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Galangal extract of an antimicrobial model for predicting the reduction in histamine concentration in minced pork

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Abstract

Histamine concentration increases in meats, such as fresh pork, when contaminated with some bacteria. Galangal extract, known for its antimicrobial properties, was studied to determine its effects in reducing bacteria in minced pork artificially inoculated with Histamine-Producing Bacteria (HPB) such as *Lactobacillus plantarum* and to predict the DR value for the Weibull model. Antioxidant activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Galangal extract was prepared by ethanol distillation and used at 10% and 15% dilutions in distilled water. Bacterial inhibition was evaluated at 4 °C and 8 °C using filter papers soaked in the extract and placed on a minced pork surface. The extract at both concentrations effectively inhibited bacterial growth for 5 days. On the 6th day, the histamine levels detected in food samples stored at 8 °C and exposed to 10% and 15% extract dilutions were 0.9935 and 0.9825 µg/g, respectively. The DR value of the Weibull model, the concentration of galangal extract required for more than 90% reduction in HPB population in minced pork, was determined to be 2.3516 mg/mL (R^2 = 0.9793). The detected and predicted histamine levels in minced pork were safe. The findings of this study can be used to reduce HPB in food by galangal extract application in packaging and suggest histamine safety levels in pork.

Keywords: Lactic acid bacteria; Biogenic amine; Weibull model; Risk estimation.

Highlights

- We developed a model to predict the DR values required to inactivate HPB in minced pork
- Experimental data and Weibull equations were used in kinetic inactivation modeling
- The findings can be used to reduce HPB in food by galangal extract application

1 Introduction

Pork is an important component of the human diet. It is also a major component of the total food expenditure in households. In 2015, pork production reached 1.06 million tons per annum. Generally, more

than 95% of the pork produced is consumed domestically (Pitakpaibulkij et al., 2016). Histamine in fresh pork is generated by the histidine decarboxylase (HDC) of some Lactic Acid Bacteria (LAB), called Histamine-Producing Bacteria (HPB) (Landete et al., 2005). Varying concentrations of histamine are present in pork: 4.7 mg/kg in raw pork; 9.9 mg/kg in pork stored at 5 °C for 15 d; and 0.5 mg/kg in pork stored at −20 °C for 15 d (Halász et al., 1994).

HPB includes a large number of strains representing various species and families isolated from a variety of food products (Koutsoumanis et al., 2010). Foods containing *Lactobacillus* strains are characterized by considerable Biogenic Amine (BA) content (Deepika Priyadarshani & Kumar Rakshit, 2014). Cooking, canning, or freezing does not reduce the level of histamine in food because it is heat-stable (Visciano et al., 2014). Histamine in food is produced by decarboxylation of free histidine, however, its content in food should be studied because histamine exerts toxicological effects in humans (Silla Santos, 1996). As can be seen, a 5–10-mg dose of histamine is considered unsafe for sensitive individuals; 10 mg is considered the tolerance limit; 100 mg induces intermediate toxicity; and 1000 mg is highly toxic (Silla Santos, 1996). The US Food and Drug Administration (FDA) has established 50 mg/kg as the upper limit for histamine in seafood; seafood products containing more histamine than this limit should not be used for human consumption and are subjected to recalls (Food and Drug Administration, 2002). Unfortunately, no limit has been established for pork products, but the limit for seafood can be used as a reference.

