Research Article

pISSN: 2345-5357
eISSN: 2423-4214

OPEN ACCESS

*APPLIED FOOD BIOTECHNOLOGY, 2024, 11 (1):e21*
*Journal homepage: www.journals.sbmu.ac.ir/afb*

# Synergistic effects of recombinant AGAAN antimicrobial peptide with organic acid against foodborne pathogens attached to chicken meat

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## Abstract

**Background and Objective:** Fresh chicken meat includes the capacity to contain foodborne pathogens. A previous study has demonstrated efficacy of recombinant AGAAN antimicrobial peptide against various bacterial strains. In general, AGAAN is a newly discovered antimicrobial peptide with a unique cationic alpha-helical structure. The peptide is originated from the skin secretions of *Agalychnis annae*. This peptide showed a significant affinity towards the negatively-charged microbial lipid bilayer, as previously demonstrated by the experimental and *in-silico* analyses. However, the major concerns include high production costs, limited expression, laborious process and potential toxicity associated with concentrated peptides. In this research, the synergistic effects with organic acid were addressed to decrease these problems while preserving its bactericidal activity.

**Material and Methods:** Recombinant AGAAN and organic acids were assessed on *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739. This was carried out by assessing minimum inhibitory concentration and fractional inhibitory concentration. In addition, effects of the combination on bacterial membrane integrity by carrying out beta-galactosidase assessment. Additionally, the potential efficacy of this combination in preserving poultry meat was investigated.

**Results and Conclusion:** Minimum inhibitory concentration of the recombinant AGAAN against the two bacterial strains was 0.15 $mg.ml^{-1}$. In contrast, the minimum inhibitory concentration of acetic acid against *Staphylococcus aureus* and *Escherichia coli* were 0.2 and 0.25% v $v^{-1}$, respectively. The combination demonstrated significant synergy, as evidenced by fractional inhibitory indices of 0.375 against the two foodborne pathogens. Based on the study, the combination effectively inhibited proliferation of these disease-causing microorganisms that led to foodborne illnesses within 300 min. Presence of intracellular beta-galactosidase indicated that the combination of factors has caused damages to the cell membrane, resulting in its compromised integrity. Red blood cells exposed to various concentrations of recombinant AGAAN and acetic acid did not result in hemolysis. Results showed significant differences ($p < 0.05$) in all the experiments on meat samples that received treatments with recombinant AGAAN and acetic acid. The current study detected that a combination of recombinant AGAAN antimicrobial peptide with organic acid could effectively inhibit growth of pathogens at lower concentrations. Data presented in this study can help food industries develop further efficient cost-effective antimicrobial uses.

**Conflict of interest:** The authors declare no conflict of interest.

**How to cite this article**
*Jiddah NU, Ajingi YS, Rukying N, Rattanarojpong T, Suntornsuk W, Pason P, Jongruja N. Synergistic effects of recombinant AGAAN antimicrobial peptide with organic acid against foodborne pathogens attached to chicken meat. Appl Food Biotechnol. 2024; 11 (1): e21.*
http://dx.doi.org/10.22037/afb.v11i1.44981

## Article Information

**Article history:**

- Received 5 Apr 2024
- Revised 12 May 2024
- Accepted 1 Jun 2024

**Keywords:**

- Foodborne pathogens
- Formic acid
- Peptide expression
- Recombinant bioactive peptide

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## 1. Introduction

Prevalence of foodborne diseases has emerged as a significant global health concern. The World Health Organization (WHO) report indicates that nearly 600 million cases of foodborne illnesses occur annually due to the consumption of food substances contaminated with microorganisms and chemicals [1]. Food contamination and increases in the risk of foodborne diseases are caused by pathogenic microorganisms [2]. Meat and meat products are important sources of nutrients for humans due to their high protein composition and other essential nutrients [3]. However, these foods provide appropriate environments for the growth of foodborne microbes due to their high water content and nutrients, [4]. A significant number of studies have shown that *Staphylococcus aureus* and *Escherichia coli* are associated with meat contamination [5-7]. The *S. aureus* is a facultative anaerobic, Gram-positive non-spore-forming bacterium [8]. It is a major problem in foodborne illnesses [9]. The *S. aureus* infections cause significant morbidity and mortality in developing and developed countries [10]. Similarly, *E. coli* is a non-spore-forming bacterium and the major cause of foodborne diseases in Gram-negative bacteria. Disease-causing strains of *E. coli* can infect the stomach, leading to serious abdominal symptoms [11]. Previous studies have primarily concentrated on spore-forming microorganisms, thereby overlooking non-spore-forming ones such as *E. coli* and *S. aureus*. Based on their contribution to foodborne illnesses, it is important to develop a cost-effective user-friendly approach to slow their rapid proliferation in food products.

