversity. *p*-values are given for diet effect at each time point.

	T0		6 Weeks		*p*-Values Effects			
					Diet		Time	
	Control	Inulin	Control	Inulin	T0 Control vs. Inulin	6 weeks Control vs. Inulin	T0 vs. T6 Control	T0 vs. T6 Inulin
ASV-Alpha-diversity	235 ± 48	219 ± 62^{a}	266 ± 73	271 ± 36^{b}	0.603	0.87	0.374	0.019

[a,b] Different subscripts indicate significant differences ($p \leq 0.05$) between times (T0, T6) in each treatment group.

- At T0, the fecal microbiota was dominated by the Firmicutes phylum (71%), followed by Actinobacteria (17–20%), Bacteroidetes (10–7%), Proteobacteria (1.5–2%), and Fusobacteria (less than 0.2%). The ten main families accounted for more than 90% of total bacteria and were Veillonellaceae (18.9%, among which were *Megasphaera*, *Megamonas*, and *Dialister*), Lachnospiraceae (16.5%, among which were *Blautia* and unclassified Lachnospiraceae), Erysipelotrichaceae (12.4%, among which were *Solobacterium*,*Catenibacterium*, *Faecalitalea*, and *Holdemanella*), Coriobacteriaceae (9.2%, mainly represented by *Collinsella*), Peptostreptococcaceae (8.3%, mainly *Peptoclostridium* and unclassified Peptostreptococcaceae), Prevotellaceae (7.3%, *Prevotella 9* and *Alloprevotella*), Ruminococcaceae (6.2%, represented by *Negativibacillus*, *Faecalibacterium*, and *Subdoligranulum*), Bifidobacteriaceae (5.2%, *Bifidobacterium*), Lactobacillaceae (4.6%, *Lactobacillus*), and Atopobiaceae (4.1%, *Olsenella*) (Supplementary Table S1).
- At T0, LEfSe analysis revealed that the fecal microbiota harbored some differences between the two groups, with higher relative abundances of Ruminococacceae and Lachnospiraceae and both members of Clostridia and Clostridiales in the inulin group, while the control group was characterized by higher relative abundances of Lactobacillales, Bacilli, and *Lactobacillus* (Figure 3). After six weeks, the fecal microbiota of cats fed with inulin was enriched in Firmicutes, *Faecalitalea*, and Family XIII and in Coribacteriia, Coriobacteriales, *Collinsella*, and Coriobacteriaceae (Figure 4). Conversely, the control group was enriched in Bacteroidetes, namely Bacteroidales, Bacteroidia, Prevotellaceae, and *Alloprevotella* (Figure 4).

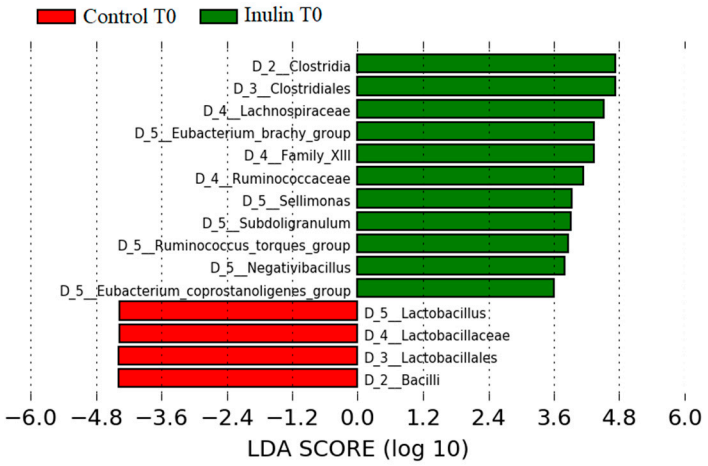

Figure 3. LEfSe analysis of the fecal microbiota at baseline (T0) in the control and inulin groups.

