In vitro digestion and fermentation of animal-derived fermentable substrates using canine and feline faecal inoculum

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Abstract

There is increasing interest in understanding the fermentative benefits of animal-derived fermentable substrates (ADFS) in pet foods. While previous research has assessed various ADFS using faecal inoculum derived from the cat, there is no published literature available for the dog. Additionally, very little is understood of the fermentation profiles of ADFS, such as skin and bone. Therefore, faeces were collected from a cohort of cats and dogs fed a complete and balanced high-protein diet and a selection of substrates were analysed in this study. Individual ADFS (tendon, bone cake, mechanically deboned meat (MDM), corium and hydrolysed collagen (a positive control)) were digested in vitro, followed by fermentation using either canine or feline faecal inoculum. Concentrations of butyrate, indole and ammonia were determined after 24 h of fermentation. Regardless of whether cat or dog faecal inoculum was used, fermentation of hydrolysed collagen produced the highest (P < 0.01) concentrations of butyrate and ammonia and the lowest concentrations of indole. For the other substrates, there were differences in the fermentation profiles between the canine and feline inocula. During the feline faecal fermentations, in comparison to the other substrates bone cake produced high (P < 0.05) butyrate concentrations, whereas in the dog faecal fermentations, MDM resulted in high (P < 0.05) butyrate concentrations. In conclusion, ADFS from different alternative co-products lead to different fermentation products, providing valuable information which may be considered in canine and feline dietary formulations.

Keywords

cooproducts – animal-derived – short-chain fatty acids (SCFA) – cat – dog

1 Introduction

As with other food industries, there is increasing interest in the environmental sustainability of pet food production (Okin, 2017; Pedrinelli et al., 2022; Swanson et al., 2013). Historically, commercial petfood production has relied heavily on ‘edible’ co-products from meat (red meat, poultry and fish) industries, most notably organs, such as liver, kidney and heart, primarily due to their nutritional value (i.e. protein, fat, minerals and vitamins). With greater competition for animal proteins, there is growing interest in alternative co-products and the functional role they can play in nutrition (Butowski et al., 2022; Depauw et al., 2012a) as well as sustainability (Acuff et al., 2021).

Gastrointestinal (GI) health, due to its role in maintaining overall health, has consistently been of interest to pet food manufacturers and pet owners for the
last few years. Domestic cats and dogs are considered obligate and facultative carnivores, respectively, evolving to consume diets high in animal proteins and fats and low in plant-derived carbohydrates. Despite this, it is well recognised that the GI microbiome of both cats (Butowski et al., 2019; De Oliveira Matheus et al., 2021; Fischer et al., 2012) and dogs (Beloshapka et al., 2012; Souza et al., 2023) can ferment a range of plant-derived dietary fibres and other carbohydrates, producing short-chain fatty acids (SCFA) in vivo. Certainly plant-derived dietary fibre plays an important role in stool formation (Butowski et al., 2019; Lee et al., 2022).

In the wild, felids consume various collagenous substrates, such as the bone and skin of their prey, that may act as a source of fermentable substrates (Depauw et al., 2013). The most abundant collagen types are I, II and III and are found in tendon, ligament, blood vessels, bones, cartilage and skin. Types of collagen are typically classified on the basis of their amino acid composition. Alternatives to traditional dietary fibres (inulin and cellulose), such as sheep’s wool (Deb-Choudhury et al., 2018), various collagens (Depauw et al., 2012a) and even the fermentation profile of cat hair have been studied (Butowski et al., 2022). These animal-derived fermentable substrates (ADFS) can be fermented in vitro, producing varying concentrations of fermentation end products (Depauw et al., 2013; Plantinga et al., 2011). For example, Depauw et al. (2012b) assessed the fermentative capacity of chicken cartilage, collagen, rabbit bone, hair and skin using cetha (Acinonyx jubatus) faecal inoculum. Collagen fermentation (assessed by total gas production) was comparable to that of cellulose, casein and fructo-oligosaccharides, whereas rabbit skin, hair and bone were all poorly fermentable. More recently, Butowski et al. (2022) identified that the fermentation of hydrolysed collagen in cat faecal inoculum resulted in butyrate concentrations similar to that of inulin, a well-known prebiotic fibre (Hussein et al., 1999). However, few studies have assessed whether this applies to dogs.