Alpinia galanga (L.) Willd., commonly called greater galangal, belongs to the family Zingiberaceae and is a rhizomatous herb distributed in various parts of India and throughout Southeast Asia. Galangal is used as a food additive in Thailand and other Asian countries. Essential oils from both fresh and dried galangal rhizomes exhibit antimicrobial activity against bacteria, fungi, yeasts, and parasites (Ghosh & Rangan, 2013). 1′-Acetoxychavicol acetate (ACA), isolated from dried rhizomes of *A. galangal*, is potentially active against several bacteria and many dermatophytes (Janssen & Scheffer, 1985). Regarding the application of galangal extract and essential oil in food, Mahae & Chaiseri (2009) suggested that the ethanolic extract of galangal is advantageous as an antioxidant in food owing to its mild odor compared with the essential oil. There is a great interest in reducing the number of HPB in fresh pork to prevent histamine synthesis; for instance, by using various bacterium-inhibiting compounds, such as extract and essential oil of galangal. Pattaratanawadee et al. (2006) found that from the spoilage bacteria tested (*L. plantarum* and *L. cellobiosus*), the galangal extract inhibited *Lactobacillus* more efficiently than other extracts (ginger, turmeric, and fingerroot). Its Minimum Inhibitory Concentration (MIC) was 4% (v/v). The action mechanism of galangal extract in inhibiting bacteria can be described as follows. The galangal extract causes both outer and inner membrane damage, as well as cytoplasmic coagulation, as observed using Transmission Electron Microscopy (TEM). The leakage of cell components, including nucleic acids, indicates that the cytoplasmic membrane characteristics are disrupted. The major ingredient of the extract was identified as d, l-1′-acetoxychavicol acetate (Oonmetta-aree et al., 2006).

Predictive models are used in the food industry to define the critical control points in food processing operations. These models can also be used by risk assessors to guide management decisions to reduce the risk of foodborne diseases (Mishra, 2011). As mentioned above, knowledge of histamine levels in minced pork containing HPB and the effects of galangal extract on HPB numbers during storage at chilling temperatures is important, as it may be used to generate equations to estimate the D-values required for the inactivation of HPB in minced pork. In the current study, it could be evaluated the effect of galangal extract on HPB inoculated in minced pork and used the data for Weibull modeling.

2 Material and methods

2.1 Materials

2.1.1 Preparation of galangal powder

Fresh galangal rhizomes were purchased from a local market in Chiang Rai, Thailand, and transported to the Faculty of Agro-Industry, Chiang Mai University, Thailand. The rhizomes were manually washed to remove dirt and other impurities. They were then sliced into 5-mm-thick pieces using a vegetable processor

and dried in a tray-dryer oven at 50 °C for 24 h (final moisture content: 10%). They were then ground in a blender (HR2071/20; Philip, Thailand) to a fine powder. The powder was stored in an amber glass bottle and kept in a dry place.

2.1.2 Preparation of galangal ethanolic extract

Galangal powder (10 g) was extracted with 100 mL of 95% ethanol (v/v) in water at a solid-to-solvent ratio of 1:10 and left at room temperature (25 ºC) overnight, as described by Mahae & Chaiseri (2009), with some modifications in solvent concentrations. The extract was concentrated using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland). The concentrated extract was dried using a freeze dryer and kept in a glass vial at –40 °C and stored at 4 °C until further use.

2.2 Microorganisms

Stock culture of *L. plantarum* (standard bacterial suspension) was used in the current study; it was provided by the Laboratory of Biotechnology (under the supervision of Assoc. Prof. Dr. Thanongsak Chaiyaso), Faculty of Agro-Industry (Chiang Mai University, Thailand). The bacteria were maintained on Trypticase Soy Agar (TSA) (TSA; Merck, Germany) slants at 4 °C. For use in experiments, the bacteria were grown separately in Triticase Soy Broth (TSB) (TSB; Merck, Germany) at 35 °C for 18–24 h. Each bacterial suspension was subsequently streaked on TSA plates and incubated at 35 °C for 48 h. A single colony was transferred to 10 mL of TSB and incubated at 35 °C for 18–24 h. The final cultures were then used in the antibacterial assays.

