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A Metabolomics Study on the Antimicrobial Compound Profiles in Rendang Seasoning against *Clostridium sporogenes* ATCC 19404

(Kajian Metabolomik Profil Sebatian Antimikrob dalam Perencah Rendang terhadap *Clostridium sporogenes* ATCC 19404)

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ABSTRACT

Rendang is a traditional food from West Sumatera, Indonesia, made from beef, coconut milk, and spices cooked for 6-7 h. Commercial rendang products have a long shelf-life at room temperatures, but they are not always processed according to commercial sterilization designed to reduce 12 log cycles of *Clostridium botulinum*. It is not known whether the rendang seasoning could assist the thermal process due to its antimicrobial activity. This study used a metabolomics approach to profile the compounds acting as antimicrobials in rendang seasoning. Due to safety concerns, *Clostridium sporogenes* was utilized as a surrogate for *C. botulinum*. Three formulas of rendang seasoning with and without coconut milk were heated for 60 or 120 min, respectively. Each seasoning was macerated in n-hexane and the extracts were tested for their antimicrobial activity toward *C. sporogenes* using agar well diffusion method. The extracts were analyzed using gas chromatography mass spectrometry to identify the compounds and a multivariate data analysis was conducted. The study showed that rendang seasoning A, B, and C with or without coconut milk inhibited *C. sporogenes* with diameters of inhibition zone ranging from 4.71 mm to 18.92 mm. The metabolomics showed a well-defined clustering of Principal Component Analysis model (Q^2 = 0.654, R^2X = 0.853) and Orthogonal Projection to Latent Structure model (Q^2 = 0.865, R^2Y = 0.95). Garlic and great galangal were identified as the leading antimicrobial spices in rendang seasoning against *C. sporogenes*, as OPLS showed their key compounds, 2-vinyl-1,3-dithiane, 2-vinyl-4H-1,3-dithiine, and (S)-4-(1-acetoxyallyl)phenyl acetate, as well as an unknown compound.

Keywords: Antimicrobial; *Clostridium sporogenes*; metabolomics; rendang seasoning; spices

ABSTRAK

Rendang ialah hidangan tradisi asal Sumatera Barat, Indonesia yang dimasak selama 6-7 jam menggunakan daging lembu, santan dan rempah-rempah. Produk rendang komersial mempunyai ketahanan simpan yang lama pada suhu bilik, tetapi tidak semuanya menjalani pensterilan komersial yang direka untuk mengurangi *Clostridium botulinum* sebanyak 12 pusingan log. Potensi aktiviti antimikrob perencah rendang masih belum diketahui dalam membantu proses terma. Kajian ini menggunakan pendekatan metabolomik untuk memprofilkan sebatian antimikrob dalam perencah rendang. Oleh kerana pertimbangan faktor keselamatan, *Clostridium sporogenes* digunakan sebagai bakteria pengganti bagi *C. botulinum*. Tiga formula perencah rendang, dengan dan tanpa santan, dipanaskan masing-masing 60 dan 120 minit. Setiap perencah rendang dimaserasi dalam n-heksana dan ekstraknya diuji aktiviti antimikrob terhadap *C. sporogenes* dengan kaedah resapan agar. Ekstrak perencah rendang dianalisis menggunakan kromatografi gas-spektrometri jisim untuk menentukan sebatian yang dilanjutkan dengan analisis data multivariat. Kajian ini menunjukkan bahawa perencah rendang A, B dan C tanpa atau dengan santan, mampu menghalang *C. sporogenes* dengan zon perencatan berukuran antara 4.71 mm hingga 18.92 mm. Metabolomik menghasilkan pengelompokan jelas dalam model Analisis Komponen Utama (Q^2 = 0.654; R^2X = 0.853) dan model Unjuran Ortogon kepada Struktur Pendam (Q^2 = 0.865; R^2Y = 0.95). Model OPLS menunjukkan bawang putih dan lengkuas sebagai rempah antimikrob dominan dalam perencah rendang terhadap *C. sporogenes*, melalui kehadiran sebatian 2-vinil-1,3-ditiana, 2-vinil-4H-1,3-ditiin dan (S)-4-(1-asetoksialil)fenil asetat, berserta satu sebatian tidak dikenali.

Kata kunci: Antimikrob; *Clostridium sporogenes*; metabolomik; perencah rendang; rempah-rempah

INTRODUCTION

Rendang is an Indonesian traditional food from West Sumatera placed first in the category of 50 best foods in the world according to CNN survey (Cheung 2017). Rendang is commonly made of beef, coconut milk and seasoning containing various spices cooked for 6-7 h at temperatures of 80-95°C (Rini et al. 2016). There are various formulas of the rendang seasoning, but they usually contain red chili, shallot, garlic, lemongrass, ginger, great galangal, lime leaves, bay leaves, turmeric leaf, nutmeg, coriander, cardamom, ginger, cloves, star anise, and *ruku-ruku* leaves, and salt (Gusnita & Fitri 2019a, 2019b). Due to the long cooking time, rendang appears dry in texture, dark brown in color and has long shelf-life (Waryono 2021).

In the Indonesia market, rendang products in sealed packaging are claimed to have a long shelf-life at room temperature. However, some may not be produced using a commercial sterilization process. According to the Indonesian Food and Drug Authority (BPOM) guidelines, a commercial sterilization process must be designed to reduce 12 log cycles of *Clostridium botulinum* (BPOM 2021). *C. botulinum* is a Gram-positive pathogenic, spore-forming, and obligately anaerobic bacteria. The spores are heat resistant and able to germinate into vegetative cells in the favorable environment which produce botulin toxin, causing botulism (Alizadeh et al. 2020). Previous study explained *C. botulinum* spores are naturally distributed in dust, soil, sediments, water and food, thus, it can be carried out via wind or human movement to contaminate food products (Yadav, Singh & Ponmariappan 2019). *C. botulinum* can contaminate red meat because it can colonize in the digestive tract of animal (Bilska et al. 2024). The dormant form of spore-forming bacteria such as *C. botulinum*, may be able to survive thermal process during the cooking of rendang. When the cooking process is inadequate, the surviving spores may germinate during storage. This raises question of whether the spices in rendang seasoning contribute to inhibit spore-forming bacteria during rendang processing and storage. Citrus extract and vinegar were reported to inhibit the growth of spore-forming *C. perfringens* in the sous vide processed chicken (Smith, Olszewska & Diez-Gonzalez 2021). Additionally, various spices in rendang seasoning have been studied for their antimicrobial activity individually (Beristain-Bauza et al. 2019; Bhatwalkar et al. 2021; Mukarram et al. 2022; Wang et al. 2023).

