Do Dogs Even Care?

Challenges in Measuring and Assessing Oxidation in Animal Co-Products

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FOUR RIVERS KENNEL, LLC

NARA

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RESEARCH PROJECT-2020-2021: THE DETERMINATION OF AROMATIC PALATABILITY OF CHICKEN MEALS AND CHICKEN BY-PRODUCT MEALS AND CORRELATING PALATABILITY INFORMATION WITH PEROXIDE VALUES, ELECTRONIC NOSE (ENOSE) DATA AND QUANTITATIVE ANALYSES OF VOLATILE AND CHEMICAL COMPONENTS IN MEAT MEALS

Dr. Craig Coon, PhD

RESEARCH PROPOSAL: COMBINING *IN VITRO* DIGESTION AND CACO-2 CELL CULTURE TO EVALUATE POULTRY MEAT AND POULTRY MEAT BY-PRODUCT MEALS CONTAINING DIFFERENT AMOUNTS OF OXIDIZED LIPIDS ON INTESTINAL MEMBRANE PERMEABILITY, CYTOTOXICITY, METABOLIC ACTIVITY, AND TRANSCRIPTOMICS.

Dr. Craig Coon, PhD

Chicken Meals and Chicken By-Product Meals

- Rendering exposes products to high levels of heat
- Meat meals with high levels of protein and fat are vulnerable to oxidation during the process
- Peroxide Values (PV) are used throughout the industry to evaluate oxidation status of food[s].

RATIONALE

Accumulation of oxidants in gut lumen can contribute to impairment of mucosal metabolic pathways, enterocyte dysfunction independent of cell injury and development of gut pathologies, such as inflammation and cancer (Tak Yee Aw, 2004).

Ingestion of dietary oxidized lipids can promote oxidative stress by making additional demands upon the antioxidant system. Oxidized lipids are absorbed and incorporated into the membrane where they alter membrane fluidity and alteration of some cellular immune functions (Turek, et al. 2003).

Depending upon raw materials and source, animal and poultry meat meals and by-product meals may require significant thermal processing for purpose of eliminating pathogens which may enhance both protein and lipid oxidation (Liu et al., 2014).

1. Aw, T.Y. (2005). Intestinal glutathione: determinant of mucosal peroxide transport, metabolism, and oxidative susceptibility. Toxico. Appl Pharmacol, 204,320-328.
2. Liu, P., Kerr, B.J., Weber, T.E., Chen, C., Johnston, L.J., & Shurson, G.C. (2014). Influence of thermally oxidized vegetable oils and animal fats on intestinal barrier function and immune variables in young pigs. J Anim Sci, 92, 2971-2979.
3. Turek, J.J., Watkins, B.A., Shoenlein, I.A., Allen K.G.D., Hayek, G.M., & Aldrich, G.C. (2003). Oxidized lipid depresses canine growth, immune function, and bone formation. J Nutr Biochem, 14, 24-31.

OBJECTIVES

Does oxidation of rendered animal proteins affect palatability for companion animals

Do oxidative products generated during rendering cause negative effects for intestinal cells

Determining acceptable PV levels for palatability and gut health for chicken meals and chicken by-product meals for consumption by companion animals

.

Outline of study

Chemical analysis (sample composition)
E-nose analysis (sample odors)
Large scale aromatic palatability trials
Traditional palatability trials

Chemical Analysis

University of Missouri Metabolomics lab performed chemical analysis through Gas Chromatography/ Mass Spectroscopy and Solid Phase Microextraction headspace analysis

- **Chicken By-Product Meal** and **Chicken Meal** acquired from Tyson Foods
- Chemical analysis of samples with an incremental increase of PV

	PV range		PV	No. of Samples
CBPM	< 10	CB_1	4.24	1
	20-50	CB_2	41.87	1
	50-100	CB_3	68.58	1
	100 - 200	CB_4	218.58	1
CM	< 10	CM_1	7.95	1
	20 - 50	CM_2	14.98	1
	50 - 100	CM_3	40.89	1
	100 - 200	CM_4	196.74	1

Summary of Chemical Analysis

- Volatile, nonpolar, and polar compounds were studied separately and qualitative and quantitative analysis were carried out.

