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Immune response and gut microbiota of mice on a diet mimicking eating habits of elderly with risk of malnutrition development

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ABSTRACT

The number of elderlies is increasing but prevalence of malnutrition has been reported. The aim of the study was to determine the significance of short-term nutritional deficiencies in mice. Immune status was assessed through flow cytometry of leucocytes in Peyer's patches (PP) and mesenteric lymph nodes (MLN), and intestinal microbiota was evaluated by terminal restriction fragment length polymorphism (T-RFLP). C57BL/6NCrl mice fed standard diet (StD) or experimental diet high in fat, and low in carbohydrates, protein, fibre, vitamins, and minerals (ExpD) for 2 or 4 weeks. ExpD-animals gained less weight, increased liver lipids, and developed splenomegaly. Diet affected regulatory T-cells, gut homing receptors and TLR2 and TLR4 in PP and MLN and the microbiota was influenced. Partial least squares models on flow cytometry- and T-RFLP data demonstrated correlations between microbial communities and immune phenotyping. Our model shows similarities to malnourished elderly and interactions between intestinal bacteria and the immune system.

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Diet; malnutrition; elderly; microbiota; immune responses

Introduction

The proportion of elderly people represents a growing share of the Swedish population and the number is continuously rising. Of Sweden's 10 million inhabitants, the residents 65 years or older are projected to exceed 3 million in 2060. Moreover, the number of persons aged 80 years or older accounts today for around 500,000 individuals and analysts predict that the number will further increase above 1 million by the year of 2040 (SCB 2021).

Much focus is given to the importance of the diet in relation to obesity, type II diabetes and cardio-vascular disease. However, for elderly people, it is generally agreed that the risk of developing under-nutrition rather than over-nutrition is the main cause for concern and substantial prevalence of protein-energy malnutrition and subclinical vitamin and trace element deficiencies have been reported in elderly people living in institutional care but also in in-home living elderly in Sweden (Fagerström et al. 2011; Borgström Bolmsjö et al. 2015; Naseer and Fagerström 2015). In addition, malnutrition is often not diagnosed unless the possibility is considered since haematological and biochemical parameters can be normal even in grossly undernourished patients (Lipski et al. 1993). The reasons for poor nutritional status are multifaceted and include physiological, psychological and social circumstances associated with aging, which affect appetite, food intake and body weight, exacerbated by the presence of morbidity and polypharmacy.

Amongst the factors affecting the nutritional intake, food quality, flavour and mealtime situations may be of considerable importance. Institutional meals in Sweden, consisting mainly of traditional food with occasional influence of modern food trends, may unfortunately be associated with a diet generally low in macro- and micronutrients, low in plant-based foods and high in saturated fat, resulting in low nutrient density insufficient to cover the nutritional needs of the residents consuming too small portions (Lammes et al. 2009; Claesson et al. 2012).

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The gastrointestinal physiology and function are affected in elderly including reduced fundal compliance contributing to early satiety, hypochlorhydria which may predispose to bacterial overgrowth, changed hormone secretion and prolonged colonic transit time related to a reduction in neurotransmitters and receptors, but surprisingly, the absorptive function of the small intestine seem to be unaffected by age (Morley 2007; Britton and McLaughlin 2013). Furthermore, the ageing processes severely cause a decline in the functionality of the immune system, indicated as immunoscenescence, resulting in changes in both the innate and adaptive immune responses and providing increased vulnerability for infectious diseases (Ostan et al. 2008). Combined with changes in dietary intake, the ageing processes also seriously affect the composition of the human gut microbiota, which may be an additional cause of malnutrition and infection-susceptibility (Claesson et al. 2012). The gut microbiota composition of elderly is distinct from that found in younger adults, characterised by unusual phylum proportions and extreme inter-individual variability (Claesson et al. 2012). The interconnection between the community structure of the microbiota and the gut associated immune system is pivotal for a homeostatic equilibrium and age associated compositional changes have been linked to immunosenescence and the state of chronic, low-grade, systemic inflammation observed in elderly (Guigoz et al. 2008; Thevaranjan et al. 2017). This condition is associated with need of care, high complication rate, increased morbidity, hospital re-admission, length of hospital stay, mortality and increased health-care costs (Ljungqvist and de Man 2009).

The aim of the present study was to determine the significance of short-term poor nutrient intake on development of malnutrition in mice. Immune status was approached through immunophenotyping of lymphocyte subsets, macrophages and dendritic cells in Peyer's patches and mesenteric lymph nodes and assessment of the diversity of colonic and small intestinal microbiota and community structure among individuals were established by the use of terminal restriction fragment length polymorphism (T-RFLP) patterns. Furthermore, to enlarge the understanding of the pathogenesis, the correlation between the microbiota composition and immune cells was evaluated by principal component analysis.

Material and methods

Animals

Wild-type female C57BL/6NCrl mice aged 14 weeks (Charles River Laboratories, Sulzfeld, Germany) were housed four per cage at room temperature of 22 °C with 12 h light/dark cycles in the animal facility and were allowed unrestricted access to feed and tap water. The animals were allowed to acclimatise to the laboratory conditions for at least 7 days before experimental inclusion. The animal experiment was performed in full compliance with the guidelines for animal welfare and minimisation of pain and stress was considered. A power calculation based on previous data was performed, to verify the number of animals included. The study was approved by the Ethics Committee for Animal Studies at Lund University (permit number and approval-ID: M 424-12).

Experimental design

Animals were randomly divided into two groups with 16 animals in each group, and were fed either standard mouse chow (R34, Lantmännen AB, Kimstad, Sweden) (StD group) or an experimental diet (ExpD group) ad libitum. The experimental diet was formulated by Lantmännen AB (Kimstad, Sweden) and a

Table 1. Composition of the standard and experimental diet.