In vitro systems are often used to assess the fermentative potential of various nutrients including non-digestible carbohydrates (Passlack et al., 2022), fruit pomaces (Holt et al., 2023), oligosaccharides (Bosch et al., 2013; Holt et al., 2023) and yeasts (Gonzalez et al., 2023; Van den Abbeele et al., 2020). However, there is growing interest in novel, potentially functional, co-products such as skin/fur and cartilage (Butowski et al., 2022; Depauw et al., 2012a). Therefore, this study examined the in vitro fermentation profile of various ADFS in both cat and dog faecal inoculum.

## 2 Materials and methods

### Ethical statement

Faecal collection for this study was conducted under the ethics approval from Massey University Animal Ethics Committee (MUAEc) protocol 20/81. All cats were housed at the Massey University Centre for Feline Nutrition (Palmerston North, New Zealand) and dogs at the Massey University Centre for Canine Nutrition (Palmerston North, New Zealand) for the duration of the study.

### Faecal collection

Faeces used in the in vitro fermentation were obtained from cats (n = 5) and dogs (n = 5) that were maintained on a commercially available complete and balanced raw air-dried food for cats (ZIWI’ Air-Dried Lamb Recipe for Cats, Auckland, New Zealand) or dogs (ZIWI’ Air-Dried Lamb Recipe for Dogs) for 10 d (Table 1). The dogs were housed in large outdoor pens (10 × 10 m) during the day and individually in indoor pens during the night.

### Table 1 Macronutrient composition of the commercial air-dried lamb recipe diets fed to the cats and dogs for ten days prior to faecal collection

<table>
<thead>
<tr>
<th>Macronutrient (%) dry matter</th>
<th>Cat1</th>
<th>Dog2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>43.14</td>
<td>43.93</td>
</tr>
<tr>
<td>Crude fat</td>
<td>41.83</td>
<td>41.78</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>1.31</td>
<td>1.18</td>
</tr>
<tr>
<td>Ash</td>
<td>9.69</td>
<td>10.20</td>
</tr>
<tr>
<td>Gross energy (kJ/g)</td>
<td>27.23</td>
<td>27.18</td>
</tr>
</tbody>
</table>

1. Lamb, lamb tripe, lamb lung, lamb heart, lamb liver, lamb bone, New Zealand green mussel, lamb kidney, lamb spleen, lecithin, lamb cartilage, inulin (from chicory root), dried apple pomace, minerals (dipotassium phosphate, magnesium sulphate, zinc amino acid complex, iron amino acid complex, copper amino acid complex, manganese amino acid complex, sodium selenite), dried organic kelp, salt, dl-methionine, preservative (citric acid, mixed tocopherols), vitamins (choline chloride, thiamine mononitrate, pyridoxine hydrochloride, folic acid, vitamin d3 supplement), taurine.

2. Lamb, lamb tripe, lamb lung, lamb heart, lamb liver, lamb bone, New Zealand green mussel, lamb kidney, lamb spleen, lecithin, lamb cartilage, parsley, dried apple pomace, inulin (from chicory root), minerals (dipotassium phosphate, magnesium sulphate, zinc amino acid complex, iron amino acid complex, copper amino acid complex, manganese amino acid complex, sodium selenite, yeast), dried organic kelp, salt, preservative (citric acid, mixed tocopherols), vitamins (vitamin e supplement, thiamine mononitrate, riboflavin, pyridoxine hydrochloride, vitamin d3 supplement, folic acid).
(2 × 2 m). Cats were housed in individual cages (80 × 80 × 110 cm) for the 10-day period. On day 10, dogs were fed at 8:00 am and then faeces were collected immediately post-defecation. For the cats, the first five faeces passed were collected. Entire faeces were placed into a 50 ml tube and snap frozen in liquid nitrogen prior to storage at −80 °C.

