

Comparison of the Antifungal Efficacy of EverWild and Citrus Extracts Challenged Against *Aspergillus flavus* in Semi-Moist Pet Treats

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Summary

There are increasing requests by pet owners to pet food manufacturers to formulate diets with fewer synthetic additives in favor of more ‘natural’ and sustainably sourced substitutes. Pet owners believe that natural alternatives are healthier and offer longevity to their pets. Therefore, the objective of this study was to investigate and compare the antifungal efficacy of two natural products, fermented whey protein (EverWild; EV) and citrus extract essential oil, when challenged against *Aspergillus flavus* inoculated in semi-moist pet treats. Semi-moist treats generally contain moisture levels of 20–30%, which is ideal for mold proliferation. The experiment was completely randomized in design. The model and nutritionally complete semi-moist pet treats were produced with three levels of EV (1.0%, 3.0%, and 5.0%), citrus extract (1.0%, 3.0%, and 5.0%), a positive control that contained 0.1% potassium sorbate, or a negative control that contained no treatment. Each treatment was replicated twice and plated in duplicate during fungal analysis. The semi-moist treats were cut into biscuits and inoculated with 0.1 mL aliquots of *Aspergillus flavus* cultures. Fungal analysis was performed at 0, 3, 6, 9, 12, 15, 18, 21, 24, and 28 days. Overall, the survivors of *Aspergillus flavus* were reduced over time in all treatments including the negative control. When determining the log reduction from d 0 to 28, the EV included at 1.0%, 3.0%, and 5.0% had a 1.90, 3.89, and 4.58 Log CFU/biscuit reduction while the positive and negative control had

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⁴ Silva, J., Pereira, M. N., & Scussel, V. M. (2018). Ozone gas antifungal effect on extruded dog food contaminated with *Aspergillus flavus*. *Ozone: Science & Engineering*, 40(6), 487-493.

⁵ Luz, C., Izzo, L., Ritieni, A., Mañes, J., & Meca, G. (2020). Antifungal and antimycotoxigenic activity of hydrolyzed goat whey on *Penicillium* spp: An application as biopreservation agent in pita bread. *Lwt*, 118, 108717.

⁶ Restuccia, C., Conti, G. O., Zuccarello, P., Parafati, L., Cristaldi, A., & Ferrante, M. (2019). Efficacy of different citrus essential oils to inhibit the growth and B1 aflatoxin biosynthesis of *Aspergillus flavus*. *Environmental Science and Pollution Research*, 26(30), 31263-31272.

⁷ Kiprotich, S., Mendonça, A., Dickson, J., Shaw, A., Thomas-Popo, E., White, S., ... & Ibrahim, S. A. (2020). Thyme Oil Enhances the Inactivation of *Salmonella enterica* on Raw Chicken Breast Meat During Marination in Lemon Juice With Added *Yucca schidigera* Extract. *Frontiers in Nutrition*, 7.

1.19 Log CFU and 0.84 Log CFU/biscuit reduction, respectively. There was a significant difference ($P < 0.05$) in log reduction between EV at 3.0% and 5.0% compared to 1.0% EV, the positive and negative controls, 1.0%, 3.0%, and 5.0% citrus extracts. Citrus extract at 1.0%, 3.0%, and 5.0% had a 1.19, 2.34, and 2.63 Log CFU/biscuit reduction compared to the positive and negative controls (1.19 Log CFU and 0.84 Log CFU/biscuit). In conclusion, a fermented whey protein could be used to inhibit mold growth in semi-moist pet treats.

Introduction

Pet owners are increasingly asking for substitution of synthetic food additives, for instance, preservatives with more “natural” and sustainable alternatives. They believe that more natural ingredients in a pet food formula are healthier and provide longevity to their companion animals.³ Semi-moist pet treats contain 25–30% moisture levels, which is ideal for the growth and proliferation of pathogenic and spoilage fungi, such as *Aspergillus flavus* that produces the mycotoxin aflatoxin. Currently, industrial production of semi-moist pet food uses synthetic antifungal chemicals such as potassium sorbate, calcium propionate, and other organic acids (e.g., butyric acids, parabens, etc.).⁴ These compounds have been successful in inhibiting mold in semi-moist pet treats and foods, but consumers and pet owners demand a shift to more “natural” alternatives.

