Ciência Animal Brasileira
Brazilian Animal Science

DOI: 10.1590/1809-6891v26e-77553E

e-ISSN 1809-6891
Animal science | Research article

Efficacy of organic acids in feed experimentally contaminated with *Aspergillus flavus*

Eficácia de ácidos orgânicos em rações experimentalmente contaminadas com *Aspergillus flavus*

Rogerio Aleson Dias Bezerra*[1], Maria Eduarda Ribeiro Ratão[1], Thainá Silva Blasques[1], Bruna Barnei Saraiva[1], Gabriel Amaral de Araújo[1], Paulo Cesar Pozza[1], Magali Soares dos Santos Pozza[1]

1 Universidade Estadual de Maringá (UEM), Maringá, Paraná, Brazil

*corresponding author: rogerioaleson@hotmail.com

Received: June 05, 2024. Accepted: January 31, 2025. Published: June 13, 2025. Editor: Rondineli P. Barbero

Abstract: Organic acids have been shown to be a promising alternative to chemical compounds due to their ability to inhibit microbial growth. The aim of this study was to evaluate the antifungal activity of organic acids in animal feed experimentally contaminated with *Aspergillus flavus*. The *A. flavus* strain (ACFV) was activated in the Sabouraud Dextrose Agar way and incubated at 25 °C for 7 days. After incubation, it was carried out the preparation of the spore suspensions and counted in a Neubauer chamber, standardizing the inoculum at 5.0 x 10^{-6} spores/mL. Spore suspensions (10 mm agar discs) were prepared in buffered peptone water (1000 mL) and the feeds were treated with commercial organic acids, including propionic acid or a blend of acids (propionic, tartaric, citric, formic, sorbic and lactic). The feed was analyzed on days 1, 3, 5 and 7 of shelf life, with microbiological analysis of mold and *Aspergillus* spp. count, in addition to measurements of water activity, dry matter, pH and temperature. Organic acids reduced the total count of filamentous fungi throughout the evaluation period. A significant reduction in *Aspergillus* spp. counts was observed in feeds treated with organic acids ($p<0.05$). Furthermore, there was a reduction in dry matter and pH in the feeds. It is concluded that organic acids have great potential to inhibit fungal growth in diets, ensuring their quality and safety during the storage.

Key-words: weak acid; antifungal activity; shelf life.

Resumo: Os ácidos orgânicos têm se mostrado uma alternativa promissora aos compostos químicos devido à sua capacidade de inibir o crescimento microbiano. O objetivo deste estudo foi avaliar a atividade antifúngica de ácidos orgânicos em rações animais experimentalmente contaminadas com *Aspergillus flavus*. A cepa de *A. flavus* (ACFV) foi ativada em meio Ágar Sabouraud Dextrose e incubada a 25 °C por 7 dias. Após a incubação, foi realizada a preparação de suspensões de esporos e a contagem em câmara de Neubauer, padronizando o inóculo em 5,0 x 10^{-6} esporos/mL. Foram preparadas suspensões de esporos (discos de ágar 10 mm) em água peptonada tamponada (1000 mL) e as rações foram tratadas com ácidos orgânicos comerciais, incluindo ácido propiônico ou um blend de ácidos (propiônico, tartárico, cítrico, fórmico, sórbico e lático). A ração foi analisada nos dias 1, 3, 5 e 7 de vida de prateleira, com análise microbiológica de bolores e contagem de *Aspergillus* spp., além de medidas de atividade de água, matéria seca, pH e temperatura. Os ácidos orgânicos reduziram a contagem total de fungos filamentosos ao longo do período de avaliação. Observou-se uma redução significativa nas contagens de *Aspergillus* spp. nas rações tratadas com ácidos orgânicos ($p<0,05$). Além disso, houve redução da matéria seca e do pH nas rações. Conclui-se que os ácidos orgânicos têm grande potencial para inibir o crescimento fúngico em rações, assegurando sua qualidade e segurança durante o armazenamento.

Palavras-chave: ácido fraco; atividade antifúngica; vida de prateleira.