Content	Standard diet Amount	Experimental diet Amount
Protein (% weight)	16.5	11.0
Fat (% weight)	4.0	21.0
Crude fibre (%)	3.5	2.0
Ash (%)	6.0	6.0
Water (%)	<12	<16
NFE[a] (%)	58.0	44.0
Phosphorus (%)	0.8	0.4
Calcium (%)	0.9	0.68
Magnesium (%)	0.2	0.05
Sodium (%)	0.7	0.3
Iron (mg/kg)	190.0	125.4
Copper (mg/kg)	30.0	8.0
Manganese (mg/kg)	100.0	70.0
Zinc (mg/kg)	110.0	30.0
Vitamin A (IE/kg)	12,000.0	10,680.0
Vitamin D (IE/kg)	1500.0	495.0
Vitamin E (mg/kg)	63.0	42.0
Vitamin K3 (mg/kg)	10.0	0.7
Vitamin B1 (mg/kg)	3.0	2.85
Vitamin B2 (mg/kg)	12.0	12.0
Vitamin B6 (mg/kg)	4.0	6.0
Vitamin B12 (mg/kg)	0.02	0.02
Vitamin C (mg/kg)	500.0	460.0
Pantothenic acid (mg/kg)	10.0	12.0
Niacin (mg/kg)	40.0	40.0
Choline (mg/kg)	1000.0	1000.0

[a]Nitrogen-free extract (NFE): consisting of carbohydrates, sugars, starches, and a major portion of materials classed as hemicellulose in feeds. When crude protein, fat, water, ash, and fibre are added, and the sum is subtracted from 100, the difference is NFE.

Ingredients (standard diet R34, Lantmännen): wheat, barley, wheat sprouts, pollard, soy, potato protein, vegetable fat, vitamins and micronutrients, 1255.0 kJ/100 g. Calorie density (calculated on the amount of feed consumed daily): 251 kJ/5 g.

Ingredients (experimental diet, Lantmännen): corn starch, cocoa butter, casein, glucose, powder sugar, cellulose flour, minerals, vitamins, cholesterol, 1560.55 kJ/100 g. Calorie density (calculated on the amount of feed consumed daily): 312 kJ/5 g.

detailed description of the composition is shown in Table 1. The experimental diet contained high amounts of fat and low levels of carbohydrates, protein, fibre, vitamins and minerals and was designed after calculation of Actual daily intake/Recommended daily intake for elderly reported in two studies performed at Skåne University Hospital (SUS) and Kristianstad Högskola, Sweden (Andrén et al. 2000; Persson et al. 2004; Larsson and Vestin 2011).

After 14 days (2 weeks) of consumption of standard mouse chow or the experimental diet, 8 animals were sacrificed from each group and the remaining 8 animals were sacrificed after another 14 days of consumption (28 days in total (4 weeks)). Body weights were registered before starting the experimental period and prior to sacrifice for all mice. Body weight differences between groups were calculated as absolute weight per animal or as body weight gain per animal and the weights were calculated in relation to amounts of feed consumed.

Sampling

At the end of the experimental period, the animals were anaesthetised with 75 mg ketamine (Ketalar 50 mg/ml, Pfizer, Sweden) and 1.0 mg Domitor (1 mg/ml, Orion Pharma Animal Health, Sweden) per kg bodyweight by intra-peritoneal injection. Under an aseptic technique, a laparotomy was performed through a midline incision, and all detectable mesenteric lymph nodes were collected. Left lateral lobe of the liver was obtained for total lipid content analysis. Small intestine, caecum and the entire colorectum from the colocaecal junction to the anal verge were excised. Peyer's patches (PP) of whole small bowel (proximal, middle and distal) were isolated and assigned for flow cytometry analysis along with mesenteric lymph nodes (MLN). The wet weight of spleen was documented. Intestinal biopsies from small intestine (ileum) and colon (descending colon) and faecal samples were saved for microbial evaluation. The weights of the samples were recorded before they were immediately frozen in liquid nitrogen and kept in −80 °C until the day of analysis.

Liver lipid content determination – Schmid–Bondzynski–Ratzlaff method

Determination of the total lipid content of the liver was done according to the Schmid–Bondzynski–Ratzlaff method (Ceirwyn 1994). In short, diethyl ether and petroleum were used to extract the lipid and by evaporating the solvents the lipid content was calculated by weight difference according to the following formula:

$$\text{Lipid content of the liver (\%)} = \frac{w1}{w2} \times 100$$

W1: mass of lipid extracted (g)

W2: weight of the original sample (g)

Terminal restriction fragment length polymorphism (T-RFLP) analysis

DNA extraction

DNA extraction from ileum and descending colon mucosal biopsies was done by using BioRobot EZ1 and DNA Tissue Kit (Qiagen, Valencia, CA) as described elsewhere (Karlsson et al. 2010).

Amplification

Using primer FAM-ENV1 (5′- AGA GTT TGA TII TGG CTC AG-3′) and ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′), the 16S rRNA genes were amplified (Wang et al. 2005). The forward primer ENV1 was synthesised and fluorescently labelled with FAM (Applied Biosystems, Foster city, CA) at the 5′ end. The total volume of the PCR reaction was 25 µl, consisting of 0.4 µM of primer FAM-ENV1 and 0.2 µM of primer ENV2, 2.5 µl of 10 x PCR reaction buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, pH 8.3), 0.2 mM of each deoxyribonucleotide triphosphate, 2.5 U of FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), and 2 µl of template DNA. The PCR was performed in an Eppendorf MasterCycler (Eppendorf, Hamburg, Germany) under the following conditions: 95 °C for 3 min, 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 45 s, and 72 °C for 2 min. Finally, an additional extension at 72 °C for 7 min was performed. Triplicate reactions were carried out for each sample and a negative control was included in all the PCR runs. After amplification, the results were confirmed by gel electrophoresis (1.5% agarose for molecular biology (Sigma-Aldrich, Munich, Germany)) in Tris Acetate-EDTA buffer (Sigma-Aldrich, St. Louis, MO). The gels were run at 120 V for 60 min and stained with GelRed (Biotium, Fremont, CA). When the verification of the results showed single bands, the PCR products of each sample were pooled and purified by MiniElute PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. However, when more than single bands were shown a higher volume of PCR products (35 µl) had to be repeatedly run in

bigger wells. Only the target band was then cut from the gel and the DNA was extracted and purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified DNA was eluted in 30 µl of sterile water and the DNA concentration was measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden).