Whey protein, a by-product of cheese production, contains bioactive peptides produced during fermentation by proteolytic lactic acid strains.⁵ Previous research demonstrated that they could increase the shelf-life of bakery products using fermented whey protein.⁵ However, citrus extracts are essential oils whose antifungal properties have been long established.⁶ Essential oils consist of complex mixtures of volatile and aromatic compounds such as terpenes, terpenoids, and phenols that can inhibit mold proliferation if applied in a food matrix.⁷ With a moisture level greater than 20%, semi-moist pet treats may be prone to mold growth. The in-process application of suitable chemical interventions in these foods might yield a long-lasting preventive action against losses due to mold growth.

To our knowledge, fermented whey protein has never been applied to semi-moist pet food, but citrus extracts have been used as antifungals in baked goods. Given the breadth and magnitude of this issue, identifying clean label strategies using fermented whey protein rather than synthetic products in the pet food industry may have significant benefits to animal health and wellbeing. Therefore, the objective of this study was to determine the antifungal efficacy of fermented whey protein (EV; EverWild, Kerry Ingredients, Beloit, WI) and citrus extracts (Kerry Ingredients, Beloit, WI) as a challenge to *Aspergillus flavus* molds in semi-moist pet treats. The secondary objective of this study was to evaluate the shelf-life of semi-moist treats treated with different levels of EV and citrus extracts.

Materials and Methods

Preparation of working culture of Aspergillus flavus

Aspergillus flavus cultures were procured from the American Type Culture Collection (ATCC) and stored at -112°F. The cultures were thawed and then streaked on potato dextrose agar (PDA) plate using an inoculation loop and incubated at 77°F for 72 h. *Aspergillus flavus* colonies (by scraping the surface with inoculation loop) were scraped

and inoculated into 10 mL potato dextrose broth (PDB) and incubated at 77°F for 72 h. The final log concentration of *A. flavus* cultures were determined by plating the culture onto PDA, then the agar plates were incubated at 77°F for 72 h.

Preparation of semi-moist pet treats

All ingredients were weighed according to the formula to produce 4.41 lb of product per batch. Dry ingredients were mixed followed by wet ingredients using a 3.5-quart planetary mixer (KitchenAid Portable Appliances, St. Joseph, MI) for 10 min at 50 RPM. A 1 cm thick layer of the batter was spread on a baking tray and baked in a convention oven at 350°F for 10 min. The product was cooled and then cut into uniform biscuits of $3 \times 3 \times 1$ cm³ using a stainless-steel knife. The biscuits were individually wrapped in sterile stomacher bags and refrigerated prior to inoculation.

Microbial challenge study

The nutritionally complete model semi-moist pet treats contained three different levels of either EV or citrus extracts (1.0%, 3.0%, and 5.0%), the positive control contained 0.1% of potassium sorbate, and the negative control contained no treatment. Each treatment was replicated twice and plated in duplicate during fungal analysis. A 0.1 mL aliquot of *Aspergillus flavus* inoculum suspended in potato dextrose agar (PDB) was applied to individual biscuits. The inoculated biscuits were then stored in an incubator set at 77°F until it was time for analysis. The final concentration of the inoculum was determined by plating 0.1 mL of the inoculum on PDA plates and incubated at 77°F for 48 h. Fungal analysis was performed on day 0, 3, 6, 9, 12, 15, 18, 21, 24, and 28. For analysis, 100 mL of 0.1% peptone water was added to biscuit in the stomacher bag and pummeled in the stomacher machine. Serial dilution, plating on PDA plates and incubation at 77°F for 72 h followed subsequently. The resulting colonies were counted and reported as Log CFU/biscuit and log reductions were calculated.

Shelf-life studies (days to mold study)

Six biscuits from both treatments (EV and citrus extracts) and their respective levels (1.0%, 3.0%, and 5.0%) including the positive control that contained 0.1% potassium sorbate and the negative control that contained no treatment were placed in sterile stomacher bags, then sealed and stored in an incubator set at a temperature of 77°F. The biscuits were monitored each day for any mold growth. The days to mold were considered as the total number of days it took for a biscuit or biscuits within a treatment to develop visible mold growth.

Statistical analysis

Data were analyzed using JMP Pro version 15 statistical software (SAS Institute, Inc., Cary, NC). One-way ANOVA was used to determine treatment levels with significant log reductions. Pairwise comparisons of different treatment levels of EV, citrus extracts, and the controls were performed. Contrasts were used to determine significance within levels of each treatment and the positive control ($P < 0.05$).