Percent Composition of Compounds from Nonpolar GC-MS Analysis

Percent Composition of Compounds from Nonpolar GC-MS Analysis								
	CM				CBPM			
	1	2	3	4	1	2	3	4
Acid (41)	59.52%	54.18%	64.52%	52.17%	54.01%	64.66%	64.61%	61.34%
Alcohol (17)	32.94%	33.32%	20.60%	35.14%	32.33%	21.79%	20.27%	23.26%
Aldehyde (1)	0.43%	0.64%	1.03%	0.51%	0.60%	1.00%	0.81%	0.84%
Amide (1)	0.42%	0.63%	0.93%	0.48%	0.65%	0.94%	0.78%	0.79%
Amino Acid (8)	1.36%	0.88%	0.36%	0.84%	1.24%	0.34%	0.37%	1.29%
Ester (11)	1.19%	1.97%	1.93%	1.97%	1.28%	2.06%	2.26%	2.55%
Ether (2)	0.08%	0.10%	0.09%	0.09%	0.07%	0.08%	0.08%	0.09%
Hydrocarbon (9)	0.84%	0.78%	0.49%	0.73%	0.76%	0.62%	0.70%	0.69%
Ketone (3)	0.20%	0.22%	0.16%	0.25%	0.24%	0.19%	0.17%	0.20%
N Organic (11)	2.65%	2.62%	2.80%	3.97%	3.05%	2.78%	2.69%	2.84%
Unknown (78)	7.99%	8.63%	8.75%	10.64%	8.59%	9.08%	9.34%	9.86%

Table :- Percent Composition of Compounds from Nonpolar GC-MS Analysis

Non- Polar Compounds Discussion

- Acids, Alcohols, Ketones, Amide, Amino Acids and N & S Organic compounds shows higher values in CBPM than CM.
- Derived from oxidation decomposition of fats
- Hydrocarbons and Esters are not considered as imporant but presence of those compounds are significant compared to the beef and pork products

Percent Composition of Compounds from Polar GC-MS Analysis

Percent Composition of Compounds from Polar GC-MS Analysis								
	CM				CBPM			
	1	2	3	4	1	2	3	4
Acid (51)	55.62%	57.12%	52.25%	58.34%	44.64%	41.60%	44.48%	44.66%
Alcohol (27)	7.22%	6.55%	8.91%	8.08%	10.93%	9.95%	10.60%	11.39%
Amino Acid (48)	33.56%	30.60%	34.04%	32.67%	38.46%	40.32%	37.66%	38.55%
Ester (11)	0.97%	0.96%	1.08%	0.99%	1.33%	1.72%	1.81%	1.73%
Hydrocarbon (11)	0.66%	0.48%	0.36%	0.22%	0.43%	0.34%	0.42%	0.54%
Ketone (5)	0.49%	0.56%	0.43%	0.51%	0.48%	0.36%	0.37%	0.32%
N Organic (31)	3.81%	5.33%	4.18%	3.76%	5.74%	5.88%	5.66%	4.82%
N and S Organic (2)	0.44%	0.34%	0.41%	0.34%	0.39%	0.53%	0.51%	0.60%
Unknown (160)	8.66%	9.62%	8.04%	7.67%	6.16%	6.32%	6.35%	6.55%

Table : Percent Composition of Compounds from Polar GC-MS Analysis

Polar Compounds Discussion

- Aldehydes, Alcohols, Ketones, Amide, Amino Acids and Esters shows higher values in CBPM than CM. The CM products contained more Acids.
- Amino acids, N-Organic and N and S Organic compounds originated from the breakdown of protein, free amino acids and nucleic acids.
- S-organic compounds may be conjugated to synthesize aromatic compounds such as thiophenes, thaozoles and thiazines.
- Some of the sulphur compounds are formed as they are prone to oxidation.

Percent Composition of Compounds from Volatile GC-MS Analysis

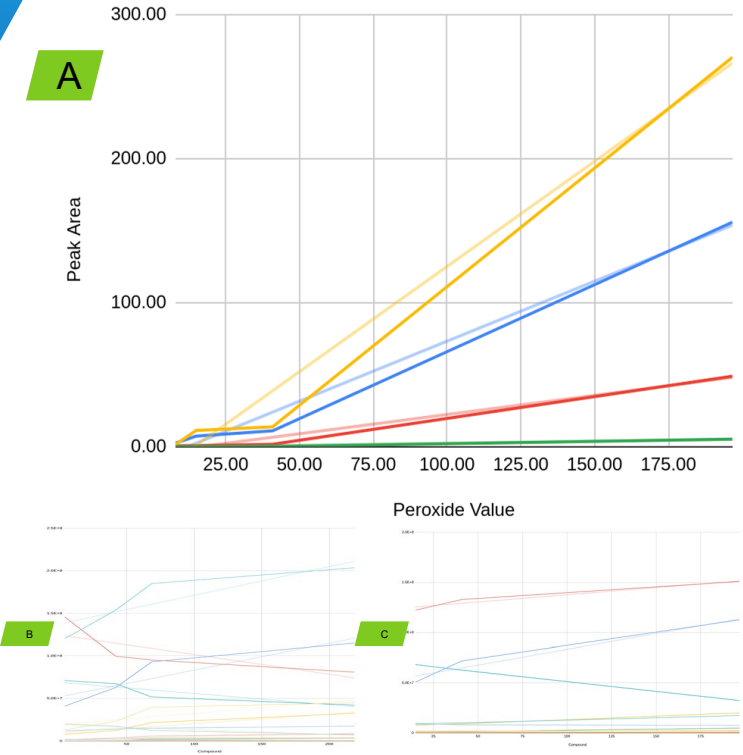
Percent Composition of Compounds from Volatile SPME Analysis								
	CM				CB			
	1	2	3	4	1	2	3	4
Acid (24)	4.07%	2.78%	1.65%	8.74%	8.00%	8.05%	11.66%	6.95%
Alcohol (21)	12.95%	8.29%	11.87%	12.76%	9.60%	8.16%	3.67%	14.47%
Aldehyde (27)	19.77%	31.38%	22.72%	27.40%	27.16%	33.10%	31.82%	25.87%
Amino Acid (2)	1.04%	0.95%	1.48%	3.43%	1.35%	2.10%	3.11%	4.35%
Ester (13)	16.36%	9.77%	12.92%	11.00%	10.20%	7.31%	9.04%	5.27%
Silicate (4)	0.21%	0.20%	0.10%	0.93%	0.11%	0.16%	0.08%	0.14%
Hydrocarbon (67)	10.26%	10.46%	10.67%	8.57%	9.70%	9.99%	10.48%	14.47%
Ketone (27)	26.73%	23.15%	25.65%	15.94%	20.56%	23.02%	17.64%	16.08%
N Organic (5)	0.14%	6.83%	6.27%	4.08%	5.69%	0.20%	5.25%	4.82%
S Organic (1)	0.17%	0.10%	0.13%	0.12%	0.16%	0.15%	0.16%	0.12%
Unknown (244)	8.30%	6.08%	6.55%	7.03%	7.72%	7.86%	7.07%	7.45%