T-RFLP analysis

Purified PCR products (200 ng) were digested using 10 U of the restriction endonuclease *Msp*I (Fermentas Life Science, Burlington, Canada) in a total volume of 10 µl for 5 h at 37 °C whereupon the enzyme was inactivated by heating at 65 °C for 20 min. After digestion, aliquots of the products were diluted 5 times with sterile water in a sterile 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and the samples were sent to Malmö University Hospital for T-RFLP analysis. The analysis was performed on a 3130 × l Genetic Analyser (Applied Biosystems, Foster city, CA) and in all samples a DNA size marker GeneScan™ LIZ 600 (Applied Biosystem, Foster city, CA) was included. Peak height, peak area and fragment size were analysed with Genemapper® software version 4.0 (Applied Biosystems, Foster city, CA) and GS600LIZ was chosen for size calling. The size range was set from 20 to 600 bp and the peak amplitude threshold was set to 50 relative fluorescence units (rfu) for samples, and 10 rfu for standards. For each sample, the total peak area was calculated by summarising the area for all peaks and the relative peak area of each peak was expressed as percentage of the total area. For the terminal restriction fragments analysis (T-RFs) the peak areas of the most prevalent peaks (at least 3 peaks present in one of the groups, threshold value 5% of total peak area) were analysed and the total amount of peaks from each group was then evaluated.

Flow cytometry

All PP and MLN were harvested, placed in Click's medium (Sigma-Aldrich, St. Louis, MO) and stored on ice before preparation. Single cell suspensions were made by mechanically disrupting the tissue by gently pressing through a 70-µm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ) into 600 µl PBS (AppliChem GmbH, Darmstadt, Germany) and the cells were washed twice with Hanks-BSS (Gibco, Invitrogen, Paisley, UK) at 250 × g for 10 min. The cells were thereafter stained with trypan blue for counting and approximately 1×10^6 cells were aliquoted for each staining. After washing the cells with 200 µL FACS buffer ((1× PBS (AppliChem GmbH, Darmstadt, Germany), 0.1% NaN_3 (Scharlau Chemie S.A., Sentmenat, Spain), 2% foetal bovine serum (VWR International, Umeå, Sweden)) at 250 × g for 1 min, the cells were stained in 25 µL antibody solution for surface markers (CD3 – Fluorescein isothiocyanate (FITC), CD 282 (TLR2) – FITC, CCR9 – FITC, CD284 (TLR4) – Allophycocyanin (APC), CD8a – APC, CD103 – Phycoerythrin (PE), CD4 – PE, F4/80 – Peridinin chlorophyll protein cyanine 5.5 (PerCP-Cy 5.5), CD69 – PerCP-Cy 5.5 and FoxP3 – APC (eBioscience, Inc., San Diego, CA) for 30 min at 4 °C in dark. After staining, the cells were washed twice with 175 µL FACS buffer at 250 × g for 1 min and re-suspended in 200 µL FACS buffer supplemented with 2% formaldehyde (Apoteket, Stockholm, Sweden). For intranuclear (FoxP3) markers, cells were fixed and permeabilized according to manufacturer's protocol and then re-suspended in 200 µL FACS buffer. Stained cells were kept in 4 °C until FACS analysis next morning. The following antibodies combinations were used: TLR2/CD103/F4/80/TLR4; CCR9/CD4/CD69/CD8a; CD4/FoxP3/CD69/CD25.

Flow cytometry analysis

The flow cytometry analysis was performed on FACS Calibur (Becton, Dickinson and Company, Franklin Lakes, NJ), and 30,000 lymphocytes in live gate were acquired for analysis. The data were analysed in FlowJo software (Treestar, Inc., Ashland, OR) and the results are presented as percentages of gated cells.

Statistical evaluations

Feed intake, body weight increase, spleen weight, liver lipid content and differences in percent gated cells were presented as medians with 25 and 75 percentiles. The statistics were conducted in SigmaPlot® version 11.0, (SYSTAT Software, Point Richmond, CA) and the differences between the two groups were assessed by a Mann–Whitney rank sum test. Bacterial diversity (T-RFLP) was estimated by calculation of richness (number of T-RFs) and of Shannon–Wiener diversity index (H') and Simpsons diversity index (1-D), as previously described (Karlsson et al. 2012). Calculations of the incidence of T-RFs were conducted in QuickStat version 2.6 and were evaluated by Fisher's exact test. Multivariate data analysis was performed on T-RFLP and flow cytometry data using Unscrambler®X software version 10.3 (32-bit) (CAMO

Table 2. Body weights (absolute, ratio of the absolute body weight to the amount of feed consumed and the ratio of the body weight increase over time to the amount of feed consumed), feed intake, liver lipid content (the ratio of the content of lipids in the liver to the body weight increase over time and amount of feed consumed) and spleen weights (the ratio of the spleen weight to the body weight increase over time and amount of feed consumed) in animals fed standard diet (StD group, $n = 8$) or experimental diet (ExpD group, $n = 8$).

