Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Functional Foods 39 (2017) 306–311

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Housing influences tissue cytokine levels and the fecal bacterial community structure in rats

Padmaja Shastri[a], Judy Green[b], Fernando Matias[b], Martin L. Kalmokoff[c], Julia M. Green-Johnson[a], Stephen P.J. Brooks[b,*]

[a] Applied Bioscience Graduate Program and Faculty of Science, University of Ontario Institute of Technology, Oshawa, Ontario L1H 7K4, Canada
[b] Bureau of Nutritional Sciences, Health Canada, Ottawa, Ontario K1A 0K9, Canada
[c] Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, Kentville, Nova Scotia B4N 1J5, Canada

ARTICLE INFO

Keywords:
Gut bacteria
Hardwood chips
Immunology
Cytokines
Wire-bottomed cages

ABSTRACT

Immune measures and the fecal bacterial community were examined in female Biobreeding rats housed in wire bottom cages (wire) or in solid bottom cages containing hardwood chips (bedding). Housing did not affect food intake, weight gain, fecal output or fibre content, serum liver enzymes, or spleen and mesenteric lymph node immune cell populations. Bedding-housed rat feces were enriched in phylotypes aligning within the phylum *Firmicutes* (families *Lactobacillaceae* and *Erysipelotrichaceae*) and had a 2-fold lower content of phylotypes aligning within the phylum *Bacteroidetes*. Feces from bedding-housed rats also contained significantly more acetic acid and less propionic, isobutyric, valeric and isovaleric acids than those housed on wire. Bedding-housed rats had significantly higher splenic concentrations of interleukin-4 ($P < 0.001$). These results demonstrate that bedding can indirectly influence systemic and mucosal immune measures, potentially adding additional complexities and confounding results to nutrition studies investigating the health effects of dietary fibres.

1. Introduction

Environmental factors, including housing, contribute to the complexity of interpreting gut microbiota-mediated effects in rodent studies (Gill & Finlay, 2011). For example, changes in the dominant lineages of the fecal microbiota were observed when mice were moved to different locations within the same facility (Friswell et al., 2010) and differences have been noted in the same strain bred in different centres (Hufeldt, Nielsen, Vogensen, Midtvedt, & Hansen, 2010). In addition, strain-dependent alterations in environmental enrichment have been linked to differences in immunological parameters in mice (Marashi, Barnekow, Ossendorf, & Sachser, 2003). Environmental considerations are important when designing immunological studies since the gut bacterial community continuously interacts with the immune system both directly and indirectly through microbial-derived antigens and metabolic products (Slack et al., 2009).

Gut commensal microbes also participate in the induction of immune tolerance in the intestine. For example, segmented filamentous bacteria and polysaccharide A of *Bacteroides fragilis* have been shown to induce T_{reg} populations (Hooper & Macpherson, 2010). Metabolic products such as propionate are involved in maintenance of a regulatory immune milieu in the colon of specific pathogen free mice (C. Smith, Rocha, & Paster, 2006) and SCFA influence immune activity at the systemic level (Maslowski et al., 2009), as do microbial components able to act as TLR or NOD ligands (Balmer et al., 2014). Factors influencing bacterial community composition may, therefore, affect immune outcomes at mucosal and systemic levels.

Housing and type of bedding are important in studies measuring the impact of gut microbiota changes but their effects on host immune status have not been fully addressed. The Canadian Council of Animal Care strongly suggests the use of plastic cages and bedding for rodents (Canadian Council on Animal Care, 1993). Recommendations also include pair housing (when possible) and replacing wire cages with solid platforms or bedding (National Research Council (NRC), 2011) to alleviate stress, which appears to affect cecal microbial diversity in mice (Bangsgaard Bendtsen et al., 2012). Ingestion of bedding material is a concern (Le Leu, Conlon, Bird, & Clarke, 2015) as is coprophagy (Levine & Saltzman, 1999) in studies examining immune and gut community changes associated with dietary fibre consumption since these can affect nutritional measures. Here, we have examined the effect of two different types of housing (woodchip bedding vs wire-bottomed cages) on various mucosal and systemic immune parameters, the fecal bacterial community structure, and fecal metabolic activity. This study was undertaken to address animal care committee criticisms concerning

* Corresponding author at: Banting Research Centre, D394 Banting Research Centre, 251 Sir Frederick Banting Driveway, Ottawa, ON, Canada.
E-mail address: Steve.Brooks@Canada.ca (S.P.J. Brooks).

http://dx.doi.org/10.1016/j.jff.2017.10.021
Received 21 December 2016; Received in revised form 27 September 2017; Accepted 12 October 2017
Available online 02 November 2017

the use of wire bottomed cages when studying the functional properties of fermentable materials. We found that the use of bedding adds additional complexities to nutritional studies by altering bacterial community composition and immune parameters in the absence of dietary change.

2. Materials and methods

2.1. Animals

A total of 24 female BioBreeding control rats (BBc; inbred line originally derived from Wistar rats obtained from Health Canada Animal Resources Division), ages 28–42 d were randomly assigned to one of two groups: wire bottomed cages with metal plates covering ¼ of the floor space (12 rats: wire) or plastic suspended housing with maple wood chips (12 rats:bedding; P.W.I. Industries, Saint Hyacinthe QC). Female rats were selected for this study to reduce the possibility of immune effects not directly related to housing. Female rats are superior to males in two respects: the intestinal tract of female rats is naturally less permeable than that of males (Homma et al., 2005; Shastri, McCarville, Kalmokoff, Brooks, & Green-Johnson, 2015), and female rats have been reported to be more resilient to stress-induced physiological effects (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004).

