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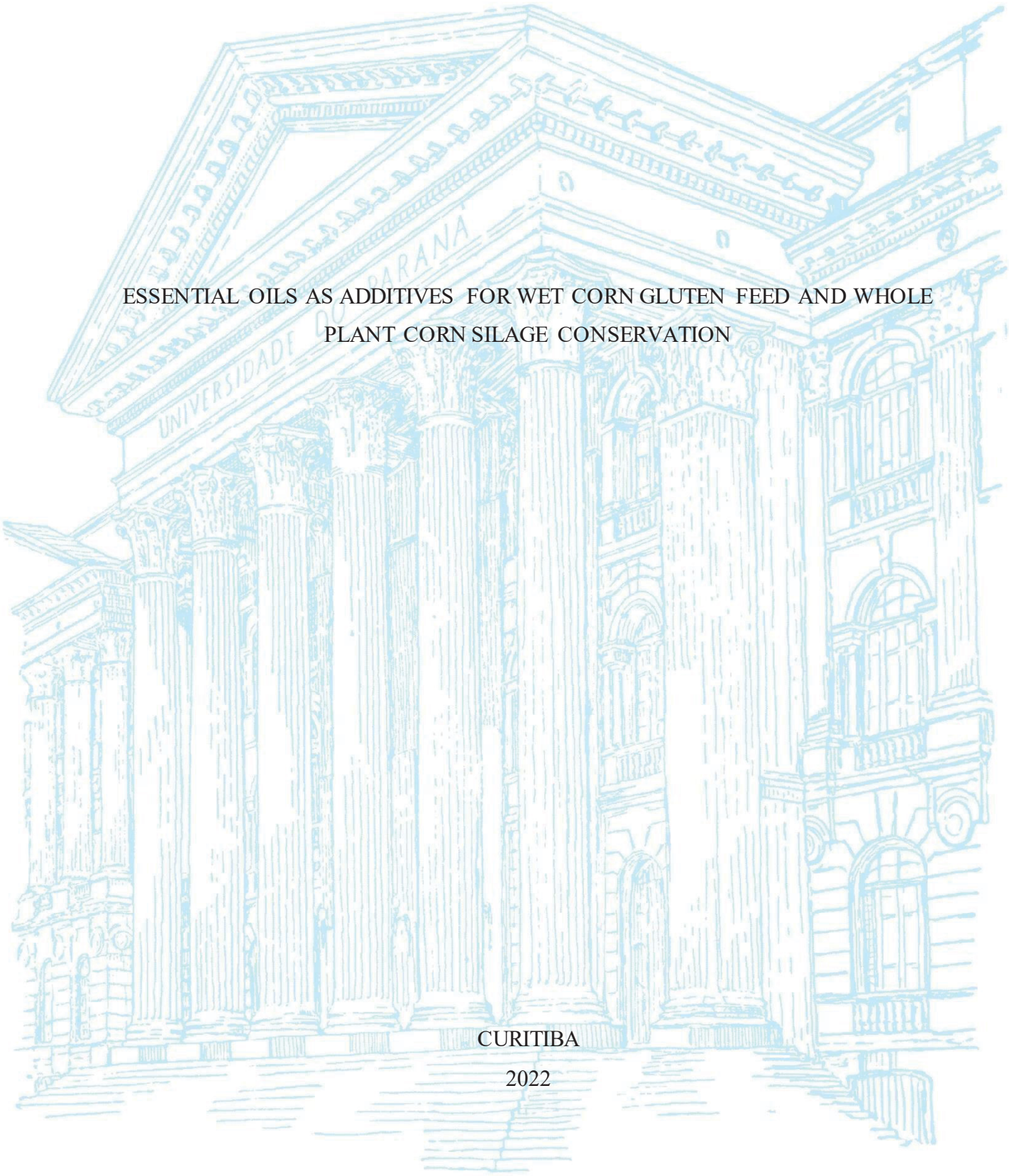

ESSENTIAL OILS AS ADDITIVES FOR WET CORN GLUTEN FEED AND WHOLE PLANT CORN SILAGE CONSERVATION

CURITIBA

2022

LUCELIA DE MOURA PEREIRA

ESSENTIAL OILS AS ADDITIVES FOR WET CORN GLUTEN FEED AND WHOLE PLANT CORN SILAGE CONSERVATION

Tese apresentada ao curso de Pós-Graduação em Zootecnia, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Zootecnia.

Orientadora: Prof (a). Dr (a). Maity Zopollatto

Coorientador (a): Dr (a). Bruna Calvo Agustinho

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“What doesn't kill us makes us stronger”.

“Amor fati”.

Friedrich Nietzsche

RESUMO

No ano de 2008 foi realizado o primeiro experimento utilizando óleos essenciais como aditivos para silagem. A partir disso, até o presente momento foram publicados aproximadamente 11 artigos com esse tema. No entanto, algumas lacunas ainda precisam ser respondidas, a respeito de doses, tipos de óleos, sua ação nos diferentes tipos de alimentos, sejam eles fermentados ou frescos. Assim, o objetivo geral desse trabalho foi avaliar a ação de cinco óleos essenciais: timol, carvacrol, cinamaldeído, eugenol e isotiocianato de alila como aditivos em silagem de planta inteira de milho e farelo de glúten de milho úmido. Desta forma, o objetivo do experimento 1 foi determinar a influência do isoticionato de alila sob a estabilidade aeróbica, análise microbiológica e perdas fermentativas em silagem de planta inteira de milho. Foram analisadas as variáveis relacionadas a perdas fermentativas, composição química, estabilidade aeróbica e curva de pH durante a exposição ao ar. Enquanto o objetivo do experimento 2 foi avaliar o uso de óleos essenciais como aditivos para controle de deterioração aeróbica em farelo de glúten de milho úmido. Nesse experimento foram realizados cinco testes de estabilidade aeróbica com seis diferentes doses (D0, D1, D2, D3, D4 and D5) de cada óleo essencial estudado (timol, carvacrol. cinamaldeído, eugenol e isoticionato de alila), e as variáveis analisadas foram conteúdo de matéria seca (MS), perdas de matéria seca após a exposição aeróbica (PMS), estabilidade aeróbica, temperatura máxima, horas para atingir a temperatura máxima e temperatura acumulada. Por outro lado, no experimento 3 o objetivo foi avaliar os efeitos da inclusão de diferentes óleos essenciais na ensilagem de farelo de glúten de milho úmido. Por fim, nesse último foram avaliadas as perdas de matéria seca, análise microbiológica, composição química, estabilidade aeróbica e curva de pH durante a exposição ao ar. De forma geral os óleos essenciais representam uma nova alternativa de aditivos para alimentos conservados. Em silagem de milho, o isoticianato de alila na dose de 20 mg/kg de forragem fresca (FF) reduziu as perdas de MS, produção de gases e fungos filamentosos. O eugenol, na dose de 350 mg/kg de FF, reduziu as perdas de matéria seca depois da estabilidade aeróbica em farelo de glúten de milho úmido (FGMU). Por outro lado, o isoticionato de alila aumentou a estabilidade aeróbica do FGMU.

Palavras-chave: Aditivos. Alimentos conservados. Compostos naturais. Coproduto. Fermentação. Milho.

ABSTRACT

In 2008 was published the first study evaluating the potential of essential oils as additives for silage. Since then, approximately other 11 articles on this topic have been published. However, some gaps still need to be addressed regarding doses, types of oils, and their use in different animal feeds, whether fermented or fresh condition. Therefore, the general objective of this study was to evaluate the potential of several essential oils in whole-plant corn silage and wet corn gluten feed. The objective of experiment 1 was to determine the influence of allyl isothiocyanate on aerobic stability, microbiology profile, and fermentative losses in whole-plant corn silage. Variables related to fermentative losses, chemical composition, aerobic stability, and pH during exposure to air were analyzed. Whereas, the objective of the experiment 2 was to evaluate the use of essential oils as additives to mitigate the aerobic deterioration of wet corn gluten feed (WCGF). Therefore, the aerobic stability, dry matter content, dry matter losses, maximum temperature, and time required to reach maximum temperature was evaluated in WCGF with inclusion of five essential oils, thymol, carvacrol, cinnamaldehyde, eugenol, and allyl isothiocyanate with several inclusion rates. On the other hand, the objective of experiment 3 was to evaluate the effects of the inclusion of different oils essential oils in wet corn gluten feed at the ensiling. In this experiment was determined dry matter content, chemical composition, aerobic stability and pH during the aerobic exposure. In general, essential oils represent a new alternative of additives for preserved feeds. In corn silage, 20 mg of AITC/kg of fresh forage reduced dry matter losses, gas production, and filamentous fungi. In addition, 350 mg of eugenol/kg FF of wet corn gluten feed, reduced the dry matter losses after the aerobic stability. On the other hand, the allyl isothiocyanate increased the aerobic stability of the WCGF.

Keywords: Additives. Co-product. Corn. Fermentation. Feed conservation. Natural compounds

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LIST OF ABREVIATIONS AND ACRONYMUS

AITC	- Allyl Isothiocyanate
GRAS	- Generally Recognized as Safe
FF	- Fresh forage
ADF	- Acid detergent fiber
NDF	- Neutral detergent fiber
AS	- Aerobic stability
CFU	- Colony-forming unit
WSC	- Water-soluble carbohydrates
DM	- Dry matter
O_2	- Oxygen
GP	- Gas production
DML	- Dry matter losses
LAB	- Lactic acid bacteria
SEM	- Standard error of the mean
Cfb	- Humid maritime temperate climate
Cfa	- Subtropical humid mesothermic climate
CP	- Crude protein
CAR	- Carvacrol
THY	- Thymol
EUG	- Eugenol
CIN	- Cinnamaldehyde
ASLoss	- Aerobic stability losses
CPFOR	- Centro de Pesquisa em Forragicultura
MAXT	- Maximum temperature
HMAXT	- Time to reach maximum temperature
°C	- Degrees centigrade
kg	- Kilograms
mm	- Millimeters
cm	- Centimeters
m	- Meters
m^2	- Square meters
ha	- Hectare
L	- Liters

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SUMMARY

1 INTRODUCTION

There are several issues related to the conservation of ingredients used in ruminant nutrition (silage, hay, and haylage). Most of the time, they are associated with maintaining product quality during storage and use. Yeasts, filamentous fungi, and *Clostridium* bacteria are the main spoiling factors of the feed (MCDONALD et al., 1991). The spoiling factors promote a decline in nutritional quality of dry matter (DM) and reduces animal intake, which reduces animal performance. The conservation of forage in the form of silage is ensured by the absence of oxygen, where lactic acid bacteria propagate and metabolize sugars, generating lactic acid as a final product. Thus, the maintenance of anaerobiosis and the drop in pH are essential factors that are responsible for the preservation of stored forage (DRIEHUIS et al., 1999; PAHLOW et al., 2003), as the microorganisms capable of deteriorating the silage are inhibited by the synergistic effect of the acids produced during fermentation, by the high osmotic pressure and the absence of oxygen (WOOLFORD, 1990).

The mass contact with oxygen is inevitable during some phases that comprise the ensiling process (silo supply, storage, and feed-out). The presence of O_2 triggers the proliferation of undesirable microorganisms present in the mass, such as yeasts, filamentous fungi, and aerobic bacteria, that develop due to energetic substrates present in the forage, causing losses in the nutritional value of the silage and reducing consumption by animals (LINDGREN et al., 1985).

The aim of with silage production, is basically to reduce aerobic spoilage, which includes the use of organic acids during ensiling (MILLS & KUNG, 2002), such as ammonia and urea (HILL & LEAVER, 2002), in addition to common methods, e.g., altering fermentation with the use of acetate and/or propionate-producing heterolactic bacteria (HIGGINBOTHAM et al., 1998; DRIEHUIS et al., 2001).

Despite this, a wide variety of preservatives have been used to inhibit microbial growth in several products intended for human or animal nutrition. However, there is a growing negative perception regarding synthetic additives, a fact that has raised the interest in the search for natural alternatives to traditional solutions (ZINK, 1997). Compounds as essential oils may be potential natural alternatives, since they are normally used to enhance the flavor of foods and inhibit microbial growth.

The constant concern of food safety involves the entire production chain, that goes from raw materials, feed supplied to animals, and animal foods, such as meat, milk or eggs. In this way, the production of food intended for animal production also seeks to meet the requirements of international market for safe products, known by the acronym GRAS (Generally Recognized as Safe).

The possibility of combining the silage additives, such as inoculates, and control of undesirable microorganisms in the production of silage using GRAS product, which is the case of essential oils, represents a new alternative to ruminant production systems.

Although it is a very incipient area of investigation, the results observed so far are promising for the use of essential oils as additives for silage (PEREIRA 2016; CANTÓIA-JUNIOR et al., 2020). However, further studies developing technology applied to the field are necessary to understand the best combination of essential oils in addition to the combination of compounds and form of administration.

1.1 OBJECTIVES

1.1.1 General objective

Evaluate the effect of different essential oils (thymol, carvacrol, cinnamaldehyde, eugenol, and allyl) as additives to improve quality and conservation of whole-plant corn silage and wet corn gluten feed.

1.1.2 Specific objectives

- Evaluate the effect of inclusion rates of allyl isothiocyanate as additive on fermentative losses, chemical composition, microbiological count of homofermentative and heterofermentative lactic acid bacteria, yeast and molds, and organic acids in whole-plant corn silage.
- Evaluate the effect of inclusion rates of thymol, carvacrol, eugenol, cinnamaldehyde and allyl isothiocyanate on dry matter composition, dry matter losses after aerobic exposure, aerobic stability, maximum temperature, hours to maximum temperature and accumulated temperature, to control the aerobic deterioration of wet corn gluten feed.
- Evaluate the effect of different essential oils (thymol, carvacrol, cinnamaldehyde, eugenol, and allyl isothiocyanate) on fermentation, chemical and microbiological composition in wet corn gluten feed and their effects on fermentative losses, microbiology, pH value and aerobic stability in wet corn gluten feed silage.

2CHAPTER I - LITERATURE REVIEW

Whole-plant corn silage

Whole-plant corn silage is a traditional ingredient in ruminant diets. The premise of this ingredient is to develop an oxygen-free environment, where the epiphytic microorganisms from the plant can transform water soluble carbohydrates in organic acids, such as lactic and acetic acid. These two compounds (lactic and acetic acid) are responsible for reducing the pH in the silage environment and minimizing the growth of spoilage microorganisms including yeasts and filamentous fungi (MCDONALD et al., 1991). Following this premise, research in the field of whole-plant corn silage has advanced over the years aiming to find more effective strains for the fermentation process, reducing dry matter losses, and increasing aerobic stability. Another research area is focused on enzymes utilization that can increase the digestibility and bioavailability of nutrients for the microorganisms inside the silo or for the animal that will consume the product after fermentation.

Mini-silos is one of the most common approaches that have been used as scientific model to test and develop new additives, inoculants, and enzymes. With mini-silos is possible to access the gases, effluent, and DM loses, as well as, fermentative profile and chemical composition (GHELLER et al., 2021).

During the use of soluble carbohydrates inside the silo, the microorganisms responsible are mostly *Lactobacillus*, *Pediococcus, Enterococcus*, *Lactococcus*, *Streptococcus* and *Leuconostoc.* During the fermentation process, *Lactobacillus* for example, convert glucose into 2 lactates, when these are homolactic fermentation with 0 % of DM losses. On the other hand, heterolactic *Lactobacillus* can promote 24% of DM losses. Other microorganisms, for example yeasts, can cause higher losses. Yeasts consume glucose generating 2 ethanol, 2 CO_2, with 48.9% DM losses and 0.2% of gross energy (BORREANI et al., 2018). If considered only the fermentation process, with the homolactic route of *Lactobacillus*. However, this material is more likely to transform into yeast and and later into filamentous fungi activity due to homolactic fermentation. This is attributed to the low antimicrobial power that lactic acid has, as it is considered a strong acid, but with a low dissociation constant pka = 3.8 (KUNG et al., 2018).

Lactobacillus buchneri is one of the most studied heterolactic strains, as this strain has been associated with greater aerobic stability in whole-plant corn silage (DRIEHUIS et al., 1999). This microorganism can ferment 1 mol of lactate without an electron acceptor to 0.5 mol of acetate, 0.5 mol of 1,2-propanediol, and 0.5 mol of carbon dioxide (OUDE ELFERINK et al., 2001). Besides the fact that this process generates losses in half mole forming carbon dioxide, it is still considered an advantage because it participates controlling yeast growth, attributed to the acetic acid. Acetic acid produced from lactate has antimicrobial action due to its dissociation constant pka = 4.76 at 25°C.

This characteristic allows this acid to cross the yeast cell membrane and alter the sodium and potassium pump in the cells. As the yeast would take time to try to rebalance its pH this would cause a delay in its growth or even cell death (BOOTH, 1985).

Other alternatives to control yeast growth that have also been widely evaluated, and the most common are the utilization of organic acids, such as formic, acetic, and propionic acid (JIANG et al., 2020).

Wet corn gluten feed

The corn milling industry produces several by-products including wet corn gluten feed (WCGF). WCGF contains high levels of protein (approximately 40 to 45% DM) and is quickly digested. Also, it is relatively cheaper when compared to other dietary protein sources because it isa co-product (MULLINS et al., 2010).

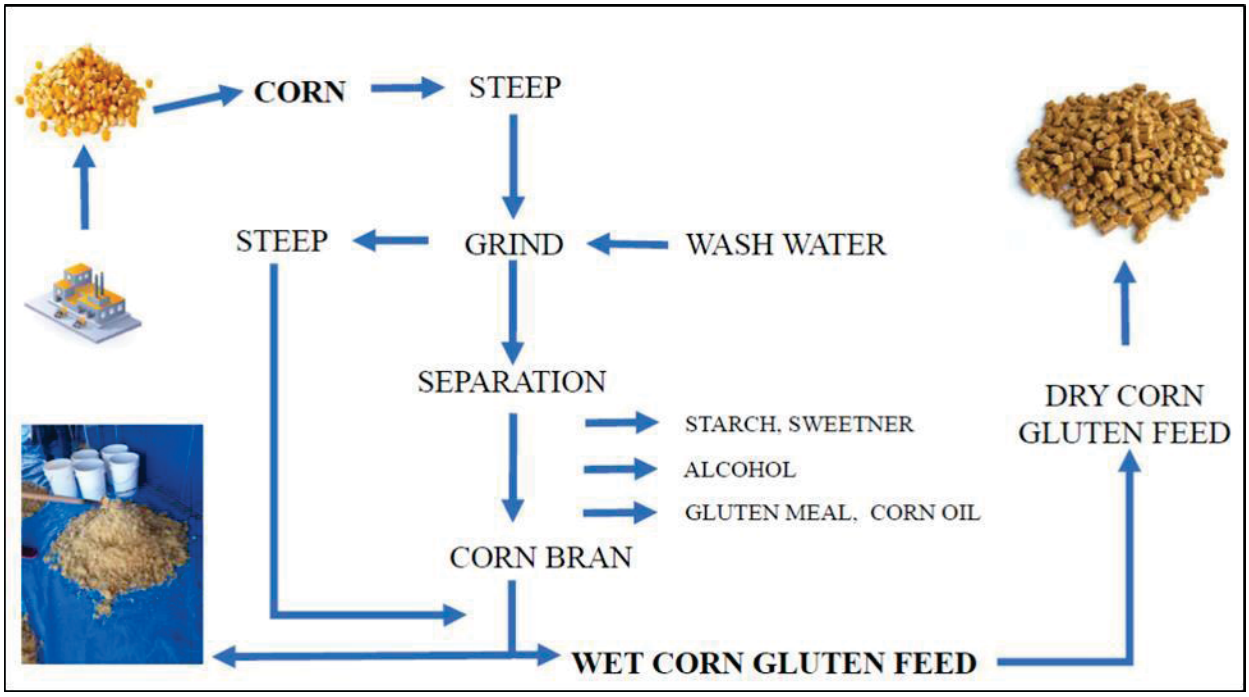
The wet corn gluten feed (WCGF) used in to provide datas for this document was provided by Cargill company. This product is basically constituted by the pericarp of the grain and soaking water (corn liquor). Corn liqueur comes from the evaporation of water used in the maceration process, therefore, concentrates a soluble corn protein. The whole grain passes through several processing steps in the industry, such as: maceration, milling, and germ separation. Maceration is conducted under controlled temperature and time, with the addition of sulfur dioxide and lactic acid. The soaking tanks are filled with whole corn kernels so that the process is not hampered. This step is crucial to efficiently separate the components of corn, physically and chemically. Milling is divided in three stages; the first stage consists of germ separation by grinding. The second step consists of fiber and residual germ separation, whereas the third step consists of separation of grain components in the third grinding. Subsequently, the separation of the grain components begins. Germ is separatedfrom fiber, starch, and gluten by hydro cyclones in an aqueous medium since it has a lower density. The separation of 16 fibers from starch and gluten components is carried out by washing and sieving. Finally, the separation of starch and gluten takes place through the density difference with the useof hydro cyclones (RIBEIRO et al., 2018; SILVA et al., 2020).

This co-product can be incorporated into a total mixed ration in diets for dairy and beef cattle, mainly because it is a food with a relatively lower price compared to other protein or forage sources. MULLINS et al. (2010), when adding different levels (0, 12, 24 and 34% DM) of WCGF in the diets of lactating cows, observed that the addition of WCGF to dairy rations can increase milk production with energy correction, and this increase appears to be driven, at least in part, by a greater dry matter intake. ZHANG et al. (2020) evaluated the inclusion of WCGF in three different diets for lactating dairy cows. These diets consisted of total mixed ration (TMR), with a partial replacement of alfalfa hay by WCGF, and a total fermented fed ration (FTMR) with partial replacement of alfalfa

hay. These authors observed that FTMR improved fermentation quality, nutrient digestibility, leading to a greater milk fat concentration, and feed efficiency, reduced fecal N excretion, feeding cost, and better yield over feed cost, while fiber content decreased.

Despite the positive results from the inclusion of WCGF, there is the challenge of conserving this product. Because it is humid and has a high concentration of nutrients, it can be easily degraded by the action of microorganisms. RIBEIRO et al. (2018), when comparing the microbial growth in wet corn gluten feed and dried corn gluten feed, observed that yeasts, molds, *Staphylococcus aureus*, and *Bacillus cereus* count in the wet co-product were 880, 880, 960, and 524 colony forming units per gram of sample (CFU/g), respectively, while dry corn gluten did not show the presence of those microorganisms. On the other hand, the drying process or material requires energy expenditure within the factory. For this reason, after the grinding process, the WCGF usually is already transported in tipping loads inside the Cargill plant. There is still no WCGF storage system after milling at the Cargill plant. Traditionally, this product is packed in silage bag stores, and transported by trucks to farms that use this feed, or to companies that manufacture TMR and sell the product in the form of feed. Thus, experiments were carried out aiming to improve WCGF storage methods, whether in the form of silage using organic additives (SILVA, 2020), addition of different levels of WCGF in whole-plant corn silage (HERMISDORFF et al., 2016), and essential oils, as demonstrated in chapters 3 and 4 of this dissertation.

FIGURE 1 - SCHEME OF THE WET MILLING INDUSTRY RESULTING IN WET OR DRY CORN GLUTEN FEED.