		Start		14 days		28 days	
	Group	Median (min–max)	*p* Value	Median (min–max)	*p* Value	Median (min–max)	*p* Value
Body weight (g)	StD	22 (21.3–23.0)		29.00 (27.60 – 29.80)		–	
	ExpD	22 (21.3–22.8)	0.878	24.50 (24.00–25.80)	<0.001***	–	
	StD	22 (21.3–22.8)				32.50 (31.00–34.00)	<0.001***
	ExpD	22 (21.3–23.0)	0.798			26.0 (23.30–29.80)	
Feed Intake[a] (g)	StD			3.60 (3.52–3.68)		2.65 (2.62–2.68)	
	ExpD			3.45 (3.38–3.52)	0.01*	3.44 (3.15–3.72)	<0.001***
Body weight (g/g feed)	StD			8.02 (7.66–8.22)		12.25 (11.82–12.66)	
	ExpD			7.10 (6.89–7.39)	<0.001***	7.71 (7.27–8.03)	<0.001***
Body weight increase (g/g feed)	StD			1.90 (1.37–2.18)		4.01 (3.37–4.47)	
	ExpD			0.72 (0.57–0.88)	<0.001***	1.11 (0.68–1.82)	<0.001***
Liver lipid content (g/(g body weight change/g feed))	StD			0.04 (0.02–0.05)		0.02 (0.01–0.02)	
	ExpD			0.06 (0.05–0.08)	0.007**	0.036 (0.02–0.06)	0.014*
Spleen weight (g/(g body weight change/g feed))	StD			0.04 (0.03–0.05)		0.02 (0.02–0.03)	
	ExpD			0.09 (0.07–0.11)	0.001**	0.07 (0.04–0.08)	<0.001***

*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between StD and ExpD groups.
[a]Due to ethical considerations animals were housed 4 per cage and the feed intake per individual was calculated as the total feed consumed per cage divided by the number of animals.
Data are presented as median values, and range is presented within brackets.

Software AS, Oslo, Norway). *p* values less than 0.05 were considered statistically significant.

Results

Feed intake and body weight increase

No adverse effects during the experimental period were observed. Feed intake and body weight increase for the two groups are summarised in Table 2. Feeding the animals, a nutrient-deprived experimental diet resulted in lower consumption of feed after 2 weeks but higher consumption after 4 weeks compared to consumption of standard diet. After 11 days of consumption, the animals in the ExpD group ($n = 16$) showed significantly lower body weights compared to the StD group ((23.0 (23.0–24.0) g and (24.0 (24.0–25.8) g, respectively) ($p = 0.004$). Further, after 14 and 28 days of consumption the animals in the ExpD group gained less weight, and the absolute body weight (g), the body weight per gram consumed feed (g/g feed) and the body weight increase per gram consumed feed (0–14 days and 0–28 days) (g/g feed) were significantly lower compared with the StD group after both 2 and 4 weeks of consumption ($n = 8$) (Table 2).

Liver lipid content and spleen weight

The liver lipid content in relation to body weight gain and feed consumption was significantly higher in the ExpD group compared to the StD group after 2 and 4 weeks (Table 2).

A difference in the absolute weight of spleen was found between the groups after 2 weeks of consumption (0.072 (0.068–0.089) ExpD group; 0.062 (0.059–0.073) StD group, $p = 0.038$) but not after 4 weeks (0.078 (0.070–0.089) ExpD group; 0.08 (0.065–0.086) StD group, $p = 0.645$). When taking body weight gain and feed consumption into account a difference in spleen weight was observed between the groups and higher weights were associated with consumption of the nutrient-deprived experimental diet compared to standard diet after both 2 and 4 weeks (Table 2).

Immunophenotyping by flow cytometry

Lymphocytes, macrophages and dendritic cells prepared from PP and MLN were analysed by flow cytometry for the presence of various cell populations after 2- and 4 weeks of consumption of the experimental diet. Lymphocyte populations were first gated based on forward (FSC)- and side scatter (SSC) properties. From this gate, CD3 + CD4+ and CD3 + CD8+ T lymphocytes were selected and further gated for CD4 + CD8− and CD8 + CD4− populations expressing CD69 and CCR9. The CD4 + CD8− and CD8 + CD4− lymphocyte gates were also used to identify the cell populations

Table 3. Changes in lymphocyte subsets in Peyer's patches presented as median with minimum and maximum values within parenthesis of percentage gated cells (animals fed standard diet (StD group), ($n = 8$ and $n = 7$ after 2 and 4 weeks of consumption respectively) or experimental diet (ExpD group), ($n = 8$)).

		Peyer's patches				
		2nd week		4th week		
Cells	Group	Median (min-max)	p^a Value	Median (min-max)	p^a Value	p^b Value
Regulatory T cells ($CD4^+CD25^+CD69^+/CD69^-Foxp3^+$)						
CD4 + CD25 + CD69 + FoxP3+	StD	59.8 (50.8–62.5)		53.9 (50.0–62.9)		0.694
	ExpD	64.9 (63.1–69.7)	0.003**	62.8 (58.2–67.4)	0.021*	0.279
$CD4^+CD25^+CD69^-FoxP3^+$	StD	26.6 (23.3–34.4)		28.9 (23.8–32.6)		0.779
	ExpD	21.6 (13.7–23.2)	0.005**	24.5 (22.8–29.1)	0.397	0.015*
$CCR9^+$ T cells ($CD4^+/CD8^+CD69^+/CD69^-CCR9^+$)						
$CD4^+CD69^+$		6.9 (5.3–8.3)		7.0 (6.6–8.0)		0.955
	ExpD	4.8 (4.4–5.5)	0.050*	6.3 (6.0–7.8)	0.397	0.038*
$CD4^+CD69^-$	StD	0.6 (0.5–0.7)		0.6 (0.4–0.8)		0.867
	ExpD	0.3 (0.2–0.4)	0.002**	0.3 (0.3–0.6)	0.054	0.195
$CD8^+CD69^+$	StD	14.3 (12.7–16.2)		16.7 (16.1–20.0)		0.054
	ExpD	13.2 (11.8–14.8)	0.505	13.1 (12.3–15.0)	0.009**	0.721
$CD8^+CD69^-$	StD	1.0 (0.7–1.3)		1.6 (0.7–2.1)		0.232
	ExpD	0.7 (0.5–1.0)	0.083	1.0 (0.5–1.1)	0.121	0.234
Toll-like receptor ($TLR2^+/TLR4^+$)						
$TLR2^+$ dendritic cells	StD	11.0 (9.8–13.7)		87.3 (86.0–88.3)		<0.001***
	ExpD	10.1 (9.3–10.7)	0.234	92.0 (90.4–93.8)	<0.001***	<0.001***
$TLR4^+$ dendritic cells	StD	3.1 (2.7–4.4)		69.0 (65.8–71.8)		<0.001***
	ExpD	1.8 (1.4–2.2)	0.002**	83.6 (80.7–85.2)	0.054	<0.001***
$TLR2^+TLR4^+$ dendritic cells	StD	73.6 (72.0–78.6)		67.2 (63.5–69.2)		<0.001***
	ExpD	78.9 (72.6–81.8)	0.279	82.1 (78.9–83.7)	<0.001***	0.105
$TLR2^+$ macrophages	StD	5.6 (4.8–6.2)		56.6 (50.3–61.3)		<0.001***
	ExpD	6.2 (5.6–7.5)	0.105	62.7 (55.7–69.2)	<0.001***	<0.001***
$TLR4^+$ macrophages	StD	23.9 (23.0–25.0)		52.4 (47.3–53.1)		<0.001***
	ExpD	19.0 (17.0–19.5)	<0.001***	69.8 (63.8–70.8)	0.054	<0.001***
$TLR2^+TLR4^+$ macrophages	StD	24.1 (22.1–27.3)		37.9 (36.1–41.7)		<0.001***
	ExpD	36.5 (27.8–40.5)	0.007**	52.3 (44.6–57.2)	0.009**	0.002**