The number of animals/housing group was determined from previous measurements in our lab showing that a 30% CV was common for many of the immune parameters measured. Power calculations indicated that 6 animals/group were required to determine a physiologically significant difference of about 1 ng/g (power of 80% with a type 1 error rate of 0.05). A total of 2 animals were required to measure all the parameters of interest. Thus, 6 × 2 = 12 animals/group were required for the study. Rats were transferred from the breeding room to the housing room (same facility) at the start of the experiment and housed individually for 63–67 d with free access to an AIN93G purified diet (Reeves, Nielsen, & Fahey, 1993) containing 5% w/w alphacel (cellulose) and reverse-osmosis treated water. This period was well in excess of the 7 days required to stabilise bacterial community changes after alterations in diet (Kalmokoff et al., 2013). Rats were subjected to a 12 h light/dark cycle at a temperature of 21 °C and cages and bedding were changed once per week. All rats were provided with shelters, glass marble toys and music. During the final two weeks of the study (d49-end), all food intake was monitored and all feces were collected (balance phase). Animals were killed by exsanguination under anesthesia. Serum samples were collected at necropsy. This study was approved by the Animal Care Committees of Health Canada and the University Of Ontario Institute Of Technology.

Health Canada maintains a specific pathogen free facility with yearly testing for lymphocytic choriomeningitis virus, mouse adenovirus FL/K87, hantavirus, *Encephalitozoon cuniculi*, and cilia-associated respiratory bacillus. Other yearly tests included bacterial cultures of nasal aspirate and cecum cultures as well as tests for endoparasites (including helminthes and protozoa) and ectoparasites. Tests repeated every three months included: sendai virus, pneumonia virus of mice, rat coronavirus, Kilham rat virus, Toolan's H-1 virus, rat parvovirus, rat minute virus, parvovirus generic assay, reovirus, rat theilovirus, and *Mycoplasma pulmonis*. All animals were negative.

2.2. Automated biochemical analyses

Serum samples (N = 6/housing group) were analysed for blood urea nitrogen (Talke & Schubert, 1965). Liver enzymes were measured using an ABX Pentra 400Automated clinical chemistry analyzer using ABX Pentra test kits (Burlington, Ontario, Canada). Specific reactions pathways as follows: alanine amino transferase (ALT) using L-alanine and 2-oxoglutarate as substrate coupled to lactate dehydrogenase and following the disappearance of NADH; alkaline phosphatase using *p*-Nitrophenylphosphate as substrate; aspartate amino transferase (AST) using 2-oxoglutarate plus L-aspartate as substrate coupled to malate dehydrogenase and following NADH disappearance; and lactate dehydrogenase using pyruvate as substrate following NADH disappearance.

2.3. Short chain and branched chain fatty acid analysis

Fecal SCFA and branched chain fatty acids were measured after acidification using sulfuric acid (Weaver, Krause, Miller, & Wolin, 1992) and analysed by gas chromatography on an Agilent 6890 gas chromatograph (Agilent Technologies Canada Inc., Mississauga ON). An internal standard (2-ethylbutryic acid) was added to the samples to correct for loss during extraction and the samples were filtered through a 0.45 µm PTFE syringe filter. The column was a 60 m × 0.25 mm, I.D. 0.25 µm film thickness Nukol column (Supleco-Sigma-Aldrich, Mississauga ON), run at 0.8 mL/min with helium carrier gas and the temperature was increased from 100 to 200 °C at 8 °C/min. Total run time was 30 min. The injector was washed in between runs by injecting acetone followed by 10 injections of water. A mixture of 2-ethylbutryic acid, sulphuric acid and volatile standard mix (Sigma-Aldrich, Mississauga ON) was injected every 10th run to ensure system calibration. Data was analysed using MSD Chemstation software (Agilent Technologies Canada Inc., ON, Canada).

2.4. Flow cytometry

Mucosal (mesenteric lymph node) and systemic (spleen) samples were analysed using a FACSCalibur flow cytometer (BD Biosciences, ON) and standard gating techniques (Shingadia, O'Gorman, Rowley, & Shulman, 2001). The following antibodies were used for cell surface staining: anti-CD45 (PE-Cy5, Clone OX-1) (Leukocyte Common Antigen), anti-CD3 (APC, Clone 1F4) (total T cell), anti-CD4 (PE, Clone OX-35) (T helper), anti-CD25 (FITC, Clone OX-39), anti-CD8a (FITC, Clone OX-8) (cytotoxic T cell), anti-CD45RA (FITC, Clone OX-33) (B cell), and anti-CD161a (PE, Clone 10/78) (Natural Killer cell). Cells were also permeabilized and intracellularly stained with anti-FOXP3 (PE, Clone FJK-16s) to detect regulatory T cell populations. Macrophage activation was analysed using anti-CD68 (Alexa Fluor 488, ED1 clone) (Onodera et al., 1997). Fluorochrome-matched isotype controls were used to assess non-specific binding. Anti-CD68 was purchased from AbD Serotec (NC, USA). All other antibodies were obtained from BD Biosciences (ON, Canada).

2.5. Tissue preparation & cytokine analysis by ELISA

Tissues were collected at necropsy, snap frozen and stored at −80 °C. The ileum, colon, cecum, and spleen were homogenized (Hoentjen et al., 2005) and then centrifuged for 30 min at 16,100*g*. Supernatants were stored at −80 °C and later analysed for the presence of cytokines: interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), transforming growth factor-β1 (TGF-β1) and cytokine-induced neutrophil chemoattractant-1 (CINC-1). ELISAs (Duo Set sandwich kits; R & D Systems, MN, USA) were performed in 96 well plates (Greiner Bio-One, NC, USA) and were read at a wavelength of 450 nm on a Synergy HTTR microplate reader (BioTek, VT, USA).