1. Introducion

The degradation of feed by fungi is a source of risk to the animal's health, due to aspects correlated with the frequency of mycotoxins produced by them[1]. This contagion can manifest itself from the primary stages of production and storage of raw materials in manufacturing, to the final stages of commercialization[2]. To avoid contamination, fungal control through preventive actions and monitoring of adverse conditions appropriate for its spread must be carried out. Aflatoxins are secondary metabolites that can be produced by fungi such as: *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominu*[2]. *A. flavus*, an aflatoxin producer and considered an opportunistic pathogen, is a filamentous storage fungus widely distributed throughout the world, mainly in tropical climate regions with ideal temperature and humidity for its development. The clinical aspect of aflatoxicosis is directly related to the degree of the product contamination , time and quantity of contaminated feed ingested by the animal and its nutritional status. Growth retardation, neoplasia, teratogenesis and acute, subacute and chronic liver diseases are reported[3]. Alternative precautions to block the action of these fungi and their toxins are pertinent, since they are highly resistant to physical and chemical procedures[3].

Therefore, the use of organic acids (OA) is a valid alternative to aspects associated with fungal contamination[4]. These acids are the result of animal and plant metabolism, being frequently found in nature[5]. These organic compounds have the carboxyl radical in their composition and, although considered weak, they are capable of entering cells with an initial non-dissociated configuration, beginning to dissociate itself inside them. This mechanism of action alters the pH range in the cell cytoplasm, causing cell death and blockage[6]. Among those most commonly used are highlighted acetic, benzoic, butyric, citric, formic, lactic, malic, propionic and tartaric acids[7]. Regarding the manner of action, undissociated organic acids can permeate through the cell membrane of bacteria and cause protection against DNA synthesis, cell division, cellular absorption of amino acids, organic acids, phosphate, and inhibitors with low pKa are particularly effective in highly acidic intestinal fluid[8].

Therefore, the objective is to evaluate the effect of propionic, tartaric, citric, formic, sorbic and lactic acids on the fungal development of *A. flavus* during the shelf life of crumbled swine feed, regarding the applicability and assertiveness of the products selected for fungal control and the physicochemical quality of the feed.

2. Material and methods

The experiment was carried out at the Iguatemi Experimental Farm (FEI), belonging to the State University of Maringá (UEM). The *A. flavus* strain (ACFV- isolated by Variani *et al.* [9] was activated in Petri plates containing Sabouraud Dextrose Agar through the streak technique. The plates were incubated at 25 °C for 7 days in a BOD type incubator. After the incubation period, spore suspensions (10 mm agar discs) were made in buffered peptone water (1000 mL) and counted in a Neubauer chamber until obtaining the inoculum standardization at 5.0×10^{-6} spores mL^{-1}.

300 kg of crumbled feed for finishing pigs (Table 1) were prepared without antifungals and 1 mL of the spore suspension was inoculated. (5.0×10^{-6} spores mL^{-1}) For every 100 g of feed, two commercial products were used, being propionic acid (PA) (propionic acid 490 g/kg pka 4.88, silicon dioxide, ammonium hydroxide, water) and acid blend (BA) (calcium carbonate, propionic acid 250 g/kg/ pka 4.88, silicon dioxide, propylene glycol, ammonium hydroxide, water, L(+)-tartaric acid pka 2.98, citric pka 3.13, formic 6800 mg/kg pka 3.75; sorbic 6000 mg/kg, pka 4.76; lactic pka 3.83, vitamin C 990 mg/kg).

The experiment was carried out in a 6x4 factorial scheme, with 6 treatments (CN; CP; AP2000; AP4000; BA2000 and BA4000) and 4 periods of evaluation (1, 3, 5 and 7 days). The evaluated treatments were: control feed without inoculum and organic acids (negative control; NC); control feed inoculated with fungus without organic acids (positive control; PC); diet inoculated with propionic acid 2,000 g/ton (PA2000); diet inoculated with propionic acid 4,000 g/ton (PA4000); feed inoculated with a blend of acids (propionic acid, tartaric acid, citric acid, formic acid, sorbic acid and lactic acid) 2,000 g/ton (BA2000); feed inoculated with a blend of acids (propionic acid, tartaric acid, citric acid, formic acid, sorbic acid and lactic acid) 4,000 g/ton (BA4000).

Table 1. Composition of crumbled feed formulated for finishing pigs.