Identification of compounds in individual spices in rendang seasoning requires a long time, hence, an alternative method approach, such as metabolomics, can shorten the time. Lately, metabolomics has gained significant attention for its potential to discover bioactive compounds from food materials and products. The individual spices used in rendang seasoning have increasingly been observed using metabolomics and those provide superb insights into their engaging biochemical profiles (Hrbek et al. 2018; Rastogi et al. 2020; Salem et al. 2022; Syabana et al. 2022). This approach combines cutting-edge analytical techniques to analyze and quantify the samples' metabolites with a molecular weight <2000 Da (Demarque et al. 2020). The steps in the metabolomics approach start with the sample preparation, compound extraction, compound identification, data mining, and multivariate data analysis. The compound identification process generally utilizes the advanced technological methods to obtain reliable results. Gas chromatography mass spectrometry (GC-MS) is an advanced analytical technique which has demonstrated its effectiveness in analyzing volatile compounds with excellent sensitivity. It also serves as the instrument or tool in metabolomics, primarily employed to identify volatile compounds from biological samples (Ahlina et al. 2020; Du et al. 2023). Rendang seasoning, which consists of various spices, is abundant in volatile compounds. Since volatile compounds are the major component in spices, GC-MS is a suitable technique for identifying the compounds in rendang seasoning.

The data of metabolomics are complex and may be difficult to interpret due to the large amount of information on metabolites. The challenge in metabolomics arises from the chemical metabolites that have varying concentration. For that reason, a comprehensive statistical analysis is needed to explain the insights of metabolomics data. Multivariate data analysis, including Principal Component Analysis (PCA) and Orthogonal Projection to Latent Structure (OPLS), was implemented as the statistical analysis in metabolomics research. The PCA may assist to elucidate the meaning or pattern of metabolites data by converting the high dimensional data into lower dimensional data (Rafi et al. 2020). Meanwhile, OPLS is operated to determine the relationship between chemical metabolites data as X variable and activity data as Y variable (Yuliana, Prangdimurti & Faridah 2018). The essential parameters to validate the models of PCA and OPLS are Q^2 value, that reflects the goodness of model prediction, and R^2 value, which shows how well the model fits the data (Novitasari, Dewanti-Hariyadi & Yuliana 2023). Other features can be utilized are loading plot, S-plot, S-plot, variables important for projection (VIP), X variables plot, permutation, and CV ANOVA.

Due to the toxicity and safety concerns related to *C. botulinum*, a surrogate bacterium is commonly used for certain studies. The ideal surrogate bacteria requirements must be non-pathogenic, non-toxigenic, and has characteristics that closely match with the target bacteria. *C. sporogenes* fulfill these criteria because of its similar physiological and morphological characteristics with *C. botulinum*. *C. sporogenes* is non-pathogenic and non-toxigenic, hence, it is widely utilized as a surrogate bacteria for *C. botulinum* in research purposes, as well as in validation of thermal processing and sterilization (Butler et al. 2017; Taylor et al. 2013). Some studies showed *C. sporogenes* demonstrated greater resistance

than *C. botulinum* in heat processing (Diao, André & Membré 2014; Reddy et al. 2016), therefore, *C. botulinum* would be likewise inhibited under the same condition. Other studies also reported the use of *C. sporogenes* as a surrogate bacteria was applied in antimicrobial activity research (Ghabraie et al. 2016; Pinelli et al. 2021).

Studies on the antimicrobial activity of rendang seasoning are very limited. Hence, studying the antimicrobial activity in rendang seasoning using metabolomics approach is very important. This study aims to profile the active compounds in rendang seasoning acting as antimicrobial against *C. sporogenes* with gas chromatography mass spectrometry (GC-MS) based metabolomics. *C. sporogenes* was utilized in this study, as the surrogate for *C. botulinum*, to ensure the safety while maintaining the relevance of the findings.

MATERIALS AND METHODS

MATERIALS AND INSTRUMENTS

Clostridium sporogenes ATCC 19404 was obtained from the SEAFAST Center, Bogor. The spices for rendang seasoning, including red chili, garlic, shallot, ginger, great galangal, turmeric, cardamom, coriander, nutmeg, clove, star anise, *kandis* acid, lemongrass, turmeric leaves, bay leaves, *ruku-ruku* leaves, coconut milk, and salt were obtained from Dramaga Market, Bogor, Indonesia. Other materials for analysis in this study included n-hexane (Merck, Germany), chloramphenicol, Mueller Hinton Agar (MHA) (Oxoid Ltd, UK), Reinforced Clostridial Medium Broth (RCMB) (Oxoid Ltd, UK), Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) solid phase microextraction (SPME) fiber, headspace vials, and analytical standard (C8-C20) (Supelco, USA).

The instruments used in this study were rotary evaporator (Rotavapor R-300, Buchi, Switzerland), anaerobic jar and instrument (Anoxomat AN2CTS, Netherlands), and gas chromatography-mass spectrometry (GCMS-QP2010 Plus, Shimadzu, Japan). The multivariate data analysis for metabolomics was performed using SIMCA software (Sartorius, Germany).

METHODS

RENDANG SEASONING PREPARATION

Three rendang seasoning formulas were based on recipes of a home industry (A), literature (B), and a recipe book (C) (Great Kitchen Team 2009; Gusnita & Fitri 2019b). The formulas without coconut milk (controls) were designated as TS, meanwhile the rendang formulas with coconut milk were described as DS (Table 1). Correspondingly, the three TS formulas were labeled as TSA, TSB, and TSC while the three DS formulas were marked as DSA, DSB, and DSC. The spices from each formula were blended and then heated at temperatures of 85-90°C for 60 min for the controls, while the formulas containing coconut milk were cooked for 120 min until each gained its fully cooked stage, resulting in rendang seasoning with consistent visual appearance, including the dark color and paste-like consistency (Rini et al. 2016).

EXTRACTION OF RENDANG SEASONING

A total of 50 g rendang seasoning was soaked and macerated in 200 mL of n-hexane solvent with the maceration time of 3×24 h, at cold temperature of 10°C (Tharukliling et al. 2021). The macerated rendang seasoning samples were filtered using Whatman filter paper and evaporated at a temperature of 42°C, pressure of 250 mBar, and 120 rpm rotation to remove the n-hexane solvent. The extracts of rendang seasoning were stored at a temperature of -18°C until further use.

Clostridium sporogenes CULTURE PREPARATION

A freeze-dried culture of *Clostridium sporogenes* ATCC 19404 was activated in 9 mL of RCMB and incubated under anaerobic conditions using anaerobic jar for 24 h at 37°C. After incubation, a loop full of broth culture was streaked onto an agar slant containing 6 mL of RCMA and incubated anaerobically for 24 h at 37°C. For antimicrobial activity test, a loopful of 1 µL of *C. sporogenes* agar slant culture was inoculated into 9 mL RCMB and incubated anaerobically for 24 h at 37°C. The culture concentration for antimicrobial activity test was ca. 10^6 CFU/mL (CLSI 2020; Pacheco et al. 2017). The concentration of *C. sporogenes* for antimicrobial activity test was confirmed by the total plate count (TPC) method. One milliliter of *C. sporogenes* culture was added to a test tube containing 9 mL of sterile 0,85% physiological NaCl solution. Three dilutions estimated to have 25-250 colonies were plated in duplicate in the Petri dish containing RCMA and incubated anaerobically for 24 h at a temperature of 37°C.