Table : Percent Composition of Compounds from Volatile SPME Analysis

Volatile Compounds Discussion

- Aldehyde and ketones show highest concentration and percentage concentration (Aldehydes 25% - 30%) and (Ketones 15% - 25%)
- Most of straight chain aldehydes are derived from oxidation of unsaturated fatty acids

Chemical Analysis Results

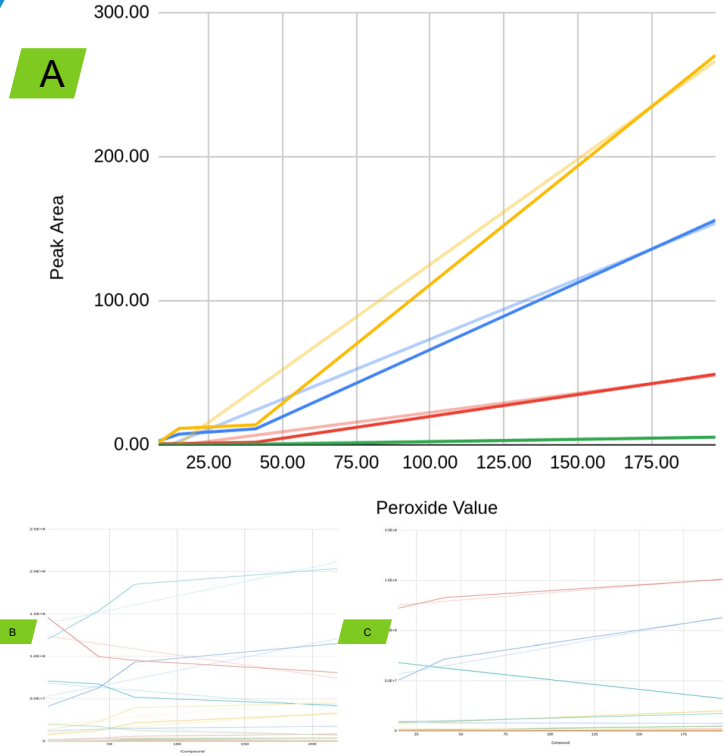


Overall:

- Over 600 compounds were identified as present in each sample
- Identified compound species significantly differed between CBPM and CM samples
- Around 7% of each sample remained unidentified

Fig A: Correlation of peak area and peroxide values of GCMS detected compounds for CM samples
Fig B: Correlation of peak area and peroxide values of SPME detected compounds for CB samples
Fig A: Correlation of peak area and peroxide values of SPME detected compounds for CM samples

Chemical Analysis Results



Potential PV Predicting Compounds:

- Around 100 compounds had correlation with PV levels
- 19 compounds had a R squared coefficient above 0.9 suggesting direct correlation between PV levels and concentration

Fig A: Correlation of peak area and peroxide values of GCMS detected compounds for CM samples
Fig B: Correlation of peak area and peroxide values of SPME detected compounds for CB samples
Fig C: Correlation of peak area and peroxide values of SPME detected compounds for CM samples

Heracles E-Nose is a state of the art system that utilizes chemical and electronic sensors coupled with an extensive odor library to identify odors present in a sample's headspace

Increasing PV increased odors labeled as :

- Fatty
- Oily
- Fermented
- Pungent
- Meaty
- Ect.