Ingredients (%)	NC	PC*	PA2000*	PA4000*	BA2000*	BA4000*
Corn grain	80.78	80.78	80.78	80.78	80.78	80.78
Soybean grain	14.08	14.08	14.08	14.08	14.08	14.08
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00
Dicalcium phosphate	0.80	0.80	0.80	0.80	0.80	0.80
limestone	0.63	0.63	0.63	0.63	0.63	0.63
Salt	0.39	0.39	0.39	0.39	0.39	0.39
L-Lysine HCL 78.4%	0.40	0.40	0.40	0.40	0.40	0.40
DL-Methionine 99.0%	0.11	0.11	0.11	0.11	0.11	0.11
L-Threonine 98.0%	0.14	0.14	0.14	0.14	0.14	0.14
L-Tryptophan 98.5%	0.05	0.05	0.05	0.05	0.05	0.05
L-Valine 99.9%	0.05	0.05	0.05	0.05	0.05	0.05
Vitamins and Minerals[1]	0.40	0.40	0.40	0.40	0.40	0.40
Enramycin	0.02	0.02	0.02	0.02	0.02	0.02
BHT[2]	0.01	0.01	0.01	0.01	0.01	0.01
Propionic acid [3]	-	-	2000	4000	-	-
Acid blend [4]	-	-	-	-	2000	4000

[1]Supplementation of vitamins, minerals and additives per kg of product: Vit A - 30000 UI; Vit D3 - 5000 UI; Vit E -120 UI; Vit B12 - 120 mcg; Vit K - 5 mg; Niacin - 150 mg; Calcium Pantothenate - 75 mg; Folic Acid - 8 mg; Choline Hydrochloride – 0.48 g; Iron - 350 mg; Copper - 15 mg; Magnesium - 250 mg; Zinc – 0.75 g; Iodine - 10 mg; Selenium 3 mg; 2 Butylated hydroxytoluene; Propionic acid (g/ton); 4 acid blend (g/ton): Propionic acid, tartaric acid, citric acid, formic acid, sorbic acid and lactic acid.

Samples were stored for a period of 7 days and analyzed over time for physicochemical and microbiological changes. Microbiological analyses of molds were performed on days 1, 3, 5 and 7. *Aspergillus* ssp. counts were performed on days 1 and 7. Analyses of dry matter, feed temperature, pH and water activity (Aw) were performed on days 1, 3, 5 and 7.

2.1 Analysis of molds and *Aspergillus* ssp

For microbiological analysis, 10 g of samples were diluted in 90 mL of 0.1% peptone water (Himedia®). From this dilution, serial decimal dilutions were performed until 10^{-6}. After the procedure, seeding by spread plate in Petri plates containing PDA agar (Potato Dextrose Agar) was performed in triplicate. The plates were incubated at 25ºC for 7 days. The reading was performed by counting colony forming units and expressed in CFU/g [(10)]. The same procedure was used for samples plated on Sabouraud Dextrose Agar to count *Aspergillus* ssp.

2.2 Determination of water activity (Aw)

The determination of water activity was performed using AQUALAB equipment (Decagon devices, WP4C). With the calibrated device, 2g aliquots of sample were weighed into the water activity meter capsules. The capsules were positioned in the equipment for reading.

2.3 Determination of dry matter

Dry matter was determined according to the methods according to the methods [11]. The crucibles were previously weighed, cleaned and dried in an oven at 105 ºC for 1 hour, cooled and weighed. Then, approximately 2 g of the crumbled feed were weighed and dried in an oven preheated to 105 ºC. The prepared samples stayed in the oven for 5 hours or until reaching a constant weight were cooled in a desiccator and weighed on an analytical balance.

2.4 pH determination

To determine the pH, 10 g of feed were dissolved in 100 mL of distilled water. It was stirred until the particles became uniform, then the pH reading was performed using a previously calibrated pH meter (NT PHM).

2.5 Determination of feed temperature

The analysis was performed using an analog mercury thermometer (Incotherm) inserted into the polyethylene bags containing 5 kg of feed. Internal temperatures were measured at three different points. The storage room temperature was also measured using a thermo-hygrometer (Incotherm) three times a day.

2.6 Statistical analysis

The data obtained in the experiment were subjected to statistical analysis in the computational package (SAEG 2000) and the averages, when significant, were subjected to comparison using the Tukey test at 5% significance.