Organic acids have been used as antimicrobial agents to inhibit foodborne pathogenic bacterial growth in chicken meats during processing [12]. Due to the potential resistance development by microorganisms, there are needs of drug alternatives that can efficiently kill resistant bacteria and enhance preservation [13]. Antimicrobial peptides (AMP) are produced by living organisms and include critical functions in protecting hosts against infections [14,15]. Likelihood of microbes exhibiting resistance to AMP is exceedingly low because of their wide range of mechanisms of action. Multiple studies have emphasized potential of AMP as a viable option for preventing meat spoilage and foodborne diseases [16-19]. In a previous investigation by the current authors, recombinant AGAAN (rAGAAN) effectively was cloned, expressed and analytically characterized [20]. Technically, AGAAN is a novel antimicrobial peptide with a cationic α-helical structure from the skin secretions of the blue-sided frogs. The rAGAAN is stable at various temperatures and pH and destroys a wide range of bacteria [20]. A hemolytic assay has shown that the peptide is relatively non-toxic to mammalian red blood cells (RBCs). Combination of these characteristics with its rapid killing kinetics demonstrates that rAGAAN includes the potential as an effective food preservative against foodborne pathogens. Nevertheless, major issues include exorbitant production expenses, labor-intensive procedures and potential toxicity of using high concentrations of peptides.

Combining two or more AMPs may boost antimicrobial activity at lower doses [21]. The present study assessed pairwise combinations of the rAGAAN with formic and acetic acids against *E. coli* and *S. aureus*. Selection of these two organic acids was based on their high effectiveness against the highlighted bacterial strains. In addition, FAO/WHO Expert Committee on Food Additives has classified acetic and formic acids as generally regarded as safe. The former chemical was assigned to an unrestricted group acceptance daily intake (ADI), while the latter was assigned to an ADI range of 0–3 $mg.kg^{-1}$ [22]. Combination of rAGAAN and these organic acids could decrease the concentration while preserving their potentially bactericidal activity. Differences in their mechanisms of action necessitate assessment of synergy in membrane permeation and kinetics of inactivation. This study could provide an additional option for poultry industries to protect chicken meats from pathogens.

## 2. Materials and Methods

### 2.1 Bacterial strains

Department of Microbiology at King Mongkut's University of Technology Thonburi in Bangkok, Thailand, supplied the foodborne pathogenic strains of *E. coli* ATCC 8739 and *S. aureus* ATCC 6538.

### 2.2 Recombinant AGAAN peptide expression and purification

The rAGAAN was produced based on the method of Ajingi et al., [20]. Briefly, the recombinant plasmid (pET-AGAAN) was transformed into *E. coli* BL21 (DE 3) competent cells. A colony of the competent cells with recombinant plasmids was inoculated into Luria-Bertani (LB) broth supplemented with chloramphenicol and ampicillin and grown at 37 °C and 200 rpm overnight. Then, 1% v $v^{-1}$ from the overnight culture was introduced into a fresh 1-l LB broth supplemented with chloramphenicol and ampicillin as well as 1% w $v^{-1}$ glucose. Culture was grown to an optical density (OD 600 nm) range of 0.4–0.6 at 37 °C and 200 rpm. Then, rAGAAN was expressed through induction with isopropyl β-D-1-thiogalactopyranoside at a concentration of 500 mM. Culture was grown at 16 °C for 18 h at 150 rpm. Cells were collected through centrifugation at 6,120× g for 30 min at 4 °C. Then, cells were suspended in 10 ml of buffer solution (10 mM Tris-HCl, 1 M NaCl; pH 8.0). These were subjected to sonication at an amplitude of 60% for 2 min, repeated for five cycles to induce cell

disruption. Supernatant was purified after sonication and centrifugation at 6,120× g for 25 min at 4 ℃ using HisTrap FF column linked to the FPLC system. The column was pre-equilibrated with binding buffer (10 mM Tris-HCl, 1 M NaCl; pH 8.0). Elution of the bound peptide was carried out using buffer B (10 mM Tris-HCl, 1 M NaCl, 250 mM imidazole; pH 8.0). Then, dialysis was carried out overnight at 4 °C using 50 mM Tris-HCl solution. Then, peptide was concentrated using 3-kDa centricon centrifugal filter tubes (Amicon, Germany). Concentration of the rAGAAN was measured using Bradford protein assay and its purity was assessed using 16% tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (tricine-SDS-PAGE).

### 2.3 rAGAAN and organic acid preparation

The rAGAAN was formulated in milligrams per milliliter ($mg.ml^{-1}$). It was dissolved in 1× phosphate-buffered saline (PBS), whereas the organic acids were formulated in percentages (% v $v^{-1}$) by dissolving in distilled water (DW).

### 2.4 Culture preparation

A volume of 20 µl of microbial stock, previously stored at -80 ◦C, were plated on LB agar. The resulting culture was incubated at 37 ºC for 18 h. Then, subculture process was carried out for each strain under identical conditions to preserve integrity and purity of the cells. On the next day, a suspension was generated by transferring isolated colonies into sterilized 10-ml LB media. The bacterial strains were cultured until they reached an OD of $10^8$ $cfu.ml^{-1}$. This measurement was achieved at 600 nm using spectrophotometer (U-2900UV/VIS Hitachi Tokyo, Japan). Concentration was modified to $10^5$ $cfu.ml^{-1}$ using sterile LB broth.

### 2.5 Minimum inhibitory concentration assessment

Briefly, 50 µl of the inoculated sample were administered into each well of the 96-well plates. Then, aliquots of 50 µl were dispensed into the wells, containing rAGAAN and organic acids at various concentrations. The 96-well plates with the lids closed were incubated at 37 °C for 18 h. Results were analyzed at 600 nm using microplate reader (BioTek, synergy H1, Winooski, USA). Control contained 100 µl of the bacterial inoculum. The MIC values included the lowest concentrations of the antimicrobial agents that cause bacterial growth inhibition.