ANTIMICROBIAL ACTIVITY TEST

The antimicrobial activity was tested with the agar well diffusion method by following previous study (Afriyanti et al. 2023). *C. sporogenes* culture (1×10^7 CFU/mL) was inoculated into sterile MH agar and subsequently poured into a Petri dish. Once the agar had solidified, a sterile cork borer with 6 mm diameter was used to make four wells at different points. The rendang seasoning extracts were applied in undiluted form (50 mg/mL) due to the high concentration of active compounds (Ghazal et al. 2022; Rathnayake et al. 2020). A volume of 60 µL of rendang seasoning extract (50 mg/mL) was placed in the well. Chloramphenicol (200 ppm) as a positive control and n-hexane as a negative control were pipetted with the same volume to other wells in the Petri dish. All Petri dishes were incubated under anaerobic conditions at the temperature of 37°C for 24 h.

TABLE 1. Formulas of rendang seasoning

Ingredients	Rendang seasoning formulas					
	TSA	DSA	TSB	DSB	TSC	DSC
Curly red chili (g)	300	300	30	30	250	250
Cayenne pepper (g)	20	20	-	-	-	-
Garlic (g)	120	120	20	20	25	25
Shallot (g)	500	500	200	200	60	60
Ginger (g)	75	75	20	20	8	8
Great galangal (g)	200	200	30	30	20	20
Turmeric leaves(g)	5	5	5	5	5	5
Bay leaves (g)	2	2	2	2	-	-
Kaffir lime leaves (g)	2	2	3	3	3	3
Turmeric (g)	-	-	-	-	10	10
Kandis acid (g)	-	-	-	-	4	4
Nutmeg (g)	-	-	1	1	-	-
Cardamom (g)	-	-	0.5	0.5	-	-
Coriander (g)	-	-	10	10	-	-
Cloves (g)	-	-	0.5	0.5	-	-
Star anise (g)	-	-	0.5	0.5	-	-
Ruku – ruku leaves(g)	-	-	13	13	-	-
Lemongrass (g)	16	16	8	8	8	8
Coconut milk (mL)	-	750	-	750	-	750
Salt (g)	20	20	20	20	20	20
Frying oil (mL)	500	500	500	500	500	500

TS = without coconut milk (controls); DS = with coconut milk

The clear zone diameters resulted from the extracts' antimicrobial activity were measured. The antimicrobial activity category of rendang seasoning extracts were determined according to CLSI (2014), where the inhibition zone diameters >11 mm indicate susceptible (strong), diameters of 6-11 mm are classified as intermediate, and diameters <6 mm are resistant (weak).

COMPOUNDS IDENTIFICATION USING HEADSPACE SOLID PHASE MICROEXTRACTION GAS CHROMATOGRAPHY MASS SPECTROMETRY (HS-SPME-GC-MS)

The HS-SPME-GC-MS analysis was prepared according to the protocols from Hardoyono et al. (2019) with some modification. Five milliliter (50 mg/mL) of rendang seasoning extract was transferred into a 20 mL headspace vial and extracted using SPME fiber at 50 °C for 30 min. The condition of GC-MS used DB-5MS column (30 m × 0.25 mm × 0.25 µm) and Helium as the carrier gas (0.95 mL/min). The initial oven temperature was set at 50 °C and gradually increased to 250 °C with rates 5 °C/min (100 °C), 8 °C/min (200 °C), and 10 °C/min (250 °C). Each rate was maintained for 5 min. The temperature of injector, interface, and ion source was conducted at 200 °C, 280 °C, and 200 °C, respectively. The electron impact mode (70 eV) and mass range (45 to 550 *m/z*) were applied. The n-alkanes standards (C8-C20) were injected to the GC-MS with same conditions. The identified volatile compounds would be validated by comparing the linear retention index (LRI) and National Institute of Standards and Technology (NIST) 17.0 database.

DATA ANALYSIS OF ANTIMICROBIAL ACTIVITY

The antimicrobial activity of rendang seasoning was expressed as inhibition zone diameters. The data analysis was conducted with one-way analysis of variance (ANOVA) test (α=95%) using IBM SPSS 22.0 for windows (SPSS Inc, USA). If ANOVA analysis shows the significant result (p-value<0.05), the data analysis will be assisted to the next test, Duncan Multiple Range Test (DMRT), to figure out the significant difference between each sample.

MULTIVARIATE DATA ANALYSIS FOR METABOLOMICS

The multivariate data analysis, including PCA and OPLS, was performed by SIMCA software 18.0 (Sartorius, Germany) (Yuliana, Prangdimurti & Faridah 2018). Both

PCA and OPLS applied Pareto scaling and an exponentially weighted moving average (EWMA) as data pre-processing (Novitasari, Dewanti-Hariyadi & Yuliana 2023). PCA was used to cluster the rendang seasoning samples according to their volatile compound profiles, whereas OPLS would find out the correlation or interaction between the antimicrobial activity (Y variable) and volatile compounds (X variable). The PCA and OPLS used Q^2 and R^2 value as the goodness of prediction and goodness of fit, with minimum values of 0.5. OPLS S-plot and variable importance for projection (VIP) value were conducted to determine the compounds associated with antimicrobial activity in rendang seasoning. Other features, such as CV-ANOVA and permutation plot, were also applied to affirm the OPLS model (Figure S1).

RESULTS AND DISCUSSION

ANTIMICROBIAL ACTIVITY OF RENDANG SEASONING AGAINST *C. sporogenes*

This study showed that all formulas of rendang seasoning have various abilities in inhibiting *C. sporogenes* (Figure 1). The statistical analysis applied to rendang seasoning antimicrobial activity also showed a significant effect ($p<0.05$).

The formula A (TSA and DSA) contained the largest amount of key rendang spices, such as red chili, shallot, garlic, ginger, great galangal, and lemongrass, than other formulas. These spices may produce active compounds that contribute to the strong inhibitory against *C. sporogenes* in rendang seasoning TSA. The synergistic interaction between the spices is also suggested to promote the inhibitory against *C. sporogenes*. Previous studies confirmed that the combination between ginger, garlic, and lemongrass significantly enhance the antimicrobial activity compared to the individual use of each component (Rajendrasozhan 2024; Yakubu, Katsa & Chrysantus 2023). The formula B (both TSB and DSB) involved a variety of spices, though the quantity of each individual spice was lower, with shallot as the predominant component. In this formula, the spices might have a synergistic interaction, but the effectiveness of active compounds as antimicrobial agents was not optimal due to the lower concentration of spices. For instance, clove essential oil at levels of 50-500 ppm has been reported to exhibit strong inhibitory against *C. perfringens* and *C. difficile* (Hu, Zhou & Wei 2018). In contrast, formula C (TSC and DSC) which showed low inhibitory activity against *C. sporogenes*, used red chili as the predominant spices, combined with *kandis* acid and turmeric as additional spices. The *kandis* acid and turmeric have been reported for their individual role as antimicrobial agent against pathogenic bacteria (Ali, Islam & Zaman 2020; Joseph, Dandin & Hosakatte 2016); however, they did not exhibit a significant effect in this study. Manohar et al. (2014) showed that the essential oil extracted from 100 grams of *kandis* acid fruits showed the antimicrobial activity at levels ranging from 0,125-16 µg/µL. On the other hand, the combination of fresh turmeric, garlic, and ginger was more effective in reducing *C. sporogenes* growth by approximately 1 log cycle CFU/g compared to using the heated turmeric (Goswami, Prabhakarn & Tanwar 2013). Therefore, the concentration of spices is crucial thing to produce the sufficient compounds that act as antimicrobial agents.