Substrates
The various substrates evaluated in this study were all of bovine origin; hydrolysed collagen (Peptan B 2000 LD: Rosselott, Kuala Lumpur, Malaysia), tendon, mechanically deboned meat (MDM), bone cake (the remnants after the MDM process), lung, skin (whole shaved bovine hide) and corium (dermis of the skin). MilliQ water was used as the control. Hydrolysed collagen (manufactured from bovine skin) was used as a positive control as it has previously been shown to be readily fermentable by feline faecal inoculum in vitro (Butowski et al., 2022) and faecal microbiome in vivo (Butowski, 2021).

In vitro digestion
The in vitro gastric and small intestinal digestion was performed as described previously (Minekus et al., 2014), with the following adaptations made to simulate the cat and dog gastrointestinal tract (Butowski et al., 2022), whereby digestion was competed at 39 °C (cat and dog body temperature), pH of the simulated gastric fluid (SGF) was reduced to 2.5. The oral phase of digestion was removed, as salivary amylase activity is significantly reduced in cats and dogs (McGeachin and Akin, 1979).

In total, 10 ml of MilliQ water plus 0.2 ml HCl (6 M) was added to each 5 g of substrate into a Schott bottle – one for each digest. Next, 9.8 ml of SGF complete was added (Butowski et al., 2022). The pH was then checked and adjusted to 2.5 before adding to the shaking incubator (100 rpm) at 39 °C for 2 h. After the allotted time, in vitro digests were removed from the incubator, 0.15 ml NaOH (1 M) and 19.85 ml of ‘complete’ simulated intestinal fluid (SIF) (Butowski et al., 2022) were added then pH was modified to 6.5. Digests were then returned to the shaking incubator for a further 2 h.

After digestion, Schott bottles were transferred to a water bath (80 °C) for two minutes to neutralise enzyme activity. The total volume following digestion was recorded, then three parts digesta to one part potassium phosphate buffer solution (pH 6.8, 0.4 mol/l) were added to dialysis tubing (Biotech CE tubing MWCO: 100–500D, New Zealand), which had previously been cut and soaked in reverse osmosis (RO) water for 30 min. Dialysis clips were used to secure the ends, then the tubes were immersed into buckets of RO water (8 l to 30 cm dialysis tubing, 140 ml starting vol) which was changed twice in the 24-h period. Once complete, total volume of retentate was recorded, then frozen at −20 °C before use in the in vitro fermentation.

Preparation of faecal inocula
A 10% faecal solution (w/v) was prepared using phosphate buffer (pH 6.8, 0.4 mol/l) by straining faeces through a mesh bag (MicroAnalytix, Auckland, New Zealand) for each animal (n = 5 cats and n = 5 dogs).

In vitro fermentation
After digestion, the substrates were fermented for 24 hours in autoclaved 10 ml glass culture (Hungate) tubes. The day prior, retentate was defrosted and 6 ml was aliquoted into a Hungate tube (one tube per animal per substrate), along with 2 ml phosphate buffer (pH 6.8, 0.4 mol/l). Tubes were then bubbled with nitrogen for 20 s to remove the dissolved oxygen then the head space topped with carbon dioxide before sealing.

Pressure was equalised in each Hungate tube, then 100 µl of 3% L-cysteine was added, then rested for 10 min to allow cysteine to equalise prior to adding the inoculum. An aliquot of 2 ml of faecal solution was then injected into each Hungate tube and the time was noted before it was placed into a shaking incubator at 39 °C for 24 h. After this period, samples were removed from the incubator, inverted, placed on ice and then centrifuged at 14,000×g for 15 min. The supernatant was then removed for fermentation end point analysis. Tubes were stored at −80 °C until analysis.

Amino acid analysis of substrates
Amino acid analysis was determined using ion exchange chromatography (Shimadzu Scientific Instruments Ltd, Columbia, MD, USA) as described previously (Bermingham et al., 2006). Briefly, 50 mg of substrate was derivatised with ninhydrin after acid hydrolysis with 6.0 M HCl at 110 °C for 22 h. Collagen content was estimated by multiplying the hydroxyproline content by 7.25 (Chaosap et al., 2021; Li et al., 2022). Protein content was estimated by multiplying the sum of alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, valine and proline by 1.13 (Reinmuth-Selzle et al., 2022).