### 2.6 Synergistic effects of rAGAAN with acetic and formic acids

Combination effects of rAGAAN with organic acids against the bacterial strains were assessed using checkerboard method. Briefly, 18-h cultures in LB broth were used to inoculate fresh LB broth to achieve a cell density of approximately $10^5$ $cfu.ml^{-1}$. Generally, 50 µl of the inoculated sample were added into 96-well microplates. Then, rAGAAN and organic acids were transferred into the 96-well microplates with increasing concentrations arranged in columns and rows, respectively. The organic acids were mixed with rAGAAN separately to assess their combinatorial effects on pathogenic bacteria. The purpose was to decrease the effective concentration of rAGAAN while preserving its antimicrobial activity. Assessment of the synergistic interactions involved the summation of the fractional inhibitory concentration indices (FICI) as Eq. 1 [23].

$$FICI = \frac{\text{MIC (rAGAAN + organic acid) mixture}}{\text{MIC (rAGAAN only)}} + \frac{\text{MIC (rAGAAN +organic acid) mixture}}{\text{MIC (organic only)}} \quad \text{Eq. 1}$$

where, FICI ≤ 0.5 indicated synergistic relationships between the rAGAAN and organic acids that increased the antimicrobial activity, FICI > 0.5–4.0 was indifferent and FICI > 4.0 was antagonistic.

### 2.7 Kinetics of inactivation

The OD of bacterial strain was measured to assess the rate of inactivation when treated with rAGAAN, acetic acids or their combination. Bacterial culture, diluted in LB broth to a concentration of approximately $10^5$ $cfu.ml^{-1}$, was added to 96 well plates. The rAGAAN and acetic acid were added at their minimum inhibitory concentration (MIC) levels, individually and in combination with their fractional inhibitory concentration (FICI) at 1×, 2× and 3× to separate wells. The 96-well plate was incubated at 37 °C. The procedure entailed monitoring the rate of inactivation for various bacterial strains by measuring the OD at consistent intervals of 1 h for 5 h. The OD was measured using spectrophotometer set at 600 nm and microplate reader (BioTek, synergy H1, Winooski, USA).

### 2.8 β-Galactosidase assay

The β-galactosidase assay was carried out to assess effects of the rAGAAN and acetic acid or their combination on membranes of the bacteria. First, *E. coli* was inoculated into lactose broth and incubated at 37 °C for 18 h to stimulate β-galactosidase production. The bacterial cells were centrifuged and the pellet was washed thrice with 1× PBS. Then, the bacterial concentration was modified to roughly $10^5$ $cfu.ml^{-1}$ in 1× PBS solution. Moreover, 50 µl of purified rAGAAN, acetic acid and their combination at 1× FICI, 2× FICI and 3× FICI were added into wells of a microplate containing 50 µl of *E. coli* cell suspension. A volume of 30 µl of O-nitrophenyl-β-D-galactoside (ONPG) were added into every well of the microplate. The microplate was incubated at 37 °C and activity was assessed by measuring the spectrophotometric absorbance at 405 nm and various time intervals.

### 2.9 Hemolysis assay

The RBC lytic assay was carried out based on a procedure by Taniguchi et al. [24] with minor adjustments. The RBCs were washed thrice in 1× PBS and centrifuged at

14,530× g for 10 min. Pellet was dissolved in 1× PBS to achieve a concentration of 4%. Generally, 500 µl of blood were mixed with 500 µl of rAGAAN and acetic acid, individually and in various combinations (1×FICI, 2×FICI, and 3×FICI). The positive control included a solution containing 0.1% TritonX-100, while the negative control included a solution containing 1× PBS. Solution was incubited in microtubes at 37°C for 1 h and centrifugation was carried out at 14,530× g for 5 min. Then, 100 µl of the supernatant were extracted from each microtube and transferred to each well of 96-well plate. Assessment of hemoglobin release was carried out by measuring the absorbance at 540 nm.

### 2.10 rAGAAN-acetic acid against chicken meat spoilage

Antimicrobial efficacy of the rAGAAN and acetic acid combination was assessed using a methodology described by Ajingi et al. [25], with minor adjustments. In brief, fresh chicken meat was purchased from a local market and immediately transferred to the laboratory. Meat was divided into approximately 10-g specimens and washed thoroughly. Specimens were transferred into a laminar flow hood and 100 µl of $10^5$ cfu of *E. coli* were divided to five separate locations. Sample was set for 1 h to promote appropriate attachment of the bacterial strains. Then, meat sample was submerged into 200-ml solution of rAGAAN/acetic acid for 1 h. Furthermore, sample was extracted, transferred into a plastic bag and incubated at 37 ºC for 3 d. The chicken meat sample was transferred into a plastic bag with solution consisting of 0.1% peptone water. Sample was mechanically pulverized using stomacher to enhance liberation of the bacterial cells. Following the process of serial dilution, a 100 µl of sample were transferred onto an LB-agar plate. Number of colonies on the plate was counted at intervals of 0, 1, 2 and 3 d. Control group was administered with DW.