Source: Adapted from Rausch and Eckholf (2016).

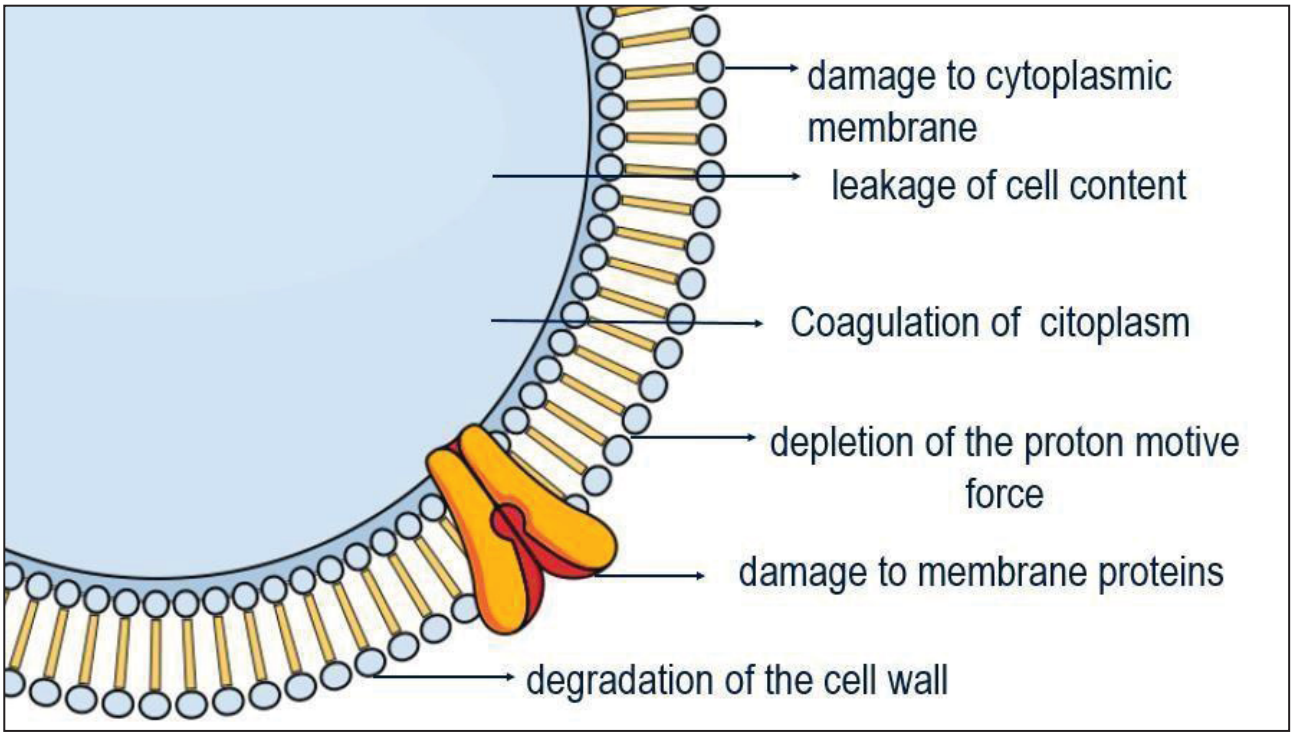
Essential oils

Essential oils (EOs) are volatile derivatives with hydrophobic characteristics, synthesized by the secondary metabolism of aromatic plants (BURT, 2004). They are extracted from different parts of the plants, like leaves, fruits, flowers, bulbs, seeds, roots, stems, and bark. The essential oils form a complex mixture of several compounds in different concentrations, consisting of terpene hydrocarbons, esters, organic acids, aldehydes, ketones, phenols, and several other compounds. They are known to have antiviral, antibacterial, antifungal, and insecticidal properties (LEE et al., 2003; Burt, 2004). In addition, essential oils have helped to improve animal performance in intensive production systems when used as feed additives (WILLIAM & LOSA, 2001; ORNAGHI et al., 2017; LEI et al., 2018).

The use of essential oils in animal feeds is authorized in Europe by Council Directive 70/524/EEC (Chapter III), which refers to aromatic and palatability substances. In the USA, the Food and Drug Administration (FDA) recognizes the group of essential oils as safe substances (GRAS: Generally Recognized as Safe) for use in animal and human food (Code of Federal Regulations title 21, v.6; Part 582).

The possible mechanisms of action of essential oils on bacterial cells is outlined in Figure 2-2. The actions of essential oils are mostly associated with the cell membrane, such as electron transport and ion gradient, protein translocation, phosphorylation, and other enzyme-dependent reactions (ULTEE, 1999; DORMAN, 2000).

FIGURE 2 - PROPOSED MECHANISMS FOR THE ANTIMICROBIAL ACTION OF ESSENTIAL OILS IN THE BACTERIAL CELL.



Source: Adapted from BURT (2004) and MARURYA et al. (2021).

KHORHIDIAN et al. (2018) observed and described other mechanisms of action of essential oils, such as extravasation of cytoplasmic constituents, membrane protein damage, cytoplasmic coagulation, and reduction in the activity of the H+ pump. The variation in the mode of action of these substances may be related to the type of EOs and the plant of origin. As demonstrated by BURT et al. (2014), extracts from oregano (*Origanum vulgare*) act by inhibiting the Quorum Sensing mechanism of microorganisms, disrupting their communication, and release of self-inducing hormones; therefore, there is a change in the behavior of the bacterial colonies (WATERS et al., 2005). BASSOLE et al. (2010) evaluated the antimicrobial activity of carvacrol, thymol, linalool, and menthol against *Listeria monocytogenes*, *Enterobacter aerogenes*, *E. coli*, and *Pseudomonas aeruginosa*. These authors observed that the most active compound was carvacrol, followed by thymol, with minimal bactericidal doses, being 300 and 800 µg/mL, respectively.

1.2 CARVACROL

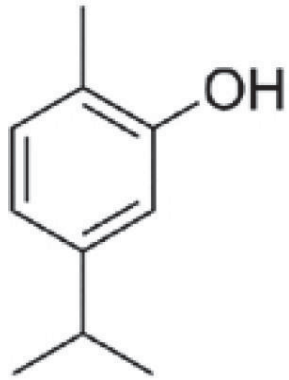
Carvacrol is the main constituent of oregano, followed by thymol, both presenting a hydroxyl group in a different position on the phenolic ring. These compounds act by causing changes in the permeability and activity of the bacterial cell membrane, changes in the activity of calcium channels, disturbance of ionic balance, and loss of K+ ions. These damages to the bacterial enzyme system are related to energy production and synthesis of structural components, making it difficult to conduct and transport intracellular ATP (NOSTRO et al., 2004; KNOWLES et al., 2005).

There are no reports in the literature on the development of bacterial resistance to essential oils, due to these different mechanisms of action on bacteria. Tests were carried out in an attempt to induce resistance with sub-doses of different essential oils (carvacrol and cineole) in *Listeria monocytogenes, Salmonella typhimurium,* and *Pseudomonas aeruginosa*. The results showed that even with non-lethal doses, when returning to standard doses, the essential oils are effective in inactivating these bacteria, concluding that there is no formation of bacterial resistance (LUZ, 2012a, b; GOMES NETO, 2012).

Among studies on the fungicidal effect of essential oils, carvacrol usually has the highest efficacy, with an average minimum inhibitory concentration (MIC) value of 154.5 µ/mL (CHAO and YOUNG, 2000; ABBASZADEH et al., 2014). Antifungal activity of essential oils (eugenol, thymol, carvacrol, cinnamaldehyde, and myristin) is also observed against the fungi *Aspergillus parasiticus* and *Fusarium verticillioides*, both mycotoxin producers (JUGLAL et al., 2002). The fungicidal action of essential oils from rosemary, onion, basil, mint, and oregano was evaluated in test colonies of *Fusarium* sp., *Aspergillus ochraclus* and *Aspergillus flavus*, noting that the essential oil of oregano,

in the concentration of 1000 mg/mL, inhibited the growth of fungi, and for the other oils the inhibition occurred from concentrations above 1500 mg/mL (PEREIRA et al., 2006).

FIGURE 3 - CHEMICAL STRUCTURE OF CARVACROL



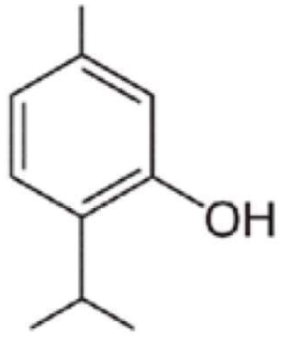
- International Union of Pure and Applied Chemistry (IUPAC) 5-Isopropyl-2-methylphenol
- Moleular formula: $C_{10}H_{14}O$;
- Molecular weight: 150.22 g/mol

NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2022). (PUBCHEM COMPOUND SUMMARY FOR CID 10364, CARVACROL.)

1.3 THYMOL

Most studies related to the antimicrobial mode of action of essential oils are mainly focused on bacteria, while little is known about their mode of action on fungi and yeasts. The thymol has molecular weight of 150.22 g/mol (Figure 2-4). The mode of action of thymol against yeast has been barely investigated, but studies point to interactions with the cell membrane. Thymol disrupted vesicles and cell membrane and impaired ergosterol biosynthesis in *Candida* strains, which consequently affected cell membrane integrity, because ergosterol regulates membrane fluidity, just as cholesterol does in animal cells (GHANNOUM & Rice, 1999; cristani et al., 2007; ahmad et al., 2011).

FIGURE 4 - CHEMICAL STRUCTURE OF THYMOL



- International Union of Pure and Applied Chemistry (IUPAC) 5-methyl-2-propan-2-ylphenol
- Molecular formula: $C_{10}H_{14}O$;
- Molecular weight: 150.22 g/mol

(NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2022). PUBCHEM COMPOUND SUMMARY FOR CID 6989, THYMOL).

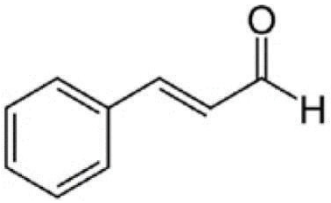
1.4 CINNAMALDEHYDE

Cinnamaldehyde (CIN) is formed as a phenylpropanoid, having a molecular formula and weight of C_9H_8O and 132.16 g, respectively (Figure 2-5). This compound has antimicrobial action

presenting several action modes in prokaryotic cells. One of them is the control of bacterial growth is through FtsZ protein shielding. This protein is essential in the beginning of cell division. FtsZ forms a ring in the cytokinetic medium cell, which serves as a support for the assembly of other cell division proteins. The formation of the Z ring is essential for the correct location of the split plane. When cinnamaldehyde shields itself to this protein, it automatically alters the cell division of bacteria (HEMAISWARYA et al., 2011).

The cinnamaldehyde can also inhibit some enzymes, for example, histidine decarboxylase, (WENDAKOON and MORIHIKO, 1995). This enzyme is responsible for the formation of histamine. Histamine, as well as tyramine, putrescine, and cadaverines are the main biogenic amines that are found in inefficient fermentation of wines (ZEE et al., 1983). VANS et al. (1996) observed that 90% of the biogenic amine present in the silage were histamine, tyramine, putrescine and cadaverines, when working with perennial ryegrass silage. The authors found that the formation of these amines took place in the first 10 days of fermentation and correlated these biogenic contents with ammonia amine and acetic acid. The formation of biogenic amines can be controlled with the rapid drop in pH during fermentation, but in plants with a high protein content, such as alfalfa, this can be achallenge caused by the high buffering capacity (MUCK, 1988).

FIGURE 5 - CHEMICAL STRUCTURE OF CINNAMALDEHYDE



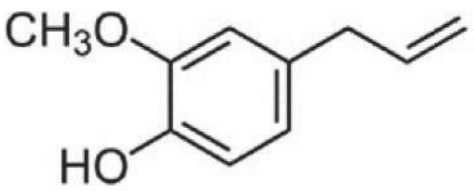
- International Union of Pure and Applied Chemistry (IUPAC)
  (E)-3-phenylprop-2-enal
- Molecular formula: C_9H_8O;
- Molecular weight: 132.16 g/mol

(NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2022). PUBCHEM COMPOUND SUMMARY FOR CID 637511, CINNAMALDEHYDE).

1.5 EUGENOL

Eugenol is an essential oil extracted from cloves and acts as a potent fungicidal agent as described by ABBASZADEH et al. (2014). Eugenol, as well as cinnamaldehyde, belongs to the phenylpropenes classification with the molecular formula and weight $C_{10}H_{12}O_2$ and 164.2 g/mol, respectively (Figure 2-6). Gill and Holley (2004) compared the effects of cinnamaldehyde and eugenol in controlling the growth of *L. monocytogenes* and *Lactobacillus sakei*. They observed that a lower dose of eugenol (MIC - only 821 and 985 µg/mL) than cinnamaldehyde (MIC - 3965 and 66080 µg/mL) is required to obtain a bactericidal effect.

FIGURE 6 - CHEMICAL STRUCTURE OF EUGENOL



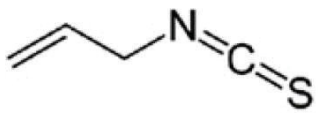
- International Union of Pure and Applied Chemistry (IUPAC) 2-methoxy-4-prop-2-enylphenol
- Molecular formula: $C_{10}H_{12}O_2$
- Molecular weight: 164.2g/mol

(NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2022). PUBCHEM COMPOUND SUMMARY FOR CID 3314, EUGENOL)

1.6 ALLYL ISOTHIOCYANATE

Allyl isothiocyanate is part of the group of essential oils. There are reports in the literature described by Kojima (1971), Jo et al. (2012), and Nazareth et al. (2020) where allyl blocked the oxygen uptake by yeasts and uncoupled the oxidative phosphorylation of cytrochrome C oxidase in the electron transport chain. This compound has the chemical formula C_4H_5NS (Figure 2-7), and like the other compounds, it has anti-oxidative, anti-bacterial, anti-fungal, anti-nematode, and anti-insect activities (MASUDA et al., 1999; KERMANSHAI et al., 2001; SHIN et al., 2004). In nature, the formation of AITC occurs from the degradation of glucosinolates present in the *Brassicaceae* family (e.g., brown and black mustard, cabbage, horseradish, wasabi) into three main compounds: nitriles, thiocyanates, and Isothiocyanates, with AITC classified in this last group of compounds (Figure 2-8).

FIGURE 7 - CHEMICAL STRUCTURE OF ALLYL ISOTHIOCYANATE



- International Union of Pure and Applied Chemistry (IUPAC) 3-isothiocyanatoprop-1-ene
- Molecular formula: C4H5NS or CH2=CHCH2N=C=S
- Molecular weight: 99.16g/mol

(NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (2022). PUBCHEM COMPOUND SUMMARY FOR CID 5971, ALLYL ISOTHIOCYANATE).

1.7 ESSENTIAL OILS IN SILAGE

Essential oils are already widely used in animal nutrition, with positive results (Lee et al., 2003; Patra et al., 2012; Ornaghi et al., 2017). In the case of silages, studies are scarce, although we know that fermentations in the silo and in the rumen are dependent on microbial activity, which can be affected by the use of essential oils. Most studies related to the antimicrobial mode of action of essential oils are mainly focused on bacteria, while little is known about their mode of action on filamentous fungi and yeasts (Lambert et al., 2001; Bakkali et al., 2006; Hyldgaard et al., 2012; Calo et al., 2015).

Yeast species that are normally associated with aerobic spoilage in silages have been inhibited by some essential oils. For example, carvacrol inhibited the growth of *Saccharomyces cerevisiae* (Knowles & Roller, 2001), and thymol inhibited the growth of *Debaryomyces hansenii* (Curtis et al., 1996). Cinnamaldehyde was responsible for reducing the growth of *Aspergillus niger* and *Aspergillus flavus* in post-harvest fruits and vegetables (Mousavian et al., 2018). Eugenol is also responsible for controlling fungi during storage in oat seeds, and reduced the growth of *Aspergillus flavus* (Kumar et al., 2020). Allyl isothiocyanate was also responsible for reducing the growth of aflatoxin b1 produced by *Aspergillus flavus* during corn seed storage (Nazareth et al., 2020).

Juglal et al. (2002) observed antifungal activity of the essential oils eugenol, thymol, carvacrol, cinnamaldehyde and myristin, against the fungi *Aspergillus parasiticus* and *Fusarium moniliforme*, producers of mycotoxins. Ahmad et al. (2011) also observed a fungicidal effect of thymol and carvacrol against *Candida albicans, Candida krusei, Candida tropicalis* and *Candida glabrata.*

Thymol has also been shown to be effective in controlling *D. hansenii* (Curtis et al., 1996) and *Pichia* and *Candida* species (Falcone et al., 2005). These yeasts have been identified as initiators of aerobic deterioration in silages (Pahlow et al., 2003). Ávila et al. (2010) demonstrated that in sugarcane silages, the predominant yeasts comprise *Torulaspora delbrueckii*, *Pichia anomala* and *Saccharomyces cerevisiae*.

2.2.1. Quality of silages with essential oils

Kung Jr. et al. (2008) evaluated the effect of a blend of essential oils containing thymol, eugenol, vanillin and limonene, in concentrations of 40 or 80 mg/kg of fresh forage, on the fermentation and aerobic stability of corn silages, not observing any effect of the essential oils compared to the control treatment. However, Chaves et al. (2012), evaluating the effect of essential oils (eugenol, thymol and carvacrol or limonene) on the fermentation and aerobic stability of barley

silage, observed that at concentrations of 120 mg/kg of dry matter (400 mg/kg of fresh forage) reduced the yeast population in relation to the control treatment, during the stability tests.

In ryegrass silage, the addition of thymol and eugenol at the highest dose (2000 mg/kg of fresh forage) inhibited deamination, an effect observed with carvacrol at a dose of 500 mg/kg, which from the point of view of preserving nutrients is interesting in the silage process. The authors report that plant enzymes are responsible for part of the protein degradation during ensiling, indicating that the possible action of essential oils would be the inhibition of the activity of these enzymes (Foskolos et al., 2016).

In barley silage, the inclusion of essential oils (eugenol, thymol and carvacrol or limonene) at concentrations of 37.5, 75 and 120 mg/kg in dry matter, decreased yeast growth during exposure of silage to air for up to 7 days (Chaves et al., 2012). On the other hand, in corn silages, no effects were observed on aerobic stability and on the process of fermentation using a mixture of essential oils containing thymol, eugenol, vanillin and limonene at concentrations of 40 and 80 mg of active ingredient per kilogram of fresh forage. However, the authors observed that there was no resistance to lowering the pH, which reached 3.68, demonstrating that the essential oils did not affect the development of lactic acid bacteria, and the consequent conversion of soluble carbohydrates into lactic acid (Kung Jr. et al., 2008).

In legume silage (forage pea), the inclusion of essential oils as additive resulted in an increase in crude protein and dry matter contents, and a decreased prduction of CO_2, in addition to inhibiting mold formation during the period of exposure to air in silages treated with 400 mg/kg of fresh forage of cinnamon extracts (41.5 g of cinnamaldehyde propylene glycol and 35.28 g of cinnamaldehyde, in 100 g of extract). The treatment containing 400 mg/kg of fresh forage of oregano extract (59 g of carvacrol and 12 g of thymol, in 100 g of extract) did not have a similar effect, but with the combination of the two essential oils, at the same dosage of 400 mg, there was a synergistic effect on acetic acid, and antagonistic during aerobic exposure, dry matter losses and mold formation (Soycan-Önenç et al., 2015).

The essential oil compounds thymol, carvacrol and their combination proved to be potential additives in sugarcane and corn silages in a study conducted in Brazil (Pereira, 2016), at doses of 600, 400 and 500 mg/kg of fresh forage, respectively, reducing dry matter losses and increasing the aerobic stability of the silages. More recently, an experiment was carried out using essential oils asadditives in alfalfa silages (caraway essential oil at a dose of 300 and 500 mg/kg of fresh forage). The silages treated with essential oils had the lowest pH, even in the treatment with the lowest dose of OE this variable was 4.77 and the control 4.88. These data still corroborate with the levels of lactic acid found in treated (44.57 DM) versus control group (34.82 g/kg DM). Anothereffect observed was ammonia concentration. Even at the lowest dose it was 50% lower than the control, demonstrating the ability

of essential oils to reduce protein deamination. Regarding pH, after seven days of aerobic exposure test, cumin to silage with 500 mg/kg additive, kept the lower values 7.04 versus 8.79 control. The lowest dose of EO obtained a lower count than the control of yeast (3.43 *vs.* 4.86 CFU/g), demonstrating that cumin EO under the conditions of the experiment was efficient in preserving silage quality even after seven days of exposure to air (Turan and Soycan-Önenç, 2018).

In total, 11 articles with the theme essential oils as additives in silage, it is possible to observe in Table 2-1, the main results of this type of additives when added in the fermentation process of corn silages, barley, sugar cane, ryegrass, alfalfa and pea, in addition to the diversification of doses and types of compounds used. Whether by choosing from plants or using the chemical compound with greater purity. From this it is difficult to estimate doses and make recommendations. Based on this premise, this work aims to evaluate the use of five compounds, thymol, carvacrol, cinnamaldehyde, eugenol and allyl isothiocyanate with a high degree of purity (more than 95%) with different doses in the fermentation process and aerobic stability of WCGF, in addition to evaluated of the purity (95%) AITC of the action o as an additive in whole-plant corn silage.

TABLE 0-1 - ARTICLES REGARDING ESSENTIAL OILS AS ADDITIVE IN FEED CONSERVATION SUCH AS SILAGE.

Articles	Essential oils source	Forage Source	Results
Kung et al. (2008)	Commercial blend 40 mg/kg FF* Commercial blend 80 mg/kg FF (40%) Bioactive compounds.	Corn silage	No effect on yeast, molds, enterobacteria acid lactic bacteria; No effect on DM recovery, fermentation, end products, nutrient content, aerobic stability;
Chaves et al. (2012)	Cinnamon 125, 250 and 400 mg/kg FF Oregano 125, 250 and 400 mg/kg FF Sweet orange 125, 250 and 400 mg/kg FF	Barley silage	Control of yeast and molds growth cinnamon and oregano (400 mg/kg FF);
Soycan-Onenç et al. (2015)	Origanum onites 400 mg/kg FF Cinnamon 400 mg/kg FF Origanum + Cinnamon 200 + 200 mg/kg FF	Field Pea Silage	Cinnamon decreased acetic acid, ammonia nitrogen and weight loss. Crude protein and dry matter (DM) increased by cinnamon essential oil; Not affect yeast growth;
Montazeri et al. (2016)	Cinnamon 120, 240 mg/kg DM Thymol 120, 240 mg/kg DM Mint 120, 240 mg/kg DM Oregano 120, 240 mg/kg DM Cumin120, 240 mg/kg DM	Corn silage	Mint 240 mg/kg/DM, increase deamination on silage protein Improve the aerobic stability; Oregano 240 mg/kg/DM promote fermentation efficiency and reduction of gas production and increasing in vitro DM degradability;
Foskolos et al. (2016)	Thymol 50, 500, 2000 mg/kg FF Eugenol 50, 500, 2000 mg/kg FF Cinnamaldehyde 50, 500, 2000 mg/kg FF Chemical compounds (99.5% Sigma Aldrich)	Ryegrass silage	Thymol, Eugenol and cinnamaldehyde 2000mg/kg/ FF inhibited deamination; Cinnamaldehyde 2000mg/kg/FF improve true protein; Increase pH value and decrease LAB growth and lactic acid concentration.
Pereira (2016)	Thymol 600 mg/kg FF Carvacrol 400 mg/kg FF Thymol + Carvacrol 500 mg/kg FF Chemical compounds (99%, Grasp company)	Sugarcane silage	Preserved the content of soluble carbohydrates; Reduces dry matter losses; Increases aerobic stability in sugarcane silage.