2.3 Antioxidant activities of galangal ethanol extract

The antioxidant activity of the ethanolic extract of galangal rhizome was studied using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and reported as milligram equivalent Trolox and milligram equivalent vitamin C per 100 g of dry weight. The DPPH antioxidant assay is based on the fact that antioxidants cause the stable free radical DPPH to shift colors. The advantage of DPPH is that it is unaffected by some adverse effects brought on by various additions, such as metal ion chelation and enzyme inhibition (Melanathuru et al., 2017). The DPPH assay was carried out as described by Kedare & Singh (2011) with some modifications. The electron-donation abilities of the extracts were measured based on the reduction of DPPH radical in the presence of hydrogen-donating antioxidants. For the assay, 0.8 mM DPPH radical solution (Sigma-Aldrich, USA) in 95% ethanol (Merck) was prepared. Four hundred microliters of the extract were diluted to 5.4 mL using deionized water and 95% ethanol (1:1) before adding 0.6 mL of DPPH solution and shaken vigorously. The decrease in absorbance was recorded at 1, 5, 10, and 30 min after mixing. Trolox (0–50 µg) (w/v) (Sigma-Aldrich) and vitamin C (0-40 µg) (w/v) (Sigma-Aldrich) were used as positive standards. Antioxidant assays were performed, and the results were analyzed by Central Laboratory (Thailand) Co., Ltd. The results are expressed as micromole Trolox equivalent per gram of dried sample (µmol TE/g dw).

2.4 Challenge test

Fresh minced pork was purchased from a local market in Mae-Hea District, Mueang (Chiang Mai, Thailand). It was placed in an icebox with a controlled temperature below 4 °C and transported to the Faculty of Agro-industry, Chiang Mai University, within 1 h of purchase. Before the experiment, duplicates of minced pork samples without the inoculum and the galangal extract were subjected to quality control to verify the possible presence of *L. plantarum*. Fresh minced pork portions (25.0 ± 0.5 g) were weighed and packed in polyethylene bags. The samples were then inoculated with *L. plantarum* (initial concentration of approximately 8 log10 CFU/g) using the inoculating method where the 0.5 McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension with a concentration of

1.5 x 10^8 Colony Forming Units (CFU/mL) (Aryal, 2021). Another 25 g of fresh minced pork was directly inoculated with *L. plantarum*. The inoculated samples were serially diluted in sterile 0.1% buffered peptone water (Merck) and *L. plantarum* was enumerated on de Man, Rogosa, and Sharpe (MRS) agar (all media were from Merck). The effect of temperature was determined at 4 ± 2 °C and 8 ± 2 °C.

The potential antimicrobial activity of the ethanol galangal extract diluted to 10% or 15% in distilled water on *L. plantarum* was primarily investigated using the agar disc diffusion method. To determine the inhibition of HPB inoculated into the minced pork and stored at 4 ± 2 °C or 8 ± 2 °C for 0, 2, 4, and 6 d, filter papers were soaked with the diluted extracts and placed on the surface of the tested food in the packaging. The positive control amoxicillin (20 µg/mL) was prepared following the manufacturer's instructions (Merck). A low-density polyethylene tray with a lid was used for storing minced pork under various conditions. The minced pork was weighed (portions of 25 ± 0.5 g) and used for the following six treatments as following: (1) control, minced pork with no extract, stored at 4 ± 2 °C; (2) minced pork with 10% extract, stored at 4 ± 2 °C; (3) minced pork with 15% extract, stored at 4 ± 2 °C; (4) control, minced pork with no extract, stored at 8 ± 2 °C; (5) minced pork with 10% extract, stored at 8 ± 2 °C; and (6) minced pork with 15% extract, stored at 8 ± 2 °C.

2.5 Microbiological analysis

Fresh minced pork inoculated with HPB in low-density polyethylene trays was aseptically sampled on days 0, 2, 4, and 6 of storage at chilling temperatures of 4 ± 2 °C and 8 ± 2 °C: 10-g composite sample from two patties per treatment. Approximately 10 ± 0.5 g of fresh minced pork was collected from each package and homogenized in 90 mL of sterile 0.1% peptone water for 2 min using a stomacher (IVL Masticator 400; IUL Instruments, Spain). Serial 10-fold dilutions were prepared in dilution test tubes containing 9 mL of 0.1% peptone water (Merck). Solutions (1 mL) for duplicate aerobic plate counts were prepared using the pouring plate method with appropriate dilutions of MRS agar (Merck) for *L. plantarum* and incubated at 37 °C for 48 h. The growth is reported as log10 of the number of CFU/g.