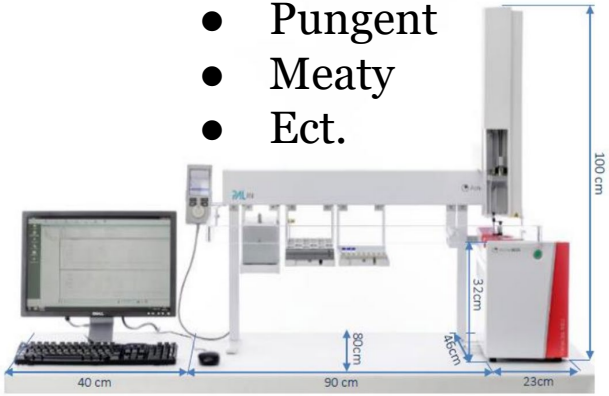


Figure 1. Heracles NEO system

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Electronic Nose Results

D

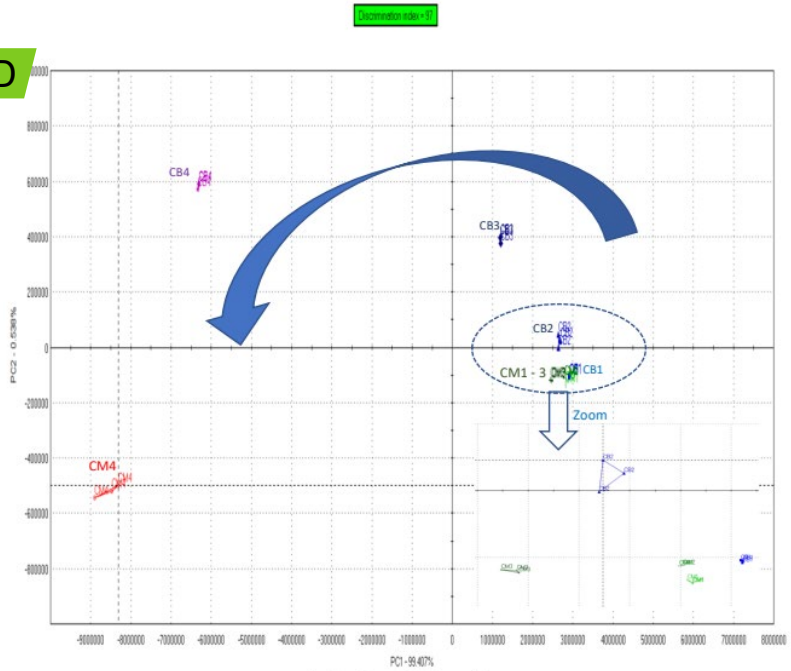


Fig 3c. CB and CM samples

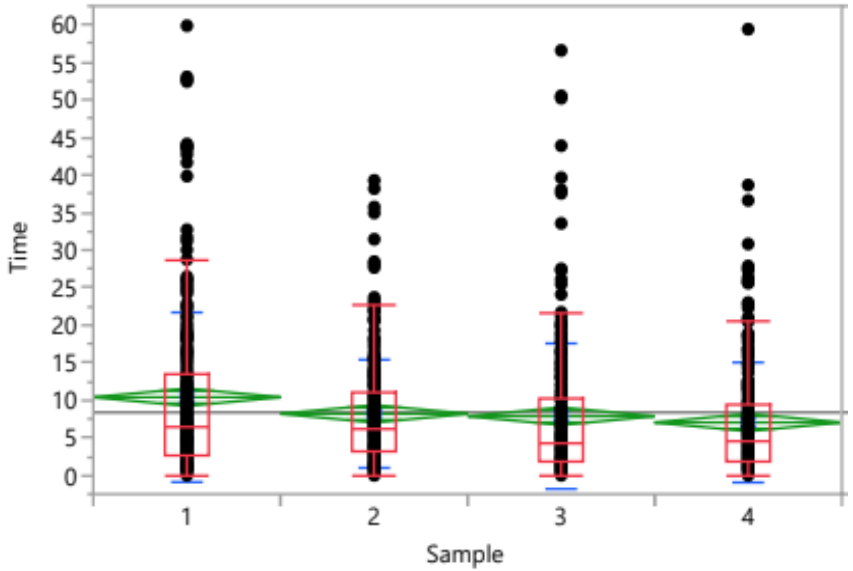
- Higher PV levels had more intense odors
- Equivalent CM and CB samples still differed significantly in odor
- The detected odor of each sample was significantly different from the others
- Odors also had good mathematical correlation with PV levels

Fig D: Primary Component Analysis of enose results for all samples

Is it feasible to create reliable aromatic palatability trials?