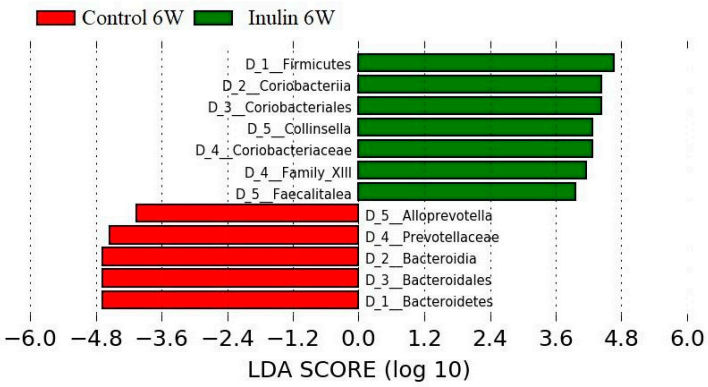

Figure 4. LEfSe analysis of the fecal microbiota after six weeks (T6) with control or inulin-supplemented diet.

- When tested at each time point independently, as a case–control study, the alpha-diversity did not change significantly. When using a longitudinal approach, the number of observed ASVs (but not the Shannon index) evolved differently depending on the diet. Indeed, the fecal microbiome of cats presented a higher richness with the inulin diet at T6 when compared to T0 ($p = 0.019$). In the control group, no difference was detected over time (Table 4).
- Similarly, no difference was observed in the beta-diversity when we tested each time point independently. When comparing beta-diversity distance matrices at paired time points, we found that the weighted UniFrac control and inulin groups presented different distances when moving from T0 to T6 ($p = 0.024$). No other differences in the distance matrix were detected.

3.2. Vaccine Response (Part 2)

Before the first vaccination, a significant time effect was observed between day −42 and day 0 ($p = 0.003$) on serum specific IgG, without a difference between treatment. After the first vaccination, a significant time effect was observed ($p < 0.0001$), while a trend for a diet effect was detected ($p = 0.09$). More precisely, compared to the control group, the inulin group had higher anti-FeLV p45 antibodies at day 5 (6.9 ± 5.7 vs. 4.0 ± 3.2%, $p = 0.04$) and day 11 (25.7 ± 18.1 vs. 14.4 ± 9.6%, $p = 0.05$) post-vaccination (Figure 5). A time effect was still observed after the second vaccine ($p < 0.0001$), without a difference between treatments. No diet-by-time interaction was observed after the vaccine or booster.