2.6 Fluorometric assay of histamine concentration in fresh minced pork

All samples were collected for the analysis of histamine concentration using a standard fluorometric technique. Column eluent (2 mL) was placed in a test tube, and then 0.4 mL of 3 M NaOH (RCI Labscan, Thailand) was added, and the tube was shaken. A 100-µL aliquot of 1% (w/v) o-phthaldialdehyde (Sigma-Aldrich, France) was added and mixed, and the reaction was allowed to proceed for exactly 4 min. The reaction was stopped by the addition of 200 µL of 3 M HCL (RCI Labscan). Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm using a fluorometer (Quantech Fluorometer, Barnstead Turner Thermo Scientific, Sweden) (Association of Official Analytical Chemists, 2012).

2.7 Kinetic model of HPB number reduction

Microbial inactivation follows the first-order kinetics, expressed as Equation 1:

$$N/N_{0=e^{-ktc}} \quad (1)$$

and the inactivation of HPB as a function of galangal extract concentration (C) can be assumed to comply with a first-order kinetic, which can be approximated by the following Equation 2:

$$log\left(\frac{N}{N_0}\right) = ktc \quad (2)$$

where N is the bacterial concentration after exposure (CFU/g of contaminated minced pork), that is, N is the number of microorganisms at galangal extract concentration C: N0 is the initial number of

microorganisms (CFU/mL); k is the concentration rate constant; and t is the exposure time. This equation is only valid for linear inactivation curves. However, because many microbial inactivation curves are non-linear, a Weibull model is used for such non-linear survival curves. The Weibull model is described according to Equation 3 (van Boekel, 2002):

$$log\left(\frac{N}{N_0}\right) = \frac{1}{2.303}\left(\frac{ct}{\propto}\right)^{\beta} \quad (3)$$

where α is the characteristic dose or scale parameter (time) and β is the shape parameter (dimensionless). The α and β parameters may be obtained using non-linear least-squares regression in Microsoft Excel 2010, and they can be used to calculate DR using Equation 4 (van Boekel, 2002):

$$D_R = \propto \times (2.303)^{\frac{1}{\beta}} \quad (4)$$

where, DR is defined as the concentration of galangal extract (mg/mL) required for a 90% reduction of the pathogen population, similar to the D-value in thermal processing.

2.8 Statistical analysis

The means with standard deviations were analyzed using SPSS version 15.0 (IBM, Armonk, NY, USA) and Microsoft Excel™ Platform (Microsoft Corporation, Redmond, WA, USA). The experiments were performed in triplicate.

3 Results and discussion

3.1 Histamine concentration in fresh minced pork

Histamine was recovered from fresh minced pork extracts using ion-exchange chromatography, derivatized with o-phthalaldehyde, and measured using the intensity of the resulting fluorophore (the results are shown in Table 1).

Table 1. Effects of galangal extract concentration on the changes in *Lactobacillus plantarum* populations and histamine concentration in minced pork.

Storage Temperature (°C)	Galangal Extract (%)	Storage Period (days)							
		0		2		4		6	
		HBP (log CFU/g)	Histamine (µg/g)	HBP (log CFU/g)	Histamine (µg/g)	HBP (log CFU/g)	Histamine (µg/g)	HBP (log CFU/g)	Histamine (µg/g)
4 °C	0	0.00	0.00	8.70 ± 0.05	0.00	7.30 ± 0.37	0.00	8.30 ± 0.39	1.9927 ± 0.19
	10	0.00	0.00	7.50 ± 0.37	0.00	8.10 ± 0.38	0.00	6.90 ± 0.36	0.0000 ± 0.00
	15	0.00	0.00	9.70 ± 0.40	0.00	8.60 ± 0.39	0.00	8.30 ± 0.39	0.0000 ± 0.00
8 °C	0	0.00	0.00	10.00 ± 0.41	0.00	9.10 ± 0.40	0.00	10.70 ± 0.42	1.9692 ± 0.19
	10	0.00	0.00	8.90 ± 0.39	0.00	10.30 ± 0.41	0.00	9.30 ± 0.40	0.9935 ± 0.12
	15	0.00	0.00	6.30 ± 0.35	0.00	7.40 ± 0.37	0.00	6.90 ± 0.36	0.9825 ± 0.12

The numbers are means from three independent replicates. The positive control was amoxicillin (20 µg/mL).

