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ANTIMICROBIAL ACTIVITY OF MUSTARD SEED MEAL POLYPHENOLS

Andrei Cristian ANGHEL[1, 2*], Ionelia ȚĂRANU[2], Alina ORȚAN[1], Narcisa Elena BĂBEANU[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania
[2]National Research-Development Institute for Animal Biology and Nutrition (IBNA),
1 Calea Bucuresti, Balotesti, 077015, Ilfov, Romania

Corresponding author email: andrei.anghel@ibna.ro

Abstract

The weaning period is critical in piglet development, marked by a weakened immune system that makes piglets susceptible to E. coli F4 infections. This bacterium disrupts the intestinal lining, causing severe diarrhea, which hinders nutrient absorption and slows growth, often leading to high mortality rates. While antibiotics and zinc oxide have helped manage these infections, stricter regulations drive the need for alternatives. Polyphenols from agro-industrial by-products are promising due to their antimicrobial, anti-inflammatory, and antioxidant properties. This study investigates the in vitro antimicrobial effects of polyphenol extracts from mustard seed meal against E. coli. Extracts were derived from both unfermented and S. cerevisiae-fermented mustard seed meal to assess fermentation's impact on antimicrobial activity. Over 24 hours, bacterial growth was monitored in varying polyphenol concentrations, with significant antimicrobial effects observed from both extracts. These findings highlight mustard seed meal's potential as an alternative to antibiotics and zinc oxide in piglet farming, promoting a sustainable, circular economy.

Key words*: agro-industrial by-products, antimicrobial, E.coli, polyphenols, weaning.*

INTRODUCTION

The weaning crisis represents a challenging period in piglet development, often associated with a range of physiological and environmental stressors that can negatively impact their health (Rhouma et al., 2017). Weaning typically occurs when piglets' immune systems are still immature, and the sudden removal of maternal milk antibodies makes them more vulnerable to infections, particularly intestinal infections such as post-weaning diarrhea, which is mostly caused by *E. coli* F4 (K88). Additionally, moving piglets to a new environment exposes them to new pathogens, further increasing their vulnerability (Rhouma et al., 2017). *Escherichia coli* F4 infection typically occurs within the first two weeks after weaning. The bacteria use their fimbriae (F4) to attach to the intestinal epithelium, leading to colonization and diarrhea. This condition is often characterized by watery stools, resulting in severe dehydration and electrolyte imbalances, which increase the piglets' vulnerability to other diseases (Luise et al., 2019). Diarrhea also reduces feed conversion efficiency and slows growth. In more severe cases, *E. coli* infection can lead to higher mortality rates. Even after recovery, the extended recovery period continues to hinder the piglets' growth and development (Luise et al., 2019). Antibiotics are essential in treating bacterial infections, improving animal health, preventing the spread of pathogens, promoting faster growth, and enhancing reproductive performance. By reducing mortality and illness rates, antibiotics help mitigate economic losses in the livestock industry (Burow et al., 2019). Due to these benefits, antibiotics are sometimes used prophylactically, which unfortunately contributes to the development of antibiotic resistance (Burow et al., 2019). Furthermore, resistant bacteria from animals can be transmitted to humans through direct contact, the environment, or the food chain, posing a serious public health risk. These infections in humans may become difficult or even impossible to treat with existing antibiotics. Moreover, countries or regions with high levels of antibiotic-resistant bacteria in their livestock might face trade restrictions or bans, impacting the agricultural economy (Burow et al., 2019). As a result, many countries have implemented

stricter regulations on antibiotic use in livestock to combat resistance, including banning certain antibiotics for growth promotion or restricting the use of critically important antibiotics (Burow et al., 2019). In response to the growing issue of antibiotic resistance, the EU took action by banning the use of certain antibiotics as growth promoters in 1997, followed by a complete ban on all antibiotic growth promoters by January 1, 2006. Restrictions on antibiotic use in Europe date back to 1985, when Sweden became the first country to introduce regulations, with Denmark following in 1990. In the United States, the FDA began implementing measures in 2013 to phase out the use of antibiotics for growth promotion. In Asia, the Indian government has issued advisories and circulars aimed at reducing the use of antibiotics in animal feed. However, the lack of strict enforcement and comprehensive legislation remains a significant challenge (Chatterjee et al., 2019).