3. Results and discussion

3.1 Microbiological analysis

The amount of inoculum used to contaminate the samples was based on the usual contamination for this type of product. Thus, the inoculum used initially contained 5.0 x 10^6 CFU/mL, when sowing on the plates the total fungal count obtained was 4.4 x 10^4 CFU/mL for the control feed and 5.35 x 104 CFU/mL for experimentally contaminated feeds. Similar quantity [4] (10^{-4} spores mL^{-1}) was used to evaluate synergism between organic acids and potassium sorbate in the control of *Aspergillus flavus* in corn and swine feed samples.

The Sabouraud culture way provided ideal conditions for isolating and quantifying the *A. flavus*, being evidenced by its morphology stained with methylene blue. As expected, PC presented a greater amount of CFU when compared to NC ($p<0.05$) (Table 2). The AP4000, BA2000 and BA4000 treatments presented equivalent averages to the negative control (NC), demonstrating the efficiency of the acids in inhibiting *A. flavus*. The AP2000 treatment was less efficient, as it presented an average equivalent to the

PC of CFU. However, the evaluation over the storage time (Table 3) demonstrated a tendency towards a reduction in the values of the microbiological counts of the feed (y= 4.277-0.096620x).

Furthermore, evaluation over time becomes valuable since the storage process provides favorable conditions for the development of fungi that require low humidity and prolonged time to develop. The longer the storage period, the greater the chance of fungal growth and possible production and contamination of the food by aflatoxins [14].

In a study carried out by Ojeda *et al.*[15] combinations of organic acids were used to control the mycotoxigenic *Aspergillus* spp.. The author demonstrated that acidifiers are effective in inhibiting the growth of fungi of the genus *Aspergillus*. Furthermore, the study found that the combination of 67% ascorbic acid, 16.5% citric acid and 16.5% lactic acid allowed a reduction in the dose applied for fungal control, associating aspects of synergy between the compounds, a promising strategy in combating microbial development, demonstrating that the inhibitor formed by the mixture of ascorbic acid, citric acid and lactic acid was highly effective in inhibiting growth in the feeds evaluated at a concentration of 1,000 ppm.

3.2 Water activity (Aw)

For water activity (Table 2), a significant effect for the treatment, time and interaction between treatment and time ($p<0.05$) were observed. The water activity of the stored feed ranged from 0.565 to 0.637. However, NC presented lower averages than the other evaluated feeds. The crumbled feed has little free water available for fungal development, therefore, the water activity values found in the specific study are below the ideal for fungal development. Spoilage fungi require a minimum Aw of 0.80 [16] more specifically, *A. flavus* requires at least 0.71 water activity for its growth, with an optimum value of 0.98 (International Commission on Microbiological Specifications for Foods - ICMSF,1996). This means that it was not possible to evaluate the effectiveness of the treatments against Aw, since the values obtained were not within the minimum limit for the development of filamentous fungi according to Rebonatto *et al*.[4].

However, it is worth mentioning that maintaining a low Aw is a useful practice to reduce fungal damage to grains and feeds during storage and the risk of potential contamination by mycotoxins [17]. Rosa *et al*. [17] when evaluating corn and brewing grains, ingredients used in swine feed, higher water activity values were found in brewing grain samples with averages ranging from 0.936 to 0.082. For corn grain and final feed samples, Aw ranged from 0.627-0.112 and 0.628-0.055, respectively.

3.3 Moisture content (%)

Moisture content influences fungal incidence during storage, with the presence of fungi being more evident at higher humidity levels (Table 2). The interaction between treatment and time was not significant ($p>0.05$). The control feed treatment inoculated with fungus and without acids (PC) showed a higher average compared to the other treatments. The ideal humidity conditions for its proliferation vary from 13% to 18%, and in this study it was found that the average values reached the minimum for its proliferation. Aflatoxins, produced by the species *A. flavus* and *A. parasiticus*, have a more pronounced effect on the liver and cause more serious health problems in swines when compared to other mycotoxins [18].

3.4 Temperature

The temperature is one of the factors that can affect the shelf life of feed, which considerably affects the speed of reactions that occur after processing, distribution and storage. For temperature (Table 2 and 4) beneficial effects for treatment, time and interaction between treatment and time ($p<0.05$) were observed.