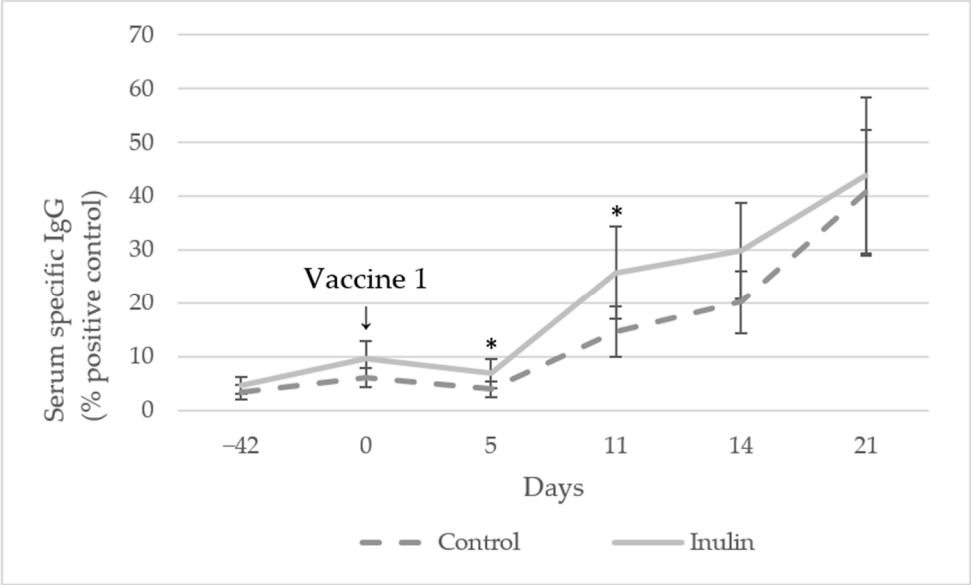

Figure 5. Serum IgG immune response against FeLV p45 before (days −42, 0) and after the first vaccination (days 5, 11, 14, 21) (mean ± SD). Vaccinations took place on day 0. * indicates a significant difference between control and inulin groups.

4. Discussion

The objective of our pilot study with 26 cats was to test the effects of a low-dose dietary inulin supplementation on gut microbiota composition and activity (Part 1) and the specific immune response after vaccination against FeLV (Part 2) using a double-blind randomized controlled trial design. We hypothesized that inulin exerted a prebiotic effect (Part 1) and that inulin may enhance the serum antibody response to FeLV (Part 2). It is suggested that inulin-type fructans immunomodulation is elicited through indirect and direct mechanisms [17,18]. Indirect mechanisms encompass stimulation of growth and activity of selected bacteria (i.e., lactate and SCFA-producing bacteria), but can also be caused by fermentation products of these bacteria, i.e., SCFAs [18]. Their production was used in this study as a marker of microbiota activity. It was decided to measure fecal SCFAs after three and six weeks of supplementation, a reasonable time to expect to have a prebiotic effect based on previous studies involving fructooligosaccharide supplementation in cats [3–8]. Given the exploratory nature of the present study, the analysis aimed to identify specific differences. To this end, we examined the differences between groups at each time point and the differences between time points for each group. We considered both significant and trend-level differences to be meaningful. From the present results, it appears that three weeks of supplementation with inulin was not sufficient at the dose tested to see a significant effect on SCFAs, but six-week inulin supplementation resulted in a significant higher fecal butyrate concentration and a trend of higher total SCFAs at T6 with inulin compared to the control group. However, the difference in butyrate concentration should be interpreted cautiously and confirmed in a larger-scale study. While a small numerical difference was observed between groups at baseline (T0), the overall diet effect was only a trend, and no significant diet-by-time interaction was detected. Although the butyrate concentration significantly increased from T3 to T6 in the inulin group, it was not compared to the baseline level (T0).

Interestingly, a general pattern emerged for the different SCFAs with a decrease at T3 compared to T0, followed by an increase at T6 in the inulin group, resulting in a significant difference in butyrate concentration between treatments at T6 and in a trend towards a difference in total SCFAs. Significant diet-by-time interactions were observed for propionate and total SCFAs, and a trend was detected for acetate.

It is crucial to acknowledge that this was a field study involving cats kept in an environment resembling an indoor multi-cat household. In such studies, a time effect is often observed, emphasizing the importance of a control group for comparison. The daily routines of the cats were modified to facilitate blood and fecal sample collection, which could have increased their stress levels [19]. In mice, stress exposure has been shown to reduce colonic SCFA levels [20]. It is plausible that this procedure contributed to the overall decline in SCFAs over time. However, inulin supplementation appears to have mitigated this decline after six weeks of treatment.

Given the observed changes in SCFAs after six weeks of supplementation, suggestive of a prebiotic effect, we proceeded to analyze the fecal microbiota at this time point.

