Metagenomic Insights into Pet Food Microbiomes and Resistomes

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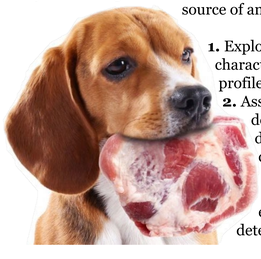
Abstract

Raw pet food is an increasingly popular dietary selection for companion animals. Comprised primarily of minimally processed animal tissues and raw vegetables, there are perceived nutritional benefits over heavily processed kibbles. As with any food, the origin of ingredients and their path through food manufacturing environments defines potential hazards. Raw animal tissues may serve as reservoirs for zoonotic foodborne pathogens and antimicrobial resistance (AMR) genes. In this study, metagenomic sequencing was used to characterize microbial communities of frozen (n=8) freeze-dried (n=8) and conventional dry (n=5) commercial pet foods. All samples were processed in triplicate. To complement the metagenomic profiles, selective enrichments for *Campylobacter*, *Listeria*, *Salmonella*, and *Enterococcus*, were also established and evaluated by metagenomic sequencing. Bacterial taxonomy and antimicrobial resistance genes were annotated using Kraken and AMRFinderPlus, respectively. Communities from culture independent frozen samples were dominated by Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes phyla, and shared a small core microbiome comprised of *Carnobacterium*, *Brochothrix*, *Bacillus*, *Acinetobacter*, *Lactococcus*, and *Psychrobacter* genera. *Enterococcus* spp. were also detected across all samples, accounting for ~0.02-1.1% of reads in culture independent and ~3-99% in enriched samples. Despite the use of traditional enrichment protocols for detection of *Salmonella*, *Campylobacter*, and *Listeria*, none were detected at levels above (> 1.0 %) for any of the raw frozen or freeze-dried products. Raw freeze-dried communities were distinct from those of raw frozen products and were dominated by *Bacillus*, *Pediococcus*, and *Lactobacillus*. Products with poultry (duck or chicken) as the primary protein source had a higher relative abundance of *E. coli* (1.54 ± 1.03%) in culture independent samples compared to non-poultry-based products (0.08 ± 0.15%; $P < 0.01$). However, no other associations between primary raw ingredient source and potential pathogens were identified. Variable profiles of AMR genes associated with resistance to aminoglycosides, tetracyclines, and folate pathway inhibitors were observed across frozen compared to freeze-dried products with increased diversity and abundance of AMR genes observed in frozen products. While metagenomics is valuable for identification of the complex macro and micro-ingredients associated with foods, it is critical to include enriched approaches when assessing potential microbial and antimicrobial food safety risks. Both data types are presented here and effectively create a high resolution methodology for the description of microbial communities, potential microbial pathogens, and antimicrobial resistance in pet foods.

Introduction

- Academic research surveys found pet owners perceived raw pet food as higher quality relative to conventional dried kibble (i.e. higher nutritional content/digestibility, and safety)
- While they may be perceived as better or healthier options, like any uncooked meat product, there is the risk of microbial contamination
- The FDA does not currently have a definition of what constitutes raw pet food but it generally includes products where the primary protein source is uncooked.
- The FDA considers a pet food to be adulterated when it is contaminated with *Salmonella* and will not undergo subsequent commercial heating (or other processes that will kill the *Salmonella*)
- Contaminated pet food can serve as a risk to humans due to shedding by infected animals and direct inoculation during product handling
- Beyond serving as a reservoir for human food-borne pathogens, raw pet is a potential source of antimicrobial resistant microbial communities.



Research Objectives:

1. Explore the use of shotgun metagenomics to characterize microbial communities and AMR gene profiles in raw pet food.

2. Assess the ability of shotgun metagenomics to detect human food-borne pathogens and determine whether these detection rates are consistent with conventional culture and molecular-based methods.

3. Investigate the impacts of limited selective enrichment on community profiles and pathogen detection rates.

Materials and Methods

- A total of 21 pet foods were purchased from commercial retailers and included several raw frozen (n=8), raw freeze-dried (n=8) and conventional dried (n=5) dog food representing multiple primary protein sources **(Table 1).**
- Samples were homogenized to generate rinsates which were used to establish selective enrichments for *Listeria*, *Salmonella*, *Enterococcus*, *E. coli* and *Campylobacter* species **(Figure 1).**

Table 1. List of products included in the study. Products were binned into animal food types: raw frozen (RF), raw freeze-dried (RD) and conventional dry kibble (D). The primary and secondary protein sources were recorded including specific tissue types when available.