PC was the only one that presented a lower average than the other treatments (Table 2). This lower temperature could limit the growth of the fungus and slow down its metabolic activity, but it is still at acceptable levels for the development of *A. flavus*. The temperature of the environment in which the bags containing the feed were stored varied from 24 to 32 ºC throughout the storage period, remaining within the range reported as ideal for the growth of *Aspergillus* ssp. Effects ($p<0.05$) were observed on temperature throughout the storage period (Table 4). The highest temperatures (28 °C) were observed at the beginning of the storage period in the BA2000 and BA4000 feed compared to the other treatments evaluated (NC; PC; AP2000; AP4000). Over the time, changes could be observed with variations of up to one percentage point for all treatments. It is important to note that both temperatures presented are within the recommended range for the development of *A. flavus*. Therefore, the temperature range for the growth of *A. flavus* varies between 24 and 40 ºC, with the optimum temperature being 35 ºC [19].

Table 2. Microbiological counts of *A. Aspergillus flavus* (log_{10}) and characteristics of crumbled feed for finishing pigs inoculated with Aspergillus flavus (5.0 x 10^{-6} spores mL^{-1}) containing organic acids

Treatment	*Aspergillus flavus* (CFU)	Water activity	% Humidity	Temperature (°C)	pH
NC	3.75 ± 0.48^{B}	0.565 ± 0.022^{B}	12.19 ± 2.12^{B}	26.80 ± 0.29^{A}	6.00 ± 0.08^{A}
PC	4.43 ± 0.90^{A}	0.637 ± 0.007^{A}	7.53 ± 0.20^{A}	26.00 ± 0.00^{B}	6.02 ± 0.09^{A}
AP2000	4.03 ± 0.41^{AB}	0.626 ± 0.035^{A}	12.63 ± 1.42^{B}	26.67 ± 0.75^{A}	5.79 ± 0.05^{B}
AP4000	3.73 ± 0.50^{B}	0.625 ± 0.032^{A}	13.37 ± 2.28^{B}	26.67 ± 0.6^{A}	5.57 ± 0.04^{C}
BA2000	$3.71 \pm 0,28^{B}$	0.626 ± 0.024^{A}	12.99 ± 3.08^{B}	26.67 ± 0.74^{A}	5.93 ± 0.10^{A}
BA4000	$3.70 \pm 0,52^{B}$	0.623 ± 0.017^{A}	13.56 ± 3.43^{B}	26.80 ± 0.74^{A}	5.82 ± 0.14^{B}

NC: negative control (feed without inoculum and organic acids); PC: positive control (feed inoculated with fungus without organic acids); AP2000: propionic acid 2000g/ton; AP4000: propionic acid 4000g/ton; BA2000: *blend of acids* 2000g/ton; BA4000: *blend of acids* 4000g/ton. Measures followed by different capital letters in the same column differ from each other by Tukey's test at the 5% significance level

3.5 pH

For pH (Table 2), the 2.000 g/ton acid mixture (BA2000) was the only treatment with acid addition where no drop in pH was observed, however, it was statistically similar to PC and NC. Controlling the hydrogen potential is very important in preventing microbiological contamination, since under specific conditions, the fungus can produce aflatoxins, making the environment less conducive to fungal development, extending the shelf life of the feed [20].

In the study carried out by Miguel *et al.*[21] the pH values of the pre-starter, starter 1 and starter 2 feeds were close to 6.0, and showed significant reductions with the use of 1.0% potassium diformate, 1.0% fumaric acid, 1.0% citric acid and 1.0% benzoic acid. The addition of acidifiers resulted in reductions ranging from 0.56 to 1.64 pH units.

The effects of dietary inclusion of mixtures of organic acids or fatty acids may depend on the composition of the mixture. It is not clear whether there is a synergistic effect of the mixtures. Dietary inclusion of 4.16 mM fumaric acid reduced the dietary pH from 4.69 to 4.41[22]. The effect of propionic, acetic and lactic acids on *A. fumigatus*, *A. nidulans*, *P. commune*, *P. roqueforti* and *F. sporotrichioides*, were investigated, the authors concluded that the fungi were inhibited in concentration ranges between 4 and 30 mM for propionic and acetic acid, while a concentration of 160 mM or higher of lactic acid was required for total inhibition [23]. At pH 5.0, all fungi were inhibited with 60 mM or less of propionic acid and 120 mM or less of acetic acid, but lactic acid concentrations above 500 mM were required to inhibit most species. In the intestine, this reduction in pH promotes the activity of digestive enzymes. Therefore, it increases the utilization of nutrients and reduces the colonization of pathogenic microorganisms. [16]. Organic acids with a high pKa value are weaker acids and, therefore, more effective preservatives for feed since, present in the diet ingredients in their undissociated form in greater proportion, they can defend the feed against fungi and bacteria. This is one of the reasons why acids such as propionic acid, with a high pKa value, are mainly used as grain or feed preservatives [24].