2.6. Fecal community analysis

Fresh, manually expressed feces were collected 1–2 days prior to the initiation of the balance phase and immediately stored at −20 °C. Fecal material from individual rats (1.0 g/rat) was pooled (N = 6/housing) to minimize inter-rat fecal community variability (Kalmokoff, Franklin, Petronella, Green, & Brooks, 2015). Fecal community DNA was prepared (Kalmokoff et al., 2015) and duplicate near full length 16S rRNA gene libraries were constructed from two independently derived community DNA samples for each housing condition. Ribosomal genes were

amplified using the primers F44/R1543 and cloned following 12 cycles of amplification (Kalmokoff et al., 2013). One hundred clones were randomly selected from each duplicate clone library (2 × 100 clones/housing) and sequenced. Near full length 16S rRNA clones were initially aligned against the Silva data base (Quast et al., 2013) and sequences demonstrating < 3% DNA sequence divergence binned into the dominant phylotype. The data was checked for chimeric sequences using subroutines available in Mothur (Schloss et al., 2009). Final binned sequences were assigned to the phylogeny using the seqmatch function available at the Ribosome Data Project (Cole et al., 2009).

Changes in community structure determined from the analysis of 16S rRNA clone libraries were confirmed using two additional analyses. First, differences in 16S rRNA gene content (as a percentage of total 16S rRNA gene copy number) within the phylum *Bacteroidetes* and the genus *Lactobacillus* (Castillo et al., 2006; Rinttila, Kassinen, Malinen, Krogius, & Palva, 2004) were quantified using quantitative PCR (qPCR). Total 16S rRNA gene content was determined using the universal primer set HDA1/HDA2 (Walter et al., 2000). Second, individual rat fecal pellets (N = 8/housing condition) were placed into separate pre-weighed 50 mL screw-cap plastic tubes containing 1.0 g of sterile glass beads, weighed, and transferred into an anaerobic hood. Following the addition of 10 mL anaerobic 0.1% peptone water, the pellets were mixed to obtain homogenous slurries, and subjected to a 10-fold dilution series. Dilutions were plated on L-10 anaerobic medium to obtain the total anaerobic count (Caldwell & Bryant, 1966) and de Man-Rogosa-Sharpe medium (De Man, Rogosa, & Sharpe, 1960) for total lactobacilli. Plates were incubated in an atmosphere of $H_2/CO_2/N_2$ (10:20:70) at 38 °C for 48 h, and the dilution plate having 30–300 colonies counted, with final counts expressed in terms of percentage of the total anaerobic count.

2.7. Statistical analysis

Immunological data were analysed after log transformation by two-way ANOVA with bedding and tissue as factors (Statistica; Statsoft, OK, USA). Fecal SCFA, food and energy consumption, feed efficiency, serum biochemical parameters, and Lactobacilli plating counts were analysed using the two-tailed Student's *t*-test (equal variance). The results are presented as mean ± standard error (SE). Results were considered significant when $P < 0.05$.

3. Results

No housing-related differences in food consumption or weight gain were observed (Table 1). Similar feed efficiencies suggested similar energy expenditure between groups. Fecal output and fecal fibre content over the 2 week balance phase were also not affected by housing. While dry weight fecal outputs were not significantly different, bedding material was present in the insoluble fraction derived from feces indicating that the rats housed on bedding ingested this material (Fig.1).

No differences in the levels of serum enzymes normally associated with liver pathology were observed. Blood urea nitrogen values were also similar between housing groups (Table 1). The proportion of fecal SCFA corresponding to acetic acid was significantly higher in bedding-housed rats. On the other hand, the proportion of propionic, isobutyric, valeric and isovaleric acids was significantly lower (Fig. 2). No differences in butyric acid were observed.

3.1. Immune parameters

Housing environment had no effect on immune cell population percentages (B cells, T cell subsets, macrophages and natural killer cells) in spleen and mesenteric lymph nodes (data not shown). Housing environment also had no effect on the levels of regulatory cytokines at the ileum (TGF-β1 and IL-10). However, bedding-housed rat ileal IL-6 levels (pro-inflammatory) tended to be higher when compared to wire-housed rats ($P = 0.052$; Table 1). No changes in cytokine levels were detected in the colon or cecum. At the systemic level, splenic CINC-1 levels were unchanged but splenic IL-4 concentrations were higher in rats housed on bedding versus those on wire ($P < 0.001$; Table 2).

Table 1
Physiological measures for rats housed on wire or on bedding.

	Wire		Bedding	
	N	Value	N	Value
Food, body weight:				
Total food consumption (g)	12	872 ± 31	10	881 ± 48
Daily food consumption (g/d)	12	19.8 ± 0.7	10	20.7 ± 1.1
Total energy consumption (kcal)	12	3447 ± 124	10	3482 ± 191
Daily energy consumption (kcal/d)	12	78.3 ± 2.8	10	81.7 ± 4.3
Total body weight gain (g)	12	85.1 ± 3.5	12	85.9 ± 3.3
Daily body weight gain (g/d)	12	1.93 ± 0.08	12	2.01 ± 0.08
Energy efficiency (g BW/kcal consumed)	12	25.0 ± 1.3	10	24.5 ± 1.0
Feces				
Fecal output (g dry weight/d)	12	1.16 ± 0.02	12	1.23 ± 0.02
Fecal fibre (%: g/g dry weight)	12	57.5 ± 0.8	12	58.2 ± 0.6
Serum biochemical parameters				
Lactate dehydrogenase (IU/L)	6	116 ± 16	6	137 ± 35
Blood urea nitrogen (mg/dL)	6	23.0 ± 0.6	6	22.3 ± 1.1
Alkaline phosphatase (IU/L)	6	201 ± 9	6	176 ± 15
Aspartate aminotransferase (IU/L)	6	58.5 ± 1.3	6	59.5 ± 2.0
Alanine aminotransferase (IU/L)	6	30.7 ± 2.1	6	29.8 ± 1.2

All P values were not significant (two-tailed *t*-test); values represent means ± SE for indicated N.