Pereira (2016)	Thymol 600 mg/kg FF Carvacrol400 mg/kg FF Thymol + Carvacrol 500 mg/kg FF	Chemical compounds (99%, Grasp company)

		Alfalfa silage	Decrease gas production; Increase protein content.
Nasab et al. (2018)	Ziziphora clinopodioides 10, 20 and 30 mL/kg FF Mentha pulegium 10, 20 and 30 mL/kg FF		
Turan and Onenç (2018)	Cummin 300 mg/kg FF Cummin 400 mg/kg FF	Alfalfa silage	Cummin 300 mg/kg provide cell wall fractionation and increase LAB; Aerobic Stability 72 hours; Decrease of deamination.
Çayiroglu et al. (2020)	Oregano 10 mL/75 cm^2 Oregano 20 mL/75 cm^2	Sugar beet pulp silage	Decrease LAB, Total live bacteria and yeast formation. Stopped mold growth up 72 hours a
Besharat et al. (2020)	Cinnamon 60 mg/kg FF Lemon seeds 60 mg/kg FF Flaxseeds 60 mg/kg FF Cinnamon+ Lemon seed+Flaxseeds) 180 mg/kg FF	Alfalfa silage	Reduced silage pH; Increase the crude protein contents; Gas production decrease in silage treated with lemon seed and flaxseed essential oils; Increased the aerobic stability of the Silage.
Cantóia-Junior et al. (2020)	Lemongrass 1 mL/kg FF Lemongrass 2 mL/kg FF Lemongrass 3 mL/kg FF	Sugarcane silage	Improve DM, organic matter, DM recovery and chemical composition; Decrease yeast and mold count; Increase the aerobic stability; Reduce ethanol concentrations.

Fresh forage – FF; Lactic acid bacteria – LAB; Dry matter – DM;

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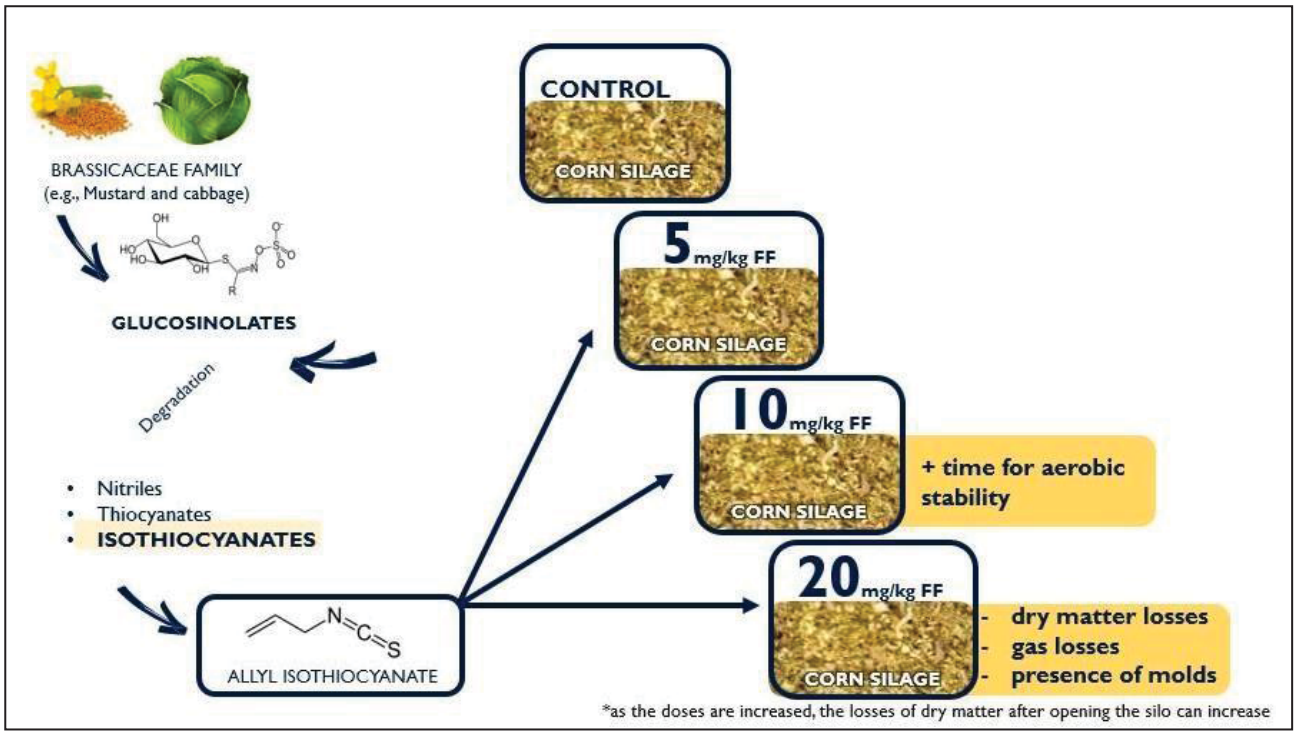
2 CHAPTER 2 – INFLUENCE OF ALLYL ISOTHIOCYANATE ON THE AEROBIC STABILITY AND THE FERMENTATIVE LOSSES OF WHOLE-PLANT CORN SILAGE

Highlights

- Allyl isothiocyanate is one of the most studied essential oils.
- The effect of allyl isothiocyanate was evaluated at ensiling of whole plant corn silage.
- The dose 20 mg/kg FF decrease gas losses and total dry matter losses.

Graphical abstract



ABSTRACT

Undesirable microorganisms can proliferate during ensiling, storage, and aerobic phase of silage, reducing nutritional quality and increasing fermentative losses. Based on this, we hypothesized that allyl isothiocyanate (AITC) can play an important role in mitigating the growth of microorganisms that cause spoilage in whole-plant corn silage. Therefore, the objective of this study was to determine the effect of AITC inclusion on the fermentative losses, microbiology counts, and aerobic stability of whole-plant corn silage. Four AITC levels were tested in a completely randomized block design: 0, 5, 10, and 20 mg/kg of fresh forage of whole-plant corn, with five replicates per treatment, totaling 20 experimental units. AITC was applied and mixed with the material at ensiling. Each experimental unit consisted of one 8-L plastic bucket with an average density of 468 kg/m^3 of fresh forage. The silos were opened 90 days after ensiling. Samples were collected to determine microbiology analysis, DM concentration, and aerobic stability. Data were analyzed using a MIXED model procedure of SAS with significance declared at $p \leq 0.05$ and the tendency at $p \leq 0.10$. The gas production, total dry matter (DM) losses, and mold population decreased linearly ($p < 0.01$; 0.01; 0.03, respectively) with the AITC inclusion levels. Although aerobic stability linearly increased ($p = 0.02$), DM losses showed a quadratic increase ($p = 0.02$) with the AITC levels during aerobic deterioration. Furthermore, pH and heterolactic count tended to linearly decrease ($p= 0.06$ for both), whereas DM concentration and yeast count tended to linearly increase ($p = 0.09$; 0.08, respectively) with AITC levels. However, the AITC inclusion levels did not affect the effluent production and homolactic count. In conclusion, the inclusion of AITC at ensiling affected the fermentative losses, microbial population, and aerobic stability of whole-plant corn silage at doses 10 and 20 mg/kg FF.

Key-words: Essential oil.feed conservation Microbiology.

3 INTRODUCTION

Allyl isothiocyanate (AITC, chemical formula C_4H_5NS) is part of the composition of plants of the *Brassicaceae* family, such as: brown and black mustard seeds, cabbage, and wasabi. This compound is considered an essential oil, and has high antioxidant and antimicrobial potential, being responsible for protecting plants from pathogens (Fallal et al., 2017). The AITC has a purging odor, is part of the organosulfur family, and has been used as an additive in foods due to its ability to control the growth of various microorganisms (e.g., *Escherichia c*oli O157:H7, *Salmonella montevideo*, *Salmonella typhimurium* and *Listeria monocytogenes)* (DELAQUIS and SHOLBERG, 1997; LIN et al., 2000a,b; LUCIANO and HOLLEY, 2009).

AITC antifungal potential has been evaluated in fermented foods, e.g., sauerkraut and kimchi (XIONG et al., 2012; KO et al., 2012). The process of producing this type of food for humans is similar to silage production, since both of them are fermented by lactic acid bacteria. During anaerobic activity, the lactic acid bacteria use the soluble carbohydrates present in the plants or vegetables and convert them into organic acids, that reduce pH, preventing the growth of undesirable microorganisms (MC DONALD et al., 1999; PAHLOW et al., 2003). This drop in pH during fermentation is responsible for food preservation.

A challenging moment for silage process is when the food is exposed to air. In this way, several additives are used to control the growth of microorganisms that cause spoilage, such as yeasts. To control adverse effects, chemical agents, such as propionic acid (QUEIROZ et al., 2013), are used, either to improve the fermentation process or the post-opening of the silage, increasing aerobic stability and controlling the growth of yeasts and filamentous fungi. In corn silage it is possible to find the same microorganisms that are found in sauerkraut and kimchi, as *Lactococcus mesenteroides, Lactobacilllus plantarum, Lactobacillus brevis*, *Enterococcus faecalis*, *Pediococcus cerevisiae*, *Debaryomyces hansenii* and *Pichia fermentans (*PLENGVIDHYA et al., 2007). For this reason, the same additives that are used in food for humans have been gaining ground within animal nutrition, such as organic acids, essential oils, natamycin, and microbial inoculants (KUNG et al. 2008; ARRIOLA et al., 2011; CANTÓIA-JUNIOR et al., 2020; JIANG et al., 2020; PINTO et al., 2020).

The first author to utilize essential oils as an additive in silage was Kung et al. (2008). After that, other authors observed the positive effects of essential oils on rumen modulation, mainly on methane reduction (CALSAMIGLIA et al., 2007; BELANCHE et al., 2020).

Regardless of where fermentation takes place, whether inside the silo or in the rumen, it is dependent on the action of microorganisms.

The use of additives aims to favor the growth of lactic acid bacteria and inhibit the growth of yeasts, molds, *Clostridium* or *Listeria*. Based on this, we hypothesized that due to the high microbiological potential of the compound allyl isothiocyanate (AITC), it can control yeast growth, influencing the aerobic stability of corn silage. Therefore, our study aimed to evaluate the effect of different doses of AITC as an additive in whole-plant corn silage.

4 MATERIAL AND METHODS

4.1 ENSILING

The experiment was conducted at the Center for Research in Forage (acronym in Portuguese CPFOR- (25º32'05"S, 49º12'23"W, 906 m altitude, Köpen-Geiger climate type Cfb) at the Federal University of Paraná. The corn (Pioneer, Corteva Agriscience™, Wilmington, USA) was planted in October 2019, and harvested in March 2020, with 37% dry matter (DM). The corn plant was chopped in a stationary forage chopper (Super 15 T, Menta Ltda., Cajuru, SP, Brazil) with an average particle size of 10 mm. The forage was homogenized, sampled, and separated into four piles to add the different levels of AITC to compose the treatments. AITC was not diluted before application. To allow the exact calculation of the dose, 100 kg of chopped fresh forage was weighed for each treatment, i.e., four piles of 100 kg of forage. From each pile, an amount of 2 kg of forage was separated, and the dose corresponding to the treatment was applied. After that, the 2 kg with the treatment was homogenized to the original pile of 98 kg. For the control treatment, only homogenization was performed without any additive or water. The treatments were: Control (CON) - whole-plant corn silage without addition of AITC; Dose 5 (D5) - corn silage with AITC, 5 mg/kg of fresh forage; Dose 10 (D10) - corn silage with AITC, 10 mg/kg of fresh forage; Dose 20 (D20) - corn silage with AITC, 20 mg/kg of fresh forage.

For all treatments, except the CON, the additive was applied to the forage and homogenized, and subsequently compacted in experimental silos (8-L plastic buckets), with an average density of 468 kg/m^3 of fresh forage. Posteriorly, the silos remained closed for 90 days at room temperature (26°C ± 2). Fermentative losses were calculated by gravimetric difference following the recommendations of JOBIM et al. (2007). After opening the silos, the content of

each replication was homogenized, and 300 g of sample for each replicate was collected to determine pH, chemical composition, and microbiological profile.

4.1.1 Chemical analysis

For chemical analysis, 200 g of each replicate were dried in a forced-air ventilation oven (55ºC) and ground in a Willey mill (Model #2, Arthur H. Thomas Co., Philadelphia, PA) with a 1 mm mesh sieve and stored for posterior analysis. Subsequently, 2 g of the samples were used to determine the DM content at 105°C (method number 934.01; AOAC, 1990), ash (method number 924.05; AOAC, 2012), crude protein (CP) by the Dumas method (FP-528, Leco, combustion N analyzer, Leco Instruments Inc., St. Joseph, MI), according to Wiles etal. (1998). Neutral detergent fiber (aNDF) with thermostable alpha-amylase and sodium sulphite (MERTENS, 2002), and acid detergent fiber (ADF) (VAN SOEST, 1973), were sequentially determined using an Ankom A200 Fiber Analyzer (ANKOM Technology, Macedon, NY). The concentration of lignin was determined using the acid detergent lignin methodology, according to VAN SOEST and WINE (1968), by submitting the material to sulfuric acid (72/28, wt/wt, in deionized water) sequentially following ADF analysis. The cellulose concentration was obtained via the difference between ADF and lignin, whereas hemicellulose concentrationwas obtained via the difference between NDF and ADF. N-NDF was evaluated according to GOERING (1970) and AOAC (1975). Ammonia nitrogen content was determined by colorimetric method, according to EEATHERBURN (1967).

The pH was determined using a digital pHmeter (PG 1400, Gehaka – Brazil) according to KUNG et al. (2000), where 25 g of samples were diluted in 225 mL of distilled water and homogenized for one minute, and the extract was used to the measurement, and another sample of fresh forage was collected before ensiling and was also analyzed for DM, CP, aNDF, ADF, and ash as described above, and the values are presented in Table 5-1.

TABLE 4-1 - CHEMICAL COMPOSITION OF FRESH FORAGE AT THE TIME OF ENSILING.

Variables	Treatments				SD[3]
	CON	D5	D10	D20	
pH	6.3	6.3	6.2	6.4	0.081
DM[2], %	37.7	37.9	38	37	0.513
aNDF, % DM	48.5	50.4	48.4	48.1	1.047
FDA, % DM	26.7	28.6	24.8	26.2	1.571
CP, % DM	6.6	6.2	6.5	6.7	0.216
Ash,% DM	2.8	2,6	2.5	2.6	0.125
N-NDF, % DM	28.1	27.1	27.8	32.1	2.255
Celullose, % DM	23.2	24	21.2	22	1.243
Lignin, % DM	3.7	5,3	3,8	4.3	0.732
Crude fiber, % DM	23.2	23.3	20.8	21.5	1.246
LAB, log CFU/g FF	1.96	1.96	1.96	1.96	0
Yeasts, log CFU/g FF	1.61	1.61	1.61	1.61	0
Molds, log CFU/g FF	1.04	1.04	1.04	1.04	0

Treatment: CON = Control, without additive, D5 = 5 mg of AITC/kg fresh forage, D10 = 10 mg of AITC/kg fresh forage; D20 = 20 mg of AITC/kg fresh forage; [2]DM= dry matter, aNDF = neutral detergent fiber assayed using amylase, ADF= acid detergent fiber, Nitrogen fixed to neutral detergent fiber assayed - N-NDF; CP= crude protein; LAB = lactic acid bacteria; [3]SD = Standard deviation.

4.1.2 Microbiological analysis

Microbial counts were performed in aqueous extracts. The extracts were prepared in sterile plastic bags containing 25 g of sample and 225 mL of saline solution (76.40% NaCl, 17.82% KCl, 4.07% $CaCl_2$, 1.69% $NaCO_3$) homogenized for four minutes in a sample homogenizer (Marconi-MA 440/CF, São Paulo, Brazil) and filtered with four layers of cheesecloth. Ten-fold dilutions in saline solution were prepared and plated in 3M™ Petrifilm™ The count of lactic acid bacteria (LAB) was done in Petrifilm™ Lactic Acid Bacteria Count Plate AC), after incubation for 48 h at 32ºC in anaerobic jars. Yeasts and molds were enumerated in Petrifilm™ YM after incubation at 23ºC for 72 h and 120 h, respectively. Microbial counts were expressed as log CFU/g of silage.

4.1.3 Aerobic stability and pH curve

For aerobic stability tests, 2 kg of silage were placed in 20-L plastic buckets (experimental silos) without compaction, and the material remained for 10 days in a temperature-controlled room at 25°C. A thermal sensor (EL-USB-1, Lascar Electronics Inc., Erie, PA, USA) was inserted into each bucket, and the temperature recording was performed every 5 minutes during the 240 hours of exposure to air. Therefore, stability break was determined when the silage reached 2°C above ambient temperature, according to Moran etal. (1999). On day 0 and at the end of 10th day (240 hours), the buckets were weighed to determine

dry matter losses during exposure to air. Furthermore, the pH curve was performed during the aerobic stability test. For this, 1 kg of silage was allocated in 8–L buckets correspondent to each replication per treatment, 25 g of samples for each replicate were collected every two days, and the pH was measured in a digital potentiometer (PG 1400, Gehaka – Brazil), according to KUNG et al. (2000).

4.1.4 Volatile compounds analysis

The concentration of volatile fatty acids (VFA), glucose, xylose, lactic acid, acetic acid, and ethanol were obtained using high-performance liquid chromatography (HPLC; model LC-20A, Shimadzu, Kyoto, Japan). The HPLC columns used in the analysis included Rezex RHM 300 x 7.8 (Phenomenex, Torrance, CA, USA) and the analytical parameters recommended by the manufacturer (Mobile Phase: H2SO4 5.0 mmol L-1; Flow Rate: 0.6 m/min; Column Temperature: 65°C).

4.2 STATISTICAL ANALYSIS

The experimental design consisted of a completely randomized, with four treatments (AITC levels: CON, D5, D10, and D20) and 5 replicates (experimental silos), totaling 20 experimental units. Proc IML was used to determine the coefficients. The linear and quadratic effects were tested for the essential oil application rates using orthogonal polynomial contrasts. Data were analyzed using a MIXED procedure of SAS (9.4; 2016) and considered $p \leq 0.05$ as a significant difference and $p \leq 0.10$ as a tendency, following the model.

$$Y_{ij} = \mu + E_{ij} + e_{ij}$$

Where: Y_{ij} is the dependent variable; μ =overall mean; E_{ii} is the fixed effect of AITC levels (i = 0 to 20 mg of AITC/kg of fresh forage); e_{ij} = residue (j = 1to 4).

5 RESULTS

The results of fermentative losses, chemical analysis, microbial analysis, volatile organic compounds, and aerobic stability are shown in Tables 6-1, 6-2, 6-3, 6-4 and 6-5.

5.1 FERMENTATIVE LOSSES

The results of fermentative losses and pH are shown in Table 6-1. The pH value tended to linearly increase ($p = 0.06$). Dry matter and effluent losses were not affected by treatments, and the average was 38.87% and 6.03%, respectively. However, gas losses (GLoss) and total dry matter losses (TDML) decreased linearly ($p < 0.05$) with AITC inclusion.

TABLE 5-1 - VALUES OF PH, AND FERMENTATIVE LOSSES IN WHOLE-PLANT CORN SILAGES ADDED WITH ALLYL ISOTHIOCYANATE.

Variables	Treatment				SEM	*p*-value	
	CON	D5	D10	D20		Linear	Quadratic
pH	3.74	3.85	3.79	3.84	0.026	0.062	0.339
Dry Matter, %	38.38	38.84	38.87	39.41	0.388	0.088	0.892
Effluent losses, kg/ton FF	5.53	6.62	5.95	6.02	1.810	0.931	0.797
Gas losses, %	4.81	4.07	4.02	0.05	0.996	0.002	0.256
Total Dry Matter losses, %	5.34	4.71	3.24	1.03	1.138	0.010	0.87

CON = Control, without additives; D5 = 5 mg/kg fresh forage; D10 = 10 mg/kg fresh forage; D20 = 20 mg/kg fresh forage (FF).

5.1.1 Chemical analysis

The chemical composition after fermentation is presented in Table 6-2. The value of N-NDF decreased linearly ($p < 0.01$) with AITC inclusion. The ADF values presented quadratic tendency ($p = 0.10$). Crude protein (CP) and NH3-N increased linearly with AITC inclusion ($p < 0.02$; < 0.04, respectively). The values of aNDF, ash, cellulose and lignin were not affected ($p = 0.13$) by treatments, and the averages were 45.73%; 3.33%; 24.15%; and 3.90%, respectively.

TABLE 5-2 - CHEMICAL COMPOSITION OF WHOLE-PLANT CORN SILAGES ADDED WITH ALLYL ISOTHIOCYANATE.

Variables	Treatment				*p*-value	
	CON	D5	D10	D20	SEM	Linear	Quadratic
N-NDF, % DM	16.04	16.20	13.36	9.14	0.962	$p < 0.01$	0.26
aNDF, % DM	45.04	46.50	45.44	45.94	0.555	0.53	0.51
ADF, % DM	27.08	29.32	27.34	27.12	0.493	0.31	0.09
CP, % DM	6.58	6.66	6.66	6.92	0.097	0.02	0.54
Ash,% DM	3.30	3.40	3.22	3.40	0.165	0.79	0.72
N-NH3 % FF	9.84	8.44	8.84	8.04	0.496	0.04	0.46
Crude fiber, % DM	23.82	25.18	24.34	23.86	0.465	0.56	0.12
Cellulose, % DM	23.38	25.54	23.72	23.96	0.432	0.85	0.13
Lignin, % DM	3.90	4.16	3.66	3.88	0.177	0.57	0.72

CON = Control, without additives; D5 = 5 mg/kg fresh forage; D10 = 10 mg/kg fresh forage; D20= 20 mg/kg fresh forage.[2] Nitrogen fixed on wall of NDF - N-NDF; aNDF - neutral detergent fiber assayed using amylase; ADF - acid detergent fiber; CP – Crude protein; NH3- N - Ammoniacal nitrogen; Standard error of means – SEM; Variables with $p < 0.05$ had significant influence of treatments;

5.2 MICROBIOLOGICAL ANALYSIS

The values of heterofermentative lactic acid bacteria (LAB), homofermentative lactic acid bacteria, yeasts, and molds are shown in Table 6-3. Heterofermentative LAB, homofermentative LAB, and yeasts counts were not influenced by the inclusion of AITC (p = 0.59; 0.91; 0.61; 0.92, respectively), and the mean value corresponded to 5.75, 2.55 and 5.04 log CFU/g, respectively. Meantime the values of molds were influenced linearly ($p < 0.03$) by AITC inclusion, where the D10 presented 2 times more colonies than D20.