Zinc oxide is commonly used as an alternative to antibiotics in animal feed, particularly for weanling pigs, to prevent diarrhea and promote growth. It works by reducing pathogenic bacteria in the gut, thereby enhancing gut health. However, its use comes with several drawbacks (Milani et al., 2017). Around 80% of ingested zinc oxide is excreted in feces, leading to environmental pollution and potentially harming local ecosystems (Chen et al., 2022; Zhang et al., 2014). Furthermore, elevated zinc levels in the environment may contribute to the emergence of antibiotic-resistant bacteria. In addition, the use of zinc oxide in pig feed can have toxic effects, as it may cause anemia in piglets by disrupting the regulation of zinc and iron concentrations in their bodies (Ciesinski et al., 2018; Milani et al., 2017). In response to these risks, the European Union (EU) has implemented significant measures to phase out the use of zinc oxide in pig farming. In 2017, the EU decided to restrict medicinal treatments based on zinc oxide, leading to a complete ban on its use in June 2022 (Pejsak et al., 2023). In response to the restrictions on antibiotics and zinc oxide, various agro-industrial residues rich in bioactive compounds have been introduced into pig feed in the hope of alleviating the symptoms of the weaning crisis and enhance piglet health. These residues offer several benefits, including a prebiotic effect, and are abundant in organic acids and polyphenols - compounds known for their significant therapeutic properties (Pejsak et al., 2023). Agro-industrial by-products are rich in bioactive compounds, fibers, antioxidants, and other nutrients that can improve gut health, strengthen the immune system, and promote growth in piglets. For instance, fermented by-products act as effective probiotics, fruit and vegetable by-products are abundant in polyphenols, grain by-products are high in fiber, and seed by-products are rich in oils. All these components work together to enhance gut health, boost immune function, and support growth in piglets (Enciso-Martinez et al., 2024). Another advantage of using agro-industrial by-products is their positive environmental impact. These materials are often disposed of in landfills or other improper sites, where they decompose slowly and contribute to pollution, greenhouse gas emissions, and potential toxicity from waste breakdown. Repurposing these by-products alleviates environmental pressure by reducing the amount of waste sent to landfills and supporting sustainable waste management practices (Enciso-Martinez et al., 2024). This approach also helps mitigate issues such as soil degradation, water contamination, and ecosystem disruption (Enciso-Martinez et al., 2024). Furthermore, utilizing agro-industrial by-products supports a circular economy by promoting recycling, reusing, and repurposing materials that would otherwise be discarded. Instead of being treated as waste, these by-products are converted into valuable resources for industries like food, pharmaceuticals, and cosmetics, fostering a more sustainable and closed-loop system. This reduces the need for raw material extraction, conserves natural resources, and lowers costs related to waste disposal, such as treatment, landfill fees, and transportation, promoting economic sustainability (Enciso-Martinez et al., 2024). Additionally, bioactive compound-rich by-products can serve as natural preservatives and antimicrobial agents in the food industry, decreasing reliance on synthetic chemicals that are harmful to both the environment and human

health. These natural compounds are biodegradable and non-toxic, leading to a smaller environmental footprint (Enciso-Martinez et al., 2024).

Agro-industrial by-products, such as fruit peels, seeds, and pomace, are commonly rich in polyphenols, as these compounds are concentrated in parts of plants typically discarded during food processing. Polyphenols, known for their bioactive properties and potential health benefits, are plant metabolites categorized into four main groups: flavonoids, phenolic acids, tannins, and stilbenes. Many of these by-products contain multiple types of polyphenols, which can work together to enhance their overall bioactivity (Abbasi-Parizad et al., 2022; Correddu et al., 2020; Hernandez-Montesinos et al., 2024; Rosales & Fabi, 2023). Polyphenols offer various beneficial effects. They act as antioxidants, neutralizing free radicals and reducing oxidative stress (Iqbal et al., 2023). Their anti-inflammatory properties are evidenced by their ability to inhibit the expression of inflammatory cytokines like TNF-α, IL-6, and IL-1β (Enciso-Martinez et al., 2024; Yahfoufi et al., 2018). Additionally, polyphenols have antimicrobial activity, disrupting microbial cell membranes and inhibiting key enzymes such as ATPase, which are essential for pathogen survival (Enciso-Martinez et al., 2024). They also promote gut health by modulating the composition of gut microbiota, encouraging the growth of beneficial bacteria while inhibiting harmful species, thereby reducing the need for antibiotics (Enciso-Martinez et al., 2024). Furthermore, polyphenols improve feed efficiency by enhancing nutrient absorption and promoting growth rate (Formato et al., 2022; Mahfuz et al., 2021).