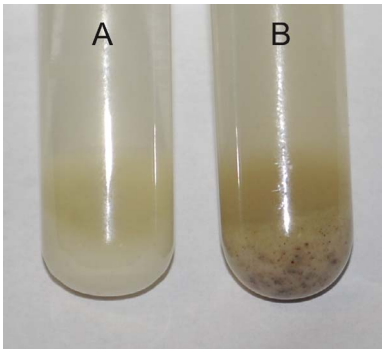

Fig. 1. Insoluble fecal fraction from rats housed on wire (A) or bedding (B). This material consists predominantly of wood cellulose added to the AIN93G diet as a source of insoluble dietary fibre.

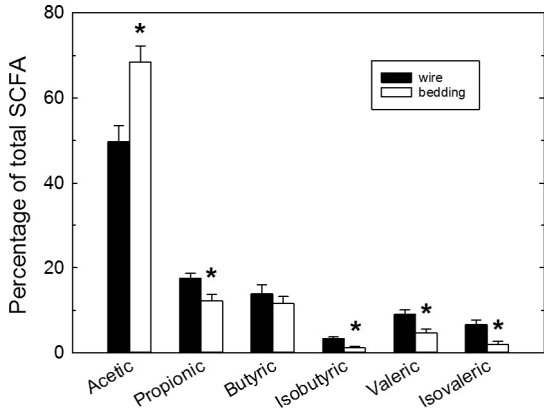

Fig. 2. Percentage of total SCFA corresponding to acetic, propionic, butyric, isobutyric, valeric and isovaleric acids in feces from rats housed on wire (black filled rectangles) or bedding (white filled rectangles). *Significantly different from wire housed rats. Values represent mean ± SE (N = 12).

Table 2
Tissue cytokine levels in rats housed on wire or bedding.

Tissue	Cytokine	Housing		ANOVA P
		Bedding (ng/g)	Wire (ng/g)	
Colon	IL-4	nd	nd	–
	IL-10	8.51 ± 1.08	8.10 ± 0.60	NS
	TGF-β1	0.510 ± 0.03	0.441 ± 0.03	NS
	IL-6	8.16 ± 7.47	7.80 ± 0.89	NS
Ileum	IL-4	0.645 ± 0.099	0.472 ± 0.134	NS
	IL-10	1.35 ± 0.05	1.89 ± 0.53	NS
	IL-6	2.18 ± 0.08	1.14 ± 0.14	0.052
	TGF-β1	< LOQ	< LOQ	–
Cecum[a]	IL-6	10.02 ± 0.61	9.79 ± 0.77	NS
	IL-10	7.92 ± 0.12	7.91 ± 0.38	NS
	TGF-β1	18.91 ± 0.88	22.11 ± 3.92	NS
Spleen	IL-4	1.25 ± 0.08	0.50 ± 0.22	< 0.001
	IL-10	4.45 ± 0.85	2.68 ± 0.09	NS
	TGF-β1	2.16 ± 0.43	1.29 ± 0.24	NS
	IL-6	< LOQ	< LOQ	––

Values represent means ± SE (N = 3–6). < LOQ denotes values that were below the limit of quantification; nd – not determined; NS-not significant.
[a] Cecal IL-4 concentrations were not evaluated due to insufficient amounts of tissue.

3.2. Fecal bacteria community

In total, 114 phylotypes were identified in feces, a level of community richness similar to previous fecal surveys of BBc rats (Abnous et al., 2009; Shastri et al., 2015). The fecal community of bedding-housed rats was enriched in clones aligning within the phylum *Firmicutes*, but depleted in clones aligning within the phylum *Bacteroidetes* compared to the community of wire-housed rats (14% versus 26% of total community respectively; Fig. 3A). The altered community structure resulted from differences in the content of clones aligning within the families *Lactobacillaceae* (14% versus 2% of total community in bedding versus wire housed respectively), *Erysipelotrichaceae* (14% versus 6%), and *Bacteroidaceae* (9% versus 1%; Fig. 3B).

Lactobacilli gene copy numbers, determined by qPCR, represented 22% and 3% of the total 16S rRNA gene copy number in rats on bedding or wire, respectively. Lactobacilli plate counts were also significantly different between bedding and wire housed animals (18 ± 11% versus 5 ± 4% of the total anaerobic plate count respectively, $P = 0.014$ two-tailed *t*-test). qPCR estimates for *Bacteroidetes* 16S rRNA gene content were higher than those found in the 16S rRNA clone libraries but followed a similar trend (29% versus 44% for bedding versus wire, respectively). We previously found that 16S rRNA gene based community analysis of samples from the rat gut tend to underestimate *Bacteroidetes* (Kalmokoff et al., 2015).

4. Discussion

In laboratory animals, the gut microbial community is sensitive to changes in a variety of environmental parameters, including diet (Abnous et al., 2009), the nutrition source provided during weaning (O'Sullivan et al., 2013), and conditions and husbandry practices used in the animal facility (Ivanov et al., 2009; Ma et al., 2012). A recent report also showed that bedding can influence the gut bacterial community and its metabolic activity (Le Leu et al., 2015). Similar to that study, we found that changes in housing affected fecal community diversity and metabolic activity. We also found limited changes in host systemic and mucosal immune parameters.