As shown in Table 1, the histamine concentration in minced pork during storage at chilling temperatures under various treatments (4 ± 2 °C and 8 ± 2 °C; and without and with 10% or 15% galangal extract) ranged between 0 and 1.9927 ± 0.19 µg/g. The extract at both concentrations effectively inhibited bacterial growth for 5 days. On 6th day, the levels of histamine detected in food samples stored at 8 °C exposed to 10% and

15% extract dilutions were 0.9935 and 0.9825 µg/g, respectively. The detected histamine production rate during 6 days of storage at 4 °C and 8 °C was below the US Food and Drug Administration safety level of 50 mg/100 g (50 ppm). Histamine levels higher than the mentioned concentration should be considered a potential hazard for human health (Food and Drug Administration, 2002).

The histamine concentration results in this study were lower than those obtained by Michalski et al. (2021), who found that the histamine concentration of 97 samples of long-ripening meat products untreated by heat in Poland ranged from below the limit of detection to 346.64 mg/kg, where 3.47 mg/kg was the lowest concentration in a positive sample. Histamine was detected in 48 samples (49.5%). The maximum amount of histamine was identified in dry ham and the minimum in traditional salami.

In our study, we examined the levels of histamine in raw meat. Even though grilled pork has increased histamine level (by 1.5 times compared with raw pork) and boiling reduces it by 10%–20%, the increased histamine level observed in this study was not sufficient to cause food illness or sensitivity (Chung et al., 2017). However, depending on the enteral environment and Diamine Oxidase (DAO) activity, each person's vulnerability to histamine varies (Maintz & Novak, 2007).

This tolerance explains the adaptation of the tested strains to the chilling temperature conditions (4 °C and 8 °C), observed as a slight increase in bacterial counts in the control samples (0 µL/mL galangal extract) (Table 1). Therefore, the lowest galangal extract concentration used was 425 mg/mL, or 0.425 µL/g (MIC). The other concentrations (10% and 15% of 425 mg/mL) used were based on the work of Pandit & Shelef (1994), who reported that essential oil concentrations 10 times greater than the MIC are necessary to achieve efficient inhibition of bacterial growth in meat products. Furthermore, concentrations higher than the in vitro MIC are required for effective inhibition of microorganisms in food because of various interactions that can occur between the food constituents and the essential oil components.

3.2 Antioxidant activities of ethanol galangal extracts

The DPPH assay was conducted with ethanol galangal extracts and both standards (Trolox and ascorbic acid). The galangal extract had the highest free radical scavenging capacity of 623.03 mg vitamin C (ascorbic acid) equivalent per 100 g of dry weight, *i.e.*, it was close to the value reported by Juntachote & Berghofer (2005). The highest Trolox equivalent antioxidant capacity of galangal extract was 811.08 mg Trolox equivalent per 100 g of dry weight. The DPPH assay is used to measure the ability of antioxidant molecules to quench DPPH free radicals. The galangal extracts in this study showed the ability to reduce free radicals, stop free radical generation, or retard free radical chain reaction in the propagation of the oxidation mechanism. Mahae & Chaiseri (2009) reported that the ethanol galangal extract contained the highest concentrations of total phenolic compounds (31.49 mg GAE/g) and flavonoids (13.78 mg CE/g).

3.3 Kinetic model for HPB reduction

The shelf life and kinetic model for HBP reduction in minced pork during refrigerated storage with galangal extract at various concentrations were also determined. K value, which describes galangal safety, was determined based on histamine generation during storage at 4 ± 2 °C and 8 ± 2 °C (Table 1).

As shown in Figure 1, the K value decreased linearly at a slow rate during storage at 4 ± 2 °C with 15% galangal extract. Based on the K values, minced pork maintained an acceptable shelf life of 6 d at 4 ± 2 °C with 10% galangal extract, 3 d at 4 ± 2 °C with 15% galangal extract, and 3 d at 8 ± 2 °C with either 10% or 15% galangal extract. The objective of the current study was to evaluate the effect of different treatments on antimicrobial kinetics (reduction in bacterial population during storage at chilling temperatures). The Weibull model, largely used to generate bacterial inactivation curves in antimicrobial processes (van Boekel, 2002), was generated (Figure 2).