TABLE 5-3 - MICROBIAL POPULATION (LOG CFU/G) IN WHOLE-PLANT CORN SILAGES ADDED WITH ALLYL ISOTHIOCYANATE.

Variables	Treatment				SEM	*p*-value	
	CON	D5	D10	D20		Linear	Quadratic
Heterofermentative LAB	6.27	4.77	6.21	5.78	0.613	0.99	0.59
Homofermentative LAB	4.20	3.01	4.20	2.80	1.744	0.66	0.91
Yeasts	4.80	5.11	4.86	5.42	0.214	0.08	0.61
Molds	2.40	2.72	0.86	ND	0.859	0.03	0.92

CON = Control, without additives; D5 = 5 mg/kg fresh forage; D10 = 10 mg/kg fresh forage; D20 = 20 mg/kg fresh forage; Standard error of means – SEM; Variables with $p < 0.05$ had significant influence of treatments;

5.3 VOLATILE ORGANIC COMPOUNDS

The volatile organic compounds are shown in Table 6-4. Glucose and ethanol were not influenced by AITC inclusion ($p < 0.09$) with mean values of 0.82% and 0.41% of dry matter, respectively. Xylose composition was affected linearly by treatments ($p < 0.02$). In the

silages with additives the average was 0.56% *vs.* 0.47% in the CON treatment. Lactic acid was affected linearly by AITC inclusion ($p < 0.01$), with an average of 5.44% DM. Meantime the acetic acid concentration was linearly affected ($p < 0.04$) by treatments. The D20 (0.93%) was 13.4% higher than CON (0.82%), without additives.

TABLE 5-4 - VOLATILE ORGANIC COMPOUNDS OF CORN SILAGES ADDED WITH ALLYL ISOTHIOCYANATE.

Variables	Treatment[1]				SEM	*p*-value	
	CON	D5	D10	D20		Linear	Quadratic
Glucose, % DM	0.79	0.83	0.84	0.82	0.041	0.64	0.44
Xylose, % DM	0.47	0.50	0.52	0.56	0.249	0.02	0.69
Lactic acid, % DM	4.83	5.23	5.32	5.56	0.153	<0.01	0.35
Acetic acid, % DM	0.82	0.86	0.86	0.93	0.033	0.04	0.98
Ethanol, % DM	0.35	0.46	0.43	0.38	0.043	0.93	0.09

CON = Control without additives; D5 = 5 mg/kg fresh forage; D10= 10 mg/kg fresh forage; D20 = 20 mg/kg fresh forage.; Standard error of means – SEM; Variables with $p < 0.05$ had significant influence of treatments;

5.4 AEROBIC STABILITY AND PH CURVE

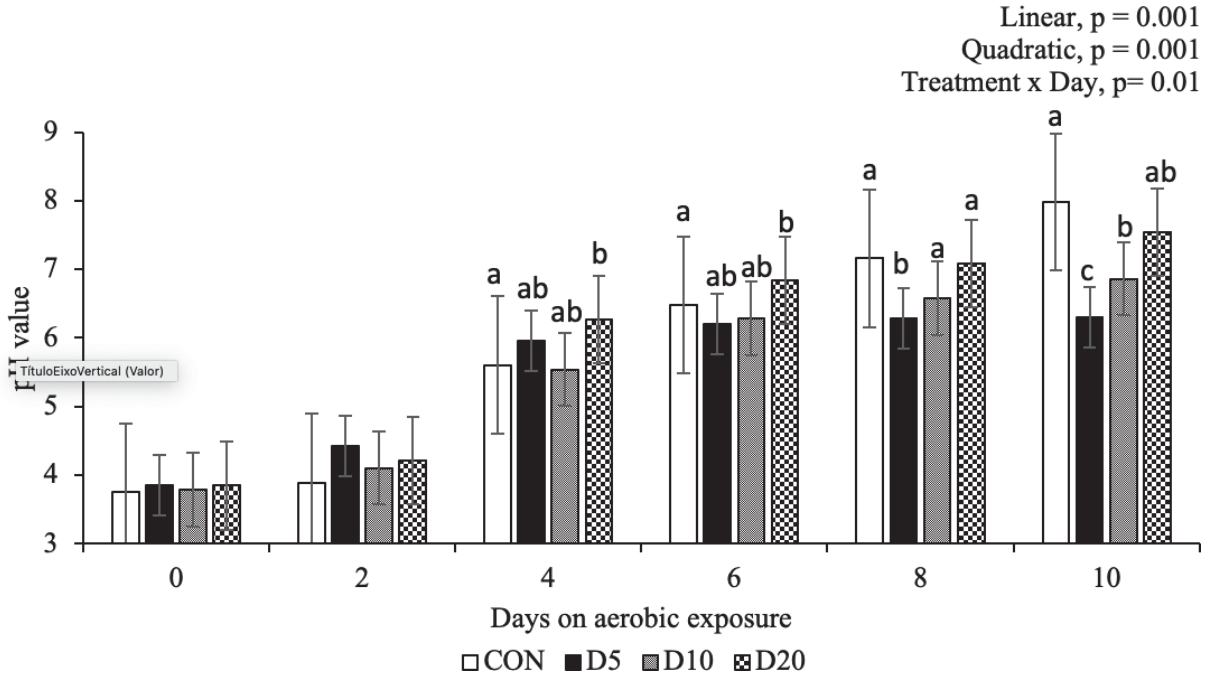
Aerobic stability is shown in Table 6-5, and pH values are presented in Figure 6-1. The aerobic stability (AS) increased linearly ($p < 0.02$), where the D20 presented 7.5 hours more stability compared to the average of CON, D10 and D20 treatments (53.0 vs. 45.6 hours, respectively). Dry matter losses after aerobic exposure (DML_{AS}) increased linearly and quadratic ($p < 0.024$) with AITC inclusion. The average of treatments D5, D10, and D20 was 15.6 % and for the CON, 9.77%, therefore the CON treatment presented 37.4% less dry matter losses after aerobic exposure. The maximum temperature, hours to maximum temperature, and accumulated temperature were not ($p = 0.29$) affected by treatments, and the averages corresponded to 39.2°C; 53.8 °C, and 919.2 °C, respectively.

TABLE 5-5 - AEROBIC STABILITY OF WHOLE-PLANT CORN SILAGES ADDED WITH ALLYL ISOTHIOCYANATE.

Variables	Treatment[1]				SEM	*p*- value	
	CON	D5	D10	D20		Linear	Quadratic
AS[2], h	45.9	47.0	49.8	53.0	2.180	0.02	0.98
DML_{AS}[3], %	9.77	16.7	14.7	15.4	1.188	0.02	0.01
T_{MAX}, ºC	40.4	40.1	37.3	39.1	1.466	0.43	0.29
HT_{MAX}, h	53.7	52.8	56.0	52.8	2.661	0.92	0.56
T_{ACU}, ºC	479.1	2395.5	534.2	267.9	1988.07	0.60	0.63

CON = Control, without additives; D5 = 5 mg/kg fresh forage; D10 = 10 mg/kg fresh forage; D20 = 20 mg/kg fresh forage; [2]AS = Aerobic Stability; [3] DML_{AS} = DM losses during aerobic stability; T_{MAX} = Maximum Temperature; HT_{MAX} = hours to reach maximum temperature; T_{ACU} = accumulated temperature; Standarderror of means – SEM; Variables with $p < 0.05$ had significant influence of treatments;

FIGURE 8 - PH CURVE OF CORN SILAGES ADDED WITH ALLYL ISOTHIOCYANATE DURING AEROBIC EXPOSURE.



CON = control, without additives; D5 = 5 mg/kg fresh forage; D10 = 10 mg/kg fresh forage; d20= 20 mg/kg fresh forage

6 DISCUSSION

In general, allyl isothiocyanate (AITC) promoted positive results during the fermentation of whole-plant corn silage. The dose 20 mg/kg FF decreased gas losses and total dry matter losses and AITC inclusion linearly decrease the N-NDF and N-NH_3, in addition to linearly increasing aerobic stability.

Fermentative losses

We hypothesized that AITC, as an additive in whole plant corn silage, can improve the quality and decrease dry matter losses. This compound has been studied as food preservatives (barley seeds, maize grains) as described by Nazareth et al. (2019) and Quiles et al. (2019), and in food fermentation, for example in sauerkraut and kimchi (Jo et al., 2012; Banerjee et al.,

2015), and to control the growth of yeasts, *E. coli*., *L. monocytogenes*, *Salmonella* and others (Jo et al., 2012). These microorganisms promote the deterioration of food and cause diseases in humans and animals (Sonnier et al., 2018; Driehius et al., 2018; Marshall et al., 2020). In food conservation, additives are utilized to minimize and improve the quality of feed. Therefore, in silage conservation these same organisms are known for promoting fermentative losses, reducing aerobic stability, and reducing DM intake by animals (QUEIROZ et al., 2018).

The present study is the first evaluating four doses of allyl isothiocyanate (AITC) as an additive in whole-plant corn silage. A positive effect on the fermentation, chemical composition, and aerobic stability, was observed in the treatment with the smallest dose (5 mg/kg fresh forage). The pH values followed a similar pattern observed by Kung et al. (2018), around 3.7 until 4.0, in whole plant corn silage with 30-40% DM. The pH is a fermentative indicator (KUNG et al., 2018). Basically, the acid lactic bacteria convert soluble carbohydrates in other compounds, such as lactic acid (WANG et al., 2021). Lactic acid is the most efficient compound to preserve food during fermentation, reducing the DM losses (WEINBERG et al., 1993). The AITC presented a tendency to increase ($p < 0.01$) the pH. The same pattern was observed by Jo et al. (2012) with AITC inclusion (0.05, 0.1, 0.15 and 0.2% w/w) as an additive in kimchi production. JO et al. (2001) observed that the control treatment, without theaddition of AITC, had a lower pH, 5.7 *vs.* 3.3, but when the doses increased from 0.05 to 0.2% w/w, there was a reduction in the pH values, 5.7 *vs*. 4.8. FOSKOLOS et al. (2016) and CANTÓIA-JUNIOR et al. (2020), when including essential oils in the fermentation process of ryegrass also observed an increase in pH (carvacol 5.77 to 6.57; thymol 5.60 to 6.48) and sugarcane (Lemon grass 3.89 to 4.03), respectively.

During the fermentation process of silage, dry matter losses occur. The total dry matter losses are considered as the sum of effluent losses + gas losses. The gas losses in silos are primarily from carbon dioxide production, in addition to other gases such as (nitrogen, oxygen and methane). These losses typically are in the range of 2 to 4% (ZIMMER, 1980). These gas losses in the form of carbon dioxide are related to different pathways due to the fermentation of LAB glucose heterofermentatively, where 1 mol of carbon dioxide is produced per mol of glucose, to 24% DM losses. If a LAB species ferments citrate or malate, carbon dioxide is produced, with DM losses, regardless of whether the LAB strain is homo or heterofermentative when fermenting glucose. (BORREANI et al., 2018). Given this fact, with the linear reduction of gas losses with increasing AITC dose, we can suggest that there was a selection of lactic acid bacteria during fermentation. Whether favoring homolactic or selecting the type of heterolactic, such as enterobacteria that use two glucose molecules producing 2 lactate, 1 acetate, 1 ethanol and 2 CO2. This pathway would result in 17% of DM losses and 11.1% gross energy, different from the heterolactic LAB that use a glucose and produce 1 lactate, 1 ethanol, 1 CO2, leading to 24 % of DM losses and 1.7 of gross energy (BORREANI et al., 2018). TAKAHASHI et al.

(2021), using different culture media containing AITC at 2000 mg/L to control microorganisms, observed that the counts of *Enterococus faecalis*, *L. brevis* and *L. plantarum* were 3.7, 3.5, and 2.5 log CFU/mL after 7 days, respectively. The bacterial count on day 14 of *E. faecalis* was 2.5 log CFU/mL, and *L. plantarum* and *L. brevis* were below the detection limit. Due to this action in selecting lactobacillus strains, the effects on gas losses can be observed, reflecting the total losses of dry matter.

Chemical analysis

The AITC can reduce losses in protein concentration, by reducing ammoniacal nitrogen losses, which was also observed by FOSKOLOS et al. (2016) when using 2000 mg/kg of eugenol, carvacrol, cinnamaldehyde and thymol, in ryegrass silage, and by CANTÓIA-JUNIOR et al. (2020), when using different doses of lemongrass in sugarcane silage. In general, essential oils in the rumen can also reduce ammonia formation (CALSAMIGLIA et al., 2007; WANAPAT et al., 2009). Although, the previous literature reports concentration of NH3-N in whole-plant corn silage with 30 to 40% DM around 5 to 7% of DM (KUNG et al., 2018), and the average values found in this experiment were 8.79% in silage with 38.87% DM. Generally, higher concentrations of NH3-N are more common in silages with high humidity, mainly due to undesirable fermentation and/or with the presence of *Clostridium*. *Clostridium* uses two molecules of lactate as a substrate for its own growth, and produces one molecule of butyrate, 2 CO2, 2 H2, causing more than 50% of DM losses in whole-plant corn silage (BORREANI et al., 2018). The inclusion of AITC promoted a linearly increase in CP values, and this finding may be linked to the fact that AITC reduced ammonia formation and allows the availability of nitrogen-fixed cellulose (N-NDF). We believe that AITC acts on the bonds of nitrogen fixed to the neutral detergent fiber (aNDF), allowing its availability. In some studies, with isothiocyanates, the power of this compound to interact with amines and thiols was demonstrated and AITC can also induce oxidative cleavage of all or part of the disulfide bonds (KAWAKISHI et al., 1983; GERENDAS et al., 2009; KISSEN et al., 2016). Our suggestion with this cleavage of the disulfide bond of L-cystine with allyl isothiocyanate, the disulfide bonds present in the corn grains can be broken, making it more digestible for microorganisms.

In our study, the ash, crude fiber, cellulose and lignin contents were not influenced by the addition of AITC. Ash and lignin concentrations agree with data from GHELLER et al. (2021) when using different compositions of organic acids in whole-plant corn silage, with averages of 3.9% and 4.8%, respectively. The cellulose value with 24% is according with BASSO et al. (2014) for whole-plant corn silage after 165 days of fermentation.

Microbiological analysis

Fermentative losses are caused by undesirable microorganisms (MCDONALD et al., 1991; MUCK et al., 2018). Therefore, the present study compared fermentative profile of homolactic and heterolactic LAB. Homolactic LAB are known to ferment glucose, producing 2 lactate, with 0% DM losses, and 0.7 gross energy. On the other hand, heterolactic LAB are known to ferment glucose, producing 1 lactate, 1 ethanol, 1 CO_2, promoting 24% DM losses and 1.7 gross energy (BORREANI et al., 2018). Meanwhile, after opening the silos the acetic acid produced by heterofermentative LAB can promote greater aerobic stability as a result of its antifungal power (KLEINSHMIDT and KUNG, 2006; FERRERO et al., 2019).

The antimicrobial action of AITC increases in lower pH. This explain why this compound is more effective in acid food as described by LUCIANO & HOLLEY (2009), and possibly in whole-plant corn silage that has a pH around 3.7 until 4.0. The growth of homo and heterolactic bacteria were not affected by the inclusion of AITC in this experiment and the LAB population count at the opening of the silos is in agreement with the results described in the literature (ZHANG et al., 2021). The AITC can select heterolactic bacteria, such as *Leuconostoc*, and this kind of microorganisms is found during the cabbage fermentation and whole-plant of corn silage, e.g., *Lactobacillus plantarum, L. brevis, Leuconostoc mesenteroides, Pediococcus pentosaceus* (ZABATH et al., 2018; JIANG et al., 2020). AITC did not prevent the growth of these organisms at doses up to 0.1% in kimchi, but increased the shelf life, without reducing food quality (JO et al., 2012).

Although AITC showed a minimum inhibitory concentration of 20.26 mg/L, as described by NAZARETH et al. (2021), to control the growth of *Aspergillus flavus,* and 95 μg/mL (Pereira et al., 2020; Nazareth et al., 2021). In the present study, AITC was not efficient in controlling yeast growth after opening the silo, as there was a tendency to increase ($p = 0.08$) the number of these microorganisms. Yeasts play an important role in the quality of silages, since these microorganisms consume the acids produced during the fermentation process (lactic acid) causing a cascade effect, increasing the pH, as it opens paths for the growth of filamentous fungi that are less tolerant to the lower pH (VYLKOVA, 2017). On the other hand, the dose of 10 mg/kg of AITC added to fresh forage was able to reduce mold growth after opening the silos. The potential capacity of AITC in controlling mold growth is attributed to its action on the cell wall, causing disorganization and easy release of K+, Ca+ and Mg2+ ions at sublethal and lethal concentrations (CHAUDHARI et al., 2020b). The presence of molds in

silages is already much discussed, in addition to increasing the qualitative losses, these microorganisms are still responsible for the production of mycotoxins, as described by OGUNADE et al. (2018), that can cause disorders in animals such as: reduction in DM intake and reduction in fertilization rate (OGUNADE et al., 2016; OGUNADE et al., 2018).

Volatile organic compounds, aerobic stability and pH curve

In general, volatile organic compounds can be used as a parameter of fermentation. The average values of lactic acid (5.23% DM) found in this study, agrees with recommendation data of KUNG et al. (2018) of 3-6% of lactic acid, while for acetic acid these values are slightly lower (0.86% DM) compared to those described by the authors (1-3% DM) for corn silages with 30–40% DM. The same authors suggested concentrations of ethanol for corn silage values between 1-3% of DM, meanwhile the ethanol value for this experiment was 0.40% DM. AITC may have selected more homolactic, compared to heterolactic bacteria, during the fermentation process, since the silos remained closed for more than 90 days. This time could favor heterolactic fermentation, that is, the microorganisms use lactic acid to produce acetic acid causing positive effects such as, increasing the aerobic stability of silages after opening the silos. In this experiment, the dose of 20 mg/kg of fresh food promoted an increase of 7.1 hours in aerobic stability in relation to the control silage (without additive).

It is discussed in the literature that the production of acetic acid carried out by heterolactic bacteria during fermentation promotes greater losses of DM, but after opening the silo this compound reduces losses of DM due to its power to control the growth of yeasts and molds (PAHLOW et al., 2003). As the production of acetic acid in this study was low (0.8% DM), there was a linear increase in DM losses during aerobic exposure with the inclusion of AITC. Higher post-opening lactic acid values may increase DM losses due to lactate assimilation by yeasts (WANG et al., 2018). In the present study, the lactic:acetic acid ratio was 6.08, which means that it is lower than 1, what may indicate abnormal fermentations inside the silos (KUNG et al., 2018).

This work was not the first to verify the increase in aerobic stability when using essential oils as additives in silage production. CHAVES et al. (2012) and CANTOIA-JUNIOR et al. (2020) previously conducted experiments with barley silage and sugarcane silage, respectively, and also observed a similar effect. AITC in this study was efficient in controlling the growth of filamentous fungi. However, the control of these microorganisms in the present study was not efficient in preventing the reduction of dry matter losses after opening the silos.

In addition to aerobic stability and DM losses, another measure that can be used after opening the silos is the pH. In general, as 0.5 points increase, there is an increase in DM losses and an increase in silage temperature.

Another extremely relevant factor is that silage is produced to compose the diet of different animal categories, so higher doses of essential oils could interfere with dry matter consumption, due to the purging odor that this compound presents. The literature is still incipient in relation to data with the use of AITC in diets for ruminant animals. However, this compound is found in garlic as described by BAUTISTA et al. (2005) and ZHU et al. (2021) did not observe differences in DM intake when they included 8% garlic skin (DM basis) from the TMR replacement for alfalfa hay in lamb diets. In addition, the garlic skin-fed supplementation improved the growth performance of lambs by modifying rumen fermentation through shifts in the rumen microbiome and metabolome.

7 CONCLUSION

The findings of this study indicate that the dose of allyl isothiocyanate of 10 mg/kg of fresh forage had positive effects, such as the increase in time of aerobic stability. While the dose of allyl isothiocyanate of 20 mg/kg fresh food also presented good results in the characteristics of the silage (dry matter losses, gas losses, presence of molds). However, as the doses are increased, the losses of dry matter after opening the silo can increase. Further investigations are needed to better understand the action of allyl isothiocyanate inside the silo and its benefits to the animals that will consume it.

CONFLICT OF INTEREST

There are no conflicts of interest.

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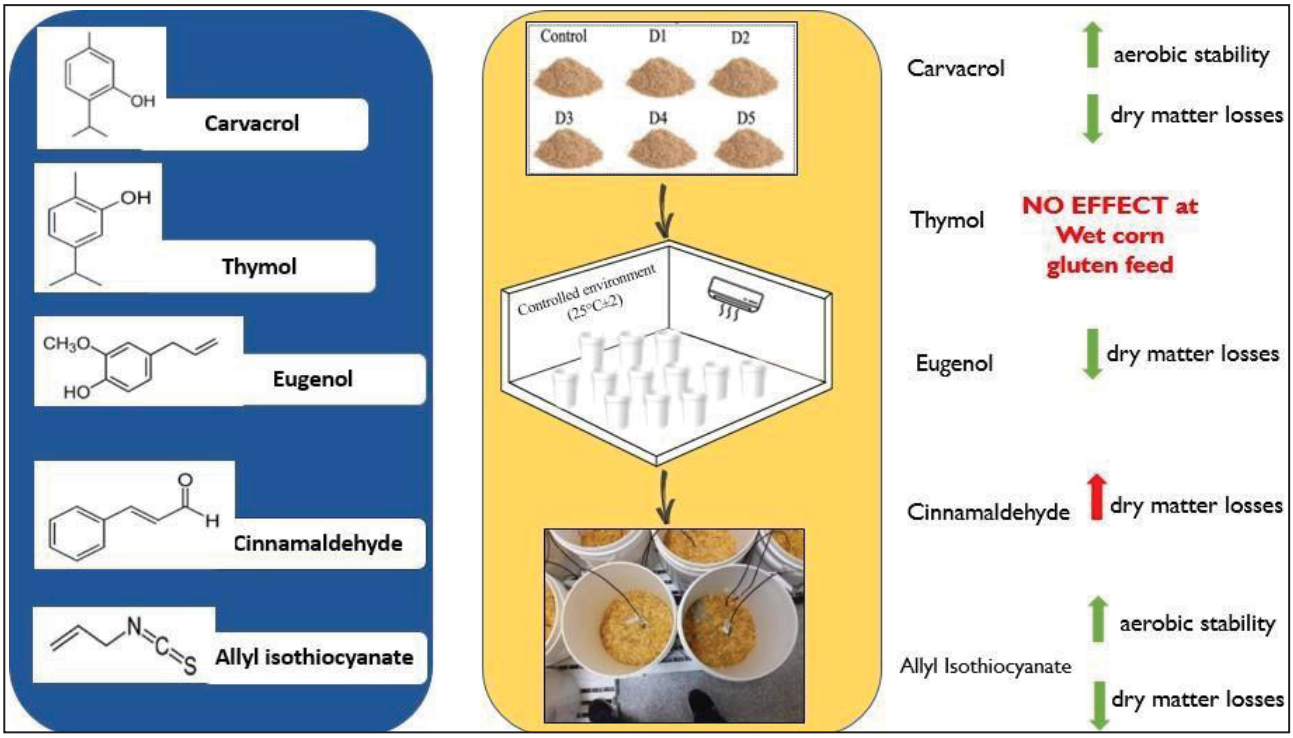
8 CHAPTER 3 - USE OF ESSENTIAL OILS AS ADDITIVES TO CONTROL DETERIORATION IN WET CORN GLUTEN FEED DURING AEROBIC EXPOSURE

Highlights

- Cinnamaldehyde increases the dry matter losses after aerobic exposure, whereas eugenol decreases dry matter losses of wet corn gluten feed (WCGF).
- Allyl isothiocyanate increases aerobic stability and decreases dry matter losses of WCGF.
- Thymol does not affect the aerobic stability of WCGF.
- Carvacrol quadratically decreases dry matter losses and increase aerobic stability of WCGF.