Housing rats on bedding altered fecal community structure by increasing *Firmicutes* content. This change resulted primarily from the enrichment of phylotypes aligning within the families *Lactobacillaceae* (*Lactobacillus*) and *Erysipelotrichaceae* at the expense of those aligning within the family *Bacteroidaceae* (*Bacteroides*). These general trends in the 16S rRNA-based analysis were confirmed by qPCR and anaerobic plating. Lactobacilli have an absolute requirement for peptidyl-nitrogen (De Man et al., 1960; Kandler & Weiss, 1986), whereas genera within the family *Bacteroidaceae* use ammonia as a source of nitrogen for growth (C. Smith et al., 2006). Thus, increases in the content of *Lactobacillus* likely reflect increases in the availability of peptidyl-nitrogen sources in the gut (Kalmokoff et al., 2015). We have observed similar phylum-level and species shifts in the feces of male rats fed various diets (Kalmokoff et al., 2013; Kalmokoff et al., 2015; Shastri et al., 2015).

In common with a previous study (Le Leu et al., 2015), we also observed a housing-related change in fecal SCFA output. In bedding-housed rats, the shift towards acetate at the expense of propionate was consistent with the decrease in the family *Bacteroidaceae*, as propionate production is common across this taxa (C. Smith et al., 2006). Furthermore, branched chain fatty acid concentrations (produced by the fermentation of valine, isovaline and lysine) were also significantly lower in feces from bedding housed rats compared to those housed on wire, which likely reflects an increased use of gut peptidyl-nitrogen to support continued growth rather than as an energy source (i.e., protein fermentation (E. A. Smith & Macfarlane, 1998)). Changes in gut community metabolic activity may also result from the fermentation of the bedding material used to house rats (Le Leu et al., 2015). However, it is unlikely that this material affected SCFA profiles for the following reasons: (i) fecal output was not affected by housing indicating that rats ingested only a very small amount of bedding material; (ii) woodchips (primarily cellulose) are fermented to a limited extent (Davies, Brown, & Livesey, 1991); and (iii) the cellulose content of the AIN93G

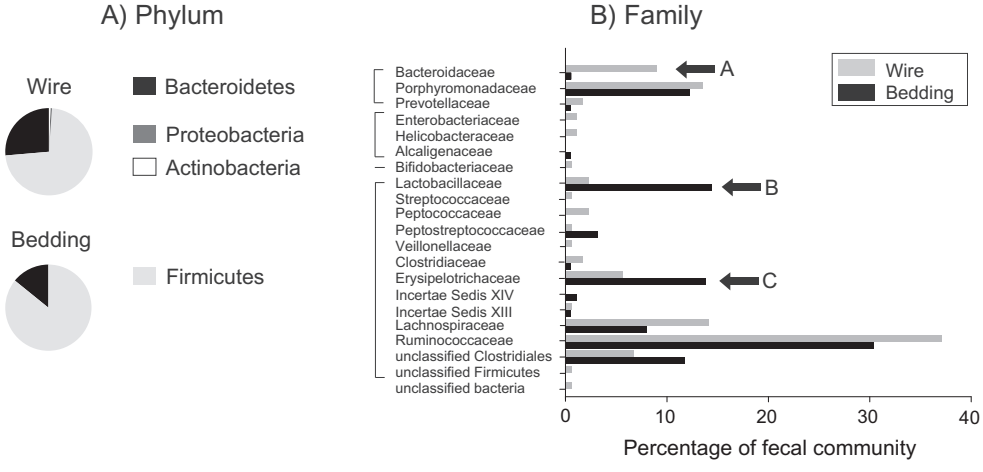

Fig. 3. Changes in fecal community structure in female rats housed on wire or bedding. Panel A: Community change at the phylum level. Panel B: Fecal community change at the family level. Taxa (family level) driving changes in community structure are indicated with arrows (A) *Bacteroidaceae* (B) *Lactobacillaceae* (C) *Erysipelotrichaceae*.

diet (5%) is in great excess to any amount of ingested bedding.

While changes in the fecal community of rats housed on bedding may be explained on the basis of increased peptidyl-nitrogen in the gut, the primary question is the source of this material. Since both groups were fed only the AIN93G diet and showed no difference in total food consumption, we suggest that the most likely source for the additional dietary protein would be from feces. In fact, coprophagy has long been known to be a concern in bedding housed rats, altering the cultivable fecal community (Fitzgerald, Gustafsson, & McDaniel, 1964) and, depending on the diet, improving weight gain by providing an additional (microbial) source of dietary protein (Midtvedt & Gustafsson, 1981; Torrallardona, Harris, & Fuller, 1996). Increasing dietary protein content via increased coprophagy could explain both the shift in community structure and changes to the overall community metabolic activity observed in rats housed on bedding. The changes in fecal community diversity are also consistent with observations in previous rodent studies. For example, anal cupping of rats to prevent coprophagy significantly reduced cultivable fecal Lactobacilli (Fitzgerald et al., 1964). Similarly, disrupting the normal behaviour of mice (i.e., coprophagy) by physical relocation resulted in a short term transition in the fecal community structure to one where phylotypes homologous with Lactobacilli decreased, while those homologous with *Flavobacterium* (Phylum *Bacteroidetes*) and aligning within the order *Clostridiales* increased (Ma et al., 2012). These changes are similar to those observed here in the fecal community of wire housed rats.