### 2.11 Statistical analysis

Results were present as mean ±SD (standard deviation) of three replicates. Statistical distinction was assessed using one-way analysis of variance (ANOVA) with Duncan's multiple-range test. Differences with $p < 0.05$ were regarded as statistically significant.

## 3. Results and Discussion

### 3.1 Minimum inhibitory concentration

The MICs of rAGAAN and organic acids against *S. aureus* and *E. coli* are present in Table 1. The MIC of rAGAAN against *S. aureus* and *E. coli* was assessed as 0.15 $mg.ml^{-1}$. The organic acids inhibited proliferation of the pathogenic bacteria at various concentrations expressed as proportions (%).

**Table 1.** Minimum inhibitory concentration of rAGAAN ($mg.ml^{-1}$), acetic acid (%v $v^{-1}$) and formic acid (%v $v^{-1}$) against *Staphylococcus aureus* and *Escherichia coli*.

| Bacteria Strains | Minimum Inhibitory Concentration (MICs) | | |
|---|---|---|---|
| | rAGAAN | Acetic acid | Formic acid |
| *S. aureus* (ATCC 6538) | 0.15 | 0.20 | 0.25 |
| *E. coli* (ATCC 8739) | 0.15 | 0.25 | 0.2 |

Acetic acid demonstrated inhibitory effects on the growth of *S. aureus* at 0.2% v $v^{-1}$ and on the growth of pathogenic *E. coli* at 0.25% v $v^{-1}$. Formic acid inhibited growth of *S. aureus* at 0.25% v $v^{-1}$ and growth of *E. coli* at 0.2% v $v^{-1}$. The findings for acetic acid were similar to those against eleven mastitis pathogens in dairy cows with MIC values ranging of 0.125–0.25% v $v^{-1}$ [26]. Similarly, Fraise et al. [27] reported antimicrobial activity of acetic acid against *Pseudomonas aeruginosa* and *S. aureus* at 0.166 and 0.312% v $v^{-1}$, respectively. Manuel et al. [28] detected that formic acid at a concentration of 0.06% v $v^{-1}$ exhibited antimicrobial effects against *E. coli*. Variations in their effectiveness against the microorganisms might be attributed to their chemical compositions. Methyl group (CH3) in acetic acid donated electron density to O-H bond, resulting in increased difficulties in removing the hydrogen atom. Consequently, acetic acid was weaker than the formic acid. Weak acids included a higher ability to pass through bacterial membranes, compared to strong acids due to the balances between their ionized and non-ionized states. The non-ionized form could easily diffuse through hydrophobic membranes. As a result, they provided proton gradients needed for ATP synthesis to collapse. This occurred because free anions such as acetate in this situation combined with periplasmic protons that were pumped out by the electron transport chain. Then, anions transported the protons back across the membrane without F1Fo ATP synthase [29].

### 3.2 Synergistic effects of rAGAAN with organic acids

The inhibitory concentration index (FICI), demonstrating combined effects of rAGAAN and organic acids, is present in Table 2. The compound rAGAAN demonstrated synergistic effects against *S. aureus* and *E. coli* when combined with organic acids. Results showed that the synergistic effects were strongest when using acetic acid for the two bacterial strains, compared to when using formic acid. The FICI values for the combination of rAGAAN with acetic acid were assessed as 0.375 ($p < 0.5$) for *S. aureus* and *E. coli*. The FICI values for the combination of rAGAAN with formic acid were assessed as 0.375 for *S. aureus* and 0.5 for *E. coli*.

**Table 2**. Synergistic effects of rAGAAN with organic acids against *Staphylococcus aureus* and *Escherichia coli*

| Strain | Individual Compound | MIC of Individual Compound | FIC of Organic acid and rAGAAN in combination | | | |
|---|---|---|---|---|---|---|
| | | | Organic Acid | rAGAAN | FIC Index | Effect of combination |
| *S. aureus* ATCC 6538 | Acetic acid | 0.2 | 0.025 | 0.0375 | 0.375 | Synergistic |
| | Formic acid | 0.25 | 0.0625 | 0.0188 | 0.375 | Synergistic |
| | rAGAAN | 0.15 | | | | |
| *E. coli* ATCC 8739 | Acetic acid | 0.25 | 0.0313 | 0.0375 | 0.375 | Synergistic |
| | Formic acid | 0.2 | 0.05 | 0.0375 | 0.5 | Synergistic |
| | rAGAAN | 0.15 | | | | |

Results indicated that sub-MICs of the antimicrobials were needed to effectively terminate the bacterial growth. Combination of rAGAAN and acetic acid resulted in a 25% decrease in the concentration of each antimicrobial, compared to their MICs. Synergism can occur when two various antibacterial agents, each with non-overlapping mechanisms of action, are combined with each other [30]. Therefore, the authors suggest that the antimicrobial effects could be strengthened using synergistic effects of combined organic acid with rAGAAN. While the precise process; by which, combination of rAGAAN with organic acid created synergistic effects is still unknown, studies have demonstrated that the cell membrane of bacteria is a shared target for the antibacterial effects of various antimicrobial peptides. Additionally, these peptides include an affinity for bacterial cellular components, including DNA [31]. In contrast, it is suggested that organic acids can delay absorption of nutrients and disrupt flow of electrons, leading to decreases in ATP production [32]. This various mechanism of action enables swift eradication of bacteria.