Figure 2 showed Formula A without coconut milk (TSA) had the largest inhibition zone diameter of 18.92 mm, suggesting that TSA had strong antimicrobial inhibitory. The inhibition zone of rendang seasoning A with coconut milk (DSA), B without coconut milk (TSB), and B with coconut milk (DSB) were categorized as intermediate at 10.18 mm, 9.14 mm, and 10.30 mm, respectively (Figure 2). Meanwhile, formula C with or without coconut milk (DSC and TSC), did not inhibit *C. sporogenes* as the inhibition zone diameters were 4.71 mm and 4.74 mm, respectively. This study suggests that the types and amounts of spices in each formula will affect the inhibition response. Previous study reported the spices combination could effectively inhibit the food pathogenic bacteria growth (Mutlu-Ingok et al. 2019). Furthermore, each compound can play a different role in inhibiting the *C. sporogenes*. A compound not effective against *C. sporogenes*, may still support overall antimicrobial activity by interacting synergistically with other compounds. For example, the interaction between eucalyptol and thymol enhances the antimicrobial activity against some pathogens, while eucalyptol alone does not effectively inhibit these pathogens (García-Díeza et al. 2017; Naveed et al. 2013).

In this present work, the rendang seasoning formulas containing coconut milk demonstrated varying inhibition responses. The addition of coconut milk in rendang seasoning A (DSA) did not enhance the antimicrobial activity against *C. sporogenes*. A drastic decrease in antimicrobial activity observed in rendang seasoning DSA may be due to the incorporation between the active compounds and coconut milk fats. This interaction forms the fat-rich environment and likely restricts the diffusion of compounds toward the cell membrane of *C. sporogenes*. According to Pernin et al. (2019), the high-fat food system is highly complex which may decrease the antimicrobial efficacy of active compounds. A large amount of active and stable compounds from spices may be required, particularly in the fat-rich food system, to maintain the antimicrobial efficacy. It was evident that the addition of coconut milk in rendang seasoning DSB clearly increased the inhibition against *C. sporogenes*, although was not significant with rendang seasoning TSB. Despite containing fewer spices amount, the compounds in rendang seasoning B are more stable in the presence of coconut milk. This work underlines the ratio of major components is not the sole factor regulating antimicrobial activity. Previous study highlighted the importance of synergistic

interaction between major and minor components might contribute towards bacteria inhibition (Chouhan, Sharma & Guleria 2017).

Most of the spices used in rendang seasoning have individually been reported of their potency of antimicrobial activity against food pathogens (Nikolic et al. 2021; Swamy, Akhtar & Sinniah 2016; Zhang et al. 2021). Garlic and shallot have sulfur-containing compounds as their major component that have been reported as antimicrobial agent. The sulfur-containing compounds will react with the sulfur atom of protein in the membrane cell that make the disulfide bond and lead to cell leakage (Bhatwalkar et al. 2021). Previous studies also showed that the Gram positive pathogenic bacteria were successfully inhibited by great galangal, ginger, and curcumin because they could perturb the cell stability and permeability (Zhang et al. 2021). The terpenes and terpenoids as major compound in spices might interfere the permeability of cell membrane which secretes the important substances (García-Díeza et al. 2017; Guimarães et al. 2019). This study highlights that the antimicrobial activity of rendang seasoning relies not only the high concentration of spices and their active compounds, but also the stability of these compounds, particularly under the fat-rich condition. Nonetheless, the synergistic or antagonist interaction between each component must be observed. When multiple compounds act through the different inhibitory mechanism but target the same spot, it raises the concern that one compound may disturb the affinity of others.

This work was carried out using *C. sporogenes* as the surrogate bacteria which has been reported to be more heat resistant as compared to *C. botulinum*. A previous study showed that *C. sporogenes* required 1.28 min to achieve 1-log reduction, while *C. botulinum* required only 0.2 min at the temperature of 121°C (Diao, André & Membré 2014), indicating the higher thermal resistance of *C. sporogenes*. In addition, other studies have also reported the use of *C. sporogenes* as a surrogate for *C. botulinum* in antimicrobial activity research (Ghabraie et al. 2016; Pinelli et al. 2021). These findings collectively support that *C. sporogenes* is reliable surrogate bacteria, as its inactivation behavior may sufficiently represent *C. botulinum*.

VOLATILE COMPOUNDS PROFILE OF RENDANG SEASONING

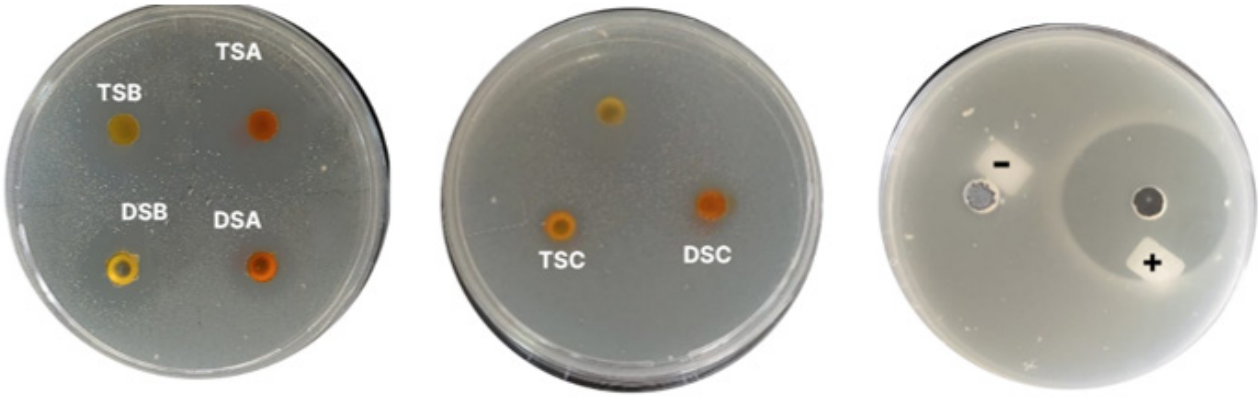
The SPME-GC-MS analysis of rendang seasoning successfully identified 186 compounds (Table 2). The volatile compounds profiles are divided into several groups, namely sulfur-containing compounds, terpenes, terpenoids, alkylbenzenes, organic peroxides, phenolics, aldehydes, alcohols esters, alkanes, alkenes, phenylpropanoids, others hydrocarbons, and unknown compounds. The GC-MS showed that the sulfur-containing compounds, terpenes, and terpenoids dominate the rendang seasoning with various concentration.