- Multiple kennels and research groups have attempted to create a protocol for testing aromatic preference
- These previous attempts had small sample sizes as well as insignificant or inconclusive results
- FRK conducted the largest aromatic trial of any method to date, utilizing 40 Labradors
- Each dog was presented with two samples, and the amount of time interacting with each was recorded
- Each of the four PV levels were tested against each other allowing for robust statistical analysis

E

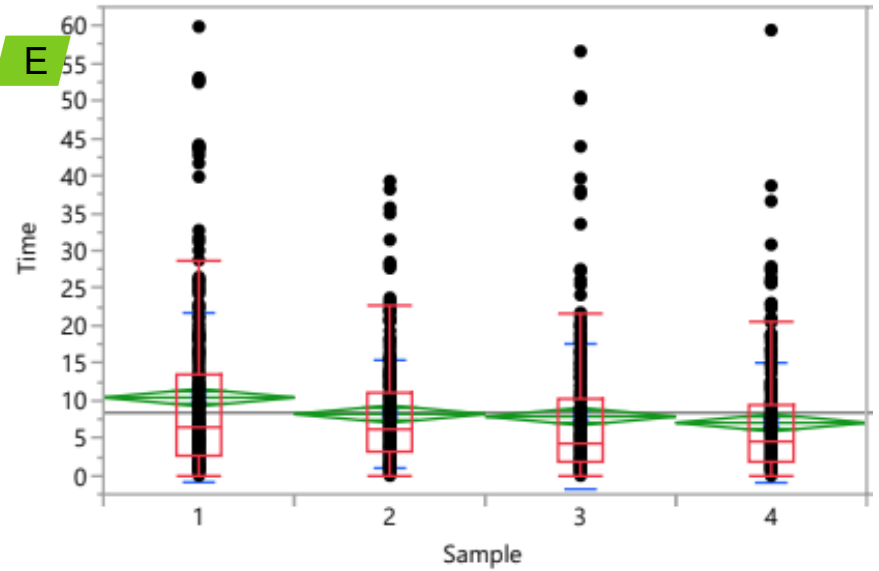


- First approach appeared unaffected by PV levels
- There was no significant difference within each trial
- However there was a significant difference with aggregated interaction
  - Total time interacting with each sample from all dogs and all trials
- Overall the dogs spent significantly more time with the lowest PV level
- The most significant difference was between the highest and lowest PV

Significant Aggregated Interaction Time			
Samples	1 vs 2	1 vs 3	1 vs 4
Difference in means	2.2	2.5	3.4
P-value	**0.0086**	**0.0026**	**<0.0001**

Fig E: Box plot of aggregated interaction time with ANOVA for aromatic palatability CM samples

E



Surprisingly, the dogs appeared to prefer the odor of the lowest PV value

Significant Aggregated Interaction Time			
Samples	1 vs 2	1 vs 3	1 vs 4
Difference in means	2.2	2.5	3.4
P-value	**0.0086**	**0.0026**	**<0.0001**

Fig E: Box plot of aggregated interaction time with ANOVA for aromatic palatability CM samples

Do aromatic and traditional palatability trials match?

- Investigated if aromatic palatability trend of dogs preferring lower PV samples would also hold for traditional palatability trials
- We followed standard two pan palatability methodology
- For this study we used five trained Labradors and CM and CBPM samples from different rendering plants
  - CBPM: PV ranged from ----1 - 25
  - CM: PV ranged from ----10 -150

INSERT VIDEOS HERE

https://drive.google.com/file/d/1G7zhhzfQc5ApP0XHCI3rEpzuw99rhi0F/view?usp=sharing
https://drive.google.com/file/d/1D_6QY38AH4nqKeXs0lSx_hB5xk0zLbNF/view?usp=sharing

Videos of the palatability trials

Once again the dogs prefered lower PV levels

Chicken Meal		
	Intake Ratio	Percent Consumed
Low PV	0.441 ± 0.074	4.07 ± 0.01
Mid PV	0.499 ± 0.091	3.52 ± 0.01
High PV	0.086 ± 0.135	1.01 ± 0.02
P-value	**0.039**	0.2694

Chicken By-Product		
	Intake Ratio	Percent Consumed
Low PV	0.57 ± 0.05	10.51 ± 0.13
High PV	0.35 ± 0.08	4.46 ± 0.19
P-value	**0.018**	**0.011**

- Interestingly the dogs approached and tasted the high PV first in 70% of trials
- But the low PV levels had a significantly higher intake ratio and % consumed
- This suggests that the dogs DIDN'T LIKE the taste of the high PV

In summary, increased peroxide values creates significant differences in chemical make up and odor…

And the dogs can tell the difference!

They consistently prefer low PV values in BOTH aromatic and traditional palatability studies.