Organic acid analysis of fermentation supernatant
A 900 µl aliquot of fermentation supernatant was homogenized in 0.01 M phosphate-buffered saline with 100 µl of 50 mM 2-ethylbutyric acid (final concentration
5 mM) as an internal standard. Internal standards containing 2-ethylbutyric acid (5 mM) were prepared in parallel. Each sample was then centrifuged at 3,000×g for 5 min at 4 °C. Next, 500 µl of this supernatant was collected and acidified with 250 µl concentrated HCl, then 1 ml diethyl ether was added. Samples were then vortexed and centrifuged at 10,000×g for 5 min (4 °C). The upper diethyl ether phase was collected and 200 µl was derivatised with 40 µl N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tertbutyldimethylchlorosilane (MTBSTFA + TBDMSCl, 99:1; Sigma-Aldrich, St. Louis, MO, USA) by heating to 80 °C for 20 min. Samples were then left for 48 h at room temperature to allow complete derivatisation before analysis.

Analysis was performed on a Shimadzu gas chromatograph system (GC-2030, Kyoto, Japan), equipped with a flame ionisation detector and fitted with a HP-1 column (30 m × 0.25 mm ID × 0.25 µm) (Agilent Technologies, Santa Clara, CA, USA). The carrier gas was hydrogen, with a linear velocity of 40 cm/second. The temperature program began at 70 °C, increasing to 80 °C at 10 °C/min, with a final increase to 255 °C at 20 °C/min, before holding for 5 min. Injector and detector temperatures were set at 260 °C. Samples were injected (1 µl) with a split injection 10:1 split ratio. The instrument was controlled and chromatograms acquired using Labsolutions software (Shimadzu, Kyoto, Japan). The acquired GC data was used to plot standard curves, providing a sample result of µmol butyrate/ml fermentation supernatant.

**Indole analysis of fermentation supernatant**

A 250 µl aliquot of fermentation supernatant was thawed and diluted to 1 ml with acetonitrile (high-performance liquid chromatography (HPLC) grade) (750 µl) then the sample was vortexed for 15 s at 2,000 rpm. Samples were then centrifuged at 1,500×g for 5 min and an aliquot was transferred to a 1.5 ml autosampler vial. A 1 µl aliquot was injected onto a Aquasil C18 column 3 µm particle size 100 × 2.1 mm (Thermo Fisher, Auckland, New Zealand). HPLC analysis was isocratic at 0.3 ml/min using 35% solvent B in A. Solvent A was 0.02 M acetic acid in MilliQ water and solvent B was 0.02 M acetic acid in 2-propanol (iso propyl alcohol) HPLC grade (Optima Thermo Fisher, New Zealand).

Detection was by fluorescence, with an excitation wavelength of 275 nm and an emission wavelength of 345 nm. The detector sensitivity range was between 0 and 6.67 µg/ml. All results were within the linear range of the standard curve. Results were calculated by the Shimadzu Lab Solutions interface and expressed as µg/ml.

**Ammonia analysis of fermentation supernatant**

A 200 µl aliquot of fermentation supernatant was thawed and vortexed, then mixed with 800 µl of 0.125 M HCl to both dilute samples within the Linear Range and drive equilibrium of endogenous ammonia to liquid ammonium ions. The samples were then filtered through a 0.45 µm cellulose acetate (CA) filter and stood on ice until analysis.

A 150 µl volume of sample was aliquoted into a HPLC vial and injected onto a HPLC (Shimadzu 20Ai), 0.3 ml/min sodium buffer: sodium cation exchange column (#II93250, 3.0 × 250 mm (Pickering Laboratories, Mountain View, CA, USA)); with detection at 540 nm. Samples were nested by Sigma Amino Acid Standard (AAS) 18 calibration standard, containing 0.5 µmol ammonium chloride. Sample signal was corrected for drift and dilution factor and reported as mmol/l.