This study investigates the *in vitro* antimicrobial effects of two polyphenolic extracts derived from mustard seed meal (MSM) and fermented mustard seed meal (FMSM) against *Escherichia coli*. Mustard seeds are recognized for being rich in polyphenols, which possess antimicrobial, antioxidant, and anti-inflammatory properties (Tian & Deng, 2020). In addition to evaluating the polyphenol extract from raw mustard seed meal, the study explores the impact of fermenting mustard seed meal with *Saccharomyces cerevisiae* known also as a source of polyphenols. Fermentation can modify the chemical composition of plant materials, potentially increasing the bioavailability and concentration of beneficial compounds. As a result, tests were conducted using polyphenolic extracts from both non-fermented (MSM) and fermented mustard seed meal (FMSM). In general, fermentation led to the formation of antimicrobial peptides and polyphenols with more potent antimicrobial effects (Palacios-Velásquez et al., 2023; Rodríguez et al., 2017; Vlassa et al., 2022). By examining these natural and sustainable alternatives, this research seeks to contribute to the development of new strategies for combating bacterial infections, reducing reliance on traditional antibiotics, and addressing the growing issue of antibiotic resistance.

MATERIALS AND METHODS

Obtation of seed extracts

The MSM and FMSM used in this study were provided as dried materials by the local distributor, S.C. OLEOMET S.R.L., Bucharest. The fermented mustard seed meal (FMSM) was produced by fermenting mustard seed meal with S. cerevisiae according to the method outlined by (Plaipetch & Yakupitiyage, 2013). For each meal, 1g was mixed with 7 ml of 80% acetone, shaken continuously for 24 hours at 200 RPM at room temperature, and then centrifuged at 8000 RFC for 10 minutes. From the resulting supernatant, 4 ml were collected, concentrated to remove acetone, and the aqueous extract was diluted and sterilized using antibacterial filters with a pore size of 0.45 µm, then prepared for in vitro experiments.

Determination of Total Polyphenol Concentration

The total polyphenol (TP) concentration in the seed meal extract was determined using the Folin-Ciocâlteu method, with gallic acid used as the standard for constructing the calibration curve. In the assay, 10 µl of seed meal extract was diluted in 790 µl of double-distilled water, followed by the addition of 50 µl of Folin-Ciocâlteu reagent. After one minute, 150 µl of 20% sodium carbonate was added, and the mixture was kept in the dark for two hours. The polyphenol concentration was then determined by measuring the absorbance at 750 nm, with

the final values expressed in mg/ml gallic acid equivalents (GAE).

E. coli Bacterial Growth

E. coli F4 (K88), kindly provided by Dr. Philippe Pinton (I.N.R.A.E, Toxalim, Toulouse, France), was cultured overnight in Luria-Bertani (LB) medium at a 1:10 ratio (1 µl/10 µl) at 37°C, following the protocol by (Roselli et al., 2003). The bacteria were then diluted 1:100 in fresh medium and incubated for another 4 hours. Bacterial growth concentration was determined by measuring the optical density at 600 nm (OD600 nm) using a Tecan Sunrise plate reader, Austria.