Before the supplementation with inulin, similar to other studies, the fecal microbiota of cats was dominated by Firmicutes [21–23], followed by Actinobacteria, Bacteroidetes, and Proteobacteria. At genus-bacterial taxa level, we found that *Megasphaera*, *Blautia*, *Collinsella*, *Prevotella 9*, *Peptoclostridium*, *Solobacterium*, *Bifidobacterium*, *Lactobacillus*, *Megamonas*, *Catenibacterium*, *Bacteroides*, *Faecalitalea*, and *Faecalibacterium* were the most abundant, which agrees with Ganz et al. [24]. This microbiota composition is consistent with that of cats fed with dry kibbles, characterized by high relative abundance of *Megaspahaera*, *Prevotella*, and *Lactobacillus* [24–26]. It makes sense, as *Lactobacillus* and *Bifidobacterium* have the metabolic machinery to use starch or glucose to produce lactate and acetate [27,28], while *Megasphaera* and *Megamonas* can use glucose or lactate to produce propionate, revealing the coexistence between lactate-producing and lactate-using bacteria [29]. *Bacteroides* and *Prevotella* have plenty of CAZymes to degrade complex polysaccharides [30]. *Prevotella*, *Catenibacterium*, *Megasphaera*, and *Megamonas* are propionate producers, and *Faecalibacterium* can use acetate to produce butyrate [31,32]. The presence of *Prevotella*, *Peptoclostridium*, and other members of the Peptostreptococcaceae family reflects that there is proteolytic activity in the microbiota, which can also achieve SCFA production [33]. Thus, our data suggest that all cats harbored a microbiota like that described in healthy cats fed with dry kibbles at the beginning of the experiment. They also highlight the importance of the trophic interactions between the bacteria and the importance of considering their coexistence.

After six weeks of supplementation with inulin, the alpha-diversity increased versus T0, while it did not with the control. Increased diversity is generally perceived to be beneficial to the host, and such a result may indicate a different microbiota trajectory between the two groups. If we focus on microbiota composition, interestingly, the Firmicutes phylum (with SCFA producing-bacteria including *Eubacterium* group family XIII) and members of the class Coriobacteriia (Coriobacteriales/Coriobacteriaceae/*Collinsella*) were found to be discriminant for the inulin-fed group, while members of Bacteroidetes were associated with the control group. This change in the phyla's relative abundances after prebiotic supplementation has been described elsewhere [21–23,34,35].

Among Firmicutes and Actinobacteria phyla, unexpectedly, we did not observe higher *Bifidobacterium* and *Lactobacillus* in the inulin group compared to the control group, although Lactobacillales/Lactobacillaceae/*Lactobacillus* was a biomarker of the control group at T0, while it was no longer present at T6. Bifidobacteria and lactobacilli do catabolize prebiotic fibers (both GOS and FOS) and generate lactate and acetate (through homo- and heterofermentation) that can fuel secondary degraders which, in turn, produce other SCFAs, like butyrate or propionate [29]. From Actinobacteria, *Collinsella* sp. appeared to be associated with the inulin group. This bacterium is able to break down di- and oligosaccharides to produce lactate, acetate, formate, H_2, and even, for one strain, butyrate [36]. Thus, in felines, it is possible that it competes with bifidobacteria and lactobacilli for the inulin substrate. In addition, the genus *Collinsella* may modulate intestinal cholesterol absorption, liver glycogenesis, and triglyceride synthesis, while the family Coriobacteriaceae has been observed as being correlated with biliary acids and biliary acids' 7α-dehydroxylation ability, making them interesting candidates to investigate in cats [37]. The biliary acids are important not only to ensure fat digestion and act on lipid metabolism, but also as signaling molecules in the gut and in various organs harboring biliary acid receptors, like the liver or the heart,

where they may exert diverse metabolic effects [38,39]. Notably, if, in dogs, *Peptoacetobacter* (formerly *Clostridium*) *hiranonis* is a key bacterium for biliary acid metabolism, in cats, there are several candidates, among which is Coriobacteriaceae, and further investigations are needed [37]. Thus, in cats, like in other species, supplementation with inulin could alter the microbiota composition, the production of SCFAs, and the biliary acid profile [40–42]. Our results suggest that *Bifidobacterium* and *Lactobacillus* could not be the most important candidates in cats fed with a diet enriched in prebiotics, and the functional role of *Collinsella* would deserve more investigation.