**Statistical analysis**

All statistical analyses were performed using R version 4.0.5 (R Core Team, 2020). Separate analyses were performed according to species. A linear mixed effect model was used with substrate as a fixed effect and faecal donor as a random effect. Pairwise comparisons with Bonferroni adjustment were then made across substrates using the ‘predictmeans’ R package (Luo et al., 2018). The R package ‘ggplot2’ was used to plot concentrations of the fermentation metabolites for each substrate. In addition, R package ‘factoextra’ was used to plot principal component analysis (PCA) for organic acids and for indole and ammonia.

Outlier samples were removed from this study based on assessment of normality. In the dog faecal fermentations, 1 sample (lung substrate) was removed from SCFA analysis. In the cat faecal fermentations, 1 sample was removed for the ammonia (tendon) analysis and 1 sample was removed from the indole (bone cake) analysis.

3 Results

**Amino acid profiles**

The amino acid profiles of the substrates investigated varied greatly between substrates (Table 2). As expected, the hydrolysed collagen had the highest collagen content (904 mg/g as is) followed by tendon (415 mg/g as...
In vitro canine and feline faecal fermentation of animal-derived substrates


Table 2 Concentrations of amino acids, collagen and protein (mg/g as is) in hydrolysed collagen, lung, mechanically deboned meat (MDM), bone cake, beef skin, corium and tendon

<table>
<thead>
<tr>
<th></th>
<th>Mechanically debone meat</th>
<th>Lung</th>
<th>Hydrolysed collagen</th>
<th>Tendon</th>
<th>Bone cake</th>
<th>Skin</th>
<th>Corium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>10.5</td>
<td>14.5</td>
<td>55.9</td>
<td>29.4</td>
<td>12.9</td>
<td>25.6</td>
<td>25.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.4</td>
<td>7.3</td>
<td>18.3</td>
<td>10.1</td>
<td>5.2</td>
<td>8.8</td>
<td>8.1</td>
</tr>
<tr>
<td>Serine</td>
<td>5.3</td>
<td>8.1</td>
<td>34.2</td>
<td>17.7</td>
<td>6.6</td>
<td>15.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>17.2</td>
<td>19.4</td>
<td>102.9</td>
<td>52.0</td>
<td>21.0</td>
<td>45.2</td>
<td>44.8</td>
</tr>
<tr>
<td>Proline</td>
<td>7.3</td>
<td>10.5</td>
<td>138.4</td>
<td>63.2</td>
<td>19.2</td>
<td>56.9</td>
<td>57.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.3</td>
<td>15.1</td>
<td>230.0</td>
<td>118.4</td>
<td>30.4</td>
<td>94.8</td>
<td>94.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.2</td>
<td>12.8</td>
<td>87.6</td>
<td>45.9</td>
<td>15.1</td>
<td>37.1</td>
<td>36.2</td>
</tr>
<tr>
<td>Valine</td>
<td>6.8</td>
<td>10.7</td>
<td>21.2</td>
<td>12.0</td>
<td>6.3</td>
<td>10.6</td>
<td>9.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.4</td>
<td>4.8</td>
<td>14.2</td>
<td>7.5</td>
<td>4.1</td>
<td>7.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.0</td>
<td>14.7</td>
<td>29.1</td>
<td>17.2</td>
<td>9.1</td>
<td>14.8</td>
<td>13.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.8</td>
<td>5.3</td>
<td>5.4</td>
<td>4.4</td>
<td>3.0</td>
<td>4.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.1</td>
<td>8.0</td>
<td>19.1</td>
<td>11.0</td>
<td>5.1</td>
<td>9.4</td>
<td>8.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.8</td>
<td>12.1</td>
<td>36.4</td>
<td>17.2</td>
<td>9.2</td>
<td>16.8</td>
<td>16.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.7</td>
<td>5.0</td>
<td>6.8</td>
<td>3.9</td>
<td>2.9</td>
<td>3.5</td>
<td>3.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.8</td>
<td>10.1</td>
<td>77.9</td>
<td>39.3</td>
<td>13.6</td>
<td>34.1</td>
<td>33.9</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>2.0</td>
<td>3.8</td>
<td>124.7</td>
<td>57.6</td>
<td>16.0</td>
<td>46.9</td>
<td>51.7</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.3</td>
<td>0.9</td>
<td>13.0</td>
<td>8.0</td>
<td>1.9</td>
<td>5.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Collagen content¹</td>
<td>14.8</td>
<td>27.6</td>
<td>904.4</td>
<td>417.4</td>
<td>116.3</td>
<td>339.7</td>
<td>374.9</td>
</tr>
<tr>
<td>Protein content²</td>
<td>96.1</td>
<td>134.7</td>
<td>805.6</td>
<td>410.6</td>
<td>143.3</td>
<td>349.8</td>
<td>343.5</td>
</tr>
</tbody>
</table>