Measurement of Antimicrobial Effects

Serial dilutions of the seed meal polyphenolic extracts derived from unfermented and fermented mustard meal were prepared in bidistilled water using a ½ dilution factor. The antimicrobial activity of each polyphenol concentration extract from the serial dilutions was tested against *E. coli* K88 in triplicate wells of sterile 96-well microplates. Each well contained 20 µl of polyphenolic extract, 3 µl of *E. coli* F4 (K88) culture, and 277 µl of LB medium, and the mixture was incubated overnight at 37°C. Bacterial growth was monitored for 28.5 hours at 37°C using a plate reader, measuring the optical density at OD600 nm every 30 minutes. Before each reading, the plate was shaken orbitally (6 mm amplitude) for 15 seconds at 141.9 RPM.

Statistical Analysis

The experiments were performed in triplicate, and the data obtained were analyzed using GraphPad Prism 10.2.0. Statistical analysis was conducted using a One-way ANOVA test, with multiple comparison methods including Tukey and Dunnett.

RESULTS AND DISCUSSIONS

Total polyphenol content of the extract obtained from RSM and FRSM

The total polyphenol concentration in the extract obtained from mustard seed meal was 14.8 mg/ml, while the total polyphenol concentration in the extract from fermented mustard seed meal was 7.3 mg/ml. The polyphenol concentrations tested for antimicrobial activity against *E. coli* from mustard seed meal were 15.4 µg/ml, 30.8 µg/ml, 61.6 µg/ml, 123 µg/ml, 246 µg/ml, 493 µg/ml, and 986 µg/ml. For the fermented mustard seed meal extract, the tested polyphenol concentrations were 7.6 µg/ml, 15.2 µg/ml, 30.4 µg/ml, 60.8 µg/ml, 121 µg/ml, 243 µg/ml, and 486 µg/ml.

Antibacterial Properties of Unfermented Mustard Seed Meal Polyphenol Extract

The highest concentration of polyphenols obtained from unfermented mustard seed meal and tested for its antimicrobial activity against *E. coli* was 986 µg/ml. The results of bacterial growth tests highlight a dose-dependent effects of polyphenols from mustard seed meal on *E. coli* growth. Treatments with MSM at concentrations of 986 µg GAE/ml and 493 µg GAE/ml significantly reduced *E. coli* growth compared to the control group, with reductions of approximately 66.53% and 41.82%, respectively. The results also indicate that lower concentrations of MSM polyphenolic extract, starting from 246 µg, no longer had a significant inhibitory effect on *E. coli* growth. Additionally, there was a significant difference between the MSM treatments at 986 µg GAE/ml and 493 µg/ml, with the higher concentration leading to a greater reduction in bacterial growth. Specifically, the 986 µg GAE/ml treatment showed an approximate 73.86% higher reduction in *E. coli* growth compared to the 493 µg GAE/ml treatment (Figure 1).

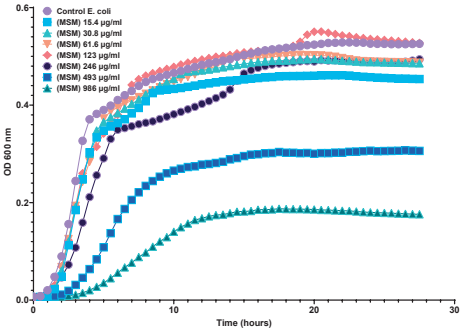

Figure 1. Effect of MSM polyphenolic extract on *E. coli* F4 (K88) growth. Various concentrations of MSM extract were prepared and mixed with *E. coli* bacteria. The mixtures were incubated for 27.5 hours, and bacterial growth was measured every 30 minutes by assessing the optical density at 600 nm