A high pH value was found for the tested feed (average pH value 5.85). For greater effectiveness of antifungals in the feeds, the pH of these should be lower than the pKa of the acids tested, to have a greater proportion of the acids in the undissociated form. The study presented a reduction in pH values throughout the feed storage period (y = 5.9014 – 0.01284x). According to Franco & Landgraf [25] filamentous fungi and yeasts are able to withstand low pH ranges, ranging from 3.0 to 6.8. The variable tolerance between fungal species is directly related to enzymatic activities and metabolic processes. Regarding *A. flavus*, it is capable of developing in pH ranges ranging from 2 to 11[26].

Fungal growth was not identified by Moon *et al.*[27] in in vitro work when the medium was treated with 0.05% benzoic acid, 0.1% sorbic acid, 0.5% acetic acid or 0.5% butyric acid. Propionic acid, butyric acid, benzoic acid, and sorbic acid also exhibited potent anti aflatoxigenic effects at a concentration of 0.1%. The inhibitory effect on fungal growth depends not only on the pH, but also on the amount of undissociated acid present. However, propionic acid is generally considered more effective against fungal growth than formic acid [28].

Among the acids evaluated, acetic acid (10%) demonstrated the greatest inhibitory effect on the growth of *A. flavus*, resulting in an inhibition of 45.21%, with a recorded final pH of 3.25. In contrast, both tartaric acid and citric acid showed a minimal inhibitory effect at a concentration of 5%, registering only 0.42% inhibition for both, with final pHs measured at 3.12 and 3.24, respectively [29].

Table 3. Total mold counts (log^{10}) in PDA Agar of crumbled feed for finishing pigs inoculated with *Aspergillus flavus* (5.0 x 10-6 spores mL-1) evaluated during shelf life.

Time (days)	NC	PC	AP2000	AP4000	BA2000	BA4000
1	3.93 ± 0.36 ^{bcB}	3.68 ± 0,49 ^{cB}	4.42 ± 0.39 ^{abA}	4.46 ± 0.11^{abA}	4.55 ± 0.05 ^{abAB}	4.36 ± 0.09 ^{abcA}
3	4.32 ± 0.23 ^{aAB}	4.34 ± 0,28 ^{aA}	4.45 ± 0.38 ^{aA}	4.08 ± 0.20 ^{aA}	4.12 ± 0.27 ^{aB}	4.42 ± 0.48 ^{aA}
5	4.37 ± 0.14 ^{aAB}	4.49 ± 023 ^{aA}	4.28 ± 0.26 ^{aA}	4.22 ± 0.15 ^{aA}	4.30 ± 0.12 ^{aAB}	4.22 ± 0.35 ^{aA}
7	4.37 ± 0.22 ^{abAB}	4.54 ± 0.33 ^{abA}	4.51 ± 0.15 ^{abA}	4.32 ± 0.11^{abA}	4.20 ± 0.18 ^{abAB}	4.06 ± 0.23 ^{bA}
Delta log_{10}	+0,44	+0.86	+0.09	-0,14	-0.35	-0.30

NC: negative control (feed without inoculum and organic acids); PC: positive control (feed inoculated with fungus without organic acids); AP2000: propionic acid2000g/ton; AP4000: propionic acid 4000g/ton; BA2000: *blend of acids* 2000g/ton; BA4000: *blend of acids* 4000g/ton; Delta (log_{10}) = (Final count – initial count). Measures followed by different lowercase letters in the same column differ from each other by Tukey's test at the 5% significance level.Measures followed by different capital letters in the same column differ from each other by Tukey's test at the 5% significance level.

3.6 Microbiological mold counts (log10)

For total mold count (Table 3) there was no significant effect for treatment or time ($p>0.05$). However, the interaction between treatment and time was significant ($p<0.05$). A higher delta value (log10) was observed in the control feed contaminated with fungus (PC) throughout the storage period when compared to the other treatments.