Graphical abstract



ABSTRACT

Aerobic stability is an important variable to be evaluated when working with different additives or inoculants in feed conservation. This variable can show, through temperature, if the food starts to deteriorate, mainly when the material is exposed to air. Several compounds, such as essential oils have an antimicrobial potential. Therefore, the aim of this experiment was to evaluate five different compounds isolated from essential oils at six doses, to identify the better dose of thymol, carvacrol, cinnamaldehyde, eugenol, and allyl isothiocyanate on wet corn gluten feed during aerobic exposure. The experiment for each essential oil was conducted separately, with six doses, and five replicates per treatment, totalizing 30 experimental units per essential oil. The experiment was conducted at Forage Research Center at Curitiba, and the wet corn gluten feed was provided by Cargill company between December 2019 and October 2020. Dry matter losses (DML), pH, aerobic stability (AS), maximum temperature (MT), hours to maximum temperature, and accumulated temperature (Tacu) were measured during aerobic exposure to evaluate the action of different additives and their respective doses. The data was analyzed using the PROC MIXED of SAS, and analyzed by contrast for linear and quadratic effect. The experiment with thymol tended to decrease DM concentration ($p=0.07$). The cinnamaldehyde increased the DML (%), accumulated temperature, and hours to maximum temperature ($p=0.03$, respectively) and decreased the aerobic stability ($p=0.01$). The essential oil eugenol decreased ($p=0.01$) the DML (%); however, carvacrol presented a quadratic effect on dry matter losses, aerobic stability, maximum temperature and accumulated temperature ($p=0.01$), and the allyl isothiocyanate increased linearly ($p=0.03$) the DM and AS and decreased DML (%). In summary, these results showed that the essential oils have a different mode of action between each other. Some may have more accentuated effects, such as allyl and carvacrol, and others just change dry matter losses, as demonstrated by cinnamaldehyde. Also, the action of essential oils is dose-dependent, and each one has a different mode of action.

Key-words: By-product. Conservation. Methodology. Temperature.

9 INTRODUCTION

The high cost of commodities has raised the price of diets for farm animals. Thus, co-products have been used as alternatives to meet the nutritional requirements of each animal category. About 85% of the world's starch is produced through corn grains, whose processing produce several products such as ethanol and gluten meal. The co-product wet corn gluten meal is obtained with the extraction of starch and sweetener.

The wet corn gluten has high protein content (25% DM), 18% of fiber, and approximately 45% DM (ZHANG et al., 2020). With the production processing, this co-product is highly susceptible to the growth and development of microorganisms that cause its deterioration. Therefore, this co-product must be fed to animals as soon as possible after arriving at the farm, to avoid the growth of undesirable microorganisms. However, with the high demand and milling of corn, and for practical and economic reasons, some alternatives are developed to store this ingredient at the farms. One way to store the wet corn gluten is conserving it in the form of silage, promoting the growth of lactic acid bacteria that will lead to a drop in the pH value and consequently preserve the ingredient (FRANÇA et al., 2015).

Additives can be used to increase the shelf life of this co-product when the ensiling is not possible. Within the category of additives used are included the organic acids, which already have demonstrated effects on improving fermentation and aerobic stability in corn silage (LI et al., 2021). Based on this, new additives are constantly studied to add or promote alternatives to farmers who wish to diversify the feed of total mixed ration and additives. In this sense, the essential oils used in cosmetics, aromatherapy, personal hygiene, and industry have already played antiseptic and antibacterial roles for a longer time (CIMINO et al., 2021). In the last ten years, the use of essential oils and active principles of plants has increased, partly due to the pressure from consumers who are looking for natural alternatives. Based on that, the objective of this work was to evaluate the action of different essential oils as additives in wet corn gluten during the aerobic exposure test.

10 MATERIAL AND METHODS

The experiment was carried out between December 2019 and October 2020 at the Forage Research Center (acronym in Portuguese: CPFOR; 25º32'05"S, 49º12'23"W, 906 m altitude, Köpen-Geiger climate type Cfb) at the Federal University of Parana, Pinhais, Paraná

state - Brazil. The wet corn gluten feed (WCGF) was collected immediately after milling the corn (Cargill; -24 .04750, -50.391950) in Castro, Paraná state - Brazil and transported to CPFOR with 12 hours of intervals, with exception to thymol assay, which collection took place 24 hours after processing at Cargill. The treatments consisted of different doses of five essential oils: thymol (THY), cinnamaldehyde (CIN), eugenol (EUG), carvacrol (CAR) and allyl isothiocyanate (AITC). Thus, each essential oil assay was considered as an individual experiment. For each experiment, the control consisted of WCGF without additive, while for each essential oil, we chose five different doses (D) per kg of fresh matter (FF) (Table 11-1).

TABLE 10-1 - DOSES (D) OF ESSENTIAL OILS USED IN EACH EXPERIMENT.

Essential oil	Doses used (mg/kg of fresh forage)					
	Control	D1	D2	D3	D4	D5
Thymol (THY)	0	50	150	250	350	450
Cinnamaldehyde (CIN)	0	25	75	100	125	250
Eugenol (EUG)	0	200	250	300	350	400
Carvacrol (CAR)	0	25	100	200	300	400
Allyl isothiocyanate (AITC)	0	5	10	15	20	30

10.1 APPLICATION OF ADDITIVES

At CPFOR, the WCGF was homogenized and subdivided into six equal (25 kg) parts. After that, the respective dose of each treatment (except in the control) was applied to each part and the WCGF was then homogenized.

10.2 AEROBIC STABILITY

Aerobic stability (AS) test followed the methodology proposed by KUNG et al. (2001). Briefly, 4 kg of WCGF were placed in 20-L buckets without lids and without compaction (5 replicates/treatment; n = 30). The experimental silos were kept in a controlled environment (25°C ± 2) for ten days (240 hours). In the center of each silo, a thermal sensor was placed (DS18b20; DENIZ et al., 2019), and the set of sensors was configured to record the temperature of the mass every 5 min. Aerobic stability breakage was defined as the moment when the internal temperature of the experimental silos reached 2°C above the ambient temperature (Kung et al., 2001). During the AS, the maximum temperature (MT, °C) of each experimental unit was measured, as well as the time (hours) that the silage mass took to reach the maximum

temperature (HMT, h), and accumulated temperature (Tacu, °C), according to NOVINSKI et al. (2012).

Samples of wet corn gluten feed (WCGF) were collected before, during, and at the end of aerobic stability trials. Before AS, two samples (n = 12) were collected from each experimental unit to determine the dry matter (DM %, determined at 65°C) and initial WCGF pH, and the results are shown in Table 11-2**.** To evaluate the pH oscillation during the AS, samples (n = 30, one sample per repetition) of 25 g were collected from the "pairs buckets" experimental silos with an interval of 24 h. The pH was determined using a digital pHmeter (PG 1400, Gehaka – Brazil) according to Kung et al. (2000), where 25 g of samples were diluted in 225 mL of distilled water and homogenized for (~1 min.), and the extract was used to the measurement. At the end of the AS, samples (n = 2) were collected from each experimental unit to determine the DM and final pH of the WCGF. With the initial and final dry matter (DM) data, we were able to determine the dry matter losses (DML), according to JOBIM et al. (2007). Although the experiments were not carried out simultaneously, the application of additives (different doses), collection of samples for DM and pH, and AS were performed in the same way, regardless of the essential oil used, as shown in Figure 11-1.

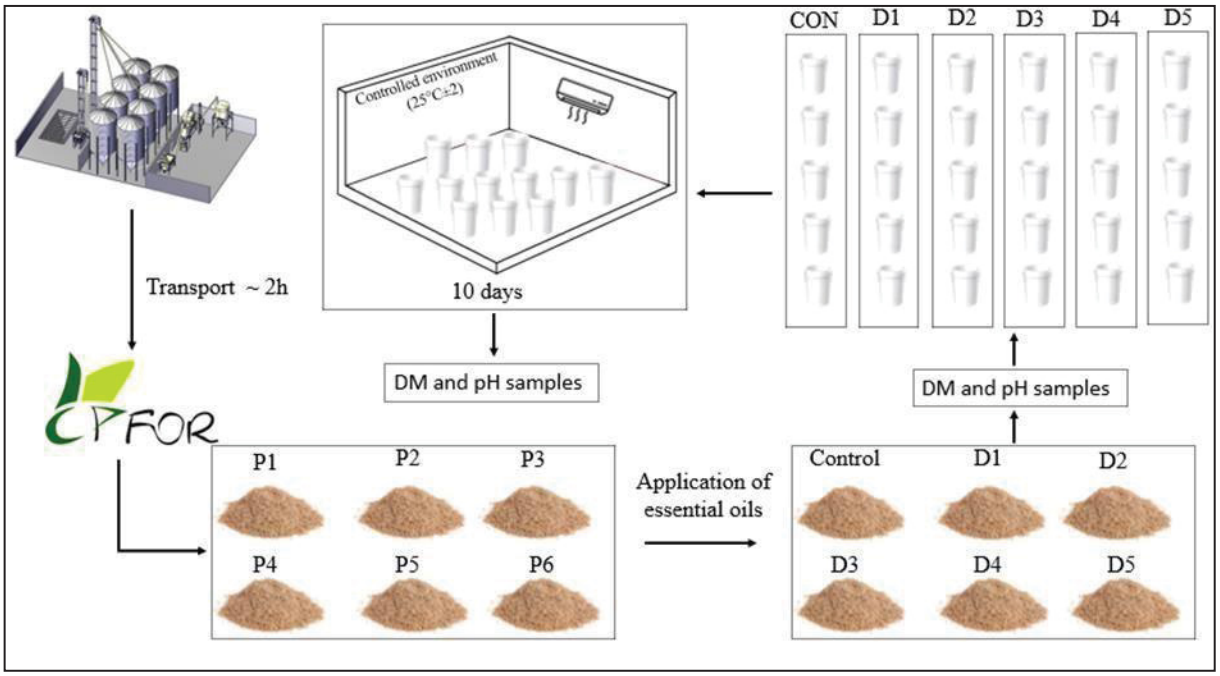
TABELA 10-2 - WET CORN GLUTEN FEED CHARACTERISTICS BEFORE THE AEROBIC STABILITY TESTS.

Date	Variable	Control	Thymol (THY)					Average	SD[1]
			D50	D150	D250	D350	D450		
04/12/2019	DM[2], %	37.9	38.0	39.2	38.3	37.7	37.6	37.9	0.58
	pH	4.0	4.0	4.0	4.0	4.0	4.0	4.0	0.00
Date	Variable	Control	Cinnamaldehyde (CIN)					Average	SD
			D25	D75	D100	D125	D250		
05/08/2020	DM, %	43.3	43.2	42.8	43.2	43.5	43.5	43.3	0.26
	pH	4.1	4.1	4.1	3.9	3.9	4.1	4.1	0.09
Date	Variable	Control	Eugenol (EUG)					Average	SD
			D200	D250	D300	D350	D400		
19/08/2020	DM, %	39.9	40.9	40.5	40.9	40.4	39.3	40.4	0.61
	pH	3.9	3.9	3.9	3.9	3.9	3.9	3.9	0.03
Date	Variable	Control	Carvacrol (CAR)					Average	SD
			D25	D100	D200	D300	D400		
03/09/2020	DM, %	44.3	44.7	43.6	44.6	44.4	43.8	44.3	0.44
	pH	3.9	3.9	3.9	3.9	3.9	3.9	3.95	0.02
Date	Variable	Control	Allyl isothiocyanate (AITC)					Average	SD
			D5	D10	D15	D20	D30		
15/10/2020	DM, %	42.4	43.2	43.1	42.8	43.1	42.4	42.9	0.37

pH	4.3	4.2	4.6	4.6	4.5	4.2	4.4	0.19

[1]SD – Standard deviation; DM[2] – dry matter.

FIGURE 9 - SCHEMATIC REPRESENTATION OF THE PROCESSES CARRIED OUT DURING THE EXPERIMENTAL PERIODS.



D1 – DOSE 1; D2 – DOSE 2; D3 – DOSE 3; D4 – DOSE 4; D5 – DOSE 5; P1- PILE 1; P2- PILE 2; P3- PILE 3; P4- PILE 4; P5- PILE 5; P6- PILE 6.

10.3 STATISTICAL DESIGN

The tests of essential oils (thymol, cinnamaldehyde, eugenol, carvacrol, and allyl isothiocyanate) were considered as replicated experiments and analyzed separately. Firstly, the dataset was submitted to the normality (Shapiro-Wilk test) and the homogeneity test. The experimental design was completely randomized, composed of six treatments (Control, Dose 1, Dose 2, Dose 3, Dose 4, and Dose 5) with 30 experimental units (five replicates by treatment).

The coefficients for orthogonal polynomial contrasts were determined using the IML procedure of SAS. The linear and quadratic effects were tested for the essential oil application rates. Data were analyzed using a MIXED procedure of SAS version 9.4, SAS Inst. Inc., Cary, NC, USA, and considered $p \leq 0.05$ as a significant difference and $p \leq 0.10$ as a tendency. The following model was used for non-repeated measures:

$$Y_{ij} = \mu + E_i + e_{ij}$$

Where: Y_{ij} is the dependent variable; μ = overall mean; E_i = the fixed effect of essential oils levels (i = D0 to D5 mg of essential oils/kg of fresh forage), e_{ij} = residue (j = 1to 4).

For the pH analyses, repeated measurements were carried out over time, where for each oil studied, there were six doses, and the collections took place every two days (0, 2, 4, 6, 8 and 10th day during the period of aerobic exposure). After that, to confirm the effect of different doses on the response variable (evaluated parameters), the data were submitted to analysis of variance (ANOVA); when significant, the mean values were compared by the Tukey test with 95% of confidence level.

11 RESULTS

11.1 THYMOL

The results during aerobic exposure of WCGF added with thymol (THY) are shown in Table 12-1. The dry matter content tended to decrease linearly (p = 0.07) with the increase on thymol doses, and the average DM was 38.1%. No differences were found between doses for dry matter losses (22.9%), aerobic stability (41.2 hours), maximum temperature (43.4°C), hours to maximum temperature (HMT - 41.7 hours), and accumulated temperature (Tacu - 24589°C).

TABLE 11-1 - CHARACTERISTICS OF WET CORN GLUTEN FEED ADDED WITH THYMOL AFTER AEROBIC EXPOSURE.

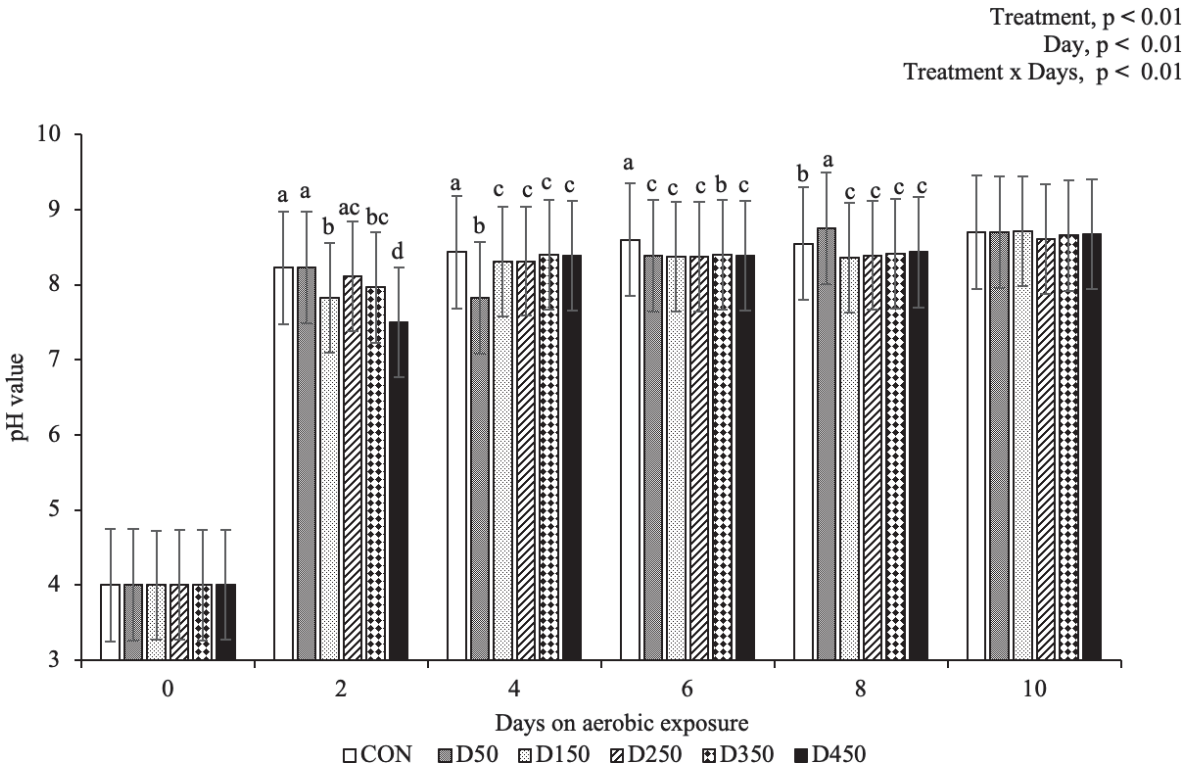
Variables[1]	Control	Treatments[2]					SEM[3]	*p*-value	
		D50	D150	D250	D350	D450		Linear	Quadratic
DM, %	38.6	38.4	39.2	38.6	36.5	37.3	0.845	0.07	0.51
DML, %	21.6	23.2	22.4	21.5	25.2	23.2	1.412	0.29	0.96
AS, hours	41.2	41.3	41.6	40.9	41.2	41.1	0.164	0.11	0.71
MT, °C	43.2	43.2	43.5	43.8	43.2	43.3	0.410	0.85	0.31
HMT, h	41.7	41.5	41.7	41.7	41.7	41.7	0.043	0.30	0.56
Tacu, °C	26486	23898	24353	25174	24620	23004	1582.52	0.32	0.88

[1]DM = Dry matter, DML= dry matter losses, AS, hours = aerobic stability, (MT, °C), maximum temperature, (HMT, hours) = hours to reach maximum temperature and (Tacu, °C) = accumulated temperature evaluated during the aerobic stability tests. CON – Without additives; D50-50 mg/kg FF); D150- (150 mg/kg FF); D250- (250 mg/kg FF); D350- (350 mg/kg FF); D450 – (450 mg/kg FF); [3]SEM = standard error of the means. Variables with $p < 0.05$ had significant influence of treatments

The pH values during 10 days of aerobic exposure are shown in Figure 12-1. The pH values during 10 days of aerobic exposure were affected by treatments, after the second day of

exposure to air (p > 0.01) the average pH was 4, and after 2 days of aerobic exposure the pH increase to 8 and, only the treatment D450 decreased the pH value to 7.5. On day 4 the D50 maintained the pH around 7.5, and at day 6 all doses decreased the pH around 8, and for the control treatment the value was 8.5. On day 8, all treatments maintained the pH values around 8, except D50 (pH 8.5). The day 0 (pH = 4) and day 10 (pH = 8.5) were not affected by treatments.

FIGURE 10 - VALUES OF pH DURING AEROBIC EXPOSURE OF WET CORN GLUTEN FEED WITH DIFFERENT DOSES OF THYMOL.



*D50 - 50 mg/ kg of fresh forage (FF); D150- 150 mg/ kg FF; D250- 250 mg/kg FF; D350- 350 mg/kg FF; D450 – 450. mg/kg FF and CON – silage without additives

11.2 CINNAMALDEHYDE

The results of different doses of cinnamaldehyde (CIN) are shown in Table 12-2. The DM (%) content presented quadratic tendency to decrease (p = 0.05); The dose D125 mg/kg FF, compared to CON (without-additive) presented 4.2% less DM. Cinnamaldehyde oils increased the percentage of DML linearly. The treatment D125 showed 38.5% more DML than CON (without-additive). The aerobic stability decreased quadratically (p< 0.01) with the doses. The treatment D250 presented approximately 45 less hours of AS when compared with the

other treatments (CON, D25, D75, D100, D125 - 135.7 hours). In addition, the WCGF treated with the dose D250 reached the maximum temperature (MT), corresponding to 47°C, after 170 hours of aerobic exposure, whereas all the other treatments showed a MT of 44.35°C after 191.2 hours of aerobic exposure. The Tacu increased linearly with the cinnamaldehyde doses ($p < 0.01$). There was an increasing of 268% when comparing the Tacu from the treatment 250 (2473°C) to CON (672 °C).

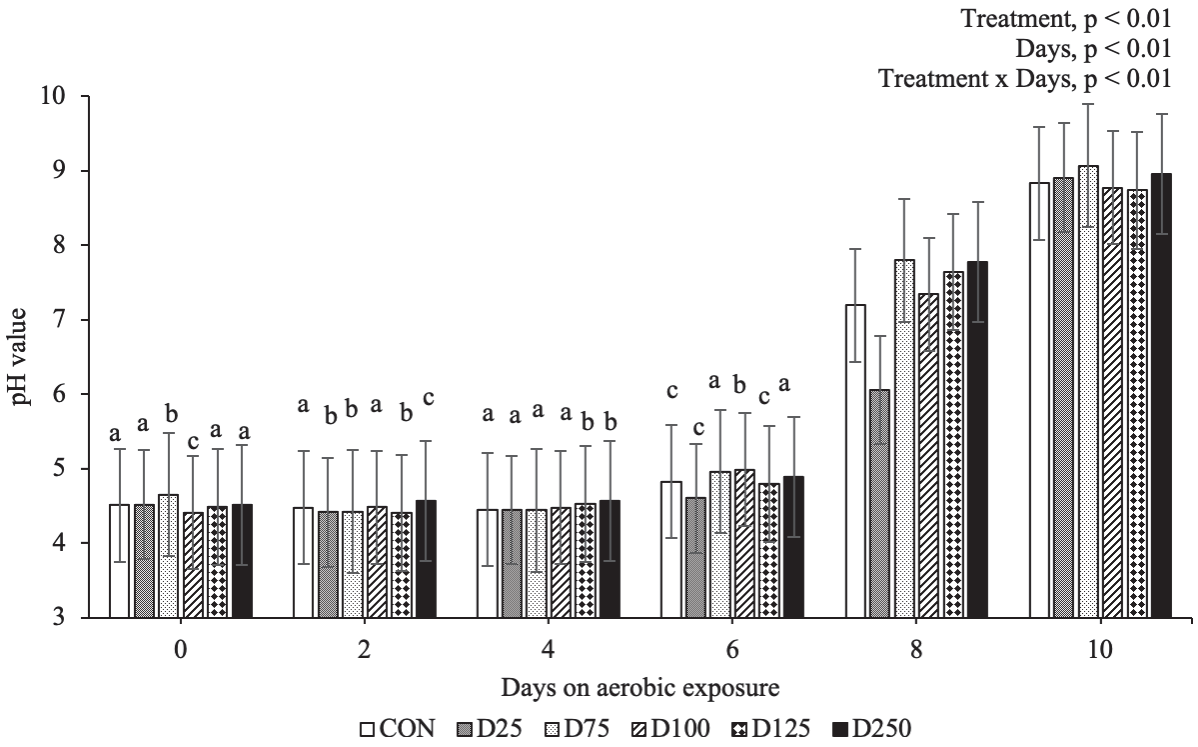
TABLE 11-2 - CHARACTERISTICS OF WET CORN GLUTEN FEED ADDED WITH CINNAMALDEHYDE AFTER AEROBIC EXPOSURE.