Turgis et al. investigated the effects of mustard essential oil (EO) on *Escherichia coli* and *Salmonella enterica* serotype Typhi and found that the minimum oil concentration required to inhibit the growth of both bacteria was 0.2% (v/v). Analysis of intracellular and extracellular ATP concentrations showed that ATP levels in the medium increased, while intracellular ATP levels decreased for both *E. coli* and *Salmonella typhi*, suggesting that the essential oil disrupted the bacterial cell membrane, causing ATP leakage from the cells. Additionally, mustard essential oil (EO) was found to lower intracellular pH, indicating that its antibacterial action may also involve disrupting intracellular homeostasis. The antimicrobial effect of polyphenols was further validated by assessing cell membrane integrity. Optical density measurements at 260 nm indicated the release of cellular constituents into the medium as a result of membrane rupture. In the experimental samples containing both bacteria and polyphenols, an increase in optical density was observed, indicating the release of cellular components caused by the presence of polyphenols. Scanning Electron Microscopy (SEM) further supported these findings, revealing damaged and deformed of bacterial membranes. The primary compound responsible for mustard EO's antimicrobial activity is believed to be allyl isothiocyanate (Turgis et al., 2009). Also, Nadarajah et al., evaluated the antimicrobial effects of mustard flour on *Escherichia coli* O157 by incorporating it into ground beef at concentrations of 5%, 10%, and 20%. Ground beef inoculated with 3 log10 cfu/g of *E. coli* O157 showed that the bacteria became undetectable after 18, 12, and 3 days in samples treated with 5%, 10%, and 20% mustard flour, respectively. The antimicrobial action was attributed to the release of allyl isothiocyanate, a compound produced when glucosinolates are hydrolyzed by the enzyme myrosinase in the presence of moisture in mustard seeds (Nadarajah et al., 2005). Investigating the effect of glucosinolates on the growth of *Escherichia coli* O157 in a dry-fermented sausage model, Luciano and Holley reported that glucosinolates, secondary metabolites found in mustard, can be converted into antimicrobial compounds known as isothiocyanates. Certain bacteria possess myrosinase-like activity, allowing them to degrade glucosinolates into isothiocyanates, which inhibit microbial growth. The study examined the ability of three bacterial strains - *E. coli* O157, *Staphylococcus carnosus*, and *Pediococcus pentosaceus* - to break down sinigrin (a glucosinolate) and produce allyl isothiocyanate. *E. coli* O157 was the most effective at degrading sinigrin, reducing its concentration by 1.02 mM, followed by *S. carnosus* (425 µM reduction) and *P. pentosaceus* (297 µM reduction). As a result, *E. coli* demonstrated the greatest sensitivity to AIT. The findings suggest that incorporating mustard flour into dry sausage production could serve as a natural antimicrobial strategy to control *E. coli* O157 contamination. Additionally, the degradation of glucosinolates also produced ρ-hydroxybenzyl isothiocyanate (ρ-HBIT), which exhibited weaker antimicrobial activity against *E. coli* O157, but was more effective against *S. carnosus* and *P. pentosaceus*. The Minimum Bactericidal Concentrations (MBC) for AIT were 1.04 mM for both *E. coli* O157 and *S. carnosus*, and 20.80 mM for *P. pentosaceus*. For ρ-HBIT, the MBC was 1.48 mM for *E. coli* O157, 0.59 mM for *S. carnosus*, and 5.92 mM for *P. pentosaceus* (Luciano et al., 2011). The same autors (Luciano et al., 2011) noticed the bactericidal activity of thermally treated yellow mustard powder against *Escherichia coli* O157 during the ripening of dry sausages. They evaluated three types of mustard powder: hot mustard powder derived from untreated mustard powder with active myrosinase, cold mustard powder in which myrosinase has been inactivated through heat treatment and autoclaved mustard powder, a mustard powder that has undergone autoclaving, which inactivates myrosinase, but may release or form antimicrobial substances. The mustard powders were tested at a concentration of 6% (wt/wt) in combination with two pairs of starter cultures. Starter culture A consists of *Pediococcus pentosaceus* UM 116P and *Staphylococcus carnosus* UM 109M, while starter culture B consists of *Pediococcus pentosaceus* UM 121P and *Staphylococcus carnosus* UM 123M. Results showed that both hot mustard powder (active myrosinase) and cold mustard powder (inactivated myrosinase) significantly reduced