A reduction in total mold (Table 3) was observed with the use of the highest concentration of organic acids (AP4000 and BA2000). It is important to emphasize that Potato Dextrose Agar (PDA) is a non-selective medium for fungi, therefore, the count includes other colonies than the experimentally inoculated *A. flavus*, such as contamination from the ingredients and the feed handling environment.

Although there was no significant reduction in total fungi, the average difference of around 14% between PC and other feeds with organic acids shows very satisfactory behavior in terms of microbial activity[12]. We can suggest that the use of organic acids was effective in stopping fungal development in feed, since their mechanism of action is due to their ability to act on the cell membrane, preventing the use of amino acids present in the substrate, and preventing microbial proliferation[13].

For total mold count, there was no significant effect for treatment and time ($p>0.05$). However, the interaction between treatment and time was significant ($p<0.05$). It was observed that the positive control presented a higher delta value (log10) throughout the storage period when compared to other treatments.

A mold reduction effect was observed with the use of a higher concentration of propionic acid (4000 g/ton) and an acid blend (2000 g/ton). It is important to emphasize that PDA agar is a non-selective medium for fungi, so the count includes other colonies than the experimentally inoculated *A. flavus*, such as contamination from the ingredients and the feed handling environment.

Although a significant reduction in total fungi was not evident in the present study, propionic acid presents a very satisfactory behavior in relation to microbial activity [12], since its mechanism of action is due to its ability to act on the cell membrane and block the use of amino acids present in the substrate, preventing microbial development [13].

The Sabouraud culture medium provided conditions for isolating and quantifying *A. flavus*. There was a significant effect for treatment and time ($p<0.05$), with the interaction between treatment and time being non-significant ($p>0.05$). As expected, the positive control presented the highest average when compared to the other treatments (Table 3). The treatments containing 4000 g/ton propionic acid and a blend of 2000 g/ton and 4000 g/ton acids presented measures equivalent to the negative control, demonstrating the efficiency of the acids in inhibiting *A. flavus*. The treatment containing 2000 g/ton propionic acid was the least efficient, because it presented a mean equivalent to the positive control.

3.7 Water activity

For water activity, there was a significant effect for treatment, time and interaction between treatment and time ($p<0.05$). The water activity of the stored feed ranged from 0.5647 to 0.6366. The negative control showed a lower average than the other treatments (Table 4).

The water activity values obtained in the present study are too low for microbial development. The crumbled feed has few free water available for fungal development. Spoilage fungi require a minimum Aw of 0.80 [25], more specifically, *A. flavus* requires at least 0.71 water activity for its growth, with an optimum value of 0.98 [30] (International Commission on Microbiological Specifications for Foods - ICMSF, 1996). This means that it was not possible to evaluate the effectiveness of the treatments against Aw, since the values obtained were not within the minimum limit for the development of filamentous fungi[4].

3.8 Temperature

For temperature, there was a significant effect for treatment, time and interaction between treatment and time ($p<0.05$). The positive control was the only one that presented a lower mean than the other treatments. This lower temperature could limit the growth of the fungus and slow down its metabolic activity, but it is still at acceptable levels for the development of *A. flavus* (Table 4). *A. flavus* has a temperature range for growth between 24 and 40 ºC, with an optimum temperature close to 35ºC [19]. The temperature of the environment in which the bags containing the feed were stored varied from 24 to 32 ºC throughout storage. This temperature range is described as ideal for the growth of *A. flavus*. It is worth noting that the temperature of a medium can be influenced by two main factors: the ambient temperature and the influence of refrigeration or heating systems. However, in this study, the storage location of the feed samples did not contain mechanisms to manipulate the temperature. This means that the temperature varied according to the ambient temperature, without this necessarily being explained by the metabolism of the fungus.

Table 4. Water activity and temperature of crumbled feed for finishing pigs inoculated with *Aspergillus flavus* (5.0 x 10^{-6} spores mL^{-1}) evaluated during shelf life.