### 3.3 Kinetics of inactivation

Acetic acid was chosen for the study because it included stronger antimicrobial effects than that formic acid with rAGAAN did. Growth inhibition kinetics of rAGAAN, acetic acid and their combination on the logarithmic phase of the pathogenic bacteria are illustrated in Figure 1. When the peptide rAGAAN was mixed with acetic acid at the FIC, there was no noticeable alteration in OD for either of the bacterial strains during 5 h. This indicated that the bacterial growth was entirely suppressed. The combination demonstrated significant inhibitory effects, greater than that of the individual antimicrobial agent and control group. The combination exhibited the capacity to inhibit proliferation of *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 at various concentrations within a few hours of exposure. Upon analyzing each treatment individually, it became clear that progressive decreases occured in OD measurements as time progressed. Nevertheless, use of rAGAAN with acetic acid led to further pronounced decreases in the turbidity level of the culture.

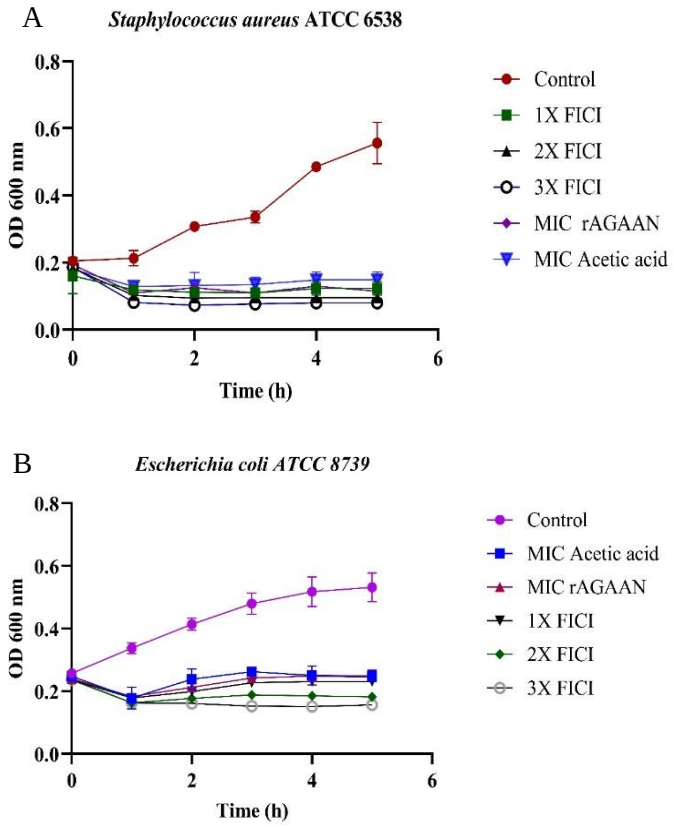


**Figure 1.** Inactivation kinetics of minimum inhibitory concentration-rAGAAN, minimum inhibitory concentration-acetic acid and their combination at fractional inhibitory concentration. (A) *Staphylococcus aureus* and (B) *Escherichia coli*

### 3.4 β-Galactosidase assay

To clarify the mechanism; by which, the combination acted, membrane permeability assay was carried out. This experiment used *E. coli* that was cultured in media containing lactose broth, which stimulated the synthesis of β-galactosidase. The β-galactosidase is an endogenous enzyme synthesized by the lac operon in bacteria. Release of this enzyme depends on disruption of the cell membrane. Release of the β-galactosidase enzyme from the disrupted cytoplasmic membrane was detected within 10 min of incubation with rAGAAN alone. In addition, cell membrane was destabilized by a combination of rAGAAN with acetic acid at 1× FICI, 2× FICI and 3× FICI, as shown in Figure 2.

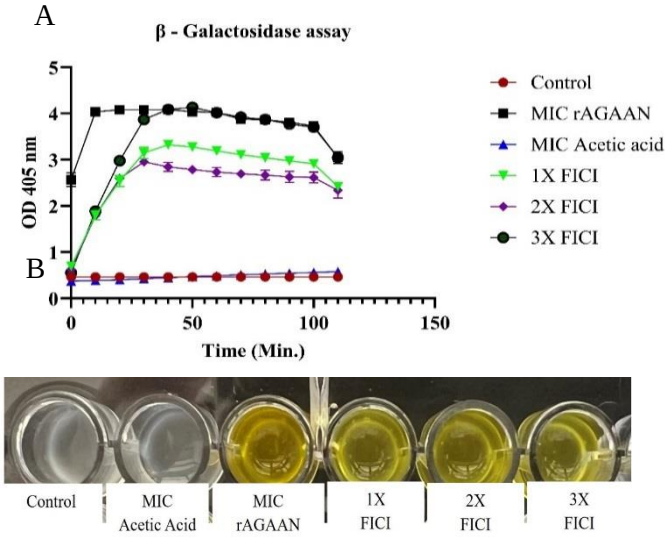


**Figure 2**. Beta-galactosidase analysis following treatment with minimum inhibitory concentration-rAGAAN, minimum inhibitory concentration-acetic acid and their combination at fractional inhibitory concentration. (A) Optical density and (B) O-nitrophenol released following membrane disruption and action of β-galactosidase on O-nitrophenyl-β-D-galactoside. 1×Fractional inhibitory concentration (0.0313% v $v^{-1}$ acetic acid & 0.0378 $mg.ml^{-1}$ rAGAAN); 2× fractional inhibitory concentration (0.0626% v $v^{-1}$ acetic acid and 0.0756 $mg.ml^{-1}$ rAGAAN); and 3× fractional inhibitory concentration (0.0939% v $v^{-1}$ acetic acid and 0.1134 $mg.ml^{-1}$ rAGAAN)