E. coli O157 viability. A reduction in the *E. coli* population occurred after 31 days with hot mustard powder and after 38 days with cold mustard powder. Remarkably, the autoclaved mustard powder, despite the inactivation of myrosinase, was the most effective, achieving a 5 log CFU/g reduction in *E. coli* populations within just 18 days. The two starter culture pairs did not show significant differences in reducing *E. coli* viability, indicating that the mustard powder played the dominant role in the antimicrobial activity (Luciano et al., 2011). Another example of mustard meal's effectiveness in inhibiting bacterial growth is the use of yellow mustard powder as a natural antimicrobial agent to control the survival of *Escherichia coli* O157 in dry-cured Westphalian ham (Nilson & Holley, 2012). The study tested mustard powder concentrations of 4% and 6% and explored the potential role of the starter culture of *Staphylococcus carnosus* in enhancing the antimicrobial effect by promoting glucosinolate hydrolysis and the production of isothiocyanates, which are lethal to *E. coli* O157. Both 4% and 6% mustard powder treatments significantly reduced *E. coli* O157 levels. The addition of the *S. carnosus* starter culture on day 45 further accelerated isothiocyanate formation, contributing to a faster reduction and better control of *E. coli* O157. The findings demonstrated that mustard powder, in combination with a starter culture, provides an effective natural method for ensuring microbial safety in dry-cured ham (Nilson & Holley, 2012).

Antibacterial properties of fermented mustard seed meal polyphenols

The highest concentration of polyphenols obtained from fermented mustard seed meal and tested for its antimicrobial activity against *E. coli* was 486 µg/ml. The treatment with 486 µg GAE/ml of FMSM extract significantly reduces *E. coli* growth by approximately 47.53% compared to the control group, with a p-value of <0.0001. By contrast, the second serial concentration, 243 µg GAE/ml of FMSM extract results in a non-significant reduction. A notable difference was observed between the effects of the first concentration, 486 µg GAE/ml, and the second, 243 µg GAE/ml, in the FMSM extract. The 486 µg GAE/ml concentration inhibited *E. coli* growth 42.59% more effectively than the 243 µg GAE/ml concentration ($p<0.0001$). These results indicate that higher concentrations of polyphenols from fermented mustard seed meal are more effective in inhibiting *E. coli* growth (Figure 2).

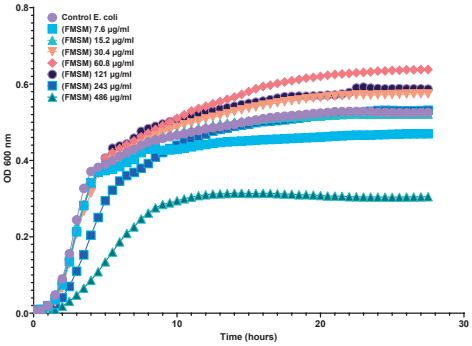

Figure 2. Effect of FMSM polyphenolic extract on *E. coli* F4 (K88) growth. Various concentrations of FMSM extract were prepared and mixed with *E. coli* bacteria. The mixtures were incubated for 27.5 hours, and bacterial growth was measured every 30 minutes by assessing the optical density at 600 nm

At the time of publishing this article, no studies had been found that specifically highlight the effects of fermentation with *S. cerevisiae* on polyphenols content in mustard seed meal. However, Park et al. (2017) studied the changes in total phenolic content (TPC) of mustard leaf kimchi extracts during different fermentation stages. Kimchi is a traditional Korean fermented vegetable dish made with lactic acid bacteria, typically using cabbage, radishes, or other vegetables such as mustard leaves. In the early stages of fermentation, TPC increases significantly, with the highest levels observed after two months. This increase in polyphenols concentration was attributed to microbial activity, which releases bound phenolic compounds by breaking down complex molecules into smaller, bioactive forms. After peaking at around two months, polyphenol content decreased, probably due to the degradation of phenolic compounds by enzymes such as polyphenol oxidase produced during prolonged fermentation. Thus, fermentation initially enhances phenolic content, boosting the antioxidant properties of kimchi, but extended fermentation can lead to a

