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Mini Review

Efficacy of Berberine as a Preservative Against Mold and Yeast in Poultry Feed

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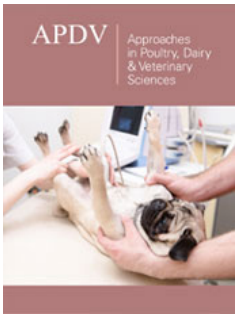

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Abstract

Preservatives are added to products to stabilize and lengthen the shelf life of a product. Classic chemical preservatives might not be as effective anymore due to the resistance of microbial organisms. There is also rising concern that chemical preservatives adversely affect animal and human health. The aim of this study was to investigate the efficacy of berberine as a natural preservatived. Berberine with a 0.03 g/kg/feed concentration was mixed into the feed. The stability of berberine was measured for 2 weeks prior to the efficacy experiment. Over a period of 4 weeks, the amount of mold and yeast cfu/g that were mixed in previously was measured and compared with the control feed that did not contain berberine. Berberine levels were stable when measured weekly for 2 weeks. The efficacy experiment showed that over the full four weeks, the concentration of yeast and mold was significantly lower ($p<0.001$) in the test group with the berberine incorporated in the feed. Berberine has an antimicrobial effect against a mix of yeast and mold strains at a concentration of 0.03 g/kg/feed in poultry feed and is stable when used in poultry feed.

Keywords: Berberine; Natural preservative; Antimicrobial; Efficacy

Introduction

Preservative agents are used to assure the stability of feed products. Preservatives are used for both animals and human food. In animal feed, antibiotics used to be the standard to lengthen the shelf life of a product and promote growth in the animals [1]. Since the use of antibiotics in feed is no longer desirable, the feed industry had to replace the antibiotics with other preservatives. The different types of preservatives currently used, can be divided into three different groups: chemical preservatives, natural preservatives and combination preservation.

The most used chemical preservatives are either weak-organic acids or hydrogen peroxide [2]. Benzoic acid and acetic acid are two examples of those weak-organic acids. Both those acids are known to inhibit fungal and bacterial cells [3,4]. Since gram positive bacteria do not have an outer cell membrane, weak acids can therefore easily penetrate those cells. Gram negative bacteria have an inner and outer membrane and for this reason, it is harder, but not impossible, for the weak acids to infiltrate the cell. When added to food, hydrogen perioxide can generate a short-lived singlet oxygen species which has antimicrobial properties [5]. Even though these types of preservatives have favourable properties, there are a few shortcomings.

A study performed by Hugo and Foster in 1964, showed that the weak acids are not always effective since some micro-organisms can break down the acids by producing specific enzymes [6]. Gram-negative bacteria strains have showed that they can survive under low pH circumstances [7,8]. Some gram-positive bacteria strains are able to induce an acid tolerance response at a low pH after exposure to a preservative at a higher pH [9]. When it comes to the resistance mechanism of bacteria against hydrogen peroxide, the microorganisms use the catalase pathway to degrade harmful levels of hydrogen peroxide.

There are a number of requirements, for a natural preservative: safety, stability during food processing and antimicrobial efficacy [10]. Natural preservatives have the ability to disturb or rupture the natural cell membrane of bacteria [5]. Membrane penetrating proteins

are found in plants and protect them against microbial infections [2]. The structure of natural preservatives varies greatly and therefore have different effects on micro-organisms [8]. In particular, plant derived compounds seem to be efficient as an antimicrobial in food applications [11-13]. Due to the variety in structure, the mechanism of action is also different per structure. Some plant compounds that are known for their antimicrobial properties are tannins, alkaloids and coumarins [14,15].

Berberine is one of those plant-derived compounds. It is a quaternary alkaloid and many studies showed that is has antibacterial properties [16-18]. Compared to antibiotics (in particular Neomycin), the bacterial cells rarely mutated to a resistant bacterial cell in the presence of berberine. Berberine had antibacterial properties to both gram positive and gram-negative bacteria strains. Although berberine's antimicrobial properties have been described it is not currently used as a feed additive or feed preservative and no studies have been done to investigates its properties as a preservative.