Findings showed that the presence of rAGAAN, independently and in combination with acetic acid, could result in permeability of the cell membrane of *E. coli*. However, acetic acid alone did not demonstrate effects on permeability of the membrane. Increasing OD measurements over time were directly linked to the rate of O-nitrophenol production from the breakdown of ONPG. The current results were similar to those of Yuan et al. [33], who observed increases in OD due to the degradation of ONPG when *Larimichthys crocea* myosin heavy chain protein-derived peptide was combined with low-intensity ultra-sound. Cell membrane disruption might lead to releases of intracellular components [34].

### 3.5 Hemolysis assay

Potential cytotoxicity of the chemical combination on human RBCs was assessed. Results of the hemolytic assay are present in Figure 3. The experimental group supernatant included clarity and transparency, which sharply contrasted to that the positive control group supernatant did. Furthermore, no substantial fluctuation was recorded in absorbance of the experimental groups. This demonstrated that combination of acetic acid and rAGAAN did not include any adverse effects on RBCs. However, absorbance of acetic acid alone resulted in approximately 50% hemolysis of the RBCs presented at high concentrations. Results suggested that the two antibacterial agents could effectively be combined as harmless food additives to combat pathogens.

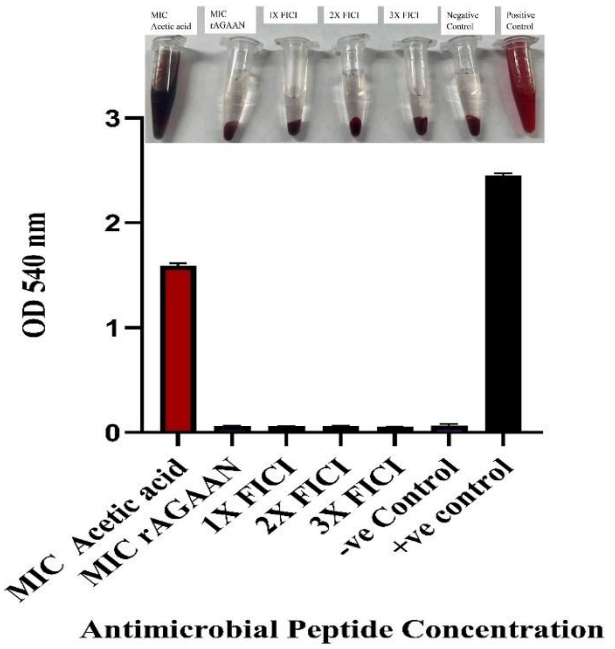


**Figure 3.** Hemolysis assay for minimum inhibitory concentration-rAGAAN, minimum inhibitory concentration-acetic acid and their combination at fractional inhibitory concentration. 1× Fractional inhibitory concentration (0.0313% v $v^{-1}$ acetic acid and 0.0378 $mg.ml^{-1}$ rAGAAN); 2× fractional inhibitory concentration (0.0626% v $v^{-1}$ acetic acid and 0.0756 $mg.ml^{-1}$ rAGAAN); and 3× fractional inhibitory concentration (0.0939% v $v^{-1}$ acetic acid and 0.1134 $mg.ml^{-1}$ rAGAAN)

### 3.6 rAGAAN-acetic acid against chicken meat spoilage

Results of the experiment are shown in Table 3 and Figure 4. Use of rAGAAN and acetic acid, either separately or in combination, led to significant suppressions of the *E. coli* growth. Inhibition was observed at various durations of exposure, in contrast to the control group (Table 3). At the beginning of the experiment (Day 0) after exposure to DW for 1 h, the control group showed decreases in the cell population to a value of 6.06 log 10 $cfu.g^{-1}$. Significant decreases ($p$ = 0.05) were observed in the bacterial count after individual treatment with rAGAAN and acetic acid (4.87 and 5.01 $cfu.g^{-1}$, respectively). Similarly, when the combination therapy was administered at concentrations of 1×, 2× and 3×, the resulting bacterial counts were 4.85, 4.82 and 4.81 $cfu.g^{-1}$, respectively. Significant differences in decreases were reported when comparing the control group to the groups that received rAGAAN treatment alone and the groups that received combination treatments at a three-fold fractional inhibitory concentration index as the duration of treatment increased. On Day 1 of the experiment, statistically significant decreases were seen in values of the microorganisms. More precisely, rAGAAN alone and its combination resulted in decreases of 1.39 and 1.40 log 10 $cfu.g^{-1}$, respectively.