Variables[1]	Control	Treatments[2]					SEM[3]	*p*-value	
		D25	D75	D100	D125	D250		Linear	Quadratic
DM, %	44.8	44.2	43.9	43.1	42.9	44.1	0.670	0.45	0.05
DML, %	12.2	14.1	14.0	15.3	16.9	16.2	1.234	0.02	0.15
AS, hours	135.1	128.0	126.1	138.4	135.7	90.1	3.730	≤0.01	≤0.01
MT, °C	43.3	40.9	45.8	45.6	46.1	47.3	2.540	0.08	0.48
HMT, hours	189.5	179.0	186.0	196.3	206.4	170.0	10.67	0.40	0.03
Tacu, °C	672.8	677.1	1217.9	853.0	1009.4	2473.1	354.77	≤0.01	0.15

[1]DM = Dry matter, DML= dry matter losses, AS, hours = aerobic stability, (MT, °C), maximum temperature, (HMT, hours) = hours to reach maximum temperature and (Tacu, °C) = accumulated temperature evaluated during the aerobic stability tests. [2]CON - without additive; D25 - 25 mg/kg FF; D75 - 75 mg/kg FF; D100 - 100 mg/kg FF; D125 - 125 mg/kg FF; D250 - 250 mg/kg FF; [3]SEM = standard error of the means; Variables with $p < 0.05$ had significant influence of treatments.

The pH values during aerobic exposure are shown in Figure 12-2. The days 8 and 10 were not affected by treatments ($p > 0.05$). On the other days (0, 2, 4 and 6) of aerobic exposure the pH values were affected by the treatments ($p < 0.05$). At day 0 the lowest pH (3.7) was observed on the treatment D100. On day 6, the D75 and D250 treatments were not efficient in decreasing the pH.

FIGURE 11 - VALUES OF PH DURING AEROBIC EXPOSURE OF WET CORN GLUTEN FEED WITH DIFFERENT DOSES OF CINNAMALDEHYDE



*CON – without additive; D25 - 25 mg/kg of fresh forage (FF); D75 - 75 mg/kg FF; D100 - 100 mg/kg FF; D125 - 125 mg/kg FF; D250 - 250 mg/kg FF

11.3 EUGENOL

The variables regarding WCGF added with eugenol are shown in Table 12-3. The average DM was 41.3%, and the doses did not affect the dry matter content. Besides that, the DML (%) were quadratically affected ($p \leq 0.01$) by treatments. The D400 presented 39.6% less DML% compared to control. However, aerobic stability, maximum temperature, hours to maximum temperature and accumulated temperature were not influenced ($p > 0.05$) by treatments.

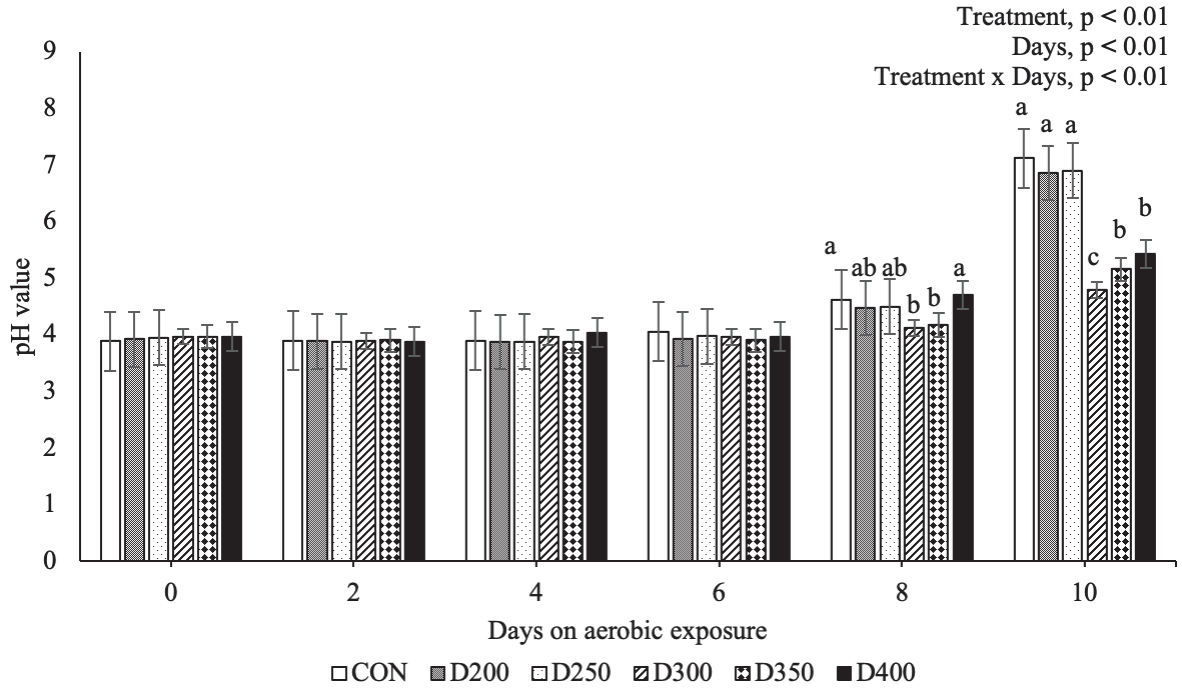
TABLE 11-3 - CHARACTERISTICS OF WET CORN GLUTEN FEED ADDED WITH EUGENOL AFTER AEROBIC EXPOSURE.

Variables [1]	Control	Treatments [2]					SEM [3]	*p*-value	
		D200	D250	D300	D350	D400		Linear	Quadratic
DM, %	40.9	42.2	40.8	41.2	42.2	40.5	0.4862	0.92	0.15
DML, %	11.1	10.8	13.5	9.0	5.2	6.7	1.173	≤0.01	≤0.01
AS, hours	161.7	149.8	144.5	164.6	177.9	162.5	15.730	0.62	0.36
MT, °C	37.6	42.5	38.1	39.6	39.5	37.7	29.493	0.95	0.31
HMT, hours	173.2	221.5	177.6	231.1	227.4	224.7	26.13	0.11	0.92
Tacu, °C	415	1358	173	824	117	414	822.01	0.52	0.25

[1]DM = Dry matter, DML= dry matter losses, AS, hours = aerobic stability, (MT, °C), maximum temperature, (HMT, hours)= hours to reach maximum temperature and (Tacu, °C) = accumulated temperature evaluated during the aerobic stability tests CON – without additive; D200 - 200 mg/kg FF; D250 - 250 mg/kg FF; D300 - 300 mg/kg FF; D350 - 350 mg/kg FF; D400 - 400 mg/kg FF; [3]SEM = standard error of the means; Variables with $p < 0.05$ had significant influence of treatments.

The pH values during aerobic exposure are shown in Figure 12-3. There was no difference ($p > 0.05$) for pH values among the days 0, 2, 4 and 6. After 8 days of aerobic exposure, the treatments D300 and D350 showed a pH value of 4, whereas the other treatments showed a pH of 5. After 10 days of aerobic exposure, the treatment D300 maintained the pH of 4, while on the other treatments (CON, D200 and D250) the pH value was around 7.

FIGURE 12 - VALUES OF pH DURING AEROBIC EXPOSURE OF WET CORN GLUTEN FEED WITH DIFFERENT DOSES OF EUGENOL



*CON – without additive; D200 - 200 mg/kg FF; D250 - 250 mg/kg FF; D300 - 300 mg/kg FF; D350 - 350 mg/kg FF; D400 - 400 mg/kg FF

11.4 CARVACROL

The results of different doses of carvacrol are shown in Table 12-4. The average DM consisted of 45.7% and was not affected by the essential oil ($p > 0.05$). However, the DML were quadratically affected by this compound, where D25 showed 51.2% more DML than the average of CON, D100, D200, D300, D400 (19.2% *vs.* 12.7%). The carvacrol quadratically affected the aerobic stability ($p < 0.01$). The treatments CON and D25 presented a difference of more than 100 hours of aerobic stability (154 *vs.* 52.8 hours). The carvacrol inclusion tended to show a quadratic effect ($p = 0.05$) for the maximum temperature, showing 46.1°C on average of all the treatments. Hours to maximum temperature (HMT) were not affected by treatments ($p > 0.05$), where the average of all treatments was 207 hours. The Tacu was quadratically affected by treatments ($p < 0.01$). The CON presented a difference of 3983°C more than D400 (6488 °C *vs.* 10471°C).

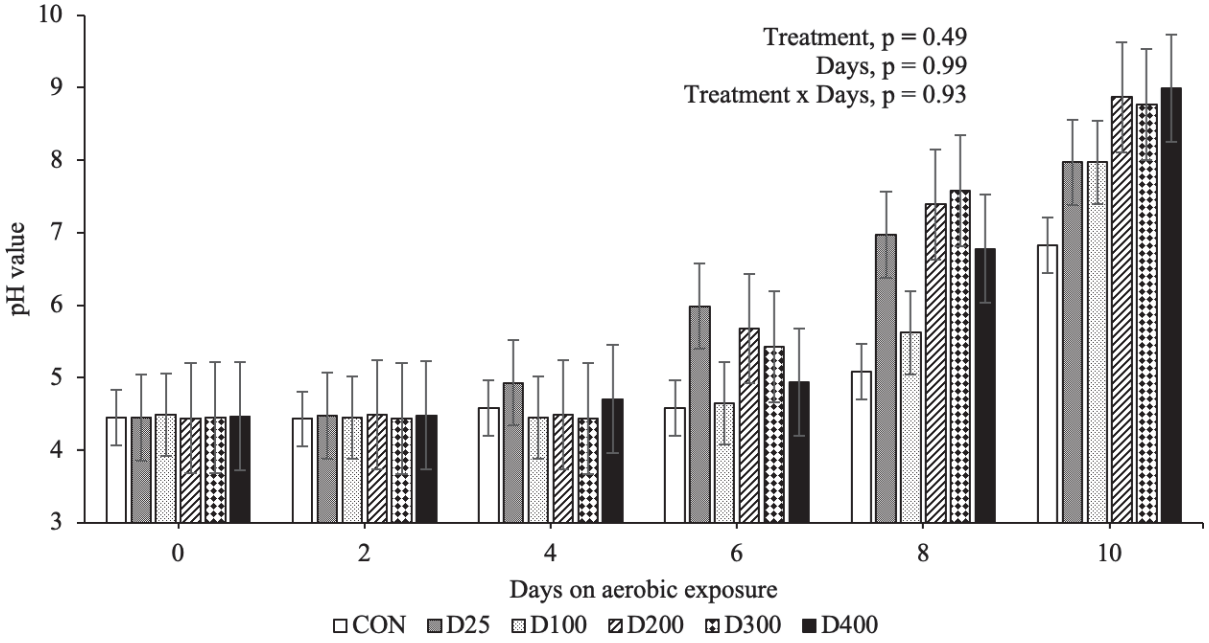
TABLE 11-4 - CHARACTERISTICS OF WET CORN GLUTEN FEED ADDED WITH CARVACROL AFTER AEROBIC EXPOSURE.

Variables[1]	Control	Treatments[2]					SEM[3]	*p*-value	
		D25	D100	D200	D300	D400		Linear	Quadratic
DM, %	46.2	45.0	46.4	45.9	44.8	45.9	0.605	0.61	0.99
DML, %	9.2	19.2	13.2	15.2	16.2	9.87	1.114	0.33	< 0.01
AS, hours	154.0	52.8	113.6	100.6	80.2	114.4	5.357	0.07	< 0.01
MT, °C	44.7	48.0	46.6	45.9	47.0	44.5	0.832	0.37	0.05
HMT, hours	219.8	220.1	218.9	195.2	171.3	217.0	0.770	0.40	0.59
Tacu, °C	6488	23820	20991	18640	22023	10471	1952	0.92	< 0.01

[1]DM = Dry matter, DML= dry matter losses, AS, hours = aerobic stability, (MT, °C), maximum temperature, (HMT, hours) = hours to reach maximum temperature and (Tacu, °C) = accumulated temperature evaluated during the aerobic stability tests. CON - without additive; D25 - 25 mg/kg FF; D100 - 100 mg/kg FF; D200 - 200 mg/kg FF; D300 - 300 mg/kg FF; D400 - 400 mg/kg FF; [3]SEM = standard error of the means; Variables with $p < 0.05$ had significant influence of treatments.

The pH values are shown in Figure 12-4. The pH values during aerobic stability were not affected by treatments ($p > 0.05$). On day 0 and 2 the average pH was around 4, and pH reached 8.5 at day 10.

FIGURE 13 - VALUES OF PH DURING AEROBIC EXPOSURE OF WET CORN GLUTEN FEED WITH DIFFERENT DOSES OF CARVACROL



*CON - without additive; D25 - 25 mg/kg FF; D100 - 100 mg/kg FF; D200 - 200 mg/kg FF; D300 - 300 mg/kg FF; D400 - 400 mg/kg FF.

11.5 ALLYL ISOTHIOCYANATE

The results of different doses of allyl isothiocyanate (AITC) are shown in Table 12-5. DM content was increased linearly by AITC inclusion ($p = 0.03$), with 3.7% more DM to treatment D30 compared to CON (44.7% *vs.* 43.1%). The DML quadratically decreased with AITC inclusion ($p = 0.01$), where one reduction of more than 64.7% was observed between

D30 and D5 (7.6 *vs.* 21.5 %). At the same time, AS was quadratically affected by AITC inclusion ($p < 0.01$). The treatment D5 presented a longer time in aerobic stability than the CON (149.6 *vs.* 107 hours). Although the maximum temperature did not differ among the treatment ($p > 0.05$), the HMT and Tacu were affected by treatments linearly and quadratically, respectively ($p < 0.01$; 0.01). The treatment D30 took 67.3 more hours to reach the maximum temperature than the CON (224.1 *vs.* 156.8 h), and for the Tacu the CON accumulated 9572°C more than D5 (18522 *vs*. 8950°C, respectively).

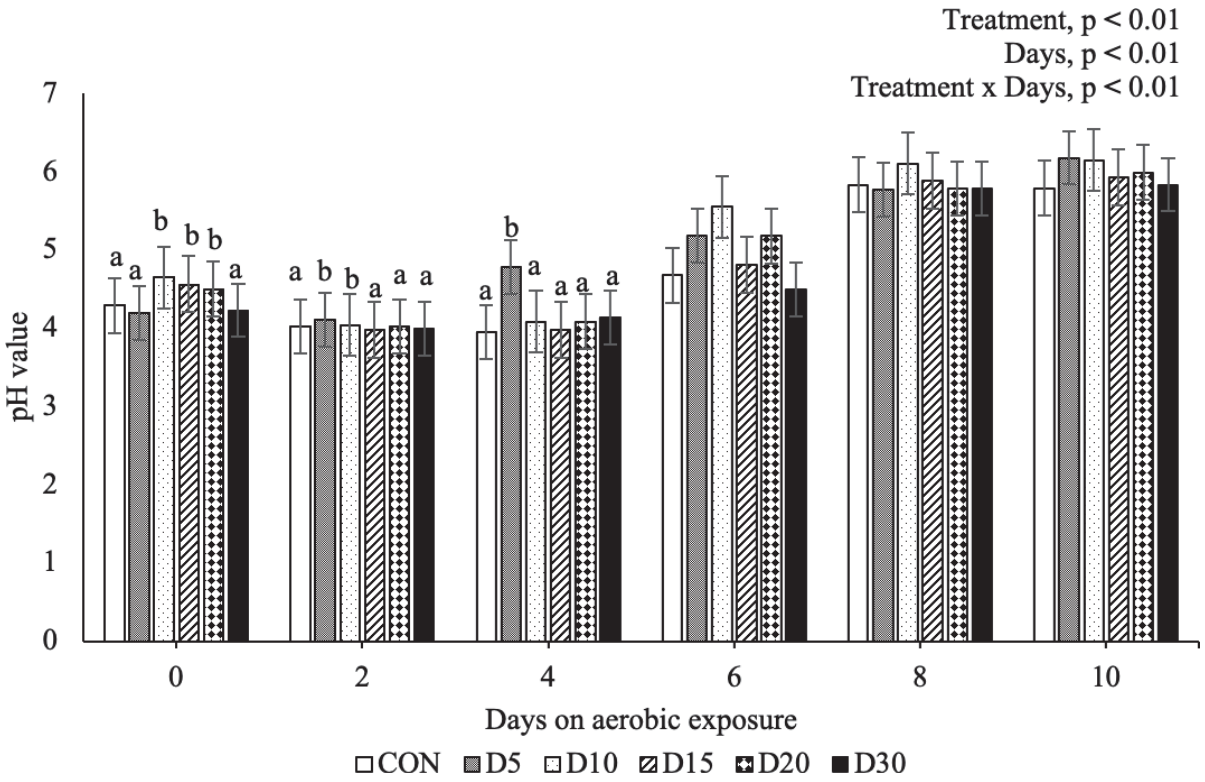
TABLE 11-5 - CHARACTERISTICS OF WET CORN GLUTEN FEED ADDITIVATED WITH DIFFERENT DOSES OF ALLYL ISOTHIOCYANATE AFTER AEROBICEXPOSURE.

Variables[1]	Control	Treatments[2]					SEM[3]	*p*-value	
		D5	D10	D15	D20	D30		Linear	Quadratic
DM, %	43.1	41.8	44.3	42.9	43.5	44.7	0.6849	0.03	0.11
DML, %	16.9	21.5	18.6	14.1	15.1	7.6	18.271	< 0.01	< 0.01
AS, hours	107.0	86.2	117.8	132.6	121.1	149.6	6.838	< 0.01	< 0.01
MT, °C	42.9	44.7	46.8	45.1	46.4	44.4	17.565	0.53	0.29
HMT, hours	156.8	126.4	202.3	206.9	190.4	224.1	14.601	< 0.01	0.05
Tacu, °C	18522	26727	21087	15574	18371	8950	4669.62	< 0.01	< 0.01

[1]DM = Dry matter, DML= dry matter losses, AS, hours = aerobic stability, (MT, °C), maximum temperature, (HMT, hours) = hours to reach maximum temperature and (Tacu, °C) = accumulated temperature evaluated during the aerobic stability tests. CON – without additive; D5 - 5 mg/kg FF; D10 - 10 mg/kg FF; D15 - 15 mg/kg FF; D20 - 20 mg/kg FF; D30 – 30 mg/kg FF; [3]SEM = standard error of the means; Variables with $p < 0.05$ had significant influence of treatments.

The pH values during aerobic exposure are shown in Figure 12-5. The treatments did not affect ($p > 0.05$) pH values on days 6, 8 and 10. On the other hand, on day 0 the treatments CON, D1, and D30 presented similar values (pH = 4), and treatment D10, D15, and D20 presented higher pH (4.5). On day 2, only D1 and D2 presented a pH around 4.5, and on day 4 the highest pH value was observed for the treatment D5.

FIGURE 14 - VALUES OF PH DURING AEROBIC EXPOSURE OF WET CORN GLUTEN FEED WITH DIFFERENT DOSES OF ALLYL ISOTHIOCYANATE



*CON – without additive; D5 - 5 mg/kg FF; D10 - 10 mg/kg FF; D15 - 15 mg/kg FF; D20 - 20 mg/kg FF; D30 – 30 mg/kg FF

12 DISCUSSION

The characteristic of shelf life is very important to determine how long the qualitative properties of food are safe to consumers, regardless of the food (humans) or feed (for animals) will be consumed by humans or animal. Therefore, a similar pattern is observed with the research comparing experiments evaluating silages additives or experiments using additives in food, guaranteeing a longer shelf life of the food, preserving its quality, and controlling the growth of undesirable microorganisms. In this same direction, the shelf life of foods is similar to the tests of aerobic stability when evaluating the preservation of silage and several by-products e.g., wet brewery waste (SOUZA et al., 2012), and orange juice processing wastes (CPFOR – internal data's).

In general, when the WCGF leaves the factory where the corn grain is processed, it has homogeneous levels of dry matter and pH. The first experiment started in December 2019 (thymol), and the last one started on October 2020 (allyl isothiocyanate). Samples were collected to determine DM and pH exactly before the material left the Cargill plant. The DM

concentration consisted of 37.9, 43.3, 40.4, 44.3, and 42.9% for the material used in the studies regarding thymol, cinnamaldehyde, eugenol, carvacrol, and allyl isothiocyanate inclusion, respectively. Except for the data from the first collection, that corresponded to 37.9 % of DM, all the other values are in agreement with the data found by Zhang et al. (2021) with 44.9% of DM.

Thymol

Thymol did not change the parameters of dry matter content, dry matter losses, aerobic stability, maximum temperature, hours to reach the maximum temperature, accumulated temperature, and pH values during the air exposure. Besides that, the values of dry matter losses (22.7%) and pH at day 0 (8.0) are numerically high compared to other trials described in this study, that might be attributed to the collection time that took place one day after the corn was milled at the Cargill factory. Therefore, one day of exposure to air can already interfere in the quality of the WCGF, such as this material has a high susceptibility, and this difference can be clear with the one day. In addition, we observed that even using the highest dose (450 mg/kg FF), it was not enough to reduce DM losses and increase aerobic stability, which probably came from the high yeast count in the material exactly before the dose's application. Ribeiro et al. (2020), when including thymol (1.25 mg/kg of DM) in lamb diets, observed a reduction in rumen pH on the 14th day of the experimental period. This may be associated with the fact that the rumen environment is anaerobic, and this condition potentialize the action of essential oils (Juven et al., 1994). The absence of thymol effects in this present research may be due to the low dosage and the high challenge from the WCGF, as it is extremely susceptible to the growth of spoilage agents such as yeasts and molds, since it is rich in nutrients (França et al., 2015).

After 10 days of exposure to air, the pH value curve during aerobic exposure, as it is altered by the growth of microorganisms, mainly yeasts, which started the deterioration process of the exposed mass. The yeasts are known for consuming acids and carbohydrates, raising the pH, and consequently giving sequence to the second wave of microorganism's growth, carried out by filamentous fungi (MCDONALD et al., 1991). Wet corn gluten feed exposed to air are more likely susceptible to deterioration than when stored in an anaerobic environment, such as silage. This effect is attributed to the lower pH in anaerobic-preserved WCGF, which can efficiently control the growth of other microorganism, such as *Bacilli* and *Listeria monocytogenes* (LINDGREN et al., 2002).