The SCFA production was used in this study as a marker of microbiota activity. Based on diet-by-time interaction, propionate, acetate, and the sum of SCFAs were or tended to be higher in the inulin group compared to the control group. A trend for diet effect was also observed for butyrate, with a higher level at T6 with inulin compared to control. Acetate was the most present SCFA. Acetate is a net fermentation product for most gut anaerobes, and it almost invariably achieves the highest concentrations among the SCFAs. In contrast, propionate and butyrate are produced by distinct subsets of gut bacteria, and their patterns are, thus, more interesting in terms of demonstrating a prebiotic effect [43]. As an example, butyrate is used as a fuel for epithelial cells and can regulate intestinal macrophage function via downregulation of proinflammatory effectors [44], ameliorate mucosal inflammation [45], and increase epithelial barrier function [46]. We observed a small but significant butyrogenic effect of inulin compared to the control group at T6, similar to what has already been reported in humans and dogs [8,47]. Barry et al. [5] also proved the effect of a high dose of fructan supplementation (4%) for 30 days on gut SCFA production in cats. The trend of an increase in acetate production suggests that inulin was efficiently used to produce acetate (and probably lactate, which was not analyzed in our study), and that acetate was used by specific butyrate-producing bacteria through cross-feeding. SCFAs, and especially butyrate, are important mediators of the crosstalk between the microbiota and the host. In our study, the higher production of butyrate is consistent with the trend of higher CD4+ T lymphocytes in the blood, as it has been shown that butyrate may trigger lymphocyte differentiation through different mechanisms, including histone deacetylation [48]. Taken altogether, our results show that a low dose of inulin given for six weeks exerts a prebiotic effect, as defined by Gibson et al. [1], through a modulation of the microbiota composition, leading to higher SCFAs and a tendency for a higher percentage of CD4+ T cells in plasma. To our knowledge, this is the first time that such effects have been demonstrated in healthy adult cats with this dose. However, with some of the results being only a trend, this should be confirmed with a larger-scale study.

In the second part of the study, vaccination was used as a model to assess the functioning of the immune system against an antigen. The selected vaccine was unknown to the cat's immune system, and vaccine-specific antibody measurement was validated [14]. The selected inulin was a mix of 75% short (<10 fructose units) and 25% long (>10 fructose units) chain fructans. Indeed, in humans, long-chain inulin has demonstrated a stronger effect after vaccination against Hepatitis B [10]. After a long washout period, cats from the inulin group were supplemented again with inulin for six weeks before the vaccination was performed. A six-month washout period was assumed to be sufficient, although this assumption has not been quantitatively verified through SCFA measurements. Six weeks of feeding was decided based on part 1's results. We measured serum anti-FeLV p45 IgG concentration as a marker of inulin-mediated immunomodulation. A significant time effect was observed after each vaccination, indicative of the immune response to the vaccine. Both groups reached their maximum immune response over time, as expected from a vaccine response. However, compared to the control group, inulin-supplemented cats had higher specific IgG responses on day 5 and day 11 post-vaccination. Although these results should be interpreted cautiously, as a small numerical difference existed between groups before vaccination, and the overall diet effect was only a trend, they suggest an earlier immune response with inulin supplementation following an immune challenge. This observation could be linked to the indirect effect of higher SCFAs [49]. Indeed,