Time (days)	NC	PC	AP2000	AP4000	BA2000	BA4000
			Water Activity			
1	0.55 ± 0.02 ^{cAB}	0.64 ± 0.00 ^{abA}	0.57 ± 0.03 ^{bcB}	0.58 ± 0.02 ^{bcC}	0.62 ± 0.01^{abBC}	0.61 ± 0.03 ^{abcB}
3	0.59 ± 0.01 ^{cAB}	0.63 ± 0.01 ^{abcA}	0.64 ± 0.02 ^{abcA}	0.61 ± 0.02 ^{abcBC}	0.59 ± 0.02 ^{cC}	0.60 ± 0.01 ^{bcB}
5	0.54 ± 0.01 ^{bB}	0.63 ± 0.01 ^{aA}	0.65 ± 0.01 ^{aA}	0.65 ± 0.01 ^{aAB}	0.64 ± 0.02 ^{aABC}	0.62 ± 0.01 ^{aB}
7	0.58 ± 0.00 ^{bAB}	0.64 ± 0.00 ^{abA}	0.63 ± 0.00 ^{abA}	0.63 ± 0.01 ^{abABC}	0.62 ± 0.01 ^{abBC}	0.62 ± 0,00 ^{abB}
			Temperature			
1	26.67 ± 0.58 ^{bcA}	26.00 ± 0.00 ^{cA}	27.33 ± 0.58 ^{abcAB}	27.67 ± 0.58 ^{abcAB}	28.00 ± 0,00 ^{abA}	28.00 ± 0.00 ^{abA}
3	27.00 ± 0.00 ^{aA}	26.00 ± 0.00 ^{aA}	26.33 ± 1.15 ^{aB}	26.67 ± 0.58 ^{aB}	26.33 ± 0,58 ^{aB}	27.00 ± 0.00 ^{aB}
5	27.00 ± 0.00 ^{aA}	26.00 ± 0.00 ^{aA}	26.33 ± 0.58 ^{aB}	26.67 ± 0.58 ^{aB}	26.67 ± 0,58 ^{aB}	26.00 ± 0.00 ^{aC}
7	27.00 ± 0.00 ^{aA}	26.00 ± 0.58 ^{aA}	27.00 ± 0.00 ^{aAB}	27.00 ± 0.00 ^{aAB}	27.00 ± 0,00 ^{aB}	27.00 ± 0.00 ^{aB}

NC: negative control (feed without inoculum and organic acids); PC: positive control (feed inoculated with fungus without organic acids); AP2000: propionic acid 2000g/ton; AP4000: propionic acid 4000g/ton; BA2000: *blend* of acids 2000g/ton; BA4000: *blend* of acids 4000g/ton; Measures followed by different lowercase letters in the same row differ from each other by Tukey's test at the 5% level of significance. Measures followed by different capital letters in the same column differ from each other by Tukey's test at the 5% level of significance.

4. Conclusion

The used acids were effective in reducing *A. flavus* counts in the feed. In addition, the acids reduced the pH of the feed, although they did not influence the temperature or water activity. Thus, the use of organic acids appears to be a promising alternative for controlling the growth of contaminating fungi in crumbled feeds, contributing to maintaining the quality and safety during the product's shelf life.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

Data will be made available on request to the corresponding author.

Author contributions

Conceptualization: Bezerra, R. A. D. Pozza, M. S. S.; Pozza, P.C. Data curation: Saraiva, B. B. Formal analysis: Saraiva, B. B. Acquiring financing: Pozza, M. S. S.: Pozza, P.C.. Project administration: Pozza, M. S. S.; Pozza, P.C.. Methodology: Bezerra, R. A. D. Ratão, M.E.R.; Araújo, G.A.; Pozza, M. S. S.. Supervision: Pozza, M. S. S.. Bezerra, R. A. D. Investigation: Bezerra, R. A. D.; Ratão, M.E.R.; Blasques, T. S.; Visualization: Bezerra, R. A. D.; Pozza, M. S. S. Writing (original draft): Bezerra, R. A. D. Pozza, M. S. S. Writing (review and editing): Bezerra, R. A. D. Pozza, M. S. S.

Acknowledgments

We appreciate and acknowledge the collaborative efforts between the Department of Animal Science, the Postgraduate Program in Animal Science of the State University of Maringá. This study was funded in part by the National Institute of Science and Technology of the Milk Production Chain (INCT-LEITE/UEL/UEM-PR), Londrina/Maringá, Paraná, Brasil.

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