Study on the Synergistic In Vitro Antibacterial Effect of Plant Essential Oil and Sodium Butyrate (Postprint)

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Abstract

This study aimed to investigate the in vitro synergistic antibacterial effects of plant essential oils and sodium butyrate. The Oxford cup method was employed to determine the antibacterial activities of cinnamaldehyde, thymol, and vanillin against four bacterial species (Escherichia coli, Salmonella, Clostridium perfringens, and lactic acid bacteria). Both individual and combined plant essential oils were formulated with sodium butyrate to assess their antibacterial efficacy. The results indicated that all three plant essential oils exhibited significant inhibitory effects against Escherichia coli, Salmonella, and Clostridium perfringens, but showed no inhibition against lactic acid bacteria. At identical concentrations, the antibacterial potency followed the order: cinnamaldehyde > vanillin > thymol. The combination of cinnamaldehyde and vanillin demonstrated superior antibacterial activity compared to individual essential oils, with optimal efficacy observed at a mass ratio of 6:1. Sodium butyrate alone did not exhibit antibacterial activity; however, its combination with plant essential oils significantly reduced the required dosage of the essential oils. In conclusion, combinations of different plant essential oils exhibit synergistic effects, and synergistic interactions also exist between plant essential oils and sodium butyrate.

Full Text

Synergistic Bacteriostatic Effect of Plant Essential Oils and Sodium Butyrate In Vitro

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Abstract

This study investigated the *in vitro* synergistic bacteriostatic effects of plant essential oils and sodium butyrate. The Oxford cup method was employed to evaluate the antibacterial activity of cinnamaldehyde, thymol, and vanillin against four bacterial species (*Escherichia coli*, *Salmonella*, *Clostridium perfringens*, and *Lactobacillus*). Additionally, both single and compound plant essential oils were combined with sodium butyrate to assess their antibacterial efficacy. The results demonstrated that all three essential oils significantly inhibited *E. coli*, *Salmonella*, and *C. perfringens* but showed no inhibitory effect on *Lactobacillus*. At equivalent concentrations, the antibacterial potency followed the order: cinnamaldehyde > vanillin > thymol. Compound essential oils comprising cinnamaldehyde and vanillin exhibited superior antibacterial activity compared to single oils, with an optimal mass ratio of 6:1. While sodium butyrate alone displayed no antibacterial activity, its combination with essential oils substantially reduced the required dosage of plant essential oils. These findings indicate synergistic effects both among different plant essential oils and between plant essential oils and sodium butyrate.

Keywords: plant essential oil; sodium butyrate; synergistic bacteriostatic effect; minimum inhibitory concentration (MIC)

Introduction

The use of antibiotics in animal feed has become increasingly restricted due to concerns over drug residues and the development of antimicrobial resistance. Consequently, identifying safe and environmentally friendly antibiotic alternatives represents a critical focus in animal nutrition research. Previous studies have demonstrated the potential of plant essential oils as effective substitutes. For instance, Fang et al. [1] reported that dietary supplementation with 1 g/kg of *Acanthopanax senticosus* essential oil significantly reduced intestinal populations of *E. coli*, *Staphylococcus aureus*, and *Salmonella* in 21-day-old weaned piglets. Similarly, Michiel et al. [2] found that thymol supplementation increased *Lactobacillus* counts in the jejunum and cecum of piglets while decreasing anaerobic bacteria, *Clostridium*, and *Streptococcus* populations in the jejunum.

Sodium butyrate plays a crucial role in maintaining cellular differentiation and colonic epithelial integrity, thereby improving animal performance. Kotunia et al. [3] observed that sodium butyrate supplementation in artificial milk for 3-day-old piglets increased villus length and mucosal thickness in both the jejunum and ileum. Manzanilla et al. [4] reported that dietary sodium butyrate increased the number of goblet cells in the ileum of weaned piglets. Furthermore, Li et al. [5] demonstrated that sodium butyrate promoted growth and immune organ development in weaned piglets, enhanced serum immunoglobulin levels, and stimulated intestinal mucosal secretion of immunoglobulin A, thereby improving immune function. Wang et al. [6] found that dietary sodium butyrate reduced stress responses in piglets infected with rotavirus.

While plant essential oils exhibit antibacterial properties that inhibit pathogenic intestinal bacteria and promote healthy animal growth, most research has focused on their individual characteristics. Studies investigating the synergistic effects between plant essential oils and butyrate salts remain relatively scarce. This study employed *in vitro* antibacterial assays to identify the most effective plant essential oil and to explore the synergistic effects between plant essential oils and sodium butyrate, aiming to provide a theoretical basis for developing novel green feed additives.