Therefore, the objective of this study was to test the stability of berberine in feed followed by a challenge study with berberine against mold and yeast strains so as to investigate the efficacy of berberine as a preservative in poultry feed.

Material and Methods

Feed

Southern Poultry Research, Inc. provided all feeds. All feeds were manufactured at SPR feed mill. Quantities of all basal feed and test products used to prepare treatment batches were documented. Each batch of feed was mixed and bagged separately. Each bag was identified with the study number, date of mix, type of feed, and the correct treatment number.

Stability

Standards with certificates of analysis were provided by Aspen. Individual analytical standards of berberine were prepared in methanol. Samples were analysed in duplicate and the sample description is found in Table 1. In addition to that, there was a blank sample tested. Sample A was prepared using the pelletized samples and sample B was prepared using the mash samples. The samples were tested at day 0, 7 and 14.

Table 1: Tested samples of berberine with identification number.

Sample Description	ARC ID	Date Received	Date(s) Extracted
Berberine Hydrochloride (97%)	52359-3	06-07-18	Method dev and standard
Berberine: 0.03g/kg feed	52359-28	9/27/2018	9/28;10/5;10/12

1g of sample was extracted with 10mL of methanol. "A" samples were crushed using a mortar and pestle prior to weighing. Samples were shaken for 30 minutes using wrist-action shaker, allowed to settle for 10 minutes, then the methanol was decanted off into a separate vial. To achieve the 0.03g/kg/feed, berberine was diluted in 10:1 methanol. All samples were filtered through a 0.2µm PTFE syringe prior to analysis. All standards and samples were injected to a LCMS using conditions described in Table 2.

Table 2: Details of the HPLC settings used for the stability experiment.

HPLC		
LC model	Agilent 1200 LC	
LC column	Luna 3u C18(2), 150mm x 2.0 mm	
LC flowrate	0.2 ml/minute	
LC injection volume	5µl	
LC column temperature	40 °C	
LC eluent A	0.1% Formic Acid	
LC eluent B	0.1% Formic Acid in Acetonitrile	
LC	**Gradient**	
Time minutes	Eluant A%	Eluant B %
0	90	10
5	90	10
20	5	95
35	5	95
37	90	10
Run time	37 minutes	
Post time	10 minutes	
Mass	**Spectrometer**	
MS model	Agilent 6210 Time of Flight MS	
ESI (electrode ionization)	Positive mode	
ESI source	300°C gas (N_2) at 8 L/minute, 30 psi nebulizer, 4000V Vcap, 110 V fragmentor	
TOF (Time of Flight) range	50-1500 amu (atomic mass unit)	
Ions of interest	229.0874, 336.1236, 391.1387, 439.3593, 457.3678 m/z	

Efficacy

All yeast and mold cultures (listed in Table 3) were grown and harvested according to Deibel Labs protocols. Harvested mold cultures were filtered through a sterile cheese cloth to form a spore suspension. An "Inoculation Cocktail" was established by combing the individual harvested spore suspensions into a single mixture. After the culture was filtered, a drop of lactophenol aniline blue was placed on a slide. A drop of culture was dispensed into the lactophenol aniline blue and mixed. A cover slip was placed over the mixture and examined under the microscope for minimal hyphae presence. If a significant amount of hyphae was observed, the culture was filtered again. Once spore culture was verified, it was diluted to achieve the target inoculum level listed below.

Table 3: Used mold and yeast strains for this study. The official name and organism ID or ATCC number are given.

Mold	Organism ID/ATCC Number	Yeast	Organism ID/ATCC Number
Aspergilus flavus	USDA NRRL 453	*Candida albicans*	ATCC 869
Penicilium glabrum	USDA NRRL 766	*Zygosaccharomyces bailli*	ATCC 2227
Mucor plumbeus	ATCC 2630	*Zygosaccharomyces rouxii*	ATCC 12691
Rhizopus oryzae	ATCC 3652	*Saccharomycescerevisiae*	ATCC 12632
Eurotium rubrum Aspergilus flavus	USDA NRRL 5000 Aspergilus flavus USDA NRRL# 5565 (Turkey feed mix)	*Dekkera bruxellensis Yeast spp.*	ATCC 1411 DLI client isolate
Talaromyces sayulitensis	NRRL# 62280 (Chicken feed)		