Results and Discussion

*Fungal challenge study of *Aspergillus flavus* inoculated in semi-moist pet treats*

The final concentration of *Aspergillus flavus* that was delivered on each biscuit during the challenge study was 4.65 Log CFU/biscuit. When calculating the log reduction from d 0 to 28, including EV at 1.0%, 3.0%, and 5.0% had a 1.90, 3.89, and 4.58 Log CFU/biscuit reduction, respectively, while the positive and negative control had a 1.19 Log CFU/biscuit and 0.84 Log CFU/biscuit reduction. Citrus extract at 1.0%, 3.0%, and 5.0% had a 1.19, 2.34, and 2.63 Log CFU/biscuit log reduction, respectively, compared to the positive and negative control reductions (1.19 Log CFU and 0.84 Log CFU/biscuit). The positive control had a 1.19 Log CFU/biscuit reduction and the negative control had 0.84 Log CFU/biscuit reduction. There was a difference ($P < 0.05$) between 1.0% EV and 3.0% and 5.0% EV as the log reductions of *Aspergillus flavus* kept increasing with an increase in the concentrations of the treatments. There was no significant difference observed between 1.0% EV, 1.0% citrus extract and both the negative and positive controls ($P > 0.05$). There was also no difference ($P > 0.05$) between EV at 3.0% and 5.0% concentration. For both EV and citrus extracts, their 3.0% and 5.0% concentrations were different ($P < 0.05$) from the other treatments in the study (Table 2). However, the higher concentrations led to product concerns about low pH and product texture even though they were excellent antifungals. For commercial production, 1.0% EV would be the concentration most probable to perform.

Days to mold study

The negative control samples showed visible mold within 12 days, 1.0% citrus extract molded in 22 days, and 3.0% extract in 53 days. The rest of the samples did not have any signs of mold growth by day 82 (Figure 2). All the EV-treated semi-moist treats showed no mold growth for 0.1% potassium sorbate treats.

Conclusion

Fermented whey protein (EV) was an effective antifungal inhibitor in the challenge study and the days to mold study, compared to the citrus extracts, despite the lack of statistical significance between 1.0% EV and 1.0% citrus extracts. This is because the generally regarded as safe (GRAS) levels for citrus extract is only 0.15%, which is a low concentration incapable of preventing mold growth. Comparing the 1.0% EV to the standard industrially applied 0.1% potassium sorbate, there was no statistical significance, but the fermented whey protein had a better log reduction and showed a more fungicidal effect, whereas potassium sorbate was more inhibitory of fungi. This improves the prospect for fermented whey proteins that might otherwise be wasted and improves the sustainability of pet food. However, the higher concentrations led to product concerns even though they were excellent antifungals. For industrial application, 1.0% EV would be the preferred concentration as it had minimum impact on the product's sensory and visual quality.

Brand names appearing in this publication are for product identification purposes only. No endorsement is intended, nor is criticism implied of similar products not mentioned. Persons using such products assume responsibility for their use in accordance with current label directions of the manufacturer.

Table 1. An in-house nutritionally complete formula* was used for the preparation of semi-moist pet treats

Ingredient	%
Water	18.0
Corn	12.6
Chicken by-product meal	12.4
Corn gluten meal	12.4
Glycerin 99.7% USP	12.5
Wheat flour	5.8
Chicken fat	5.8
Corn syrup	5.0
Gelatin 250 bloom	2.5
Rice flour	4.3
Soybean meal	4.3
Molasses	1.0
Dry-digest (flavoring)	0.5
Salt	0.5
Dicalcium P	1.4
Vitamin premix	0.2
Potassium Cl	0.2
L-Lys	0.1
Trace mineral premix	0.1
Calcium carbonate	0.1
Choline Cl (60%)	0.1
Potassium sorbate	0.1
Antioxidant	0.1

*Base formula was used to produce 8 treatments of semi-moist treats that contained different mold inhibitors and levels: 1.0, 3.0, and 5.0% (w/w) EV (EverWild, Kerry Ingredients, Beloit, WI) and citrus extracts at 1.0, 3.0, and 5.0% (w/w), a positive control, which contained 0.1% potassium sorbate and a negative control without antimicrobics.