The relative peak areas showed in Table 2 indicate the compound concentration in each formula. The concentration and unique compounds are affected by the use of type and amount of spices in each rendang seasoning formula. The sulfur-containing compounds are primarily found in shallot and garlic that are used in the rendang seasoning formulas (Wang et al. 2023). Rendang seasoning samples also contain terpenes and terpenoids groups that are dominant in spices. Terpenes are hydrocarbons with the isoprene structure, also have their derivatives form as terpenoids containing the functional group and oxidized methyl group (Novitasari, Dewanti-Hariyadi & Yuliana 2023). Terpenes, terpenoids and sulfur-containing compounds were reported about their ability as antimicrobial agents (Bhatwalkar et al. 2021; Guimarães et al. 2019). The data mining was conducted to verify and extract the compounds data. The verification process involved comparing the mass spectra of each compound with reference library by evaluating the matching score, patterns similarities, and number of peaks (Stettin, Poulin & Pohnert 2020). This study found 14 unknown compounds in rendang seasoning which were detected according to their consistency of peaks, retention time, and reproducible ion fragments. These unknown compounds could be formed through the transformation or degradation during cooking. Moreover, interactions among the ingredient components may also generate new derivatives. Even if these compounds did not match with the mass spectra database, each exhibited unique and distinctive fragmentation profiles. Therefore, the further studies to evaluate these compounds are required.

RENDANG SEASONING CLUSTERING RESULT USING PRINCIPAL COMPONENT ANALYSIS (PCA)

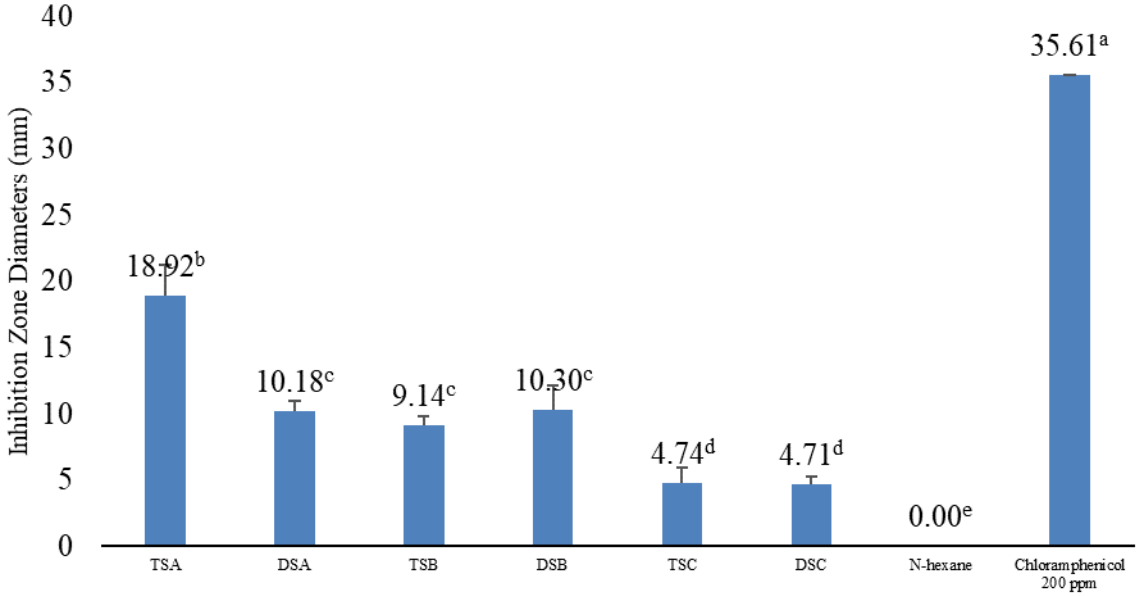
The clustering of rendang seasoning samples was showed using PCA score plot (Figure 3). PCA score plot explains the discrimination of the samples according to their volatile compound profiles with the Q^2 and R^2X cumulative values of 0,654 and 0,853, respectively, which indicates that the model is well founded. PCA is very useful statistical analysis to find out the pattern of large big data by transforming high dimension to low dimension without changing the meaning (Rafi et al. 2020).

According to Figure 3, the group separation observed in PCA score plot was assigned with different color for each group. The PCA model clustered three groups of rendang seasoning based on their volatile compound profiles. Each group in PCA model indicates the three formulas of rendang seasoning having the different and unique of volatile compound profiles. The model also describes that these compounds are successful in contributing to distinguish the clustering patterns. Because of PCA is an early step in multivariate data analysis, the reliable result in the PCA model will determine the next analysis, such as OPLS or others multivariate data analysis (Afriyanti et al. 2023).



Note: TSA = formula A without coconut milk; DSA = formula A with coconut milk; TSB = formula B without coconut milk; DSB = formula B with coconut milk; TSC = formula C without coconut milk; DSC = formula C with coconut milk; - = n-hexane; + = chloramphenicol (200 ppm)

FIGURE 1. Inhibition zones of rendang seasoning against C. sporogenes



TSA = formula A without coconut milk; DSA = formula A with coconut milk; TSB = formula B without coconut milk; DSB = formula B with coconut milk; TSC = formula C without coconut milk; DSC = formula C with coconut milk; the different superscripts indicate significant difference ($p<0.05$)

FIGURE 2. Inhibition zone diameters of rendang seasoning extracts against C. sporogenes

IDENTIFICATION OF ANTIMICROBIAL COMPOUNDS IN RENDANG SEASONING USING OPLS ANALYSIS

The OPLS score plot clearly demonstrated the separation of rendang seasoning samples according to the antimicrobial activity (Figure 4(A)). The rendang seasoning TSA are the strongest group associated with antimicrobial activity as characterized by the intense size and red color. The OPLS score plot also showed that TSC and DSC were located in the left area with faint size and color, indicating the groups with low antimicrobial activity. The OPLS analysis was operated to find out the correlation between the X and Y data (Yuliana, Prangdimurti & Faridah 2018).

The OPLS model showed the Q value of 0,865 and R^2Y of 0,95, explaining the model is well-suited to represent the dataset. Syabana et al. (2022) stated that the Q^2 and R^2 values closer to 1 will present a better model fit and predictive accuracy. The correlated antimicrobial compounds in rendang seasoning were also described by OPLS S-plot and validated using variable importance in projection (VIP) (Figure 4(B)).