Product Code	Type	Primary Protein	Secondary Protein(s)
A	RF	Beef (muscle, liver, kidney, spleen)	-
B	RF	Pork (muscle, liver, heart, bone)	-
C	RF	Duck (muscle)	Turkey (bone)
D	RF	Venison (muscle)	Beef (heart, liver)
E	RF	Lamb (muscle)	Beef (liver, kidney, spleen)
F	RF	Chicken (muscle, liver, bone)	-
G	RF	Beef (muscle, liver, bone)	-
H	RD	Beef (muscle, heart, liver, kidney, bone)	-
I	RD	Chicken (muscle, bone, liver, gizzard)	-
J	RD	Pork (muscle, liver, bone, kidney)	-
K	RD	Lamb (muscle, heart, liver, bone)	-
L	RD	Rabbit (muscle, liver, heart, kidney, lung)	-
M	RD	Venison (muscle, liver, lung)	Lamb (muscle, liver, kidney, heart)
N	RD	Duck (muscle, neck, heart, wing, liver)	-
O	RF	Turkey (heart, muscle, liver)	-
P	RD	Salmon (muscle)	-
Q	D	Lamb (muscle)	Menhaden (meal)
R	D	Chicken (muscle)	-
S	D	Beef (meal)	-
T	D	Chicken (muscle, meal)	-
U	D	Beef (muscle)	Chicken (meal)

- The presence of viable pathogens in the enrichment cultures was determined using pathogen-specific biochemical tests and selective/differential medias.
- Species identification of presumptive pathogens was performed using MALDI-TOF mass spectroscopy
- Isolates confirmed as pathogens of interest were subject to antimicrobial susceptibility testing according to methods established by the FDA National Antimicrobial Resistance Monitoring System (NARMS)
- DNA was extracted from both enrichments and rinsates and subject to shotgun metagenomic analysis and loop-mediated isothermal amplification (LAMP) for the detection of specified pathogens.
- Microbial community and AMR gene profiles were established for the shotgun metagenomic data using Kraken2 and AMRFinderPlus pipelines respectively

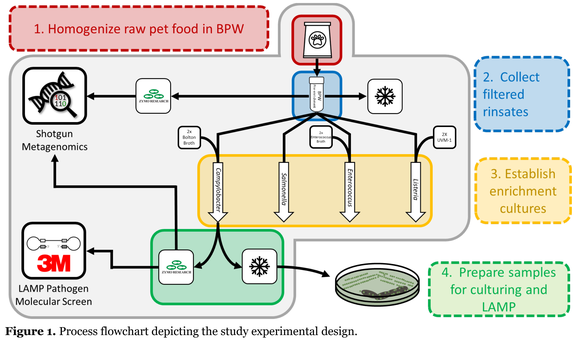


Figure 1. Process flowchart depicting the study experimental design.

Results and Discussion

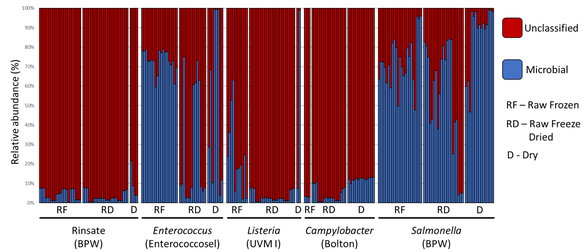


Figure 2. Bar chart depicting the relative abundance of reads classified as either "unclassified" (presumptive eukaryotic matrix DNA) or microbial in origin using Kraken2 with the standard Kraken database.

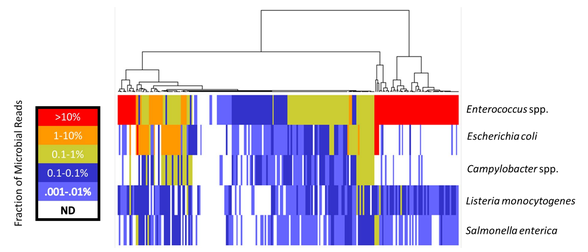


Figure 3. Heat map displaying the relative abundance five food-borne pathogens detected in rinsates and enrichment cultures by shotgun metagenomic analysis. Temperatures indicate the log relative abundance of each pathogen as a fraction of the total number of microbial reads identified in the sample.