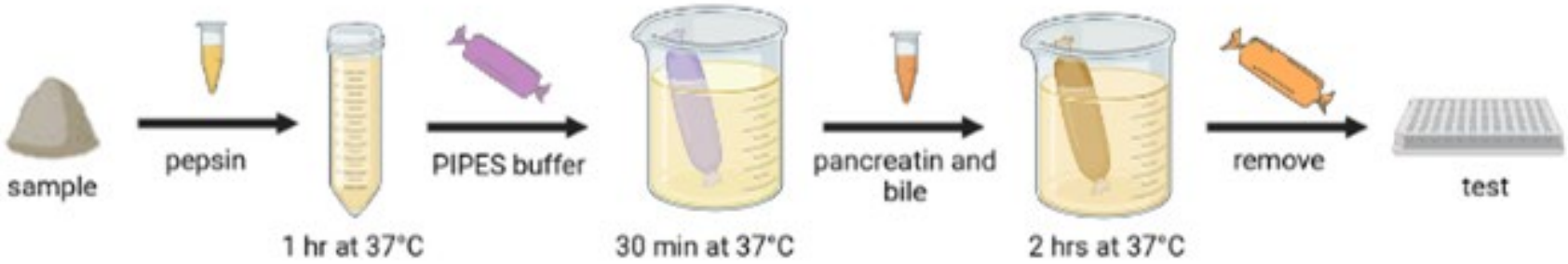
What are the next steps for investigating PV and ?

At Four Rivers Kennel, we have expanded our capabilities and are excited to continue investing the effects of oxidation.

This includes:

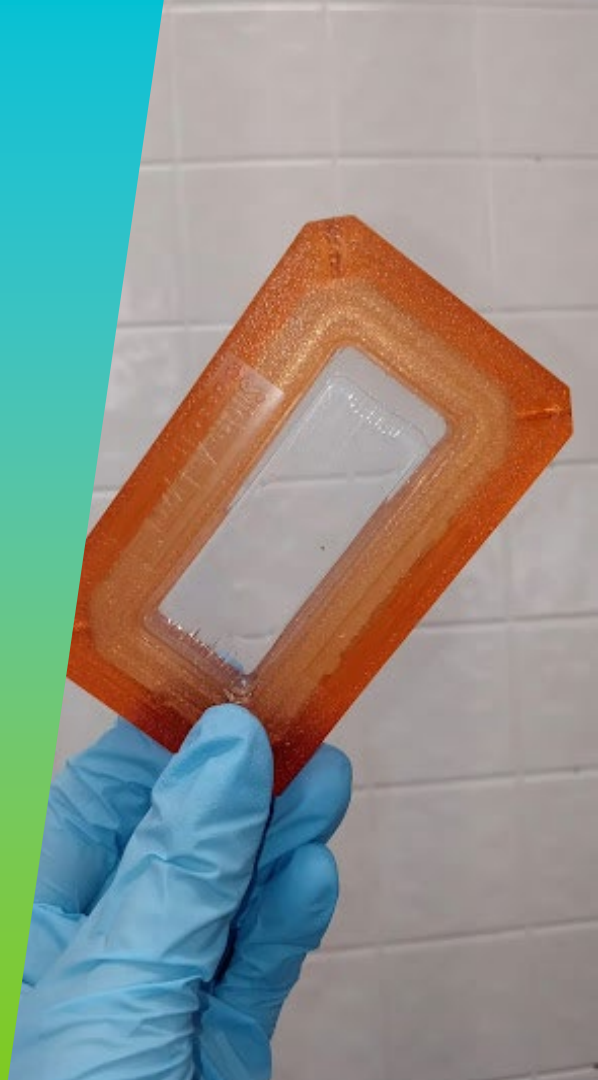
- Development and validation of an *in vitro* digestion method
- Development of intestinal cell cultures in house
- Analysis of the effects of PV on nutrient digestibility
- Investigation of PV related cellular damage
- Investigation of PV cause gut permeability

Importance of in vitro digestion protocols

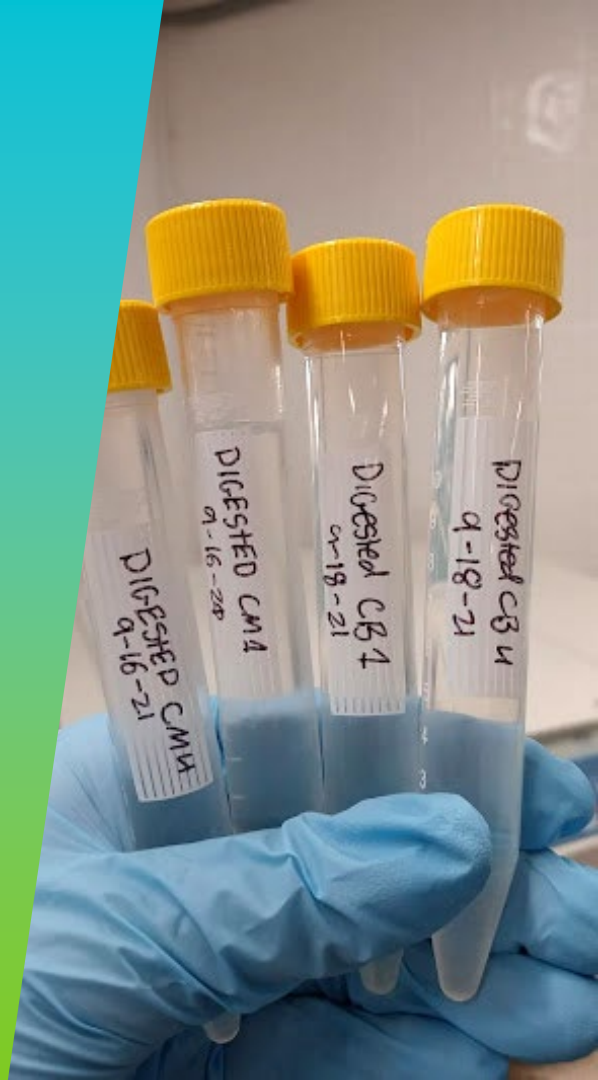


- Bile extract and an acidic pH mimics the gastric stage of digestion
- Samples started out as thick brown solutions with heavy precipitation