Housing also affected host mucosal and systemic immune parameters but the effect was minor. At the systemic level, splenic IL-4 concentrations were higher in bedding-housed rats relative to wire-housed rats. Both dietary and gut bacterial components can influence systemic immune measures at the spleen (Neyrinck, Mouson, & Delzenne, 2007). The ileum is more permeable than the colon and translocated bacteria and bacterial components from the ileum have previously been shown to affect host immune parameters in distal locations (Schulz & Pabst, 2013). Although ascertaining whether gut-derived factors had translocated across the epithelium into the circulation was outside of the scope of the current study, effects at the systemic level would be in keeping with the possibility of bedding-associated damage and IL-6-mediated repair at the ileum. Tissue injury stimulates intestinal epithelial cells to produce IL-6 (Rakoff-Nahoum et al., 2004) and the rodent gut mucosa has been shown to be sensitive to abrasion by fibrous substrates such as wheat bran (Perrin et al., 2001). One possible explanation is that the ingested wood chip material resulted in minor abrasive injuries during transit through the gut. The gut of female rats is more resistant to inflammation-induced injury (Homma et al., 2005) and β-estradiol ordinarily regulates levels of pro-inflammatory cytokines (including IL-6) in the intestinal tissue of female rats (Kilicdag et al., 2016) suggesting that the bedding-associated increase in ileal IL-6 levels reflected epithelial repair rather than inflammation.

On the other hand, fecal acetate levels also increased in bedding-housed rats. SCFAs can influence the immune system within (Smith et al., 2013) and external to the gut (Tan et al., 2016), and acetate has been shown to modulate immune activity and inflammatory responses at the systemic level after absorption (Maslowski et al., 2009). Acetate absorption can occur anywhere along the gut, so ingestion and absorption of microbial-derived fermentation products represents a reasonable explanation for the observed increases in splenic IL-4 levels.

Studies have shown that mice housed on hardwood chips (vs. isoPAD cotton bedding) had increased numbers of Peyer's patches and increased virus-specific IgA production by Peyer's patches and mesenteric lymph nodes (Sanford, Clark, Talham, Sidelsky, & Coffin, 2002). Although the authors did not report on cytokine levels, their results suggest a compromised barrier function in animals housed on wood bedding, which agrees with our observation of a local mucosal and systemic immune effect. The type of wood is also important. For example, softwood bedding induced liver drug-metabolising enzymes as well as reduced time spent asleep in rats and mice, and this was reversed by housing on hardwood bedding (Vesell, 1967). In agreement with these observations, we observed no effect of hardwood bedding on liver enzyme levels. Hardwood chip housing can be beneficial; it is superior in reducing ammonia volatiles when animals are housed in static air-flow cages (the cages used for bedding in this study) (Smith, Stockwell, Schweitzer, Langley, & Smith, 2004). Cotton bedding and paper can be problematic if total fecal output is to be measured, as we have observed considerable ingestion of these types of materials in other experiments.

Wire grid housing is known to induce stress in rodents (Giral, Garcia-Olmo, & Kramer, 2011) and this has been reported to alter certain behavioural and immune parameters as well as cecal community diversity in mice (Bangsgaard Bendtsen et al., 2012). In our study, a partial solid floor was provided for the wire-housed rats as well as environmental enrichment. These routine measures are carried out to reduce the stress of housing on bare wire housing. Since we did not determine circulating corticosterone levels, we cannot rule out the possibility that stress may also be contributing to the observed changes in the fecal community and immune parameters.

5. Conclusion

Rodent models remain an important tool for investigating and substantiating mechanisms by which functional foods or food components may affect host health. While the use of bedding is important for the welfare of laboratory animals, ingestion of bedding is known to impact various nutrition determinations. Here we have shown that the inclusion of bedding indirectly influences gut community structure as well as mucosal and systemic immune parameters, most likely by facilitating coprophagy. While alternative approaches such as rectal cups or constrained tube enclosures can be applied to prevent this, wire bottom cages likely remain the most humane and practical approach for nutritional studies where this may be of concern. It is apparent that several considerations must be taken into account when deciding on appropriate housing conditions and when interpreting results from experiments using differently housed rodents.

Acknowledgements

This work was supported through grants from the National Science and Engineering Research Council (JG-J), and by Agriculture and Agri-Food Canada (MK) and Health Canada (SB).

Conflict of interest statement

This work was undertaken with financial support from A-base funding from Agriculture and Agri-Food Canada (MK) and Health Canada (JG, FM, SB) as well as a grant from the National Science and Engineering Research Council (PS, JG-J). We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced the outcome.

References

Abnous, K., Brooks, S. P. J., Kwan, J., Matias, F., Green-Johnson, J., Selinger, L. B., ... Kalmokoff, M. (2009). Diets enriched in oat bran or wheat bran temporally and differentially alter the composition of the fecal community of rats. *Journal of Nutrition, 139*(11), 2024–2031. http://dx.doi.org/10.3945/jn.109.109470.

Balmer, M. L., Schurch, C. M., Saito, Y., Geuking, M. B., Li, H., Cuenca, M., ... Macpherson, A. J. (2014). Microbiota-derived compounds drive steady-state granulopoiesis via MyD88/TICAM signaling. *The Journal of Immunology, 193*(10), 5273–5283. http://dx.doi.org/10.4049/jimmunol.1400762.

Bangsgaard Bendtsen, K. M., Krych, L., Sorensen, D. B., Pang, W., Nielsen, D. S., Josefsen, K., ... Hansen, A. K. (2012). Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. *PLoS ONE, 7*(10), e46231. http://dx.doi.org/10.1371/journal.pone.0046231.