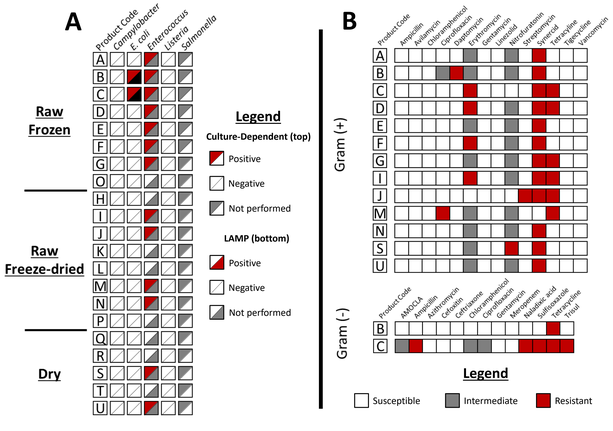


Figure 4. Tile plots depicting the presence-absence of pathogens and observed AMR phenotypes for organisms isolated from product enrichment cultures. (A) Products were screened for the presence of specified pathogens by culture-dependent biochemical tests (top half of tile) or LAMP (bottom half of tile). Colored fill indicates positive results for culture-dependent (red) and LAMP (black) assays. No fill (white) indicates negative results while gray fill indicates the assay was not performed. (B) Antimicrobial susceptibility testing was performed on enrichment isolates (Gram-positive panel for suspected *Enterococcus* and Gram-negative panel for suspected *E. coli*). Tile fill color indicates whether isolates showed susceptibility (white), intermediate resistance (gray), or resistance (red) to the specified antimicrobial.

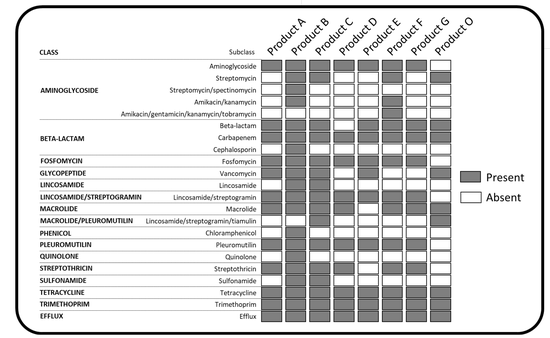
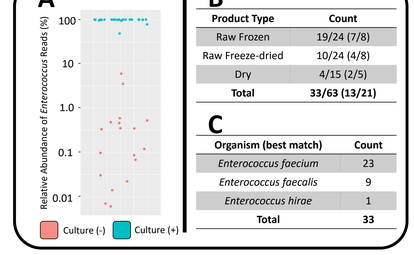


Figure 5. Tile plot showing the presence (gray) or absence (white) of specified AMR gene classes in raw pet food samples using shotgun metagenomics. Sequences were classified using AMRFinderPlus.



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Product Type	Count
Raw Frozen	19/24 (7/8)
Raw Freeze-dried	10/24 (4/8)
Dry	4/15 (2/5)
Total	**33/63 (13/21)**

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Organism (best match)	Count
Enterococcus faecium	23
Enterococcus faecalis	9
Enterococcus hirae	1
Total	**33**

Figure 6. (A) Scatter plot showing the distribution of the relative abundances of *Enterococcus* spp. identified in product enrichments by shotgun metagenomics. Datum point color indicates whether samples screened culture-positive. (B) Table summarizing the distribution of samples screening culture-positive for *Enterococcus* across the different product types. (C) Table summarizing the closest species match for the 33 *Enterococcus* isolates. Speciation was performed by MALDI-TOF mass spectroscopy (min. identification score >1.7.

Conclusion

- Shotgun metagenomics is a promising tool for profiling microbial communities and AMR genes in diverse pet food samples.
- While no *Campylobacter*, *Listeria*, or *Salmonella* was detected in the limited number of samples examined by LAMP screening and culture work, raw pet food samples demonstrated higher occurrences of *E. coli* and *Enterococcus* indicator organisms
- Raw pet food appears to serve as a reservoir of AMR genes and elevated AMR gene diversity was associated with raw pet food samples that screened positive for both *E. coli* and *Enterococcus* suggesting they may serve as the primary reservoirs.
- Limited enrichment is necessary for pathogen detection in raw pet food due to low microbial loads and high concentrations of matrix DNA.
- Until it's possible to reliably link AMR genes to specific host bacteria by shotgun metagenomics, culture-dependent analysis will likely remain an essential component of any microbiome AMR genes characterization workflow.

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