¹ Collagen content was estimated from hydroxyproline concentration using a conversion factor of 7.25 (Chaosp et al., 2021; Li et al., 2022).
² Protein content was estimated by multiplying the sum of alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, valine and proline by 1.13 (Reimnuth-Selzle et al., 2022).

is), corium (375 mg/g as is) and skin (340 mg/g as is), whereas lung (28 mg/g as is) and MDM (14 mg/g as is) were the lowest.

In vitro feline faecal fermentations
The fermentative profiles from feline inocula of the co-products are shown in Figure 1A. Fermentation of the hydrolysed collagen and bone cake produced the highest concentrations of butyrate in the cat faecal inoculum (P < 0.05; Figure 2A). Butyrate concentrations were lowest in the control (no substrate) and corium fermentations (P < 0.05; Figure 2A). Indole concentrations were highest in the bone cake fermentation samples (P < 0.05; Figure 2B) and lowest in the hydrolysed collagen samples. Ammonia concentrations were highest in the hydrolysed collagen and corium fermentation samples and lowest in the control samples (Figure 2C). Principle component analysis (PCA) of the fermentative metabolites indicated that bone cake and hydrolysed collagen samples cluster distinctly (Figure 3).

In vitro canine faecal fermentations
The fermentative profiles from canine inocula of the co-products are shown in Figure 1B. In the canine faecal fermentations, hydrolysed collagen and MDM produced the highest concentrations of butyrate (P < 0.05; Figure 4A). Indole concentrations were highest in the fermented lung tissue (Figure 4B) and ammonia concentrations were highest in the hydrolysed collagen and tendon samples (Figure 4C). The PCA analysis of fermentation products similarly showed that hydrolysed collagen had a distinct profile (Figure 5).
Figure 1  Barplot showing means of faecal metabolites after 24 h of *in vitro* fermentation using (A) cat faecal inoculum from n = 5 cats or (B) dog faecal inoculum from n = 5 dogs.
**FIGURE 2**
Boxplot of (A) butyrate (mmol/l), (B) indole (µg/ml) and (C) ammonia (mmol/l) concentrations after 24 h of *in vitro* fermentation using cat faecal inoculum from *n* = 5 cats. Letters indicate significant differences according to pairwise comparison. In the boxplots, the solid black line denotes the median and the top and bottom boundaries of boxes indicate 75th and 25th percentiles and whiskers show ±1.5× the interquartile range. Circles show outliers defined as data points beyond 1.5× interquartile range.
A principal components analysis (PCA) plot of fermentation products from substrates fermented in vitro using cat faecal inoculum. Yellow circles denote Control samples, blue triangles denote MDM samples, grey plus denote Lung samples, brown crosses denote Hydrolysed Collagen, orange diamonds denote Tendon samples, green upside-down triangles denote Bone Cake, dark blue squares denote Skin, red stars denote Corium. Ellipses represent 95% confidence intervals.
A boxplot of (A) butyrate (mmol/l), (B) indole (µg/ml) and (C) ammonia (mmol/l) concentrations after 24 h of in vitro fermentation using dog faecal inoculum from n = 5 dogs. In the boxplots the solid black line denotes the median and the top and bottom boundaries of boxes indicate 75th and 25th percentiles and whiskers show ±1.5× the interquartile range. Circles show outliers defined as data points beyond 1.5× interquartile range. Letters indicate significant differences according to pairwise comparison. Solid black line denotes the mean.
Discussion

This study showed that a range of co-products, defined as ADFS, can be fermented in vitro to produce butyrate in both canine and feline faecal inoculum. However, there appeared to be a difference between the capacity of the cat and dog faecal microbiome, as indicated by different fermentation profiles of the substrates between the canine and feline faecal inoculum. This was an important finding as it emphasised the need for distinction between the two species with regard to potential effects of ADFS on gastrointestinal health.