**Table 3**. Effects of minimum inhibitory concentration-rAGAAN, minimum inhibitory concentration-acetic acid and their combination at fractional inhibitory concentration on inactivation of *Escherichia coli* growth in chicken meat (log cfu.g$^{-1}$)

| Days | Control | MIC rAGAAN | MIC Acetic acid | 1× FICI[§] | 2× FICI[†] | 3× FICI[‡] |
|---|---|---|---|---|---|---|
| 0 | 6.06 ± 0.02$^{A}$ | 4.87 ± 0.01$^{C}$ | 5.01 ± 0.01$^{B}$ | 4.85 ± 0.02$^{C}$ | 4.82 ± 0.02$^{C}$ | 4.81 ± 0.02$^{C}$ |
| 1 | 6.29 ± 0.00$^{A}$ | 4.9 ± 0.00$^{C}$ | 5.04 ± 0.00$^{B}$ | 4.89 ± 0.00$^{C}$ | 4.84 ± 0.00$^{D}$ | 4.81 ± 0.01$^{E}$ |
| 2 | 6.38 ± 0.00$^{A}$ | 4.97 ± 0.03$^{C}$ | 5.1 ± 0.01$^{B}$ | 4.92 ± 0.02$^{D}$ | 4.9 ± 0.02$^{DE}$ | 4.85 ± 0.00$^{E}$ |
| 3 | 6.49 ± 0.01$^{A}$ | 4.98 ± 0.02$^{C}$ | 5.14 ± 0.01$^{B}$ | 4.94 ± 0.01$^{D}$ | 4.94 ± 0.01$^{D}$ | 4.86 ± 0.01$^{E}$ |

[§]1× FICI (0.0313% v v$^{-1}$ acetic acid + 0.0378 mg.ml$^{-1}$ rAGAAN); [†]2× FICI (0.0626% v v$^{-1}$ acetic acid + 0.0756 mg.ml$^{-1}$ rAGAAN); [‡]3× FICI (0.0939% v v$^{-1}$ acetic acid + 0.1134 mg.ml$^{-1}$ rAGAAN). Mean that share the same letter along the row are not significantly different ($p < 0.05$).

On Day 2, the 3× FICI combination group showed statistically significant decreases of 1.53 log 10 cfu.g$^{-1}$, compared to the control group ($p = 0.05$). Furthermore, significant decreases of 1.63 log 10 cfu.g$^{-1}$ were reported on Day 3, compared to the control group. Results of this study demonstrated that use of rAGAAN and acetic acid included combined antibacterial effects on *E. coli* in meat samples stored at 37 °C for 3 d. The synergistic effect was detected as superior to the antibacterial effects of rAGAAN when used separately. Results supported changes in the color of the meat. The transformation is documented through the alteration in color of the meat (Figure 4). The control group demonstrated a significant color change and included an unpleasant smell. This might be attributed to the proliferation of microbial organisms. Katiyo et al. [35] reported strong correlations between the odor and growth of microorganisms in chicken legs. The putrid smell of spoiled meat might be due to the existence of various compounds such as sulfur compounds, carbonyls, ketones, diamines and alcohols [36].

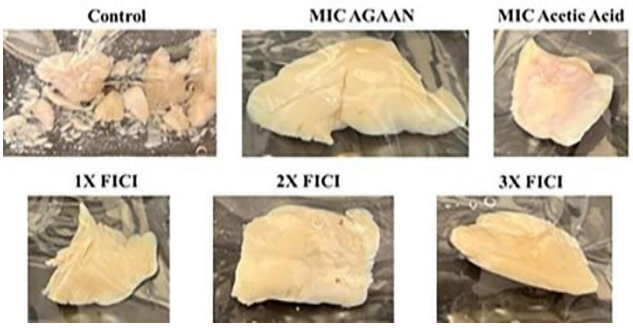


**Figure 4.** Effects of minimum inhibitory concentration-rAGAAN, minimum inhibitory concentration-acetic acid and their combination at fractional inhibitory concentration against *E. coli* on meat spoilage. 1× Fractional inhibitory concentration (0.0313% v v$^{-1}$ acetic acid and 0.0378 mg.ml$^{-1}$ rAGAAN); 2× fractional inhibitory concentration (0.0626% v v$^{-1}$ acetic acid and 0.0756 mg.ml$^{-1}$ rAGAAN); and 3× fractional inhibitory concentration (0.0939% v v$^{-1}$ acetic acid and 0.1134 mg.ml$^{-1}$ rAGAAN)

## 4. Conclusion

This study provided evidence on the synergistic effects of combining rAGAAN antimicrobial peptides with organic acids to inhibit growth of two prevalent foodborne pathogens of *S. aureus* and *E. coli*. Significantly, this integrated approach created inhibitory effects at decreased concentrations, compared to separate uses of these substances. The study revealed that rAGAAN included the highest beneficial synergy when combined with acetic acid. Simultaneous administration of rAGAAN and acetic acid caused disruption of the bacterial cell membrane without causing detectable harms to mammalian RBCs. Furthermore, implementation of multiple treatments led to decreases in the presence of microorganisms in chicken meats after 3 d, compared to the group with no treatments. The study results can be used as secure economically feasible alternatives for the preservation of chicken meats. While the current study assessed two pathogens, further studies are necessary to investigate effects of the chemical combinations on a wider range of microorganisms as well as assessing precise mechanisms of the synergistic interactions.