The pH value was 4.0 during aerobic exposure of thymol assay on the first day; however, on days 2, 4, 6, 8, and 10 the values extensively increased, probably because of the time between the collection of the sample, that was 24 hours after the corn milling. These results can demonstrate the instability and susceptibility of WCGF to the action of microorganisms. On day 8 of aerobic exposure, the doses D150, D250, D350, and D450 were more efficient in controlling the pH, where the treatments D150, D250, D350, and D450 reduced by 1.5 points the pH when compared to Control and D50.

Cinnamaldehyde

Cinnamaldehyde (CIN) play an important role controlling both yeast and filamentous fungi, as previously observed for *Candida albicans* and *Aspergillus niger* at doses from 62.5 µL/mL (BAKHTIARI et al., 2019) to 500 µg/mL (SUN et al., 2020). Yeasts cause DM losses and lower aerobic stability in silage (BORREANI and TABACCO, 2010). In this trial, although the count of yeast and filamentous fungi were not measured, the data of DML (Table 12-2) can be associated with this type of microorganism since the doses of CIN linearly decreased the DML and consequently increased the aerobic stability. The highest dose at this trial, corresponding to 250 mg/kg of fresh forage, was not enough to decrease the DML; however, increased the aerobic stability. Cinnamaldehyde has a particular effect on the enzyme histidine decarboxylase, inhibiting this enzyme activity on microorganisms. Histidine decarboxylase is responsible for histamine production (important biogenic amine), as well as, is considered a key inflammatory mediator, derived from L-histidine that administrates vital cellular processes beyond inflammation (DELITHEOS et al., 2010). Also, histidine decarboxylase has shown recent evidence actions in growth of both prokaryotes (*Escherichia coli)* and eukaryotes (*S. cerevisiae*). DELITTHEOS et al. (2010), when evaluating the histamine as a modulator of cellular stress response in yeast, found that this biogenic amine may also be responsible for the heat shock response in yeast and modulation of highly versatile heat shock proteins (Morano et al., 2012). The authors observed that histamine may be capable of inducing the adaptive phenotype, responsible for heat stress in yeast. However, in this experiment, this seems not to have happened, since the D125 treatment reached a maximum temperature of 47.3°C and the stability was 90.16 hours.

The maximum temperature and accumulated temperature are important ways to measure the aspects related to feed temperature. According to BORREANI and TABACCO

(2010), the high temperature in silage can be associated to the metabolism of yeasts, and when silages present a high temperature, it can affect the consume by animal, mainly in warm days (MAHANNA and CHASE, 2003).

After day 6 during aerobic exposure period, the values increased until 8.5, that can be explained by the aerobic stability with an average of 125.56 hours for all treatments. When dividing this value by 24 hours, we obtained the result of 5.23 days in aerobic stability, and when measuring the pH on that day (6) the values were 4 and 4.5.

Eugenol

The use of eugenol has been regulated in several countries, such as United States, and China, as well as to the whole European Union. One common utilization usually is related to dental plaque, since this compound can efficiently control microorganism growth, e.g, *Streptococcus* (HU et al., 2018). Basically, this compound plays a role as antimicrobial and antiseptic. Therefore, it has been used for food and feed preservation (HYLDGAARD et al., 2012) and can modulate ruminal fermentation (CALSAMIGLIA, 2007; HASSAN et al., 2020).

The doses of eugenol decreased the DML quadratically ($p < 0.01$). In the same direction, Ju et al. (2020) observed that eugenol and citral (0.23 mg/mL) decreased the growth of *Aspergillus niger* when measuring the shelf life of bread. The elevated antimicrobial potential of eugenol can increase the permeability of the cell membrane and its OH group can significantly inhibit the activity of related enzymes, ATPase for example (WORANUCH & YOKSAN, 2013). When the eugenol changes the permeability of the cell, as well as the microorganism develops a mechanism to balance its functions. These adjustments cause a delay in cell growth and often death (BURT, 2004). Our hypothesis is that eugenol was able to promote changes in microorganisms that increased dry matter losses (eg. yeast and filamentous fungi), such as in the trial with cinnamaldehyde, although microbiological analysis was not measured during the 10 days of aerobic exposure. The DML data can suggest this possible cause and can be supported by the literature. THOROSKI et al. (1989), when evaluating the action of eugenol on *Bacillus cereus* strains, realized that this compound causes the death of these microorganisms due to the stimulation on essential enzymes, for example, production of alpha-amylase and ATPase. In yeast, ATPase plays a role in maintaining the Na+ pump, in addition to other essential processes in cell maintenance stimulation (HYLDGAARD et al., 2012). The yeast count was not accounted in this experiment; however, the data on dry matter losses after

aerobic stability are an indication that eugenol may have altered the production of some important enzymes in the growth of microorganisms, since the dry matter losses reduced as eugenol doses increased.

The same pattern of cinnamaldehyde response occurred with eugenol, where the value of pH until day 6 was 4.0 and 4.5, and after day 8 it increased to 6.5.

Carvacrol

Carvacrol presents a strong antimicrobial activity, with a minimum inhibitory concentration of carvacrol ranging from 64 to 256 µg/mL (MAGI et al., 2015). In this trial, the carvacrol quadratically affected DML, AS, and accumulated temperature. These variables in silage are related to the action of microorganisms (e.g., yeast, filamentous fungi, *Clostridium*) and the antibacterial action of carvacrol, which is stronger against gram-positive (e.g., *Listeria*, *Bacillus*) than gram-negative bacteria (eg. *E. coli*, *Pseudomonas aeruginosas*), mainly due to the bacterial membrane damage. It results in the dissolution of the proton motive force and subsequent reduction in ATP synthesis, which leads to the reduction of other energy-dependent cell processes, including the synthesis of enzymes and toxins (NOSTRO and PAPALIA, 2012). It is possible to see this effect on variables, for example, DML, when the carvacrol, characteristic to control undesirable microorganism, reflected a greater recovery of dry matter.

Allyl isothiocyanate

WCGF stability data without using additives are in agreement with OROSZ et al. (2013). AITC was the additive that showed the best results, with a dosage of 30 mg/kg of fresh forage, which was able to maintain the aerobic stability of the WCGF for about 6 days, possibly by its antimicrobial potential. The aerobic stability is very important for silage production, since silages with more hours under aerobic stability can be related to a less dry matter loss attributed to the consume of substrates produced during the fermentation by microorganisms (TABACCO et al., 2009). An increase in temperature is a convenient indicator of the extent and intensity of aerobic deterioration, even in experimental and practical conditions. Moreover, greateraerobic stability could help to preserve the metabolites such as palmitic acid, stearic acid, linoleic acid, phenylalanine, alanine, beta-alanine, and asparagine during aerobic exposure (CHUNSHENG et al., 2020). These compounds are extremely important to ruminants since they are related to

several metabolisms. One example of that is the effect of supplementing palmitic acid in dairy cattle, that have improved feed efficiency in dairy cows (RICO et al., 2014a).

AITC has been used to improve the shelf-life of foods for humans. The addition of 0.1% AITC has been effective to improve the shelf-life and improve the quality, without changing the qualitative characteristics in kimchi fermentation (KO et al., 2012). On the other hand, the AITC inclusion, such as an additive in kimchi, as described by Ko et al. (2012), promoted an increase in pH, diverging from the results found in this work, where the pH values were not altered with the inclusion of AITC. The AITC can be easily degraded under specific conditions, such as, in the environment that is exposed to high temperatures, alkaline pH, and in the presence of water, giving rise to various compounds such as: allyl dithiocarbamate, diallyl tetra- and penta-sulphide, sulfur and N, N′-diallylthiourea (KAWAKISHI et al., 1969; TSAO et al., 2000). These aspects may explain why AITC inclusion did not affect at day 6 and on, during aerobic exposure, WCGF reached a temperature of 46.8 °C, probably this temperature increase caused volatilization and degradation of AITC molecules and AITC doses were efficient in quadratically reducing dry matter and increasing aerobic stability.

13 CONCLUSION

In summary, with the results presented in these five experiments, it is possible to conclude that the compounds thymol and cinnamaldehyde do not represent an alternative as additives in the aerobic stability of the wet corn gluten feed. Thymol had no effect on the measured variables and cinnamaldehyde promoted an increase in dry matter losses during aerobic stability. However, eugenol and carvacrol, with a maximum dose 400 mg/kg of FF reduced the dry matter losses. Concomitantly, the compound that at the same time reduced dry matter losses and increased aerobic stability was the allyl isothiocyanate, with the maximum dose of 30 mg/kg FF.

Conflict of interest

All authors declare there are no conflicts of interest.

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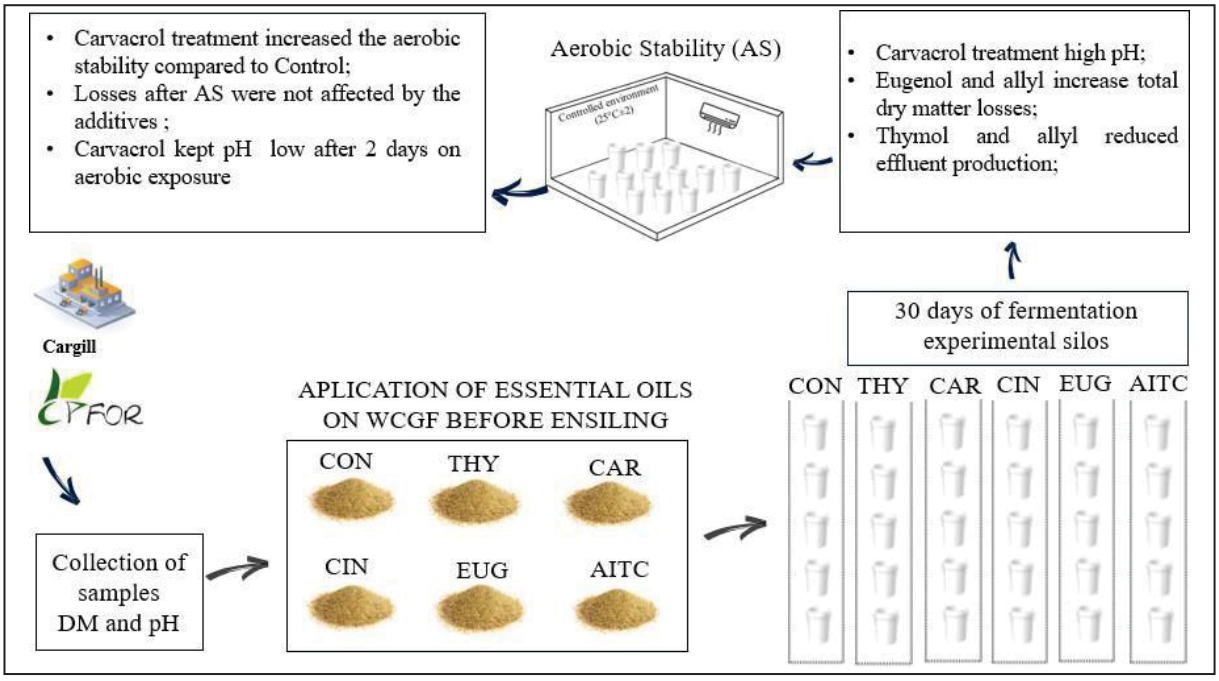
14 CHAPTER 4 - EFFECT OF INCLUSION OF DIFFERENT ESSENTIAL OILS AT ENSILING OF WET CORN GLUTEN FEED

Highlights

- Wet corn gluten feed is one of the most common by-products of corn processing.
- Essential oils are well known for controlling microbial growth.
- Allyl isothiocyanate improve total dry matter losses.
- Carvacrol and thymol improve the aerobic stability.
- After 2 days of aerobic exposure the treatment carvacrol decrease the pH value.

Graphical abstract



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ABSTRACT

Wet corn gluten feed (WCGF) is a by-product from corn processing produced during the extraction of starch and oil. Therefore, it contains a high concentration of protein and fiber, and can be used in dairy cow diets. For preserving the quality of WCGF, it is necessary to use additives when it is stored as silage. Thus, the objective of this study was to determine the effect of the inclusion of different essential oils on the fermentative losses, aerobic stability, and pH value during aerobic exposure. The experiment was a completely randomized design with six treatments: Control (without additive), thymol, carvacrol, eugenol, cinnamaldehyde, and allyl with 99% purity (150, 400, 350, 100, 30 mg/kg of fresh forage of each respective compounds, respectively) with five replicates per treatment, totaling 30 experimental units. The additives were applied and homogenized to the material at ensiling. Each experimental unit consisted of one 8-L plastic bucket with a density of approximately 748 m^3/ton. Silos were opened 35 days after ensiling to evaluate the dry matter, fermentative losses, aerobic stability, and pH. Samples were collected on day 0 after opening the silos and every two days during 10 days of aerobic exposure. Data were analyzed using PROC MIXED on SAS with significance declared at $p \leq 0.05$. Silage with carvacrol showed a greater dry matter (DM) concentration than silage with allyl (41.9% DM *vs.* 40.8% DM, $p < 0.03$). Whereas eugenol inclusion increased the production of effluents when compared with thymol (11.76% *vs.* 6.14%, $p < 0.01$), and had a greater DM loss when compared to carvacrol (7.96 % *vs*. 5.63%, $p < 0.04$). Carvacrol inclusion increased the aerobic stability compared to Control (22 *vs.* 15 h, $p < 0.01$). AITC inclusion showed a greater gas production compared to carvacrol ($p < 0.01$; 6.9% *vs*. 4.84%). However, losses after aerobic stability were not affected by the additive's inclusion ($p = 0.70$). There was an interaction between treatment × days of aerobic exposure on the pH value ($p < 0.01$). Overall, the inclusion of essential oils at ensiling of WCGF affected the fermentative losses, aerobic stability, and pH value during the aerobic exposure. For this experiment carvacrol treatment was more efficient to increase the aerobic stability.

Key-words: Aerobic stability. By-product. Corn. Terpenoids

15 INTRODUCTION

Protein and energy sources represent a high cost in TMR for dairy cows, thus, producers are constantly looking for alternatives and cheaper sources, to reduce feeding costs. Given this fact, the wet corn gluten feed (WCGF) is co-produced in wet corn milling, with high levels of protein 23.9% DM and fiber 44.7 % DM, and represents one great alternative to composing the TMR (ZHANG et al., 2021). However, unlike dry corn gluten feed (DCGF), which is easy to store because it is a dry and stable product, WCGF represents a challenge when considering storage. The degradation process is very fast, so additives are used to increase aerobic stability. Faced with this challenge, some types of additives reduce dry matter losses and increase aerobic stability, including organic acids and some foods with higher dry matter (e.g. wet corn gluten feed) content (ZHANG et al., 2020).

Thus, WCGF storage must be carried out in an anaerobic environment (silage) to maintain its nutritional characteristics, but after the contact with oxygen, this by-product rapidly changes its characteristics, such as texture and smell, among other properties. Such changes cause the animals to reject the feed. Thus, the use of additives during fermentation and after opening can minimize these effects, often caused by the growth of spoilage microorganisms. Given this problem, the objective of this experiment was to evaluate the use of five essential oils (thymol, carvacrol, cinnamaldehyde, eugenol, and allyl isothiocyanate) as additives during WCGF fermentation in experimental silos and the aerobic stability of this co-product after the fermentation.

Essential oils are studied as additives in human foods and food packaging applications (Sharma et al., 2021), due to their antimicrobial capacity and the acceptance by consumers because they are classified within the GRAS category, according to the FDA. The essential oils can be divided into four categories: Terpenes (Monoterpenes, e.g., Limonene and *P*-cymene), Terpenoids (monoterpenoids, e.g., Thymol and Carvacrol), Phenylpropanoids (e.g., Cinnamaldehyde and Eugenol) and the Allyl isothiocyanate and Allicin. However, the lasttwo compounds do not have a specific category (HYLDGAARD et al., 2012). Basically, this difference between the chemical structures is defined by the site of synthesis and formation of the compound. As described by CABALLERO et al. (2013), inside of cytoplasm of plant cell and through mevalonic acid pathway starting from acetyl-Co. These same authors describe that terpenoids are derived terpenes when this compound is submitted to biochemical modifications

caused by enzymes that add or remove methyl groups. On the other hand, phenylalanine is an important component to protein formation in plants. The amino acid precursor of this compound is synthetized and produces phenylpropanoids. This differences between the synthesis of the essential oils, for example carbon, methyl group and hydrocarbon backbone, which can be rearranged into cyclic structures by cyclases, can explain the differences in antimicrobial effect on different microorganisms.

Therefore, the objective of this experiment was to determine the effect of the inclusion of different essential oils on the fermentative losses, chemical and microbial analysis, aerobic stability, and pH value during aerobic exposure.

16 MATERIAL AND METHODS

16.1 LOCAL

The experiment was carried out in January 2021 at the "Centro de Pesquisa em Forragicultura" (acronym in Portuguese CPFOR; -25.38700262836833649.12664794483578), Pinhais, Paraná - Brazil. The wet corn gluten feed was immediately collected (Cargill; 4.04673053811548, -50.39232530223162 – Castro, Parana - Brazil) and transported to CPFOR.

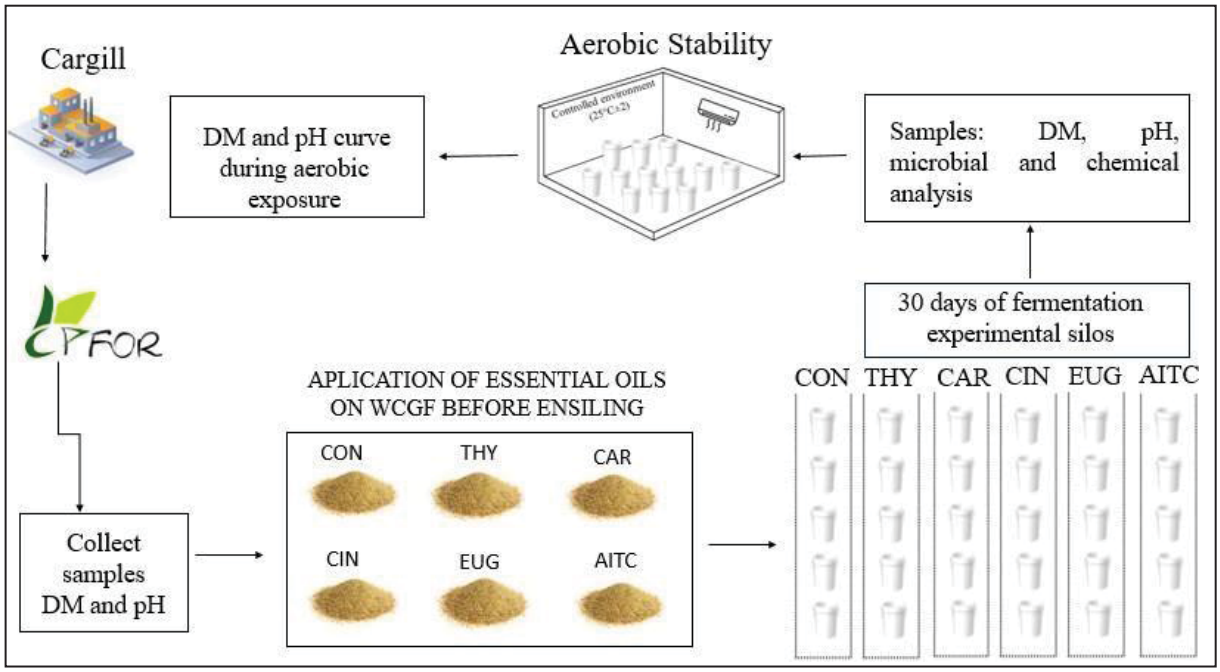
16.2 TREATMENTS

The treatments were composed of five essential oils and their respective doses and the doses were applied in mg/kg of fresh forage (FF): Thymol (THY) - 150 mg/kg FF (fresh forage), cinnamaldehyde (CIN) - 100 mg/kg FF, eugenol (EUG) - 350 mg/kg FF, carvacrol (CAR) - 400 mg/kg FF, and allyl isothiocyanate (AITC) - 30 mg/kg FF., and the form of application was similar to the experiment 2 – chapter 3 (Figure 17-1). However, differently from the other experiment, on this, the silos remained closed for 30 days. At the time of loading the WCGF at the Cargill company, a sample was collected in order to verify the pH, that was 3.99. After two hours of transportation to the CPFOR and at the time of setting up the experiment, another sample was collected to determine the pH, that corresponded to 4.09. Before ensiling, samples were collected to measure the values of DM, microbiological, and chemical composition. In Table 17-1 it is possible to verify the characterization of the wet corn gluten feed of each treatment before ensiling. The mean compaction of the ensiled mass was 748 kg/m^3. The analyzes of fermentative losses were performed according to Jobim et al. (2007).

For chemical analysis, 200 g of each replicate were dried in a forced-air ventilation oven (55 ºC) and ground in a Willey mill (Model #2, Arthur H. Thomas Co., Philadelphia, PA) with a 1 mm mesh sieve and stored for posterior analysis. Subsequently, 2 g of the samples were used to determine the DM content at 105°C (method number 934.01; AOAC, 1990), ash (method number 924.05; AOAC, 2012), crude protein (CP) by the Dumas method (FP-528, Leco, combustion N analyzer, Leco Instruments Inc., St. Joseph, MI), according to Wiles et al. (1998). Neutral detergent fiber (aNDF) with thermostable alpha-amylase and sodium sulphite (MERTENS, 2002), and acid detergent fiber (ADF) (VAN SOEST, 1973), were sequentially determined using an Ankom A200 Fiber Analyzer (ANKOM Technology, Macedon, NY). Ammonia nitrogen content was determined by colorimetric method, according to Eweatherburn (1967).

The pH was determined using a digital pHmeter (PG 1400, Gehaka – Brazil) according to Kung et al. (2000), where 25 g of samples were diluted in 225 mL of distilled water and homogenized for one min., and the extract was used to the measurement, and another sample of fresh forage was collected before ensiling and was also analyzed for pH, DM, CP, aNDF, ADF, and ash as described above, and the values are presented in the Table 17-1.

FIGURE 15 - SCHEMATIC REPRESENTATION OF THE PROCESSES CARRIED OUT DURING THE EXPERIMENTAL



CONTROL-CON; THYMOL – THY; CARVACROL – CAR; CINNAMALDEHYDE – CIN; EUGENOL – EUG; ALLYL ISOTHIOCYANATE – AITC; DRY MATTER – DM;

For aerobic stability tests, 2 kg of silage were placed in 20-L plastic buckets (experimental silos), and the material remained for 10 days in a temperature-controlled room at 25 °C. A thermal sensor (EL-USB-1, Lascar Electronics Inc., Erie, PA, USA) was inserted into each bucket, and the temperature recording was performed every 5 minutes during the 240 hours of exposure to air. Therefore, stability breakage was determined when the silage reached 2°C above ambient temperature, according to MORAN et al. (1999). On day 0 and at the end of 10th day (240 hours), the buckets were weighed to determine dry matter losses during exposure to air. Furthermore, the pH curve was performed during the aerobic stability test. For this, 1 kg of silage was allocated in 8–L buckets correspondent to each replication per treatments, 25 g of samples for each replicate were collected every two days, and the pH was measured in a digital potentiometer (PG 1400, Gehaka – Brazil), according to KUNG et al. (2000).