reduction in these beneficial compounds. The study also identified specific polyphenolic compounds during the fermentation of mustard leaf kimchi, including gallic acid, chlorogenic acid, caffeic acid, epigallocatechin, catechin, epicatechin, epigallocatechin gallate, p-coumaric acid, gallocatechin gallate, ferulic acid, epicatechin gallate, rutin, catechin gallate, naringin, and quercetin. These compounds showed varying levels throughout fermentation, with some increasing initially and others decreasing over time. For instance, chlorogenic acid, which was initially abundant, decreased by half after three months of fermentation. Caffeic acid, epicatechin, and epigallocatechin gallate increased significantly after one month before showing a slight decline. Ferulic acid and rutin steadily increased during the early stages, peaking at two months before declining. Overall, most phenolic compounds exhibit an initial increase during the early stages of fermentation (especially within the first two months), followed by a decrease as fermentation progresses. This pattern suggests that fermentation enhances the release of phenolic compounds early on, but extended fermentation may lead to their degradation (Park et al., 2017). This could potentially explain the reduction in polyphenol levels in the final extract from fermented mustard meal observed in this study. On the other hand, mustard and other plants are part of a broader ecosystem where microorganisms, including yeast, are present on their surface. Tilahun et al. (2019) isolated yeast strains of *Saccharomyces cerevisiae* from Ethiopian mustard and compared their fermentation efficiency with commercial yeast strains. Two strains of *Saccharomyces cerevisiae* were identified, named AMS1 and MMS1. These strains exhibited ethanol tolerance and temperature resistance, key features for industrial fermentation processes. The fermentation efficiency of these isolates was tested by baking bread, and results showed that 80% of individuals preferred the dough fermented by the isolated strains over commercial yeast, indicating that these indigenous *S. cerevisiae* strains could have potential applications in fermentation processes (Tilahun, 2019). Wang et al. (2015) isolated the following lactic acid bacteria (LAB) species from fermented mustard (Suan-tsai): *Lactobacillus plantarum* strains B0040 and B0110, and *Weissella cibaria* strain B0145. During mustard fermentation, *Lactobacillus plantarum* and *Weissella cibaria*, like other LAB, play key roles in converting raw mustard into a fermented product. These bacteria are known for their potential probiotic properties, and their presence in fermented mustard may offer health benefits. These strains can enhance immune function by stimulating the production of cytokines and nitric oxide, thereby supporting improved immune responses (Wang, 2015). Mustard is traditionally fermented in China to improve its flavor, texture, and color, as fresh mustard has a naturally bitter taste that makes it less appealing for consumption. Fermentation also extends its shelf life. Yu et al. (2021) isolated thirteen bacterial strains from fermented mustard samples, most of which were identified as lactic acid bacteria. The specific strains included *Lactobacillus plantarum* (Strain GZ-2), *Lactobacillus brevis* (Strain SC-2), *Leuconostoc carnosum* (Strain GD-2), and Lactobacillus sp. (Strain JX-3). These strains were selected for their ability to produce or degrade biogenic amines (BAs) and nitrite, making them promising candidates as starter cultures to control harmful compounds that may form during mustard fermentation. Notably, *Lactobacillus plantarum* GZ-2 and *Lactobacillus brevis* SC-2 showed strong potential for reducing BAs and nitrite levels (Yu et al., 2021). Chen et al. (2006) investigated the lactic acid bacteria involved in the fermentation of suan-tsai, a traditional fermented food from Taiwan. They identified two key species, *Pediococcus pentosaceus* and *Tetragenococcus halophilus*. *Pediococcus pentosaceus* was predominant during the early stages of fermentation, but as the salt concentration increased, *Tetragenococcus halophilus,* being more salt-tolerant, took over. Both strains produce lactic acid, which lowers the pH of the mustard, giving it its distinct sour flavor. The combination of reduced pH and high salt levels also inhibits the growth of harmful bacteria and spoilage organisms, effectively extending the mustard's shelf life (Chen et al., 2006). Shiou-Huei Chao et al. conducted a study to examine the microbial diversity, particularly lactic acid bacteria

(LAB), in suan-tsai and fu-tsai, traditional fermented mustard products from Taiwan. They identified 500 LAB isolates, which were categorized into 119 representative strains across 5 genera and 18 species. The genera included Lactobacillus, Leuconostoc, Weissella, Enterococcus, and Pediococcus. The study highlighted that traditional fermentation processes promote high microbial diversity, influenced by factors such as salt concentration and environmental conditions. The identification of potentially novel species highlights the abundant diversity of microbial communities present in these fermented foods. Some of the identified bacterial species included *Enterococcus faecalis*, *Lactobacillus alimentarius*, *Lactobacillus brevis*, *Lactobacillus coryniformis*, *Lactobacillus farciminis*, *Lactobacillus plantarum*, *Lactobacillus versmoldensis*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Pediococcus pentosaceus*, *Weissella cibaria*, and *Weissella paramesenteroides* (Chao et al., 2009).