*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between groups (p^a) or between the 2nd and the 4th week in each group (p^b).

expressing CD25 followed by gating for Foxp3 and CD69. Mononuclear cells and dendritic cells were also gated by their characteristic FSC and SSC, but as dendritic cells have an intermediate size that falls between that of monocytes and lymphocytes, the FSC versus SSC gate for these cells included both populations. Positive staining for F4/80 or CD103 identified macrophages and dendritic cells respectively and the gates were then further used to analyse expression of TLR2 and/or TLR4.

Regulatory T-cells

After 2 weeks, the percentages of $CD4^+CD25^+$ cells, expressing Foxp3 and CD69, were higher in the ExpD group meanwhile the population of $CD69^-$ cells were lower in both PP and in MLN compared with the StD group (Tables 3 and 4). After 4 weeks, the percentages of $CD4^+CD25^+CD69^+FoxP3^+$ cells were significantly higher in the ExpD group but only in PP (Table 3). Over time and within the same group significantly higher populations of $CD4^+CD25^+CD69^+FoxP3^+$ were found in both groups in MLN. Furthermore, significantly higher populations of $CD4^+CD25^+CD69^-FoxP3^+$ cells were found in PP in the ExpD group, but lower populations were observed in MLN in the StD group after 4 weeks compared to 2 weeks (Tables 3 and 4).

Expression of gut homing receptors

The percentage of $CD4^+$ and $CD8^+$ cells expressing gut homing chemokine receptor CCR9 was significantly affected specifically in the ExpD group. The population of $CD4^+CD69^-CCR9^+$ lymphocytes were lower in PP. The same population of cells was, however, higher in MLN after 2 and 4 weeks, but over time a decrease was found (Tables 3 and 4). For $CD4^+CD69^+CCR9^+$ cells, the percentage was significantly lower in PP but only after 2 weeks of consumption and the percentage significantly increased within the group over time (Table 3). In MLN and for both groups, $CD4^+CD69^+CCR9^+$ cells decreased over time (Table 4). For the population of $CD8^+CD69^+CCR9^+$ cells, a significantly lower percentage was found in PP after 4 weeks in the ExpD group compared to the StD group. In the same group, the percentage of both activated and non-activated cells was higher in MLN after 2 and 4 weeks. Over time $CD8^+CD69^+CCR9^+$ cells decreased in the ExpD group in both lymphoid

Table 4. Changes in lymphocyte subsets in mesenteric lymph nodes showing median with minimum and maximum values within parenthesis of percentage gated cells (animals fed standard diet (StD group) or experimental diet (ExpD group)).

		Mesenteric lymph nodes				
		2nd week		4th week		
Cells	Group	Median (min-max)	p^a Value	Median (min-max)	p^a Value	p^b Value
Regulatory T cells ($CD4^+CD25^+CD69^+/CD69^-Foxp3^+$)						
CD4 + CD25 + CD69 + FoxP3+	StD	22.7 (19.5–25.8)		30.6 (29.6–32.3)		<0.001***
	ExpD	28.5 (24.6–29.4)	0.028*	33.7 (29.2–36.6)	0.195	0.015*
$CD4^+CD25^+CD69^-FoxP3^+$	StD	58.7 (54,6–63.4)		52.7 (51.2–53.2)		0.007**
	ExpD	48.3 (44.8–54.3)	0.007**	50.3 (48.9–52.8)	0.279	0.328
$CCR9^+$ T cells ($CD4^+/CD8^+CD69^+/CD69^-CCR9^+$)						
$CD4^+CD69^+$	StD	14.1 (12.1–18.4)		10.9 (10.3–11.6)		0.021*
	ExpD	15.3 (14.4–16.9)	0.645	12.3 (11.0–13.9)	0.195	0.007**
$CD4^+CD69^-$	StD	2.3 (1.9–2.7)		2.0 (1.7–2.0)		0.105
	ExpD	3.8 (3.3–4.0)	<0.001***	2.7 (2.4–3.6)	0.001**	0.021*
$CD8^+CD69^+$	StD	44.0 (40.7–45.4)		36.5 (33.0–37.4)		0.005**
	ExpD	54.4 (51.0–58.0)	<0.001***	46.3 (43.0–48.0)	<0.001***	0.001**
$CD8^+CD69^-$	StD	6.7 (5.8–7.3)		7.6 (7.1–8.2)		0.015*
	ExpD	14.9 (13.4–16.3)	<0.001***	10.3 (9.4–11.7)	<0.001***	<0.001***
Toll-like receptor ($TLR2^+/TLR4^+$)						
$TLR2^+$ dendritic cells	StD	7.9 (7.4–8.6)		45.3 (40.6–48.7)		<0.001***
	ExpD	5.8 (4.7–8.8)	0.382	55.8 (54.9–60.0)	0.001**	<0.001***
$TLR4^+$ dendritic cells	StD	6.6 (4.6–8.3)		40.73 (35.3–43.8)		<0.001***
	ExpD	7.0 (5.0–7.8)	1.000	56.8 (54.0–58.3)	0.010*	<0.001***
$TLR2^+TLR4^+$ dendritic cells	StD	34.7 (32.3–37.3)		36.2 (30.4–39.0)		0.721
	ExpD	38.7 (32.1–39.1)	0.442	50.2 (48.5–51.6)	<0.001***	<0.001***
$TLR2^+$ macrophages	StD	3.4 (3.2–3.7)		17.9 (16.7–18.7)		<0.001***
	ExpD	2.9 (2.5–3.3)	0.065	15.6 (15.2–16.2)	0.002**	<0.001***
$TLR4^+$ macrophages	StD	23.6 (22.7–26.8)		32.0 (29.2–32.8)		<0.001***
	ExpD	25.8 (24.7–26.0)	0.105	40.3 (35.2–44)	<0.001***	<0.001***
$TLR2^+TLR4^+$ macrophages	StD	8.7 (7.8–9.3)		12.1 (11.0–13.0)		0.007**
	ExpD	8.5 (8.1–9.6)	1.000	13.0 (11.6–13.4)	0.279	<0.001***