The OPLS S-plot aims to discover the compounds that powerfully associated with the groups separation (Yuliana et al. 2020). This study found that unknown 2, 2-vinyl-1,3-dithiane, 2-vinyl-4H-1,3-dithiine, and (S)-4-(1-acetoxyallyl)phenyl acetate were the compounds that emerged as the strong contributors in inhibiting *C. sporogenes*, which were also supported by their VIP values exceeding 1. In the OPLS analysis, a VIP value

TABLE 2. Volatile compounds profile of rendang seasoning

Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
Sulfur containing compounds										
(E)-1-Methyl-2-(prop-1-en-1-yl)disulfane	940	RI, MS	120	23838-19-9	0.00	0.00	0.05	0.09	0.00	0.00
(Z)-1-Methyl-2-(prop-1-en-1-yl)disulfane	941	RI, MS	120	23838-18-8	0.39	0.43	0.05	0.00	0.00	0.00
3H-1,2-Dithiole	958	RI, MS	104	288-26-6	0.28	0.2	0.00	0.00	0.00	0.00
Dimethyl trisulfide	970	RI, MS	126	3658-80-8	0.32	0.23	0.00	0.00	0.00	0.00
Diallyl disulfide	1081	RI, MS	146	2179-57-9	0.86	1.01	0.00	0.00	0.26	0.09
(E)-1-Allyl-2-(prop-1-en-1-yl)disulfane	1095	RI, MS	146	122156-02-9	0.74	0.83	0.06	0.04	0.00	0.00
(Z)-1-Allyl-2-(prop-1-en-1-yl)disulfane	1096	RI, MS	146	122156-03-0	0.00	0.00	0.00	0.02	0.00	0.00
3-Methyl-3H-1,2-dithiole	1100	MS	118	118023-96-4	0.16	0.00	0.00	0.00	0.00	0.00
Disulfide, dipropyl	1107	RI, MS	150	629-19-6	0.21	0.00	0.00	0.00	0.00	0.00
(E)-1-(Prop-1-en-1-yl)-2-propyldisulfane	1118	RI, MS	148	23838-21-3	0.37	0.27	0.00	0.00	0.00	0.00
Trisulfide, methyl 2-propenyl	1136	RI, MS	152	34135-85-8	3.57	4.06	0.33	0.4	0.76	0.7
Trisulfide, methyl propyl	1148	RI, MS	154	17619-36-2	0.19	0.06	0.00	0.15	0.00	0.00
(E)-1-Methyl-3-(prop-1-en-1-yl)trisulfane	1158	RI, MS	152	23838-25-7	0.47	0.39	0.29	0.1	0.62	0.00
3-Vinyl-1,2-dithiacyclohex-4-ene	1187	RI, MS	144	62488-52-2	3.32	3.69	1.29	1.52	2.45	1.99
4H-1,2,3-Trithiine	1200	MS	136	290-30-2	0.00	0.12	0.00	0.00	0.00	0.00
2-Vinyl-4H-1,3-dithiine	1215	RI, MS	144	80028-57-5	9.23	8.56	1.91	1.3	3.31	2.07
2-Vinyl-1,3-dithiane	1220	RI, MS	146	61685-40-3	0.9	0.47	0.12	0.05	0.00	0.00
Trisulfide, di-2-propenyl	1297	RI, MS	178	2050-87-5	3.98	5.87	0.8	1.4	2.04	2.59
1-Allyl-3-propyltrisulfane	1302	RI, MS	180	33922-73-5	0.66	0.98	0.00	0.00	0.02	0.00
(Z)-1-Allyl-3-(prop-1-en-1-yl)trisulfane	1331	RI, MS	178	382161-75-3	0.25	0.12	0.06	0.00	0.02	0.00
(E)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	1334	RI, MS	180	23838-27-9	0.01	0.00	0.00	0.00	0.00	0.00
(E)-1-Allyl-3-(prop-1-en-1-yl)trisulfane	1335	RI, MS	178	382161-78-6	0.88	0.7	0.11	0.00	0.00	0.00
1,3-Di((E)-prop-1-en-1-yl)trisulfane	1348	MS	178	115321-81-8	0.00	0.00	0.14	0.00	0.00	0.00
(Z)-1-(Prop-1-en-1-yl)-3-propyltrisulfane	1348	MS	180	23838-26-8	0.00	0.07	0.00	0.00	0.00	0.00
Monoterpenes										
α-Pinene	932	RI, MS	136	80-56-8	1.61	1.33	0.7	1.6	1.18	1.14
Camphene	946	RI, MS	136	79-92-5	1.21	0.82	0.2	0.33	0.00	0.00
Sabinene	973	RI, MS	136	3387-41-5	1.11	0.74	0.4	1.36	0.62	0.00

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Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
β-Pinene	974	RI, MS	136	127-91-3	0.8	0.16	2.69	1.26	3.95	2.68
β-Myrcene	991	RI, MS	136	123-35-3	1.03	0.65	0.28	0.47	0.68	0.00
α-Phellandrene	1003	RI, MS	136	99-83-2	3.06	2.45	1.21	0.7	4.92	5.21
3-Carene	1009	RI, MS	136	13466-78-9	0.09	0.1	0.05	0.05	0.00	0.02
cis-β-Ocimene	1010	RI, MS	136	3338-55-4	0.00	0.01	0.00	0.00	0.02	0.00
2-Carene	1015	RI, MS	136	554-61-0	0.04	0.00	0.00	0.00	0.00	0.18
(+)-4-Carene	1016	RI, MS	136	29050-33-7	0.2	0.18	0.04	0.00	0.14	0.00
α-Terpinene	1017	RI, MS	136	99-86-5	0.00	0.00	0.00	0.17	0.00	0.00
p-Cymene	1025	RI, MS	134	99-87-6	0.06	0.00	0.37	0.49	0.00	0.04
o-Cymene	1026	RI, MS	134	527-84-4	0.2	0.43	0.27	0.3	0.47	0.29
D-Limonene	1028	RI, MS	136	5989-27-5	1.28	1.67	0.73	0.94	0.92	0.85
m-Mentha-6,8-diene	1029	RI, MS	136	1461-27-4	0.00	0.00	0.00	0.14	0.29	0.53
trans-β-Ocimene	1040	RI, MS	136	3779-61-1	0.00	0.00	0.01	0.00	0.02	0.02
β-Ocimene	1052	RI, MS	136	13877-91-3	0.22	0.00	0.00	0.00	0.00	0.00
γ-Terpinene	1060	RI, MS	136	99-85-4	0.52	0.4	1.18	1.43	0.84	2.52
Terpinolene	1088	RI, MS	136	586-62-9	1.55	0.22	0.00	0.85	2.56	2.4
Linalool	1101	RI, MS	154	78-70-6	0.00	0.00	11.76	16.68	1,00	1.05
1,3,8-p-Menthatriene	1144	RI, MS	134	18368-95-1	0.1	0.00	0.00	0.00	0.00	0.00
Monoterpenoid										
Eucalyptol	1031	RI, MS	154	470-82-6	5.52	4.08	2,00	1.31	2.51	2.91
Linalool oxide	1075	RI, MS	170	60047-17-8	0.04	0.00	0.15	0.2	0.59	0.00
trans-Linalool oxide (furanoid)	1077	RI, MS	170	34995-77-2	0.00	0.00	0.37	0.29	0.04	0.00
Camphor	1111	RI, MS	152	76-22-2	0.06	0.00	0.00	0.00	0.00	0.00
cis-2-p-Menthen-1-ol	1121	RI, MS	154	35376-39-7	0.07	0.00	0.00	0.00	0.00	0.00
2,8-p-Menthadien-1-ol	1122	RI, MS	152	22771-44-4	0.00	0.01	0.00	0.00	0.00	0.00
(+)-2-Bornanone	1142	RI, MS	152	464-49-3	0.00	0.11	1.02	1.22	0.00	0.00
Citronellal	1151	RI, MS	154	106-23-0	2.82	4.92	5.71	4.88	14.37	12.25
Isoborneol	1164	RI, MS	154	124-76-5	1.31	0.68	0.00	0.00	0.00	0.00
endo-Borneol	1165	RI, MS	154	464-45-9	0.00	0.5	0.88	0.56	0.00	0.00
trans-Linalool 3,7-oxide	1174	RI, MS	170	39028-58-5	0.00	0.00	0.17	0.16	0.00	0.00