## 5. Acknowledgements

This study was supported by research funds from Petchra Pra Jom Klao Research Scholarship (contract no. 74/2563) and Faculty of Science, King Mongkut's University of Technology Thonburi.

## 6. Conflict of Interest

The authors declare no conflict of interest.

## 7. Authors Contributions

Investigation, writing-original draft, data curation and formal analysis, N.U.J.; formal analysis, validation, Y.S.A.; Data curation, formal analysis, N.R.; project administration and supervision, T.R.; methodology, supervision, W.S.; project administration and supervision, P.P.; supervision, conceptualization, funding acquisition, resources, methodology, N.J.

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Research Article
pISSN: 2345-5357
eISSN: 2423-4214
OPEN ACCESS

APPLIED FOOD BIOTECHNOLOGY, 2024, 11 (1):e21
Journal homepage: www.journals.sbmu.ac.ir/afb

# اثرات هم‌افزایی پپتید ضد میکروبی AGAAN نوترکیب با اسید آلی بر میکرب‌های بیماری‌زای غذازاد مرتبط با گوشت مرغ

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## چکیده

**سابقه و هدف:** گوشت مرغ تازه مستعد رشد میکرب‌های بیماریزای غذازاد های است. گزارش‌های قبلی اثربخشی پپتید ضدمیکروبی نوترکیب AGAAN را در برابر سویه‌های مختلف باکتری نشان داده است. به طور کلی، AGAAN یک پپتید ضدمیکروبی تازه کشف شده با ساختار آلفا-مارپیچ کاتیونی منحصر به فرد است. منشا این پپتید از ترشحات پوستی آگالیکنیس آنایه است. این پپتید تمایل قابل توجهی نسبت به دولایه‌ای لیپیدی میکروبی با بار منفی نشان داد، همان‌طور که قبلاً توسط آنالیزهای آزمایشگاهی و *in silico* نشان داده شده بود. به هر حال، محدودیت‌های عمده در مورد آن شامل هزینه‌های تولید بالا، بیان محدود، فرآیند پر زحمت و سمیت بالقوه مرتبط با پپتیدهای غلیظ است. در این تحقیق اثرات هم‌افزایی با اسید آلی برای کاهش این مشکلات و حفظ فعالیت باکتری‌کشی آن مورد توجه قرار گرفت.

**مواد و روش ها:** AGAAN نوترکیب و اسیدهای آلی بر روی *استافیلوکوکوس اورئوس* ATCC 6538 و *اشرشیا کلی* ATCC8739 مورد ارزیابی قرار گرفتند. این کار با ازمون حداقل غلظت مهاری و غلظت مهاری جزیی انجام شد. علاوه بر این، اثرات این ترکیب بر روی یکپارچگی غشای باکتریایی با انجام ارزیابی بتا-گالاکتوزیداز بررسی شد. به علاوه، اثربخشی بالقوه این ترکیب در نگهداری گوشت طیور مورد بررسی قرار گرفت.

**یافته‌ها و نتیجه‌گیری:** حداقل غلظت بازدارنده AGAAN نوترکیب در برابر دو سویه باکتریایی ۰/۱۵ میلی‌گرم در میلی لیتر بود. در مقابل، حداقل غلظت بازدارنده اسید استیک در برابر *استافیلوکوکوس اورئوس* و *اشریشیا کلی* به ترتیب ۰/۲ و ۰/۲۵ درصد حجمی بود. ترکیب این دو هم افزایی قابل توجهی را نشان داد، به طوری که شاخص های بازدارنده کسری ۰/۳۷۵ در برابر دو میکرب غذازاد مشاهده شد. بر اساس این مطالعه، این ترکیب به طور موثر تکثیر میکروارگانیسم‌های بیماری‌زا غذازاد که در عرض ۳۰۰ دقیقه عارضه ایجاد میکنند ، را به طور موثر جلوگیری کرد. حضور بتا گالاکتوزیداز درون سلولی نشان داد که ترکیب دو ماده باعث آسیب به غشای سلولی و در نتیجه به خطر افتادن یکپارچگی آن شده است. در معرض قرارگرفتن گلبول های قرمز در برابر غلظت های مختلف AGAAN نوترکیب و اسید استیک منجر به همولیز آن‌ها نشد. نتایج نشان داد که در تمام آزمایش‌ها بر روی نمونه‌های گوشتی که تیمارهای AGAAN نوترکیب و اسید استیک را دریافت کرده بودند، تفاوت معنی‌داری وجود داشت ($P \leq ۰/۰۵$). مطالعه حاضر نشان داد که ترکیب پپتید ضد میکروبی نوترکیب AGAAN با اسید آلی می‌تواند به طور موثری رشد بیماریزاها را در غلظت‌های پایین‌تر مهار کند. داده های ارائه شده در این مطالعه می تواند به صنایع غذایی کمک کند تا کاربردهای ضد میکروبی مقرون به صرفه بیشتری را توسعه دهند.

**تعارض منافع:** نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

**تاریخچه مقاله**
دریافت ۵ آوریل ۲۰۲۴
داوری ۱۲ مه ۲۰۲۴
پذیرش ۱ ژوئن ۲۰۲۴

**واژگان کلیدی**
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