TABLE 16-1 - CHEMICAL COMPOSITION OF WET CORN GLUTEN FEED BEFORE ENSILING.

Variables[1]	Treatments[2]						SD[3]
	CON	THY	CAR	CIN	EUG	AITC	
DM, %	43.61	43.61	43.61	43.61	43.61	43.61	0
Ash, % DM	5.47	5.8	6.21	4.73	5.25	6	0.541
CP, % DM	22.5	23.8	24.9	21.2	22.9	24.3	1.343
EE, % DM	1.3	1.3	1.23	1.37	1,36	1,21	0.065
NDF, % DM	43.7	43.7	42	47.8	45.8	43,3	2.072
ADF, % DM	11.7	11.7	10.8	12.9	12.4	11,8	0.714
WSC, % DM	24.3	25.4	25.5	24.9	24.6	25,1	0.463
NH3-N-, % DM	6.51	7.85	8.04	8.48	6.51	8,88	0.997

[1] Dry matter (DM); Crude protein (CP); Ether Extract (EE); Neutral detergent fiber (aNDF); Acid detergent fiber (ADF); Water soluble carbohydrates (WSC); N-NH_3 ; [2] Control (CON - without additives); Thymol (THY – 150 mg/kg FF); Carvacrol (CAR- 400 mg/kg FF); Cinnamaldehyde (CIN- 100 mg/kg FF); Eugenol (EUG – 350 mg/kg FF); Allyl isothiocyanate (AITC – 30 mg/kg FF). [3]SD = Standart deviation.

16.3 STATISTICAL ANALYSIS

The experimental design was completely randomized, composed of six treatments (CON, THY, CAR, CIN, EUG, and AITC) and 30 experimental units (five replicates by treatment). The normality was tested by Shapiro-Wilk test and homogeneity of all data were analyzed by the PROC UNIVARIATE using SAS. To confirm that the treatments influenced the response variable (evaluated parameters), the data were submitted to analysis using a MIXED model at a 95% level of confidence, following the model:

$$y_{ijk} = \mu + h_i + T_j + e_{ijk}$$

Where: y_{ijk} is the response variable (evaluated parameters), μ is the average of the replicates; h_i is the random effect of hours; T_j is the fixed effect of treatment, and e_{ijk} is the residual effect.

Tukey test was used to compare treatment means, and significance was declared at $P < 0.05$. All analysis (descriptive and confirmatory) were performed using the SAS statistical software, version 9.1.3 for Windows (SAS, 2002).

For the pH analyses, repeated measurements were carried out over time, for each oil studied, and the collections took place every two days (0, 2, 4, 6, 8 10 during the period of aerobic exposure). After that, to confirm the effect of different doses on the response variable (evaluated parameters), the data were submitted for analysis of variance (ANOVA); when significant, the mean values were compared by the Tukey test with 95% of confidence level.

17 RESULTS

17.1 FERMENTATIVE LOSSES

Dry matter (DM), pH, effluent losses, gas losses, and total dry matter loses (TDMLosses) are presented in Table 18-1. All variables (DM, pH, gas and TDMLosses) were significantly affected by treatments ($P < 0.05$), except gas losses ($P = 0.04$). The average DM of WCGF was 4.24%, while the treatment AITC presented 2.6% less DM than the CAR. The average of pH, effluent and TDMLosses was (4.10, 7.46, and 6.11; with $P = 0.01$, 0.01 and 0.01, respectively). The pH values of the AITC (pH = 4.07) and CON treatments had a percentage reduction of 2.6% compared to the CAR treatment (pH 4.06 *vs.* 4.17). Regarding gas losses, the CAR treatment showed a reduction of 24% in losses when compared to other treatments. When we evaluated the TDMLosses, the EUG and AITC treatments together presented an average of 7.68% of total dry matter losses, that correspond to an increasing of 38% compared to the average of CAR and CON treatments with 5.57%.

TABLE 17-1 - FERMENTATIVE LOSSES OF WET CORN GLUTEN FEED WITH DIFFERENT ADDITIVES.

Variables[1]	Treatment[2]						SEM[3]	p-value
	CON	THY	CAR	CIN	EUG	AITC		
DM, %	41.45^{ab}	41.16^{ab}	41.90^{a}	41.33^{ab}	41.01^{ab}	40.80^{b}	0.221	0.03
pH	4.06^{c}	4.08^{bc}	4.17^{a}	4.11^{bc}	4.12^{ab}	4.07^{c}	0.013	0.01
Efflosses, % kg FF	6.61^{ab}	6.14^{b}	8.20^{ab}	6.90^{ab}	11.76^{a}	5.20^{b}	1.252	0.01
Gas losses, % DM	5.79	6.50	4.84	5.81	6.85	6,92	0.488	0.05
TDMlosses, % DM	5.51^{b}	7.08^{ab}	5.63^{b}	6.47^{ab}	7.96^{a}	7.41^{a}	0.401	0.01

[1] Dry matter (DM); Effluent losses (EFlosses); Total dry matter losses (TDMlosses). [2] Control (CON - without additives); Thymol (THY – 150 mg/kg FF); Carvacrol (CAR- 400 mg/kg FF); Cinnamaldehyde (CIN- 100 mg/kg FF); Eugenol (EUG – 350 mg/kg FF); Allyl isothiocyanate (AITC– 30 mg/kg FF). [3] Standard error of mean. Variables with $p < 0.05$ had significant influence of treatments.

17.2 CHEMICAL ANALYSIS

The chemical composition of WCGF after 30 days of fermentation are showed in Table 18-2. Ash, Crude protein (CP), ether extract (EE), aNDF, and ADF were affected by treatments ($P < 0.05$).

TABLE 17-2 - FERMENTATIVE LOSSES OF WET CORN GLUTEN FEED WITH DIFFERENT ADDITIVES.

Variables[1]	Treatment[2]						SEM[3]	*p*-value	
	CON	THY	CAR	CIN	EUG	AITC			
Ash, % DM	5.45^{b}	5.58^{b}	6.08^{a}	5.74^{ab}	6.11^{a}		6.12^{a}	0.104	< 0.01
CP, % DM	23.72^{b}	24.08^{ab}	25.00^{ab}	24.02^{ab}	25.20^{a}		25.06^{a}	0.305	< 0.01
EE, % DM	1.19^{bc}	1.15^{c}	1.14^{c}	1.37^{a}	1.26^{abc}		1.34^{ab}	0.039	< 0.01
aNDF, % DM	45.52^{ab}	44.96^{ab}	44.32^{ab}	45.98^{a}	43.76^{b}		43.98^{ab}	0.494	<0.02
ADF, % DM	12.28^{ab}	11.74^{b}	11.56^{b}	12.86^{a}	12.08^{ab}		12.08^{ab}	0.223	< 0.01
WSC, % DM	24.10	24.16	23.40	22.84	23.56		24.40	0.426	<0.13
N-NH_3, % DM	8.07	7.89	7.53	8.31	8.10		7.58	0.318	<0.47

[1] Dry matter (DM); Crude protein (CP); Ether extract (EE); Neutral detergent fiber (aNDF); Acid detergent fiber (ADF); Water soluble carbohydrates (WSC); Ammoniacal – N (NH_3-N); [2] Control (CON) without additives; Thymol (THY – 150 mg/kg FF); Carvacrol (CAR- 400 mg/kg FF); Cinnamaldehyde CIN- 100 mg/kg FF); Eugenol (EUG – 350 mg/kg FF); Allyl isothiocyanate (AITC – 30 mg/kg FF). [3] Standard error of mean; Variables with $p < 0.05$ had significant influence of treatments.

17.3 MICROBIAL ANALYSIS

The population of homofermentative and heterofermentative bacteria, yeasts, and moulds are shown in Table 18-3. Heterofementative bacteria, yeast, and moulds, after 30 days of fermentation WCGF were not affected significantly by treatments.

TABLE 17-3 - MICROBIAL POPULATIONS OF WET CORN GLUTEN FEED AFTER 30 DAYS OF FERMENTATION.

Variables[1] (log cfu/g FF)	Treatment[2]						SEM[3]	p-value
	CON	THY	CAR	CIN	EUG	AITC		
LAB homo	1.43	1.35	2.00	1.45	0.86	3.17	0.857	0.51
LAB hete	0.69	1	1.38	0.60	1.67	2.97	0.744	0.11
Yeasts	4.86	4.81	4.65	4.73	4.75	2.84	0.476	0.05
Moulds	1.26	2.27	2.10	2.13	1.86	0.75	0.830	0.76

[1] Homolactic Lactic acid bacteria (LAB homo); Heterolactic Lactic acid bacteria (LAB hete); Yeasts; Moulds; [2] Control (CON) without additives; Thymol (THY – 150 mg/kg FF); Carvacrol (CAR- 400 mg/kg FF); Cinnamaldehyde (CIN- 100 mg/kg FF); Eugenol (EUG – 350 mg/kg FF); Allyl isothiocyanate (AITC – 30 mg/kg FF). [3] Standard error of mean; Variables with $p < 0.05$ had significant influence of treatments.

17.4 AEROBIC STABILITY AND PH CURVE

The values of aerobic stability, maximum temperature (MT), hours to maximum temperature (HMT), accumulated temperature, and total dry matter losses after 10 days of aerobic stability ($TDML_{AS}$) are presented in Table 18-4. The aerobic stability was affected by treatments ($p < 0.05$). The treatments (CAR, CIN, and AITC) presented an average of 20.84 hours of aerobic stability, 5 hours more than CON (15.44 hours). The average HMT corresponded to 34.19°C, during 240 hours of exposure to air.

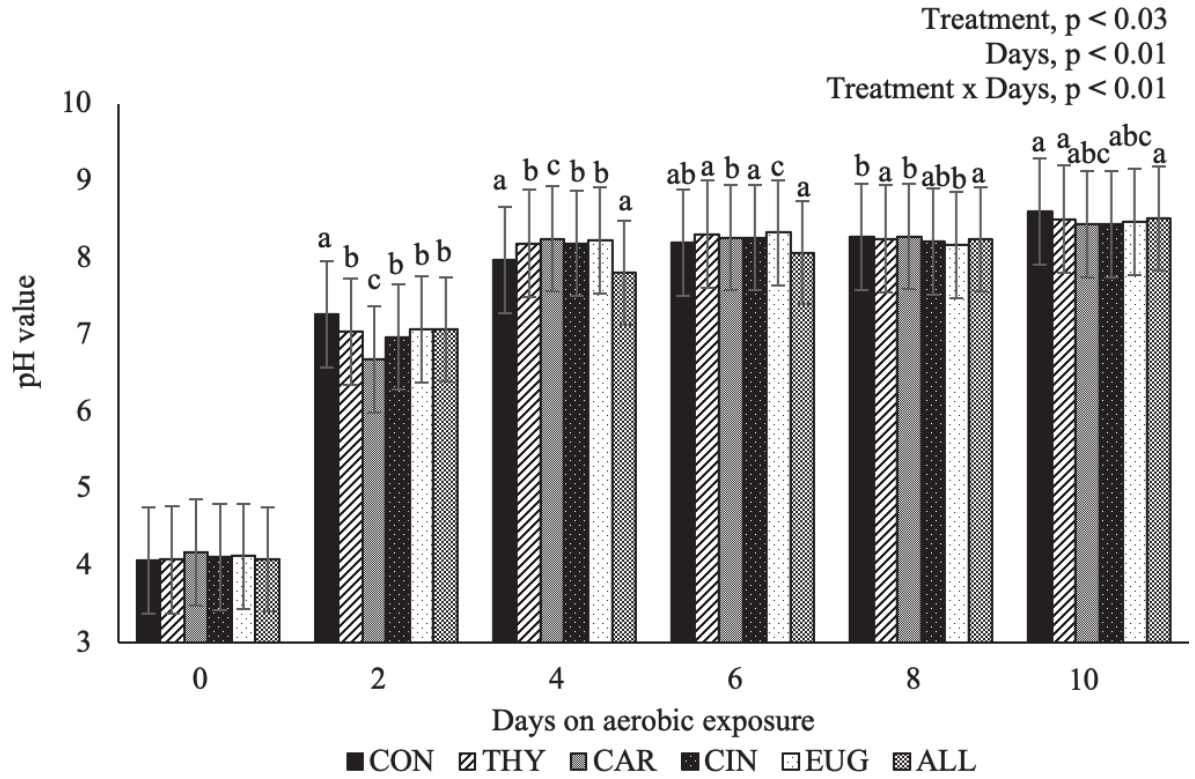
TABLE 17-4 - AEROBIC STABILITY OF WET CORN GLUTEN FEED AFTER FERMENTATION.

Variables[1]	Treatment[2]						SEM[3]	p-value
	CON	THY	CAR	CIN	EUG	AITC		
AS, h	15.44^{c}	17.08^{bc}	22.41^{a}	19.78^{ab}	18.43^{abc}	20.34^{ab}	1.065	0.01
MT, ºC	49.10^{a}	47.70^{ab}	46.20^{ab}	44.40^{b}	46.85^{ab}	45.87^{ab}	3.917	0.08
HMT, h	26.31^{b}	24.90^{b}	30.89^{ba}	35.43^{ab}	35.43^{ab}	52.21a	6.281	0.01
Tacu, ºC	16625	15758	13508	12736	15732	13944	966.23	0.06
$TDML_{AS,}$ % DM	13.66	12.96	13.63	11.81	11.50	12.39	2.348	0.96

[1] Aerobic stability (AS); Maximum temperature (MT); Hours to Maximum temperature (HMT); (Tacu, °C) = accumulated temperature evaluated during the aerobic stability tests;Total dry matter losses after aerobic stability ($TDML_{AS}$); [2] Control (CON) without additives; Thymol (THY – 150 mg/kg FF); Carvacrol (CAR- 400 mg/kg FF); Cinnamaldehyde (CIN- 100 mg/kg FF); Eugenol (EUG – 350 mg/kg FF); Allyl isothiocyanate (AITC – 30 mg/kg FF). [3] Standard error of mean; Variables with $p < 0.05$ had significant influence of treatments.

The pH value during aerobic exposure is presented in Figure 18-1. On the first day after opening the silos and exposure to air, there was no difference ($p > 0.05$) in pH values averaging 4 for all treatments. From day 2 of exposure to air, it was observed a statistical difference between the treatments ($p < 0.05$), where carvacrol was the additive that was more effective in maintaining the pH.

FIGURE 16 - PH CURVE DURING AEROBIC EXPOSURE



*Control (CON) without additives; Thymol (THY – 150 mg/kg FF); Carvacrol (CAR- 400 mg/kg FF); Cinnamaldehyde (CIN- 100 mg/kg FF); Eugenol (EUG – 350 mg/kg FF); Allyl isothiocyanate (AITC – 30 mg/kg FF)

18 DISCUSSION

The wet corn gluten feed can compose the diet of different animal categories due to its nutritional value. In the literature, there are examples of several experiments where wet corn gluten feed was included in the composition of the total mixed ration (Mullins et al., 2010; Zhang et al., 2020). Conserving this food after the extraction of corn oils is another challenge for the industry. For this reason, the objective of this experiment was to evaluate the addition of essential oils as additives in WCGF fermentation.

Fermentative losses

The average DM concentration (43.6% before ensiling and 41.2% after 30 days of fermentation) for all treatments in this experiment is lower than those described by Mullins et al. (2010), França et al. (2015), and ZHANG et al. (2020) that in both studies the WCGF

contained DM concentrations of approximately 61%. However, these DM values do not affect the fermentation process, a great indicator is the pH, in this experiment the average value was 4.10, while Zhang et al. (2020) found values of 4.29 in silos with 30 days of fermentation.

The pH values above 4.5 presented a great challenge, as this value favors the growth of yeasts, which causes a decrease in the nutritional value. In this experiment, the yeast count was on average 4.44 CFU/g FF, that is in agreement with the results observed by França et al. (2015) in WCGF silages with 21 days of fermentation. On the other hand, Silva (2020) found values of 6.5 CFU/g FF for WCGF silage after 30 days of fermentation, that is justified by the high concentration of epiphytic yeasts in the WCGF. In corn silage, these yeast counts are also associated with the DM losses that occur within the silo (Pahlow et al., 2003). The WCGF pH values collected at Cargill with 3.99 and the values collected at CPFOR after two hours of transport (4.09), indicate that even in this pH range promoted by lactic acid from the maceration process, it is not efficient in controlling the growth of spoilage microorganisms. In addition, there was a variation in compaction density between the present study, that corresponded to 748 kg/m^3 compared to the one adopted by FRANÇA et al. (2015) and SILVA, (2020), 671.3 and 750 kg/m^3, respectively.

The combination of losses through the production of gases + the losses through the production of effluent was performed to obtain the total losses of dry matter during fermentation. These characteristics are parameters to evaluate how the additives can interfere in the fermentation process. In this experiment, the average gas losses were 6.17%, that was close to the values found by FRANÇA et al. (2015) (5.08 %) in WCGF silages with 28 days of fermentation. The effluent losses are mainly related to the theories of DM and compaction density of the ensiled material, in this article the initial dry matter was the same for all treatments, and as silages were filled all treatments at the same time, that is, the effect of an effluent production from the Eugenol treatment compared to AITC and THY was due to some mechanism caused during the fermentation process. To date, Eugenol is only related to cell lysis of bacteria and fungi filamentous, and nothing has been described in the literature about this action on components of plants such as NDF and lignin.

Chemical analysis

The WCGF chemical composition values before ensiling are similar to the values after 30 days of fermentation, except for aNDF in the AITC treatment, that before ensiling was 45.8%

and after 30 days of fermentation this value reduced to 32.7%. This value of aNDF was much lower than the values found by França et al. (2015) when they performed the experiment testing different fermentation times (1, 3.7, 14, 21, 28, and 42) that the average aNDF of 49.5%. This maintenance of similar chemical composition values before and after the fermentation period may indicate that the conservation method in the form of silage is efficient in maintaining the nutritional qualities of the WCGF. On the other hand, the treatment containing AITC causes changes in the aNDF content during the fermentation process. AITC can cause changes in the cell membrane of corn, causing the bioavailability of soluble carbohydrates. Regarding the CP contents, Eugenol and AITC compared to the control after 30 days of fermentation obtained higher protein values. This finding may be related to the ability of essential oils to control the presence of specific enzymes, such as Eugenol inhibiting histidine decarboxylase, responsible for the production of histamine, an important biogenic amine or proteases (GILL and HOLEY, 2006b). Our hypothesis is that this action may preserve the protein levels in WCGF, when Eugenol interrupts this system in bacteria and yeast.

Regarding the ADF values, the thymol and carvacrol are isomers compounds that differ only by the position of the aromatic ring hydroxyl group, showed a lower concentration when compared to CIN. One hypothesis of this possible difference would be the action of the hydroxyl radical capable of degrading hemicellulose and lignin. This hydroxyl radical effect was described by Green III and HIGHLEY (1997) in brown rot fungi such as *C. puteana*, where hydroxyl radicals attack the hemicellulose enveloping cellulose microfibrils that probably represent the crucial step in the initial stages of brown-rot wood decay.

Microbial analysis

The literature describes that the lactic acid bacteria population to obtain a good fermentation is approximately 10^8 CFU/g FF (Muck et al., 2010). In this experiment the LAB values were lower. The microorganism count was performed only on the day of opening the silos, but during the fermentation process it may have occurred differently. The fermentation process is complex, and the colonies of microorganisms change according to the substrate and the medium. In general, the average number of microorganisms (LAB, yeast, and molds) was very low, especially when compared to the data presented by FRANÇA et al. (2015) with 28

days of fermentation (5.8 CFU/g FF Lab; yeast or molds). To explain this value, perhaps the characteristics of plants, or during the process extraction can alter the microbiological results.

Aerobic stability and pH curve

The aerobic stability and pH value are indicative of the fermentation process (MUCK et al., 2018). Carvacrol may have selected heterolactic bacteria during the fermentation process. In general, the wet corn gluten feed represents a challenge in the production of silages, especially after exposure to air. SILVA (2020), when evaluating the use of propionic acid and sodium benzoate in WCGF, observed an increase in aerobic stability and a reduction in dry matter losses after 10 days of exposure to air, reinforcing the hypothesis that the use of additives in WCGF silage is essential, especially when the silo is exposed to air. This fact can be observed by the pH values at the first day of aerobic exposure, that was 4 and on the second day of aerobic exposure the value increased to 7.

19 CONCLUSION

Overall, the inclusion of essential oils at ensiling of WCGF affected the fermentative losses, aerobic stability, and pH value during the aerobic exposure. The treatments containing carvacrol (400 mg/kg FF) were more efficient in improving aerobic stability, followed by cinnamaldehyde (100 mg/kg FF) and allyl isothiocyanate (30 mg/kg FF). The effects of the compounds happened in an isolated way, and as a suggestion for the next articles would be the combination of oils in order to obtain a synergism, resulting in the potentiation of results, for example the combination of different groups of terpenes e.g., carvacrol and eugenol or thymol and cinnamaldehyde.

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20 FINAL CONSIDERATIONS

An empirical consideration on the action of essential oils as an additive in corngluten feed. This co-product is susceptible to degradation, and after milling in the first 24 hours the pH remains around 4, but after 6 days exposed to air this value doubles. At the same time for all experiments, from the 6th day it was possible to visualize the growth of filamentous fungi, the color and texture of the wet corn gluten feed were changed over the 10 days of exposure to air. In addition, the conservation of wet corn feed under exposure to air becomes a major challenge.

21 FUTURE DIRECTIONS

The first author to use essential oils as an additive in corn silage was Kung et al. (2008). As suggested by other authors, up to the time of writing this material, 11 papers were published on these subjects.

In each work, the authors used different doses, some used essential oils with a high purity index and others extract from plants, where the composition is varied according to pollinators and climate. For this reason, the data found in the literature are divergent, and the effects of compounds on fermentation are still incipient.

Based on this, the proposal for future work is to use essential oils with 95% purity, in addition to testing different combinations, in order to observe the synergism between different types of essential oils.

22 APPENDIX 1 – HIGH PURITY COMPOUND COSTS

Additive	Price per g ($)
Allyl isothiocyanate	5.98
Cinnamaldehyde	0.78
Eugenol	7.22
Carvacrol	5.75
Thymol	0.47

The value of essential oils was provided at Sigma-Aldrich, Day 07-08-2022.

Dollar exchange rate 07-08-2022, $1.00 - R$ 5.31

Wet Corn gluten Feed	$132.00/ per ton

23 SUPPLEMENT A – PICTURES OF WET CORN GLUTEN FEED DURING AEROBIC EXPOSURE

Sensors to measure temperature of wet corn gluten feed during aerobic exposure

24 SUPPLEMENT B – PICTURES OF WET CORN GLUTEN FEED DURING AEROBIC EXPOSURE

Wet corn gluten feed,10 days after aerobic exposure.

Wet corn gluten feed after 30 days of fermentation

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