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Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
Terpinen-4-ol	1176	RI, MS	154	562-74-3	0.37	0.3	0.33	1.21	0.00	0.00
3,6-Octadienal, 3,7-dimethyl-	1183	RI, MS	152	55722-59-3	0.03	0.13	0.12	0.00	0.34	0.00
α-Terpineol	1192	RI, MS	154	98-55-5	0.31	0.15	0.45	0.28	0.00	0.00
L-α-Terpineol	1193	RI, MS	154	10482-56-1	0.59	0.52	0.15	0.00	0.09	0.03
Neral	1247	RI, MS	152	106-26-3	8.62	11.29	6.21	6.99	12.75	14.78
Geraniol	1266	RI, MS	154	106-24-1	0.06	0.01	1.56	2.12	1.96	1.36
Citral	1281	RI, MS	152	5392-40-5	0.1	6.01	5.14	5.57	7.02	6.76
Bornyl acetate	1289	RI, MS	196	76-49-3	0.18	0.23	0.00	0.09	0.00	0.00
Sesquiterpenes										
α-Cubebene	1354	RI, MS	204	17699-14-8	0.28	0.01	0.35	0.23	0.00	0.00
α-Longipinene	1365	RI, MS	204	5989-08-2	0.03	0.03	0.00	0.00	0.02	0.00
Cyclosativene	1368	RI, MS	204	22469-52-9	0.15	0.09	0.01	0.00	0.00	0.00
Copaene	1379	RI, MS	204	3856-25-5	0.28	0.29	1.17	0.57	0.00	0.00
α-Bourbonene	1386	RI, MS	204	5208-58-2	0.00	0.00	0.11	0.06	0.00	0.00
β-Elemene	1394	RI, MS	204	515-13-9	0.29	0.62	0.08	0.00	0.39	0.61
α-Barbatene	1412	MS	204	53060-59-6	0.00	0.00	0.01	0.01	0.00	0.00
trans-α-Bergamotene	1422	RI, MS	204	13474-59-4	0.49	0.08	0.37	0.18	0.12	0.35
Caryophyllene	1423	RI, MS	204	87-44-5	1.23	1.24	5.28	5.84	0.89	1.01
α-Guaiene	1442	RI, MS	204	3691-12-1	0.00	0.02	0.02	0.01	0.00	0.00
α-Himachalene	1447	RI, MS	204	3853-83-6	0.00	0.00	0.00	0.00	0.02	0.00
β-Barbatene	1448	RI, MS	204	39863-73-5	0.00	0.00	0.00	0.01	0.00	0.00
cis-β-Farnesene	1453	RI, MS	204	28973-97-9	0.25	0.00	0.00	0.00	0.23	0.00
Humulene	1459	RI, MS	204	6753-98-6	0.05	0.04	0.16	0.15	0.00	0.01
Aromadendrene	1461	RI, MS	204	489-39-4	0.09	0.04	0.1	0.00	0.00	0.81
(E)-β-Famesene	1462	RI, MS	204	18794-84-8	1.68	2,00	0.51	0.31	0.23	0.00
Alloaromadendrene	1464	RI, MS	204	25246-27-9	0.00	0.00	0.00	0.03	0.00	0.00
Valencene	1465	RI, MS	204	4640-07-3	0.00	0.00	0.00	0.02	0.00	0.00
β-Longipinene	1466	MS	204	41432-70-6	0.04	0.01	0.00	0.00	0.00	0.00
γ-Gurjunene	1467	RI, MS	204	22567-17-5	0.00	0.00	0.00	0.03	0.00	0.00

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Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
β-Guaiene	1468	RI, MS	204	88-84-6	0.00	0.18	0.00	0.00	0.00	0.00
δ-Guaiene	1480	RI, MS	204	3691-11-0	0.04	0.00	0.00	0.00	0.00	0.00
Ylangene	1481	MS	204	14912-44-8	0.00	0.00	0.01	0.67	0.00	0.08
γ-Muurolene	1482	RI, MS	204	30021-74-0	0.00	0.07	0.00	0.01	0.00	0.00
β-Selinene	1486	RI, MS	204	17066-67-0	0.09	0.13	0.03	0.05	0.00	0.00
Germacrene D	1487	RI, MS	204	23986-74-5	0.77	0.83	0.38	0.2	0.02	0.00
α-Curcumene	1488	RI, MS	202	644-30-4	0.93	0.00	0.31	0.14	0.34	0.52
Cuparene	1489	MS	202	16982-00-6	0.00	0.77	0.00	0.00	0.00	0.00
7-epi-Sesquithujene	1491	MS	204	159407-35-9	2.31	0.85	0.26	1.01	2.63	3.36
α-Farnesene	1493	RI, MS	204	502-61-4	1.14	1.73	0.34	0.19	0.27	0.07
(-)-Zingiberene	1498	RI, MS	204	495-60-3	0.5	1.1	0.00	0.00	0.18	1.38
γ-Curcumene	1501	RI, MS	204	451-55-8	0.00	0.00	0.00	0.22	0.00	0.00
γ-Cadinene	1501	RI, MS	204	1460-97-5	0.00	0.00	0.21	0.00	0.00	0.00
β-Bisabolene	1512	RI, MS	204	495-61-4	0.79	0.33	0.52	0.16	0.00	0.16
(E,Z)-α-Farnesene	1513	RI, MS	204	26560-14-5	0.00	0.00	0.26	0.00	0.00	0.00
(-)-α-Panasinsen	1524	RI, MS	204	56633-28-4	0.02	0.01	0.01	0.00	0.00	0.00
7-epi-α-Selinene	1525	RI, MS	204	123123-37-5	0.00	0.00	0.01	0.32	0.00	0.04
δ-Cadinene	1530	RI, MS	204	483-76-1	0.00	0.00	0.06	0.05	0.00	0.00
γ-Bisabolene	1566	RI, MS	204	53585-13-0	0.17	0.04	0.00	0.00	0.00	0.00
γ-Elemene	1566	MS	204	29873-99-2	0.1	0.03	0.00	0.00	0.00	0.00
β-Sesquiphellandrene	1529	RI, MS	204	20307-83-9	0.48	0.27	0.00	0.00	0.1	0.00
Sesquiterpenoid										
Tumerone	1682	RI, MS	218	180315-67-7	0.00	0.00	0.00	0.00	0.54	0.00
Organic peroxides										
Hydroperoxide, 1-methylpentyl	962	MS	118	24254-55-5	0.00	0.77	0.63	2.88	0.34	0.00
Hydroperoxide, 1-methylhexyl	949	MS	132	762-46-9	0.00	0.00	0.03	0.00	0.01	0.00
Hydroperoxide, 1-ethylbutyl	952	RI, MS	118	24254-56-6	0.08	0.93	0.53	2.55	0.71	0.43
Alcohols										
1-Hexanol, 2-ethyl-	1033	RI, MS	130	104-76-7	0.00	0.00	0.00	0.03	0.07	0.03