Caldwell, D. R., & Bryant, M. P. (1966). Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Applied Microbiology, 14*(5), 794–801.
Canadian Council on Animal Care (1993). *Guide to the care and use of experimental animals, 1*, 298. Retrieved from <http://www.ccac.ca/en_/standards/guidelines>.
Castillo, M., Martin-Orue, S. M., Manzanilla, E. G., Badiola, I., Martin, M., & Gasa, J. (2006). Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Veterinary Microbiology, 114*(1–2), 165–170. http://dx.doi.org/10.1016/j.vetmic.2005.11.055.
Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., ... Tiedje, J. M. (2009). The ribosomal database project: Improved alignments and new tools for rRNA analysis. *Nucleic Acids Research, 37*(Database issue), D141–D145. http://dx.doi.org/10.1093/nar/gkn879.
Davies, I. R., Brown, J. C., & Livesey, G. (1991). Energy values and energy balance in rats fed on supplements of guar gum or cellulose. *British Journal of Nutrition, 65*(3), 415–433 doi: S0007114591000466 [pii].
De Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*(1), 130–135. http://dx.doi.org/10.1111/j.1365-2672.1960.tb00188.x.
Fitzgerald, R. J., Gustafsson, B. E., & McDaniel, E. G. (1964). Effects of coprophagy prevention on intestinal microflora in rats. *Journal of Nutrition, 84*, 155–160.
Friswell, M. K., Gika, H., Stratford, I. J., Theodoridis, G., Telfer, B., Wilson, I. D., & McBain, A. J. (2010). Site and strain-specific variation in gut microbiota profiles and metabolism in experimental mice. *PLoS ONE, 5*(1), e8584. http://dx.doi.org/10.1371/journal.pone.0008584.
Gill, N., & Finlay, B. B. (2011). The gut microbiota: Challenging immunology. *Nature Reviews Immunology, 11*(10), 636–637. http://dx.doi.org/10.1038/nri3061.
Giral, M., Garcia-Olmo, D. C., & Kramer, K. (2011). Effects of wire-bottom caging on heart rate, activity and body temperature in telemetry-implanted rats. *Laboratory Animals, 45*(4), 247–253. http://dx.doi.org/10.1258/la.2011.010071.
Hoentjen, F., Welling, G. W., Harmsen, H. J., Zhang, X., Snart, J., Tannock, G. W., ... Dieleman, L. A. (2005). Reduction of colitis by prebiotics in HLA-B27 transgenic rats is associated with microflora changes and immunomodulation. *Inflammatory Bowel Diseases, 11*(11), 977–985 doi: 00054725-200511000-00004 [pii].
Homma, H., Hoy, E., Xu, D. Z., Lu, Q., Feinman, R., & Deitch, E. A. (2005). The female intestine is more resistant than the male intestine to gut injury and inflammation when subjected to conditions associated with shock states. *American Journal of Physiology. Gastrointestinal and Liver Physiology, 288*(3), G466–G472. http://dx.doi.org/10.1152/ajpgi.00036.2004.
Hooper, L. V., & Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nature Reviews Immunology, 10*(3), 159–169. http://dx.doi.org/10.1038/nri2710.
Hufeldt, M. R., Nielsen, D. S., Vogensen, F. K., Midtvedt, T., & Hansen, A. K. (2010). Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. *Comparative Medicine, 60*(5), 336–347.
Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., ... Littman, D. R. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell, 139*(3), 485–498. http://dx.doi.org/10.1016/j.cell.2009.09.033.
Kalmokoff, M., Franklin, J., Petronella, N., Green, J., & Brooks, S. P. J. (2015). Phylum level change in the cecal and fecal gut communities of rats fed diets containing different fermentable substrates supports a role for nitrogen as a factor contributing to community structure. *Nutrients, 7*(5), 3279–3299. http://dx.doi.org/10.3390/nu7053279.
Kalmokoff, M., Zwicker, B., O'Hara, M., Matias, F., Green, J., Shastri, P., ... Brooks, S. P. (2013). Temporal change in the gut community of rats fed high amylose cornstarch is driven by endogenous urea rather than strictly on carbohydrate availability. *Journal of Applied Microbiology, 114*(5), 1516–1528. http://dx.doi.org/10.1111/jam.12157.
Kandler, O., & Weiss, N. (1986). Regular, nonsporing gram-positive rods. *Bergey's manual of systematic bacteriology, 2*(2), 1208–1234.
Kilicdag, H., Daglioglu, Y. K., Sencar, L., Erdogan, S., Zorludemir, S., Polat, S., ... Coskun, G. (2016). Impacts of beta-estradiol on intestinal injury in newborn rats. *The Journal of Maternal-Fetal & Neonatal Medicine, 29*(12), 1935–1940. http://dx.doi.org/10.3109/14767058.2015.1068286.
Le Leu, R. K., Conlon, M. A., Bird, A. R., & Clarke, J. M. (2015). Housing experimental rats in solid-based cages with digestible bedding may confound outcomes of nutritional studies. *Journal of the Science of Food and Agriculture, 95*(10), 2155–2158. http://dx.doi.org/10.1002/jsfa.6919.
Levine, S., & Saltzman, A. (1999). Effects of coprophagy on serum urea and the weight of the gastrointestinal tract of fed or fasted rats. *Laboratory Animals, 33*(3), 265–268.
Ma, B. W., Bokulich, N. A., Castillo, P. A., Kananurak, A., Underwood, M. A., Mills, D. A., & Bevins, C. L. (2012). Routine habitat change: A source of unrecognized transient alteration of intestinal microbiota in laboratory mice. *PLoS ONE, 7*(10), e47416. http://dx.doi.org/10.1371/journal.pone.0047416.
Marashi, V., Barnekow, A., Ossendorf, E., & Sachser, N. (2003). Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. *Hormones and Behavior, 43*(2), 281–292.
Maslowski, K. M., Vieira, A. T., Ng, A., Kranich, J., Sierro, F., Yu, D., ... Mackay, C. R. (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature, 461*(7268), 1282–1286. http://dx.doi.org/10.1038/nature08530.