Control and treated group were aseptically weighed out into large sterile whirl-pak bags in 50g units and labelled with each pull day. The treated group consisted of feed that had 0.03g/kg berberine mixed into it. Separate 50-gram samples of product, in sterile whirl pak bags, were inoculated with 0.25ml of the mold cocktail (0.5% of total volume). Into each whirl-pak bag, the mold cocktail inoculum was added drop-wise to several different locations within the bag to aid in homogenizing the inoculum. Bags were then massaged to mix the inoculum throughout the product. The bag was rolled closed. The target inoculum level will be approximately 10^2 – 10^3CFU/g of product.

At day 0, the sample bags were immediately plated. On each successive pull day, 450ml of Butterfields Buffered Phosphate Diluent was added and homogenized for 1-2 minutes. The samples were then plated immediately. These plates were spread-plated onto Potato Dextrose Agar with chlortetracycline additive for enumeration of mold and YM agar for enumeration of yeast. The plates were incubated at 25 °C for 5 days, after which they were enumerated.

The subsequent inoculates samples were stored under at ambient temperature in a high humidity chamber (a closed chamber with an open pan of water to ensure high ambient humidity), and pulled on weeks 1,2,3 and 4 following the procedure outlined above. All pulls consisted of triplicate samples. Samples were also observed for visible mold growth.

Negative controls, consisting of uninoculated product, were sampled and plated for yeast and mold at the beginning and end of the study to determine background flora presence. As with the inoculated samples, yeast and mold control samples were incubated at 25 °C for 5 days before enumeration.

Statistics

To compare the control group and the test group at a particular time point, an unpaired T-test was executed, results were considered significant if $p<0.05$. To compare the control group and the test group over the full 12 weeks, a one-way ANOVA was executed, results were considered significant if $p<0.05$. The graphs were generated in GraphPad Prism version 8 (GraphPad software Inc., USA).

Results

Stability

The used method was capable of detecting 0.005g/kg of the additives. If the response was below that, the compound was reported as not detected (N.D). Besides the concentration of berberine in the container, containers that were in the same room were also tested on the presence of berberine. Based on the results showed in Table 4, berberine is stable when stored at room temperature over the two-week storage time.

Table 4: Concentrations of berberine present in the blank samples and test samples in both the mash and pellet forms.

ARCID	Sample type	Berberine Conc (g/kg) T-0	Berberine conc (g/kg) T-0 +7	Berberine conc (g/kg) T-0 + 14	
A52359 blank A	Basal pellet	Pellet	N. D	N. D	N. D
A52359 blank B	Basal Mash	Mash	N. D	N. D	N. D
52359-28A	Berberine; 0.03g/kg feed	Pellet	0.02	0.02	0.02
52359-28B	Berberine; 0.03g/kg feed	Mash	0.03	0.04	0.03

Yeast

At day 28, the yeast concentration was still detectable in the test group and the control group. When comparing the control group with the test group (berberine 0.03g/kg) over the full timeline a one way ANOVA test showed that there was a significance difference in the amount of yeast present in the feed ($F(9,50) = 57.83$), $p<0.001$ (Figure 1). On the test days itself, there is a significant difference between the test group and the control group on day 14 ($p= 0.0041$) ,21 ($p= 0.0497$) and 28 ($p = 0.0023$).

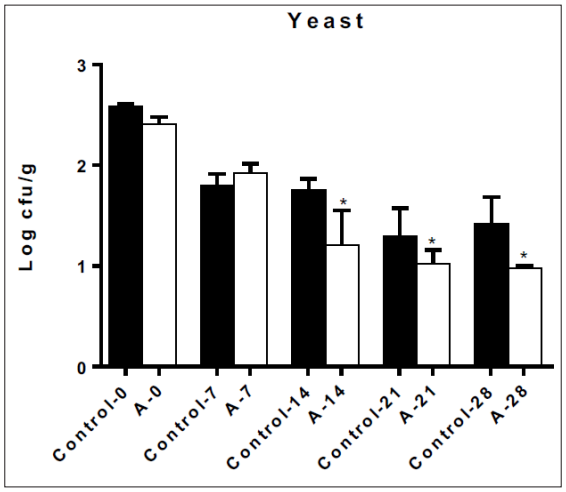

Figure 1: Concentration of yeast, given as log cfu/g, at different time points until day 28. There was a significant difference during the whole study and also at different time points: day 14,21 and 28. *=p<0.05.