- Dialysis cassettes were used in place of dialysis tubing
- The cassettes allow for a smoother diffusion process and provides accurate retrieval volume
- The cassettes are filled with PIPES buffer and placed in the previously digested sample
- Pancreatin and bile extract are added to mimic intestinal digestion



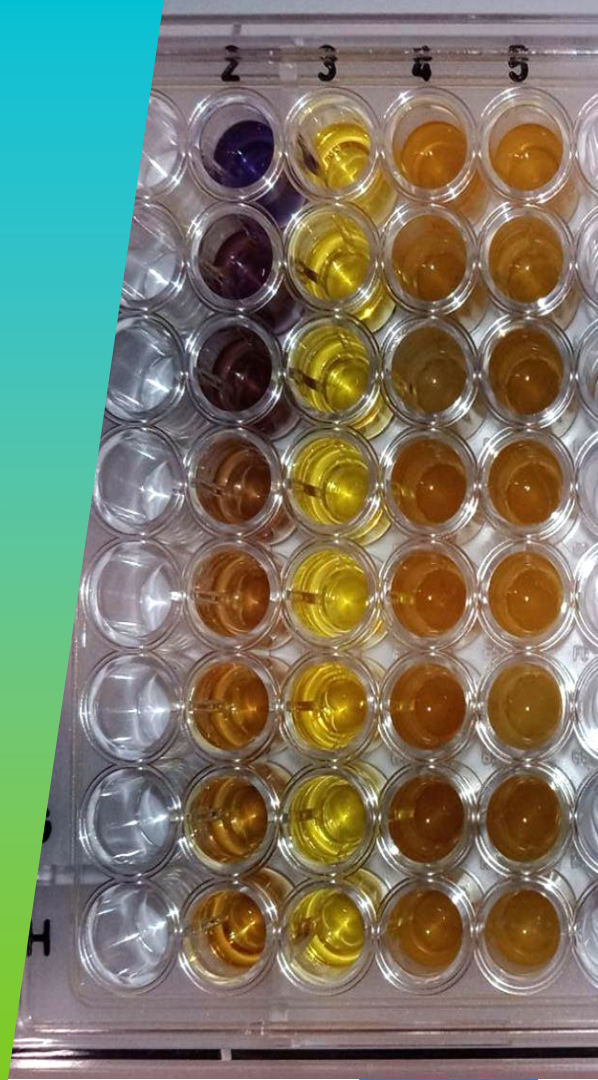
- After the complete process very little solid remains
- The solution from inside the dialysis cassette only contains digested molecules that diffuse through the membrane
- By separating out the digestion enzymes we can prevent harm to the cells



- Lipids were confirmed to be present in dialysis samples
- Lipid concentrations were equitable between protocols

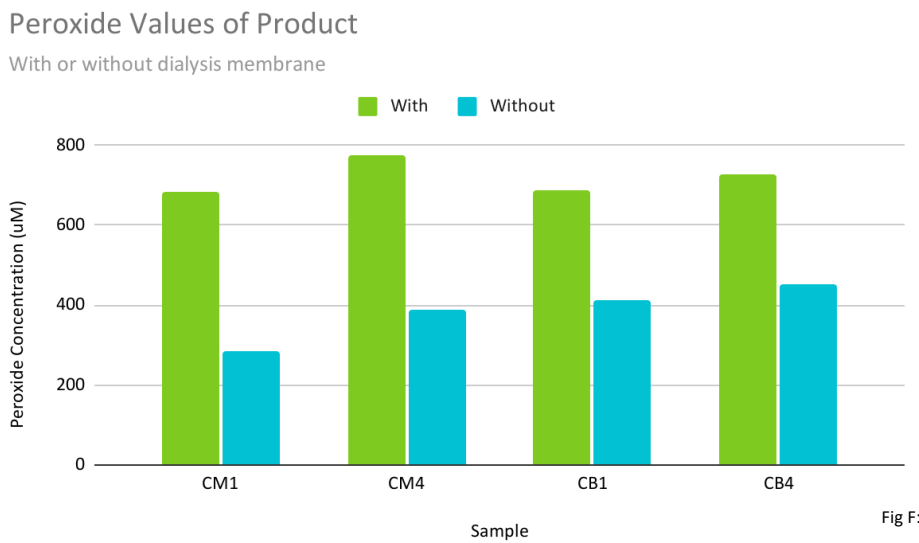
With : 90 - 170 mg/dL

Without : 95 - 200 mg/dL



Sample differences between PV levels was preserved even after the completed digestion protocol