The health benefits of collagen are typically associated with skin and joint health in humans (Fu et al., 2019) and joint health in dogs (Beynen et al., 2010; Comblain et al., 2016; D’Altilio et al., 2007; Deparle et al., 2005; Peal et al., 2007). However, there is increasing evidence to support the use of collagen peptides for promoting GI health following results from studies which showed that collagen peptides improved intestinal barrier function in vitro (Chen et al., 2017). The current study focussed on three important fermentation end-products namely, butyrate, ammonia and indole. Butyrate is considered to be one of the most beneficial metabolites, as it is a major energy source for colonocytes in the gastrointestinal tract (Clausen and Mortensen, 1995; Fleming et al., 1991) and improves intestinal tight junction function (Peng et al., 2009; Wang et al., 2020). While butyrate production is typically associated with the fermentation of carbohydrates (especially dietary fibres), microbial fermentation of amino acids, including glutamate and lysine, results in butyrate synthesis (Louis and Flint, 2017). In the current study, the highest concentrations of butyrate were observed for hydrolysed collagen in both canine and feline faecal inoculum. Lysine was found in the greatest concentrations in the hydrolysed collagen, which may have contributed to this finding. However, in the feline faecal fermentations, bone cake produced equally high concentrations of butyrate, despite relatively lower concentrations of lysine, which suggested that other nutrients remaining in the non-digested substrate within the bone cake may have played a role. Previous results have shown that hydrolysed collagen can promote butyrate production in both feline (Butowski et al., 2022) and human (Larder et al., 2021) in vitro models of GI-fermentation. In vivo, inclusion of 4% hydrolysed collagen resulted in similar concentrations of faecal butyrate, as compared to plant dietary...
fibres in cats (Butowski, 2021). Interestingly, reduced faecal butyrate concentrations were observed when the cats were fed 6% hydrolysed collagen, which suggested a maximal inclusion rate of hydrolysed collagen (Butowski, 2021).

Ammonia is derived from bacterial proteolysis of amino acids and peptides and is considered detrimental to colonicocytes in high luminal concentrations, as it inhibits mitochondrial oxygen consumption and SCFA oxidation in in vitro porcine cell models (Darcy-Vrillon et al., 1996; Davila et al., 2013). Additionally, high ammonia concentrations have been observed to decrease intestinal barrier function in vitro (Yokoo et al., 2021). In the current study, higher ammonia concentrations were associated with the fermentation of hydrolysed collagen and corium in the both the feline and canine fermentation samples. The fermentation of tendon tissue in the canine inoculum caused higher levels of ammonia. Previous research has indicated that fermentation of hydrolysed collagen with both cat (Butowski et al., 2022) and cheetah faecal inoculum (Depauw et al., 2012b) resulted in high ammonia concentrations, compared to other ADFS. Hydrolysed collagen is high in proline and hydroxyproline and it is likely that the high ammonia concentrations produced were a result of fermentation of these amino acids. Interestingly, Larder et al. (2021) observed that concentrations of ammonia gas were affected by the fermentation of collagen peptides, but this was dependent on the region of the human colon that was being modelled in vitro. After hydrolysed collagen, corium and tendon had the greatest collagen content; 417.4 mg/g and 374.9 mg/g, respectively. These findings agreed with others where the collagen content of bovine tendon (96.7% of total protein (Reutersward et al., 1985) and cow hides (76.7% of total protein content (Naff, 2017) was relatively high. Therefore, given the high collagen (and thus hydroxyproline) content, this may have contributed to the increased ammonia concentrations in vitro.