Mold

At day 28, the mold concentration was still detectable in the test group and the control group. When comparing the control group with the test group (berberine 0.03g/kg) over the full timeline a one way ANOVA test showed that there was a significance difference in the amount of mold present in the feed ($F(9,50) = 35.10$), $p<0.001$ (Figure 2). On the test days itself, there is a significant difference between the test group and the control group on day 7 ($p= 0.0009$), 14 ($p= <0.001$), 21 ($p= 0.0017$) and 28 ($p=<0.001$).

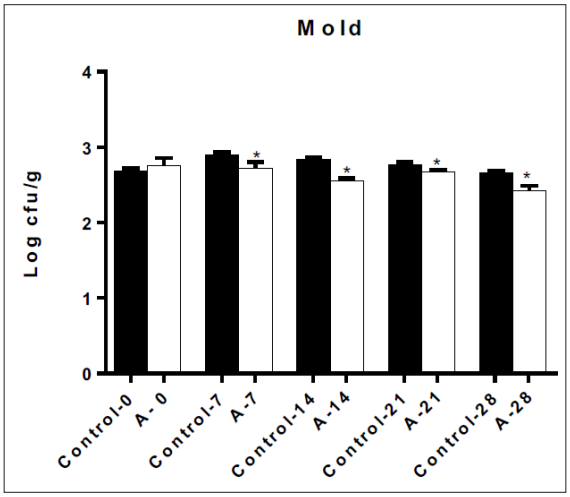

Figure 2 : Concentration of mold, given as log cfu/g, at different time points until day 28. There was a significant difference during the whole study and also at different time points: day 7,14,21 and 28* = p <0.05.

Discussion

There are a number of requirements for a natural preservative: safety, stability during food processing and antimicrobial efficacy (Lee and Paik, 2016). Natural preservatives have the ability to disturb or rupture the natural cell membrane of bacteria (Helander et al., 1998). Membrane penetrating proteins are found in plants and protect them against microbial infections (Broekaert et al., 1995) (Segura et al., 1999). The structure of natural preservatives varies greatly and therefore have different effects on micro-organisms (Savoia, 2012). In particular, plant derived compounds seem to be efficient as an antimicrobial in food applications (Cowan,1999; Tajkarimi et al., 2010; Hayek et al., 2013). Due to the variety in structure, the mechanism of action is also different per structure. Some plant compounds that are known for their antimicrobial properties are tannins, alkaloids and coumarins (Ciocan and Băra, 2007; Lai and Roy, 2004).

Berberine is one of those plant-derived compounds. It is a quaternary alkaloid and many studies showed that is has antibacterial properties (Eduardo and Groisman, 1996; Yi et al, 2007; Dai et al, 2010). Compared to antibiotics (in particular Neomycin), the bacterial cells rarely mutated to a resistant bacterial cell in the presence of berberine. Berberine had antibacterial properties to both gram positive and gram-negative bacteria strains. Although berberine's antimicrobial properties have been described it is not currently used as a feed additive or feed preservative and no studies have been done to investigate its properties as a preservative.

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Acknowledgement

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Conflict of Interest

No declaration of competing and conflicting interests. This research was supported by iRiccorgpharm Health Pty Ltd in its initiative to reduce antibiotic usage in livestock. They had no influence in the results and statistical analysis.

Authors' Contributions

Zhiyong He design the experiment. The study was conducted upon request from Sa Xiao and Zhi-Cheng Xiao. Lotte Geerlofs wrote the manuscript on consultation with Zhiyong He and made revisions when necessary. Lotte Geerlofs and Sa Xiao analysed the data. All authors have read and approved the manuscript.

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