Midtvedt, T., & Gustafsson, B. E. (1981). Digestion of dead bacteria by germ-free rats. *Current Microbiology, 6*(1), 13–15.
National Research Council (NRC) (2011). *Guide for the care and use of laboratory animals* (8th ed.). Washington DC: National Academies Press.
Neyrinck, A. M., Mouson, A., & Delzenne, N. M. (2007). Dietary supplementation with laminarin, a fermentable marine beta (1–3) glucan, protects against hepatotoxicity induced by LPS in rat by modulating immune response in the hepatic tissue. *International Immunopharmacology, 7*(12), 1497–1506. http://dx.doi.org/10.1016/j.intimp.2007.06.011.
Onodera, S., Suzuki, K., Matsuno, T., Kaneda, K., Takagi, M., & Nishihira, J. (1997). Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. *Immunology, 92*(1), 131–137.
O'Sullivan, A., He, X., McNiven, E. M., Haggarty, N. W., Lonnerdal, B., & Slupsky, C. M. (2013). Early diet impacts infant rhesus gut microbiome, immunity, and metabolism. *Journal of Proteome Research, 12*(6), 2833–2845. http://dx.doi.org/10.1021/pr4001702.
Perrin, P., Pierre, F., Patry, Y., Champ, M., Berreur, M., Pradal, G., ... Menanteau, J. (2001). Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. *Gut, 48*(1), 53–61.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... Glockner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research, 41*(Database issue), D590–D596. http://dx.doi.org/10.1093/nar/gks1219.
Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., & Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell, 118*(2), 229–241.
Reeves, P. G., Nielsen, F. H., & Fahey, G. C., Jr. (1993). AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition, 123*(11), 1939–1951.
Rinttila, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of Applied Microbiology, 97*(6), 1166–1177. http://dx.doi.org/10.1111/j.1365-2672.2004.02409.x.
Sanford, A. N., Clark, S. E., Talham, G., Sidelsky, M. G., & Coffin, S. E. (2002). Influence of bedding type on mucosal immune responses. *Comparative Medicine, 52*(5), 429–432.
Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., ... Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environment Microbiology, 75*(23), 7537–7541. http://dx.doi.org/10.1128/AEM.01541-09.
Schulz, O., & Pabst, O. (2013). Antigen sampling in the small intestine. *Trends in Immunology, 34*(4), 155–161. http://dx.doi.org/10.1016/j.it.2012.09.006.
Shastri, P., McCarville, J., Kalmokoff, M., Brooks, S. P. J., & Green-Johnson, J. M. (2015). Sex differences in gut fermentation and immune parameters in rats fed an oligofructose-supplemented diet. *Biology of Sex Differences, 6*(1), 13. http://dx.doi.org/10.1186/s13293-015-0031-0.
Shingadia, D., O'Gorman, M., Rowley, A. H., & Shulman, S. T. (2001). Surface and cytoplasmic immunoglobulin expression in circulating B-lymphocytes in acute Kawasaki disease. *Pediatric Research, 50*(4), 538–543. http://dx.doi.org/10.1203/00006450-200110000-00019.
Slack, E., Hapfelmeier, S., Stecher, B., Velykoredko, Y., Stoel, M., Lawson, M. A., ... Macpherson, A. J. (2009). Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science, 325*(5940), 617–620. http://dx.doi.org/10.1126/science.1172747.
Smith, P. M., Howitt, M. R., Panikov, N., Michaud, M., Gallini, C. A., Bohlooly, Y. M., ... Garrett, W. S. (2013). The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science, 341*(6145), 569–573. http://dx.doi.org/10.1126/science.1241165.
Smith, E. A., & Macfarlane, G. T. (1998). Enumeration of amino acid fermenting bacteria in the human large intestine: Effects of pH and starch on peptide metabolism and dissimilation of amino acids. *FEMS Microbiology Ecology, 25*(4), 355–368.
Smith, C., Rocha, E., & Paster, B. (2006). The medically important *Bacteroides* spp. in health and disease. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.). *The prokaryotes* (pp. 381–427). New York: Springer.
Smith, E., Stockwell, J. D., Schweitzer, I., Langley, S. H., & Smith, A. L. (2004). Evaluation of cage micro-environment of mice housed on various types of bedding materials. *Journal of the American Association for Laboratory Animal Science, 43*(4), 12–17.
Talke, H., & Schubert, G. E. (1965). Enzymatic urea determination in the blood and serum in the Warburg optical test. *Klin Wochenschr, 43*, 174–175.
Tan, J., McKenzie, C., Vuillermin, P. J., Goverse, G., Vinuesa, C. G., Mebius, R. E., ... Mackay, C. R. (2016). Dietary fiber and bacterial SCFA enhance oral tolerance and protect against food allergy through diverse cellular pathways. *Cell Reports, 15*(12), 2809–2824. http://dx.doi.org/10.1016/j.celrep.2016.05.047.
Torrallardona, D., Harris, C. I., & Fuller, M. F. (1996). Microbial amino acid synthesis and utilization in rats: The role of coprophagy. *British Journal of Nutrition, 76*(5), 701–709.
Vesell, E. S. (1967). Induction of drug-metabolizing enzymes in liver microsomes of mice and rats by softwood bedding. *Science, 157*(3792), 1057–1058. http://dx.doi.org/10.1126/science.157.3792.1057.
Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., & Alatossava, T. (2000). Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Applied and Environment Microbiology, 66*(1), 297–303.
Weaver, G. A., Krause, J. A., Miller, T. L., & Wolin, M. J. (1992). Cornstarch fermentation by the colonic microbial community yields more butyrate than does cabbage fiber fermentation; cornstarch fermentation rates correlate negatively with methanogenesis. *American Journal of Clinical Nutrition, 55*(1), 70–77.