Materials and Methods

Experimental Materials

Cinnamaldehyde (pale yellow liquid), thymol (colorless translucent crystals), vanillin (white powder), and sodium butyrate (white powder) were provided by Zhejiang Wanfang Biological Technology Co., Ltd. and stored at 4 °C under dark conditions before use. *Escherichia coli* (ATCC 25922), *Salmonella* (CICC 21913), and *Clostridium perfringens* (ATCC 13124) were purchased from the Guangdong Microbial Culture Collection Center, while *Lactobacillus* (ATCC 7469) was obtained from the China Microbial Culture Collection Center.

Bacteriostatic Test

The Oxford cup method was used for antibacterial testing. Five sterilized Oxford cups were placed on the culture medium surface, with three containing the three essential oils (thymol, cinnamaldehyde, and vanillin at 16 mg/mL), one containing 95% ethanol as a blank control, and one containing tiamulin (1,280 g/mL) as a positive control. Three replicates were performed for each bacterial strain, followed by incubation at 37 °C for 24 hours.

Minimum Inhibitory Concentration (MIC) and Compound Essential Oil Test

Based on preliminary screening via the Oxford cup method, gradient dilution was employed to determine the MIC of selected essential oils. Compound essential oils were prepared using cinnamaldehyde and vanillin at various mass ratios (6:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:6) to identify the optimal proportion with the strongest antibacterial effect.

Synergistic Test of Essential Oils with Sodium Butyrate

Following the method described in section 1.2.1, sterilized Oxford cups were loaded with plant essential oil (16 mg/mL), sodium butyrate solution (2.2 mg/mL), or a 1:1 (mass ratio) mixture of plant essential oil and sodium butyrate. Controls included 95% ethanol (blank) and tiamulin (1,280 g/mL, positive). Each bacterial strain was tested in triplicate and incubated at 37 °C for 24 hours.

Results and Analysis

Single Essential Oil Antibacterial Effects

As shown in and [Figure 1: see original paper], cinnamaldehyde and vanillin exhibited strong inhibitory effects against *E. coli*, *Salmonella*, and *C. perfringens*. Tiamulin, an antibiotic sensitive to enteric bacteria, effectively inhibited the proliferation of *E. coli*, *Salmonella*, and *Lactobacillus*.

Synergistic Antibacterial Effect of Essential Oils with Sodium Butyrate

and [Figure 2: see original paper] demonstrate that cinnamaldehyde alone and a 1:1 mixture of cinnamaldehyde and sodium butyrate produced similar inhibition zones against *E. coli*, *Salmonella*, and *C. perfringens*, indicating that combining sodium butyrate with essential oils can reduce essential oil usage by at least 50% while maintaining equivalent antibacterial efficacy.

Minimum Inhibitory Concentrations of Individual Essential Oils

According to and [Figure 3: see original paper], cinnamaldehyde showed the strongest antibacterial activity against *C. perfringens*, followed by *Salmonella*. The MIC against *E. coli* was 312.50 g/mL. Vanillin also demonstrated potent activity against *C. perfringens*, with MIC values of 625.00 g/mL against both *E. coli* and *Salmonella*. The 1:1 combination of cinnamaldehyde and vanillin was more effective than vanillin alone.

Minimum Inhibitory Concentrations of Compound Essential Oils

and [Figure 4: see original paper] reveal that the most effective compound formulation was cinnamaldehyde and vanillin at a 6:1 mass ratio. Generally, antibacterial efficacy increased with higher proportions of cinnamaldehyde in the mixture.

Synergistic Effect of Compound Essential Oils with Sodium Butyrate

As presented in and [Figure 5: see original paper], compound essential oils (cinnamaldehyde:vanillin = 6:1) and their mixtures with sodium butyrate at ratios of 1:1, 1:2, and 1:3 showed comparable antibacterial activity against *E. coli*, *Salmonella*, and *C. perfringens*. This demonstrates a synergistic interaction between compound essential oils and sodium butyrate, enabling a 75% reduction in compound essential oil usage while preserving antibacterial effectiveness.