Table 2. Total log reduction of *Aspergillus flavus* when exposed to semi-moist pet treats containing a fermented whey protein (EverWild) or citrus extract after 28 days post-inoculation

Treatments	Log reduction ¹	SEM
1.0% EverWild ²	1.90 ^{BC}	0.1
3.0% EverWild	3.89 ^A	0.625
5.0% EverWild	4.58 ^A	0.075
1.0% Citrus extract	1.19 ^{CD}	0.015
3.0% Citrus extract	2.34 ^B	0.135
5.0% Citrus extract	2.63 ^B	0.02
Positive control-potassium sorbate	1.19 ^{CD}	0.165
Negative control-no treatment	0.84 ^D	0.115

^{A, B, C, D} Means that do not share the same letter differ ($P < 0.05$).

¹Total log reduction is obtained by subtracting the initial inoculum from the final Log CFU counts that had been obtained on day 28.

²EverWild and citrus extract from Kerry Ingredients, Beloit, WI.

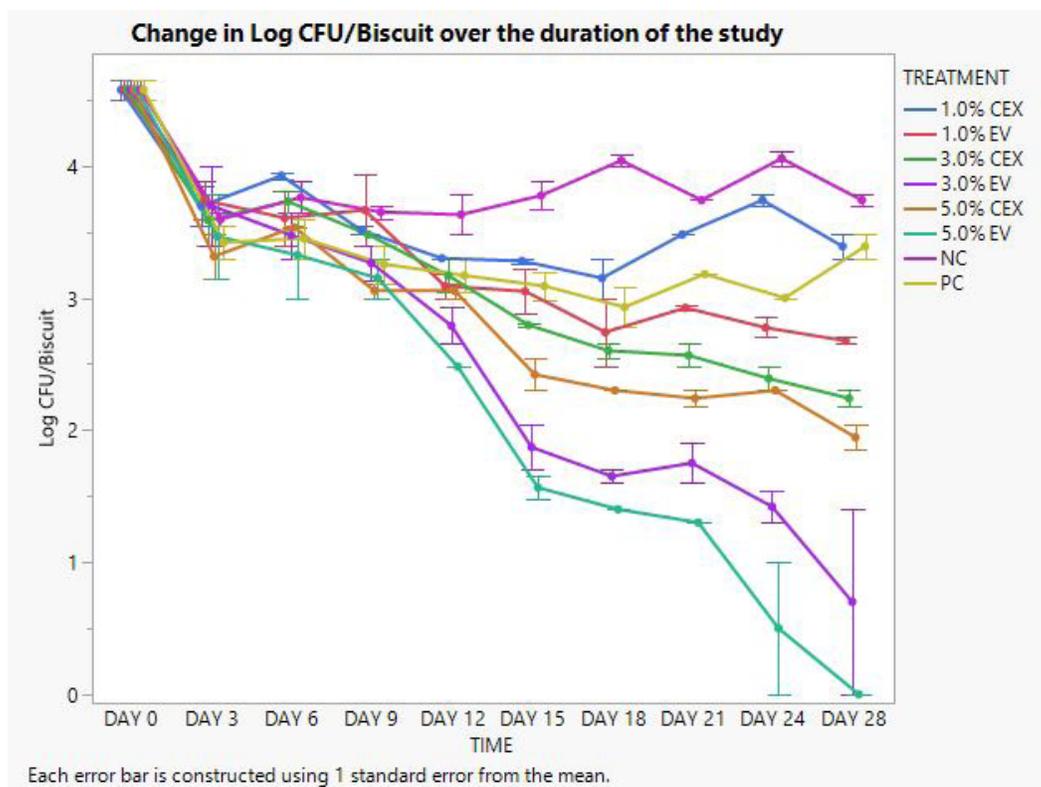


Figure 1. The Log survivors of *Aspergillus flavus* from a fungal challenge study, which were inoculated in semi-moist pet treats for dogs, treated with citrus extract and EverWild over a period of 28 days.

EV = EverWild treatment.

CEX = citrus extract treatment.

NC = negative control (without any antifungal compounds).

PC = positive control (treated with potassium sorbate).



Figure 2. Different stages of mold growth in semi-moist pet treats treated with different levels of citrus extracts and EverWild after 82 days of storage at 77°F.

DF = EverWild treatment.

CEX = citrus extract treatment.

PC = positive control (treated with potassium sorbate).

NC = negative control (without any antifungal compounds).