Comparison between MSM and FMSM antibacterial effect

The results emphasize the comparative effectiveness of MSM (mustard seed meal) and FMSM (fermented mustard seed meal) extracts in inhibiting *E. coli* growth. Both MSM at 493 µg GAE/ml and FMSM at 486 µg GAE/ml significantly reduced bacterial proliferation compared to the control group, with reductions of approximately 41.82% and 42.00%, respectively, each supported by p-values of <0.0001. The lack of a significant difference between MSM 493 µg GAE/ml and FMSM 486 µg GAE/ml indicates that both forms are similarly effective in reducing bacterial growth. At lower concentrations, MSM at 246 µg GAE/ml did not show a statistically significant difference from the control, suggesting that MSM at this concentration does not notably inhibit *E. coli* growth. Similarly, no statistically significant difference was observed between the control and FMSM at 243 µg GAE/ml, indicating that FMSM at this concentration also does not significantly inhibit bacterial growth. Comparisons within the MSM treatments reveal that MSM at 493 µg GAE/ml is significantly more effective than MSM at 246 µg GAE/ml, demonstrating a substantial reduction in E. coli growth at the higher concentration ($p < 0.0001$). Moreover, MSM at 493 µg GAE/ml is significantly more effective than FMSM at 243 µg GAE/ml, with a 73.69% greater inhibition of E. coli growth, highlighting the enhanced efficacy of higher MSM concentrations ($p < 0.0001$). For FMSM, the extract at 486 µg GAE/ml is significantly more effective than MSM at 246 µg GAE/ml, reducing *E. coli* growth by 61.93% more at the higher concentration ($p < 0.0001$). Furthermore, FMSM at 486 µg GAE/ml is significantly more effective than FMSM at 243 µg GAE/ml ($p < 0.0001$). There is no statistically significant difference between MSM at 246 µg GAE/ml and FMSM at 243 µg GAE/ml, indicating that these concentrations have similar effects on *E. coli* growth. Similarly, there is no statistically significant difference between MSM at 123 µg GAE/ml and FMSM at 121 µg GAE/ml. These findings suggest that fermentation does not significantly alter the efficacy of polyphenols from mustard seed meal in inhibiting *E. coli* proliferation, and both treatments are equally potent at the tested concentrations (Figure 3).

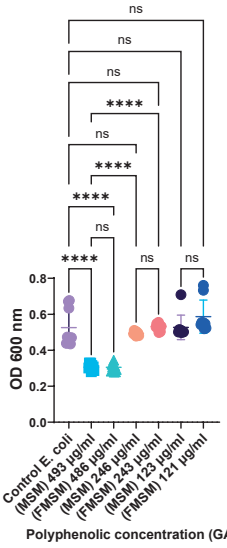

Figure 3. Comparison between similar polyphenolic concentrations extracted from MSM and FMSM against *E. coli* F4 (K88). Serial dilutions of MSM and FMSM extracts with different total polyphenol (TP) concentrations were prepared, and bacterial growth inhibition was observed over a period of 27.5 hours. This inhibition was measured by recording the optical density at 600 nm every 30 minutes

CONCLUSIONS

Fermentation with *S. cerevisiae* reduced the total polyphenol concentration in the final extract of mustard meal by half. Nevertheless, polyphenol extracts from both mustard seed meal and fermented mustard seed meal with *S. cerevisiae* demonstrated antimicrobial activity against *E. coli* F4 (K88). A comparison of their effectiveness showed no significant difference in inhibiting *E. coli* growth, indicating that fermentation does not adversely affect the polyphenol profile responsible for the antimicrobial activity in mustard seed meal. These results highlight the potential of polyphenols from agro-industrial by-products as alternatives to antibiotics and zinc oxide in piglet rearing. Further *in vivo* research is required to better understand these effects.

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