*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between groups (p^a) or between 2^{nd} and 4^{th} week in each group (p^b).

organs. For the population of $CD8^+CD69^-CCR9^+$ cells in MLN, the percentage increased over time in the StD group but decreased in the ExpD group (Tables 3 and 4).

Expression of TLR2 and TLR4

The percentage of dendritic cells and macrophages expressing $TLR2^+$ ($CD103^+/F4/80^+/TLR2^+$) were not significantly affected after 2 weeks of consumption. However, after 4 weeks significantly higher values were found for both dendritic cells and macrophages in PP in the ExpD group. On the other hand, higher and lower values were found for dendritic cells and macrophages respectively in MLN in the same group (Tables 3 and 4).

In Peyer's patches, the population of dendritic cells and macrophages expressing $TLR4^+$ ($CD103^+/F4/80^+/TLR4^+$) was significantly decreased after 2 weeks in the ExpD group (Table 3). No significant differences were found in MLN (Table 4). After 4 weeks of consumption, a higher percentage of both dendritic cells and macrophages isolated from PP and MLN and expressing $TLR4^+$ was observed in the ExpD group compared to the StD group (Tables 3 and 4).

Furthermore, the percentage of double positive macrophages ($F4/80^+/TLR4^+/TLR2^+$) was significantly higher in PP in the ExpD group after 2 weeks of consumption (Table 3). After 4 weeks, the percentage of double positive dendritic cells as well as macrophages was significantly higher in both PP and MLN (Tables 3 and 4). Over time, the proportion of populations of double positive cells increased in both lymphoid organs and in both groups (Tables 3 and 4).

Mucosal and faecal microbial communities

Diversity index

Five, thirteen and six T-RFs of different size were detected with significantly different occurrence between the two groups in samples obtained from ileum, descending colon and faeces, respectively. Of these T-RFs, three, seven and four, respectively, were only detected in the ExpD group while two, three and two T-RFs, respectively, were not detectable after consumption of the nutrient-deprived experimental diet for 4 weeks (Table 5).

Based on the analysis of the mucosal and faecal bacterial communities by T-RFLP patterns, the

Table 5. Size of T-RFs (bp) showing unique and/or significantly different frequency of occurrence and diversity indexes (medians with percentiles) detected in ileum, descending colon and faecal samples from the two groups (animals fed standard diet (StD group) or experimental diet (ExpD group)).

	Incidence of T-RFs				Shannon-Wiener diversity index			Simpson's diversity index		
	T-RFs	StD	ExpD	Significance	StD	ExpD	Significance	StD	ExpD	Significance
Ileum	81.1	6	0	$p < 0.001^{***}$	1.18 (1.10-1.44)	1.73 (1.5–2.1)	$p = 0.043^{*}$	0.82 (0.63–0.86)	0.89 (0.84–0.90)	$p = 0.038^{*}$
	83.3	2	7	$p = 0.108$						
	86.2	0	7	$p = 0.005^{**}$						
	117.3	6	4	$p = 0.142$						
	159.5	0	7	$p = 0.005^{**}$						
	273.8	5	0	$p = 0.008^{**}$						
	281.1	0	6	$p = 0.02^{*}$						
Descending colon	81.1	8	0	$p < 0.001^{***}$	1.89 (1.59–2.26)	2.48 (2.33–2.63)	$p = 0.002^{**}$	0.55 (0.49–0.66)	0.75 (0.65–0.84)	$p = 0.059$
	86.2	0	6	$p = 0.01^{*}$						
	117.3	7	0	$p = 0.002^{**}$						
	132	0	7	$p = 0.002^{**}$						
	159.5	0	5	$p = 0.038^{*}$						
	193.6	0	6	$p = 0.01^{*}$						
	206.4	0	5	$p = 0.038^{*}$						
	219.4	7	2	$p = 0.038^{*}$						
	225	5	0	$p = 0.038^{*}$						
	265.3	7	2	$p = 0.038^{*}$						
	269.3	0	5	$p = 0.038^{*}$						
	300	1	6	$p = 0.038^{*}$						
	500.3	0	5	$p = 0.038^{*}$						
Faeces	82.4	0	7	$p < 0.001^{***}$	1.8 (1.43–1.90)	2.28 (1.98–2.76)	$p = 0.026^{*}$	0.72 (0.61–0.78)	0.88 (0.80–0.91)	$p = 0.053$
	86.2	0	7	$p < 0.001^{***}$						
	94.1	0	6	$p = 0.004^{**}$						
	132	0	7	$p < 0.001^{***}$						
	227.4	5	0	$p = 0.026^{*}$						
	266.1	6	3	$p = 0.209$						
	293.6	5	0	$p = 0.026^{*}$						

$^{*}p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between StD and ExpD groups.

microbial diversity was calculated using the peak area of each sample, expressed as the proportion of the total area for a sample. The median values from the Shannon–Wiener diversity index (H') were significantly higher in the ExpD group compared to the StD group in ileum, in descending colon and in faecal samples (Table 5). Furthermore, the values from the calculation of Simpson's diversity index of descending colon were also significantly higher in the ExpD group compared to the StD group and even though no significant difference between the groups were found for ileal and faecal samples higher diversity index in the ExpD group was observed (Table 5).