Lastly, indole is a metabolite of microbial tryptophan fermentation (Tennoune et al., 2022) which is synthesised endogenously by various bacterial taxa. Indole is considered a putrefactive factor and may contribute towards faecal odour (Urrego et al., 2021). It is converted to indoxyl sulphate (a protein-bound uremic toxin) in the liver, then excreted in the urine and has been correlated with severity of chronic kidney disease in cats and dogs (Cheng et al., 2015). While indole may cause increased bacterial toxicity, drug resistance, spore and biofilm formation (Chant and Summers, 2007; Lee and Lee, 2010), it has been implicated in improving intestinal barrier function both in vitro (Bansal et al., 2010) and in vivo in humans (Shimada et al., 2013). Therefore, indole appears to play a complex role in the colon (Wang et al., 2023; Ye et al., 2022), whereby it may have beneficial effects in preserving the intestinal barrier, balanced with conversion to indoxyl sulphate. In the current study, indole concentrations were highest in the fermentation of bone cake (feline inoculum) and lung (canine inoculum), although the reasons for this were unclear, as tryptophan was not assessed in the ingredients. In vitro fermentation using dog faecal inoculum with substrates such as readily fermentable carbohydrate-rich ingredients (e.g. fructooligosaccharides or sugar beet pulp) produced less indole than protein-rich ingredients (e.g. poultry meat meal, soybean meal; Bosch et al., 2013).

Based on these metabolites, PCA analysis indicated that, for the feline faecal inoculum, bone cake and hydrolysed collagen fermentation patterns were distinct from the other ADFS studied. In comparison, dog faecal fermentation profiles from hydrolysed collagen and corium showed distinct patterns. Corium is the dermal layer of the skin (the thicker layer underlying the epidermis of the skin), made up of collagenous material (Safiya and Casparus, 2020). It has a different collagen profile than other materials, such as tendon, and this may explain why its fermentation profile differed from the other ADFS tested. While less studied, bone cake is a product produced during the compression of bones to make MDM and is comparatively low in collagenous material (116 mg/g as is) when compared to hydrolysed collagen (900 mg/g as is). Despite this, both bone cake and hydrolysed collagen produced high butyrate concentrations. In the dog, MDM and hydrolysed collagen had the highest butyrate concentrations and again the collagen content of MDM was low compared to hydrolysed collagen (c. 15 vs 900 mg/g as is). Interestingly, while hydrolysed collagen is composed of small peptides, both the bone cake and MDM are complex ingredients, so observing similar levels of butyrate, despite lower collagen levels (and potentially less available peptides), suggested that other undigested components within MDM could be fermented to butyrate. However, the faecal inoculum used in this study was obtained from animals consuming diets that contained bone cake and MDM, so it was possible that the microbiome had already adapted to ferment these products. Regardless, these results showed that the inclusion of MDM and bone cake in pet foods may be beneficial. Indeed, rodents fed...
bone collagen peptide powder from Yak tissue appeared to have anti-fatigue properties, attributed in part due to changes in the antioxidant capacity and gut microbiome (Feng et al., 2023).

Future in vitro work should include a control sample which includes only the digestive enzymes, and/or is assessed in conjunction with the digestion of a relevant pet food. The ADFS substrates are more likely to be consumed as part of a complete and balanced diet, which needs to be investigated. Undertaking a full nutritional profile of the substrates used in future work is necessary to understand the mechanisms that may underpin the fermentation pattern observed. Microbial profiles should be assessed to determine which microbes are likely responsible for the fermentation and subsequent production of metabolites from the ADFS substrates.

5 Conclusions

In conclusion, this study showed that a range of co-products can be fermented by the faecal microbiome of both the cat and dog. Specifically, bone cake and hydrolysed collagen appear to be readily fermentable, leading to butyrate production using the feline faecal inoculum, while MDM and hydrolysed collagen were readily fermentable in the canine faecal inoculum. This highlights the importance of optimising diets for each species. Additional research is required to further assess the inclusion of ADFS in a complete and balanced diet and optimise the inclusion rates for in vivo studies.

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Conflict of interest

The authors have declared no conflict of interest.

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