Discussion

The potent antibacterial properties of certain plant essential oils have been well documented. Wang et al. [7] evaluated the *in vitro* antibacterial activity of three feed-grade essential oils (cinnamon oil, oregano oil, and thyme oil) against porcine enterotoxigenic *E. coli* K88 and *Salmonella choleraesuis* A72, reporting efficacy in the order: cinnamon oil > oregano oil > thyme oil. Zhang et al. [8] analyzed the chemical composition of essential oils and found that many contain benzene rings or conjugated double bond structures, such as cinnamaldehyde, vanillin, and citral. These structures bind to ion channel proteins on microbial cell membranes, altering membrane permeability and accelerating bacterial death through metabolic disruption. Our findings align with these reports, showing cinnamaldehyde as the most effective, followed by vanillin, with thymol being the least potent.

Research on essential oil synergy primarily focuses on two aspects. First, synergistic effects among different essential oils enable multi-pathway, multi-target actions that enhance overall efficacy compared to single oils. Imming et al. [9] enumerated potential target sites for essential oils in vivo, including metabolic enzymes, substrates, transporters, proteins, receptors, cytokines, and DNA/RNA. Galindo et al. [10] found that polyphenols in essential oils, despite their ambiguous physical properties, can enhance the solubility and absorption of primary active components. Aldehydes can penetrate cell membranes and bind to cytoplasmic proteins, thereby inhibiting key enzymes and achieving bacteriostatic effects [11]. Roldán-Gutiérrez et al. [12] reported that essential oil combinations effectively resist most pathogenic bacteria, including *Listeria monocytogenes*, which exhibits tolerance to single essential oils. Compound formulations not only improve inhibition but also reduce required dosages. Bassolé et al. [13] demonstrated that rose essential oil, combining carvacrol (which disrupts outer membranes) and thymol (which binds bacterial proteins), showed superior antibacterial activity compared to either component alone. Bozkurt et al. [14] found that an essential oil mixture containing carvacrol, thymol, eucalyptol, and limonene alleviated stress and reduced mortality in laying hens. Arczewska-Wlosek et al. [15] reported that mixed essential oils (thymol and garlic extract) mitigated coccidiosis severity in broilers. Rahimi et al. [16] observed that a blend of thymol, echinacea, and garlic essential oils significantly reduced *E. coli* populations in the ileum and cecum of broiler chickens. Numerous synergistic combinations have been identified, such as cinnamaldehyde with vanillin and perillaldehyde with polygodial, all of which reduce individual MIC values [17]. Liu et al. [18] emphasized that selecting appropriate essential oil combinations maximizes benefits at minimal dosages while minimizing potential side effects. Therefore, investigating synergistic antibacterial interactions among essential oil components holds significant scientific importance. Our study is the first to identify the 6:1 ratio of cinnamaldehyde to vanillin as optimal for inhibiting *E. coli*, *Salmonella*, and *C. perfringens.*

The second aspect of synergy research involves combining compound essential oils with other antimicrobial agents to enhance activity. Bassolé et al. [13] identified that in rose essential oil, components such as menthone and eucalyptol potentiate the overall antibacterial effect beyond the primary active substances (carvacrol and thymol). Wang et al. [19] reported that sodium butyrate could reduce antibiotic usage without compromising antibacterial efficacy and exhibited synergistic effects with antibiotics. Wang et al. [20] demonstrated *in vitro* that combining compound essential oils with tannic acid produced strong inhibition against five common pathogens (*Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *L. monocytogenes*), with reduced MIC values for each component. Identifying optimal ratios can enhance antimicrobial activity while decreasing essential oil requirements. Li [21] found that the feed additive Natesse (containing sodium butyrate and blended essential oils) reduced oocyst excretion in broilers. Qi et al. [22] suggested that adding butyric acid to essential oils enhances their antimicrobial activity. Jerzsele et al. [23] reported that dietary supplementation with essential oils or a sodium butyrate-essential oil mixture significantly increased villus height-to-crypt depth ratios and reduced *C. perfringens* counts in broilers. Our results confirm a synergistic interaction between compound essential oils and sodium butyrate, enabling a 75% reduction in compound essential oil usage (cinnamaldehyde:vanillin = 6:1) while maintaining antibacterial efficacy and mitigating the strong aroma of essential oils.

Conclusion

Compound essential oils composed of cinnamaldehyde and vanillin exhibit stronger antibacterial activity against *E. coli*, *Salmonella*, and *C. perfringens* than single essential oils, with a synergistic effect between the two components and an optimal ratio of 6:1. Sodium butyrate demonstrates synergistic interaction with essential oils, enabling a 75% reduction in compound essential oil usage (cinnamaldehyde:vanillin = 6:1) while preserving antibacterial effectiveness.

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Note: Figure translations are in progress. See original paper for figures.