F

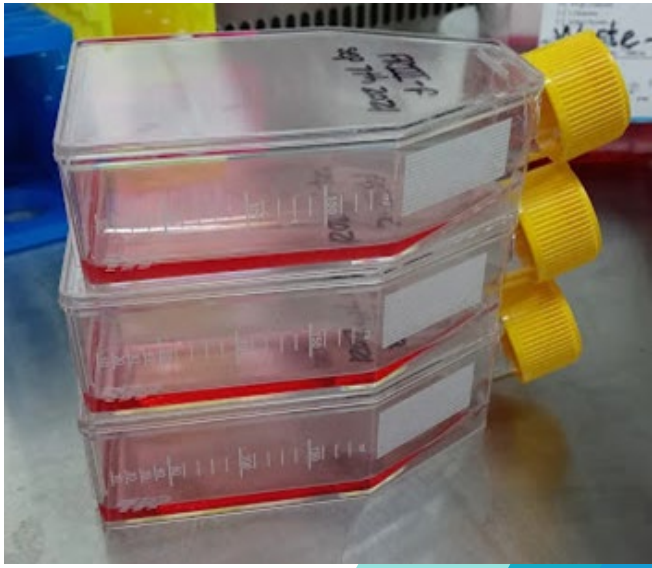


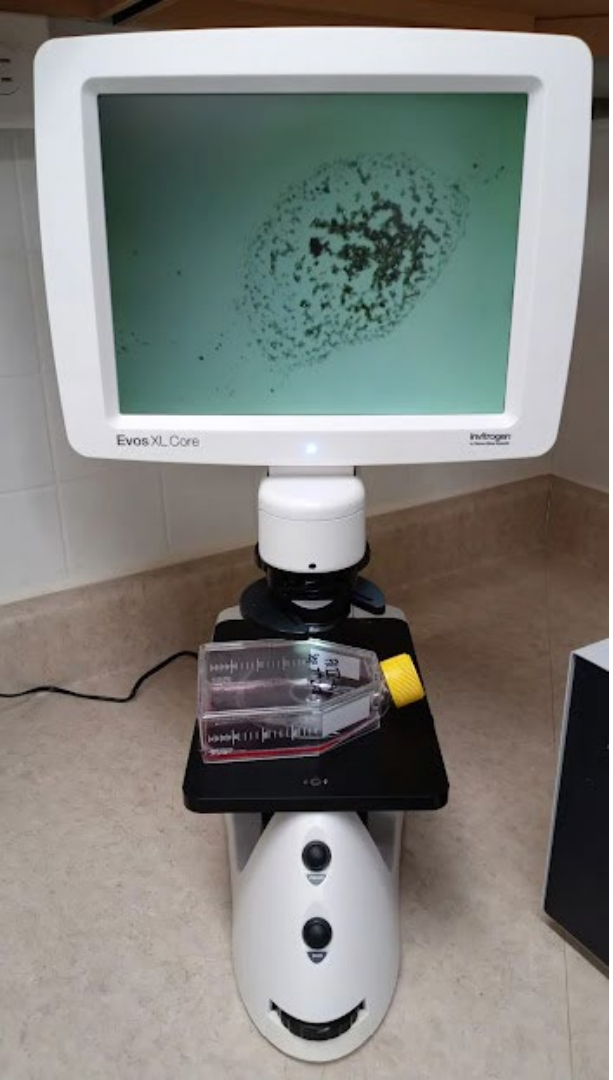
- Excluding digestion enzymes through use of a dialysis cassette led to better preservation of peroxide activity

Fig F: Comparison of peroxide levels after two digestion methods

Epithelial Cell Cultures

- FRK is maintaining healthy Caco-2 cell lines
- These epithelial cells differentiate into monolayers that mimic the intestinal barrier
- Initiating the experimental effects of PV from in vitro digestion of CM and CBPM on cells
- Determine the amount of PV (concentration and cummulative exposure time) required for negative response on intestinal cells





- We will test the cells for:
  - Proliferation
  - Cell damage
  - Cell death
  - Morphology changes
  - Permeability
- Utilizing fluorescent microscopy, cytometry, microplate readers and MagPix technology

This will expose potential health consequences of oxidized food products on the cells themselves

Future Projects

- FRK is currently installing HPLC, GC-MS, and an IRMS
- This will allow FRK to evaluate
  - Nutrient digestibility
  - Protein digestibility
  - Amino acid digestibility
  - And more

  Using the *in vitro* digestion method

Additional Discussion and Conclusions

Additional discussion

Peroxide values are correlated with chemical and aromatic changes

Dogs prefer food with lower PV

Peroxide values are preserved during *in vitro* digestion and thus have potential to harm gut cells

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