PCA of microbial communities

The PCA, with the first three principal components (PCs) applied on the relative area of the T-RFs obtained from animals in all sample group of origin, showed that the distribution of the microbial communities was clearly distinct between the StD and ExpD groups. The microbiota in ileum from both groups were separated by PC1, whereas in faecal samples, the T-RFs were separated by PC2. In descending colon, T-RFs from the StD group were found only in one quadrant whereas the T-RFs from the ExpD group were scattered in three different quadrants.

The PCA bi-plot shows the correlation between T-RFs representing different bacterial groups and the similarity of the microbiota between the individual animals based on the T-RF distribution. The ExpD group harboured high abundance of T-RF 281.1 and T-RF 83.3 on ileal mucosa meanwhile the bacterial group represented by T-RF 117.3 was suppressed. This group was on the other hand one of the dominating in the StD group. In the descending colonic samples, T-RF 117.3 and T-RF 81.1 were abundant and only found in the StD group. In the faecal samples, T-RF 266.1 was detectable in both groups, however, the number decreased in the ExpD group. Furthermore, the ExpD group also had high abundance of T-RF 86.2 which was not found in faecal samples of the StD group.

PLS of immunophenotyping and microbial communities

Partial least squares (PLS) models were built on both flow cytometry data and T-RFLP data. The PLS scatter plot showed a correlation between cellular findings in PP and T-RFs in ileum originating from the two groups respectively and the different patterns were separated by PC1. These distinct correlation patterns were also shown between the groups for cellular

findings in MLN and T-RFs in descending colon but could not be found for faecal samples nor for T-RFs in ileum and cellular findings in MLN.

Discussion

The functional integrity of the immune system is extremely dependent upon optimal nutrition and of the exogenous factors affecting the composition of the gut microbiota, long-term diet also appears to have the largest effect (Gershwin et al. 1985; Xu and Knight 2015). Poor nutritional status is widespread among elderly and the risk of malnutrition is increasing with aging. The elderly persons living in nursing homes represent the frailest part of the population and the prevalence of residents suffering from malnutrition at nursing homes in Sweden has been estimated to reach almost 18% (Borgström Bolmsjö et al. 2015). In the present study, the effect of short-term consumption of a nutrient-deprived diet mimicking a poor nutrient intake observed among elderly under care was evaluated in mice.

The underlying cellular mechanisms of aging are complex and involve different biological pathways and despite the advantages of mouse models of aging, there are some important age-related differences between mice and humans. In contrast to humans, mice can synthesise vitamin C and they have long telomeres and high telomerase activity in many organs (Vanhooren and Libert 2013). Although mice have both innate and adaptive immune systems highly similar to those in humans, differences in immune cell subpopulations such as TLR4 expression on monocytes have been observed and the immunological response of $CD4^+$, $CD8^+$ cells and macrophages was found to not always differ between adult and old mice, as is the case in humans (Smithey et al. 2015). Furthermore, aging mice do not demonstrate all the typical age-related diseases seen in humans such as cardiovascular- and Alzheimer's disease (Vanhooren and Libert 2013) neither aging-induced morphological changes of the intestine nor aging-related changes of the microbiota (Steegenga et al., 2012; Langille et al., 2014). Therefore, and also due to the complicatedness of interpreting and validating experimental observations between aged mice models and elderly humans, mature, adult C57/BL6 mice were chosen for the examination.

The risk of undernutrition in institutionalised and/or hospitalised patients is according to Swedish guidelines delineated as occurrence of at least one of the following states: involuntary weight loss, body mass index (BMI) below a certain limit (<20 if ≤ 69 years and <22 if ≥ 70 years) and eating difficulties (Cederholm et al. 2015). In the present study, a nutrient deprived feed designed based on calculations of actual dietary intake compared to recommended daily allowances of elderly subjects living in nursing homes was given to mice for 2 or 4 weeks and an unintentional lower weight gain was observed after consumption (Table 2). Even though a higher feed intake was actually observed after 4 weeks of consumption, the ratio of the absolute body weight to the amount of feed consumed as well as the ratio of the body weight increase over time to the amount of feed consumed were still significantly lower in the ExpD group after both two and four weeks (Table 2). Furthermore, by consumption of the nutrient deprived diet, the content of lipids in the liver, in relation to body weight increase and feed intake, was significantly increased (Table 2). Fatty liver is the most common liver disease in the general population, and it has been considered closely linked to obesity, altered glucose metabolism, hyperlipidaemia and hypertension, all components of the metabolic syndrome (Marchesini et al. 2003). However, although less common, protein malnutrition is also an aetiology associated with fatty liver and it has been considered to be involved in inflammation and malnutrition in elderly (Lonardo 1999; Mikolasevic et al. 2014).

In tissues with a high rate of protein turnover, such as the haematopoietic system, protein malnutrition can furthermore disrupt numerous processes in the haematopoiesis, causing damage to the haematopoietic niche, the stromal cells, and the extracellular matrix (Santos et al. 2017). In the present study, the mice had splenomegaly in the ExpD group (Table 2). Beside the spleen, other lymphoid organs are also affected by protein malnutrition through modification of physiological responses and induction of cellular disturbances such as increased rate of cellular renewal and proliferation resulting in impaired function of the immune system (Santos et al. 2017).