Kim et al. [49] demonstrated that SCFAs, produced by the gut microbiota as fermentation products of dietary fiber, support host antibody responses by regulating gene expression and enhancing cellular metabolism and plasma B cell differentiation. More precisely, in B cells, SCFAs increase acetyl-CoA production and regulate metabolic sensors to increase oxidative phosphorylation, glycolysis, and fatty acid synthesis, which produce energy and building blocks supporting antibody production. In parallel, SCFAs control gene expression (through regulation of histone deacetylation for example) to express molecules necessary for plasma B cell differentiation. In that way, SCFAs boost mucosal and systemic antibody responses during the steady state and infection. Mice with low SCFA production due to reduced dietary fiber consumption or microbial insufficiency are defective in homeostatic and pathogen-specific antibody responses, resulting in greater pathogen susceptibility [49]. However, SCFA or dietary fiber intake restores this immune deficiency. This B cell-helping function of SCFAs was detected from the intestines to systemic tissues and conserved among mouse and human B cells, highlighting its importance [49]. In our study, we observed, during the first phase, an increase in butyrate production and a trend to increase all SCFAs. However, as the present study was conducted in two distinct phases, with the first phase being dedicated to microbiota and its function and the second phase to the humoral response, we were unable to make direct correlations between the microbiota and its metabolites and humoral response.

Besides an indirect possible effect of inulin on humoral answer through SCFA production, the increased IgG response may also be due to a direct effect of inulin-type fructans. Indeed, they can be detected by gut dendritic cells (DCs) through receptor ligation of pathogen recognition receptors (PRRs) such as Toll-like receptors, nucleotide oligomerization domain-containing proteins (NODs), C-type lectin receptors, and galectins, eventually inducing pro- and anti-inflammatory cytokines. DCs may also exert antigen-presenting capacity toward effector cells, such as B cells, T cells, and natural killer cells, either locally or in the spleen. Inulin-type fructans may also ligate PRRs expressed on the gut epithelium, which could influence its barrier function [2,10,18]. To the authors' knowledge, this is the first time that an effect of inulin has been observed on adult cat humoral immunity. It has been shown recently that kittens fed a diet containing nucleotides, short-chain fructooligosaccharides, xylooligosaccharides, β-carotene, and higher levels of vitamin E had higher specific antibody titers against feline parvo (FPV) and herpesvirus (FHV) 23 and 27 weeks after vaccination, respectively, resulting in higher adequate seroconversion rates against FHV and a higher number of kittens reaching protective antibody titers against FHV compared to a control group [50]. Another study with dogs fed 1% inulin from chicory root showed that supplemented dogs had improved antibody responses to sheep erythrocytes compared to control, indicative of better humoral immunity [51]. Further studies will be required to elucidate the mode(s) of action, but our study suggests that the inulin-supplemented diet supports immune defense, enabling an earlier response after vaccination in adult cats.

The main strength of the current study was the use of a randomized, controlled, double-blind design with the previous confirmation that the low dose of inulin supplementation for the determined time was able to modify the fecal microbiota and its function. However, there are also limitations. First, the relatively small sample size may have limited the ability to identify clear effects of inulin supplementation. We indeed noticed a high inter-individual variability. Therefore, this study is considered as a pilot and should be repeated with a larger group of animals. In addition, the application of our model to privately owned pet cats could be more representative of the environmental conditions, also known to influence gut microbiota and immunity. Thus, a follow-up study with a larger cohort of owned cats would be relevant and complementary to our study. Second, the selected cats were healthy and middle-aged; although they represent a target group for prebiotics, the findings cannot be generalized to the senior population, kittens, or individuals with disease. Further investigations are required to establish the effects of low-dose inulin in those different contexts. Third, we only examined indirect mechanisms and the specific IgG response as

markers of immunity. It could be useful to more deeply investigate the mechanism of action in a one-phase study and to adapt nutritional strategies for more tailored nutrition.

5. Conclusions

Taken together, these results suggest that a six-week dietary supplementation with a low dose of inulin in cats is able to exert a prebiotic effect by modulating the fecal microbiota composition and activity. The trend of an earlier immune response with inulin supplementation after the first vaccination (used as a model of immune challenge) should be confirmed in a larger-scale study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pets1030033/s1, Table S1: Description of the most abundant phyla, families, and Amplicon Sequence Variants (ASVs) (% mean ± SD) found in the fecal microbiota of cats fed with either a control or an inulin-supplemented diet at the start of the experiment (T0) and six weeks after.

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