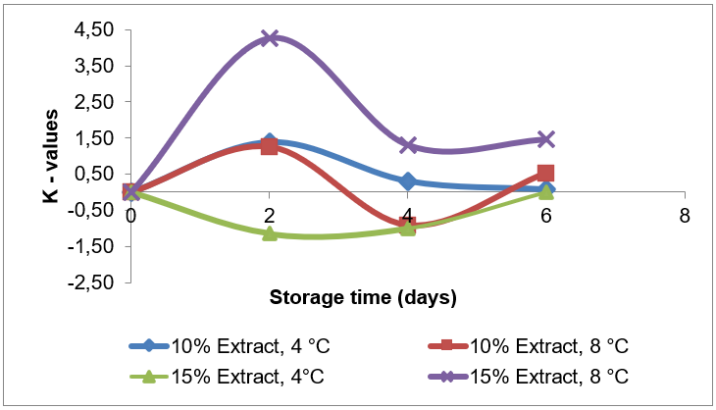

Figure 1. The Kinetic Model of HBP in Minced Pork.

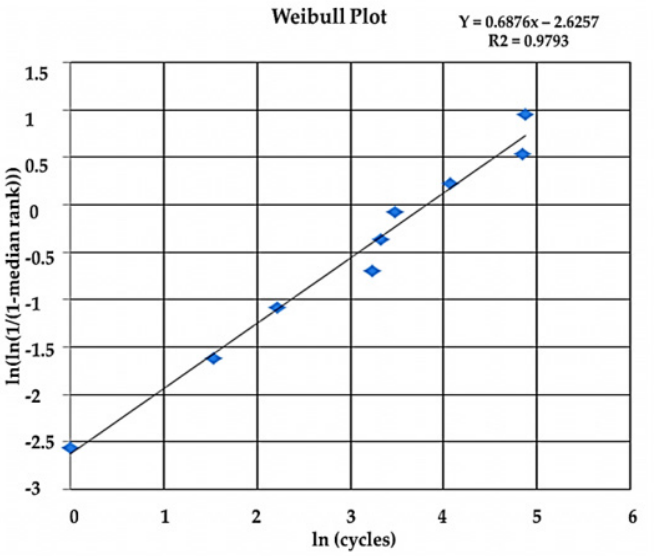

Figure 2. The Weibull Model for Inactivation of HBP in minced Pork. The estimated α and β parameters of the Weibull model were 1.6089 and 2.1980.

The estimated α and β parameters of the Weibull model were 1.6089 and 2.1980, respectively. The DR value, the concentration of galangal extract required for more than 90% reduction in HPB population in minced pork, was determined to be 2.3516 mg/mL (R^2 = 0.9793). These findings demonstrated the potential use of essential oils, natural compounds with strong antibacterial activity, to control the growth of pathogenic and spoilage bacteria in processed meats. However, it is widely documented that the use of high concentrations of essential oils can elicit undesirable sensory alterations in meat (Hayouni et al., 2008). Furthermore, the results emphasized that the HBP population in fresh minced pork was successfully reduced with galangal extract application in packaging during chilling storage.

4 Conclusions

The Weibull kinetic models can be used to explain the inactivation of bacteria by natural antimicrobials such as galangal extract during refrigerated storage. In the current study, we showed that galangal extract inhibited the growth of bacteria in the food model challenge tests (minced pork). Refrigeration is the storage method of choice for perishable foods such as ground meat. In the current study, we showed that refrigeration alone cannot control the growth of bacteria if the bacteria were already present at the start of incubation.

Natural preservation methods, such as galangal extract application, could present a feasible alternative for ascertaining the safety of highly perishable foods maintained under refrigeration during shelf life. Furthermore, the risk of histamine poisoning might be controlled by applying basic good manufacturing and hygiene practices in conjunction with an appropriate hazard analysis critical control points system, such as appropriate storage temperature and sampling plan for fresh minced pork. To control the concentration of BAs in food, decarboxylase activity for amino acids could be regulated. The levels of BAs can be reduced using several methods such as packaging, additive supplementation, hydrostatic pressure application, irradiation, pasteurization, smoking, starter culture use, and temperature control.

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