Among the changes characteristic of immunosenescence, an immune system pattern of high percentages of CD8 and simultaneous low percentages of CD4 in peripheral blood lymphocytes have been shown to associate with higher mortality (Wikby et al. 1998; Strindhall et al. 2013). In this context, it is important to note that a low CD4/CD8 ratio is common in the blood at protein malnutrition but is proven uncharacteristic of secondary lymphoid organs which generate acquired immune responses (Lee and Woodward 1996). In the present study, flow cytometric analysis

was applied to study lymphocytes, macrophages and dendritic cells isolated from Peyer's patches and mesenteric lymph nodes aiming at immunological changes occurring based on nutrients and microbiota composition. Here, we found a decreased proportion, that was increasing over time, of $CD4^+$ T cells co-expressing CCR9 in PP meanwhile the opposite situation was observed in MLN. It can be hypothesised that the findings may be due to lymphocyte trafficking. Similar results were also found for $CD8^+$ T cells (Tables 3 and 4). Cellular phenotyping further demonstrated increased percentage of activated $CD4^+CD25^+FoxP3^+$ cells in both PP and MLN (Tables 3 and 4) which are in line with previous findings of regulatory T cells in blood of both elderly and aged mice (Gregg et al. 2005; Chiu et al. 2007). The relevance of these expanded population of cells in relation to the immune senescence seen in the elderly still remains unclear.

The findings of populations of dendritic cells and macrophages expressing TLR2 and TLR4 are partly in contrast to the findings of Strohacker et al. (2012) who observed increased expression of TLR2 and decreased expression of TLR4 on monocyte subsets in blood in aged mice. In the present study, the population of dendritic cells and macrophages expressing TLR2 was generally lower meanwhile the populations of cells expressing TLR4 was higher in both PP and MLN in the ExpD group after four weeks consumption of the nutrient deprived diet (Tables 3 and 4). As mentioned above, response in blood is not reflected in responses initiated in secondary lymphoid organs. It may be hypothesised that the observed immunological response is the outcome of the interdependence of diet, immune and commensal interactions, which is of utmost importance for elderly. Diet influences both the composition and metabolic capacity of commensal bacteria as well as the immune system while dysbiosis and immunodeficiency impairs nutrient absorption and energy harvest. The immune system is further able to affect the microbiota and host–microbe signalling is critical for development and maintenance of the intestinal immune system.

By the use of T-FRLP, microbial community structures were compared, and microbial diversity assessed. A more diverse microbiota is in general regarded as preferable although a changed variety is not necessarily associated with health-promoting microbiota alterations, as may be indicated by findings of significantly higher diversity in ileum, descending colon and faecal samples in the ExpD group after four weeks of consumption (Table 5). This is confirmed in a study comprising 371 elderly subjects in which Jeffery et al. (2016) find that maximal microbiota diversity is not the variable that is most strongly associated with health but rather that a particular microbiota composition typifies healthy community dwelling subjects. In accordance with these findings, we found several T-RFs of different size that were detected with significantly different occurrence between the two groups in samples obtained from both ileum, descending colon and faeces (Table 5). T-RFs unique to the ExpD group were also found (Table 5), and especially for the descending colon, similar results have previously been shown for elderly (Hayashi et al. 2005). PCA based on T-RFLP data indicated distinct patterns of the microbiota between the two groups which also differed between sample origins. The PCA bi-plot analysis supports the results of the T-RFs incidence and is representing the correlation between T-RFs and the similarity of the microbiota between individuals based on the distribution. The results verify the importance of certain bacterial groups and their abundance.

Table 6. Correlations based on partial least squares models between T-RFLP (T-RFs) of samples originating from ileum and descending colon and flow cytometry data (immune response) of Payer's patches and mesenteric lymph nodes, (macrophages (MØ), dendritic cells (DCs)) from the two groups (animals fed standard diet (StD group) or experimental diet (ExpD group)).

	Immune response	T-RFs	Correlation
Ileum – Payer's patches	$CD4^+CD25^+CD69^+FoxP3^+$	52.5	Positive
	MØ $TLR2^+$	52.5	Positive
	DCs $TLR2^+$	52.5	Positive
	DCs $TLR4^+$	117.3	Negative
Descending colon – Mesenteric lymph nodes	$CCR9^+CD4^+$	83.3	Positive
		86.3	Positive
	DCs $TLR2^+$	159.5	Positive
		300	Positive
	DCs $TLR4^+$	159.5	Positive
		300	Positive
	$CD4^+CD25^+CD69^+FoxP3^+$	206.4	Positive
		292.8	Positive
	$CCR9^+CD8^+$	192.6	Negative
		219.4	Negative

In the present study, partial least squares (PLS) models were built on both flow cytometry data and T-RFLP data to evaluate the complex relationship between multiple immune parameters and the composition of the microbiota in different parts of the gastrointestinal tract. From the PLS scatter plot, a correlation between cellular findings in PP and T-RFs in ileum was observed and the different patterns originated from the two groups, respectively (Table 6). Distinct correlation patterns were also found between the groups for cellular findings in MLN and T-RFs in descending colon (Table 6) but was not observed after analysis of faecal samples, which was not expected due to the scarcity of immunological tissues in the distal part of colon and rectum.

In conclusion, feeding mice nutrient-deprived diet show similarities in appearance to malnourished elderly in many ways, and our results indicate that both the immune system and the microbiota are affected by the diet and that the composition of intestinal bacteria correlates with the immune responses. The results highlight the importance to further evaluate the significance of even a short-term poor nutrient intake on development of malnutrition in elderly and the subsequent composition of the microbiota as well as the correlation between quantitative changes in enteric microbial composition and immunological parameters to decrease the risk of low-grade, systemic inflammation and morbidity observed in elderly.

Consent to participate and consent for publication

All authors agreed on both participation and publication.

Author contributions

Åsa Håkansson (Assoc. Prof.): conceptualization, methodology, validation, formal analysis, investigation, resources, writing – original draft, supervision, project administration. Afina Nuur Farma Megaelectra (M.Sc.) and Jenny Persson (M.Sc.) formal analysis, investigation, writing – review and editing, visualizationvisualisation. Su Zhang (Ph.D.) investigation, writing – review and editing. Beatrix Alsanius (Prof.) and Marie Olsson (Prof.) conceptualizationconceptualisation, writing – review and editing, project administration. Bengt Jeppsson (Prof.) conceptualizationconceptualisation, methodology, resources, writing – review and editing, supervision, project administration, funding acquisition.

All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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