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Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
2-Nonen-1-ol	1051	MS	142	22104-79-6	0.00	0.07	0.00	0.00	0.00	0.00
2-Decen-1-ol, (E)-	1252	MS	156	18409-18-2	0.00	0.00	0.00	0.00	0.00	1.84
1-Tetradecanol	1389	MS	214	112-72-1	0.3	0.00	0.18	0.00	0.00	0.00
1-Hexadecanol	1690	MS	242	36653-82-4	0.03	0.00	0.00	0.00	0.00	0.00
Aldehydes										
Octanal	1006	RI, MS	128	124-13-0	0.00	0.02	0.00	0.01	0.00	0.00
Benzeneacetaldehyde	1048	RI, MS	120	122-78-1	0.00	0.09	2.04	1.23	5.68	6.37
Nonanal	1109	RI, MS	142	124-19-6	0.41	0.62	0.22	0.53	0.23	0.23
Decanal	1206	RI, MS	156	112-31-2	0.00	0.07	0.00	0.00	0.00	0.00
2-Decenal, (E)-	1270	RI, MS	154	3913-81-3	0.07	0.06	0.06	0.00	0.21	0.00
E-14-Hexadecenal	1677	MS	238	330207-53-9	0.06	0.03	0.00	0.00	0.00	0.00
Alkanes										
Decane	999	RI, MS	142	124-18-5	0.17	0.12	0.18	0.21	0.48	0.04
Undecane	1099	RI, MS	156	1120-21-4	0.27	0.03	0.1	0.18	0.29	0.02
Decane, 3,7-dimethyl-	1123	RI, MS	170	17312-54-8	0.00	0.00	0.04	0.05	0.09	0.00
Dodecane	1198	RI, MS	170	112-40-3	0.06	0.00	0.14	0.14	0.13	0.00
Tetradecane	1399	RI, MS	198	629-59-4	0.00	0.00	0.00	0.09	0.02	0.00
Alkenes										
1-Decene	990	RI, MS	140	872-05-9	0.00	0.00	0.25	0.37	0.39	0.8
1-Pentadecene	1679	MS	210	13360-61-7	0.19	0.01	0.00	0.00	0.00	0.00
Esters										
2-Heptanol, acetate	1046	RI, MS	158	5921-82-4	0.00	0.01	0.00	0.00	0.00	0.00
Limonen-6-ol, pivalate	1120	MS	236	-	0.02	0.00	0.00	0.00	0.00	0.00
2-Nonanol, acetate	1241	MS	186	14936-66-4	0.07	0.00	0.00	0.00	0.00	0.00
Carvyl acetate	1347	MS	194	97-42-7	0.32	0.00	0.00	0.00	0.00	0.00
Phenol, 4-(2-propenyl)-, acetate	1359	RI, MS	176	61499-22-7	2.41	0.93	0.34	0.1	0.1	0.00
Citronellyl acetate	1361	RI, MS	198	150-84-5	0.00	0.00	0.00	0.13	0.00	0.00
Citronellyl propionate	1362	MS	212	141-14-0	0.00	0.57	0.18	0.11	0.00	0.00
cis-Carvyl propionate	1366	MS	240	629-78-7	0.04	0.06	0.00	0.00	0.00	0.00
Eugenol acetate	1379	MS	206	93-28-7	0.00	0.00	0.21	0.03	0.00	0.00

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Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
Geranyl acetate	1388	RI, MS	196	105-87-3	0.23	0.2	0.73	0.99	0.00	0.00
Diethyl Phthalate	1618	RI, MS	222	84-66-2	0.86	0.93	0.14	0.00	0.18	0.00
(S)-4-(1-acetoxyallyl)phenyl acetate	1663	MS	234	52946-22-2	3.3	1.01	0.00	0.00	0.00	0.00
Alkylbenzenes										
Benzene, (1-methylethyl)-	925	RI, MS	120	98-82-8	0.00	0.39	0.02	0.00	0.11	0.00
Benzene, propyl-	954	RI, MS	120	103-65-1	0.21	0.46	0.64	0.52	0.92	0.67
Benzene, 1-ethyl-3-methyl-	963	RI, MS	120	620-14-4	0.83	1.05	1.03	0.6	2.2	2.51
Benzene, 1,2,3-trimethyl-	968	RI, MS	120	526-73-8	0.3	0.55	0.54	0.91	0.8	1.97
Benzene, 1-ethyl-2-methyl-	980	RI, MS	120	611-14-3	0.37	0.97	0.00	0.54	2.08	2.54
Benzene, 1-ethyl-4-methyl-	994	RI, MS	120	622-96-8	0.14	0.19	1.06	0.03	0.95	0.00
Benzene, 1,2,4-trimethyl-	1023	RI, MS	120	95-63-6	0.4	0.05	0.37	0.01	0.91	0.00
Benzene, tert-butyl-	1025	MS	134	98-06-6	0.00	0.03	0.00	0.00	0.6	0.52
Benzene, 1-methyl-2-propyl-	1067	RI, MS	134	1074-17-5	0.00	0.00	0.01	0.06	0.00	0.00
Benzene, 1-methyl-4-propyl-	1068	RI, MS	134	1074-55-1	0.06	0.00	0.36	0.32	0.01	0.05
Benzene, 2-ethyl-1,4-dimethyl-	1078	RI, MS	134	1758-88-9	0.00	0.00	0.00	0.11	0.00	0.00
Benzene, 4-ethyl-1,2-dimethyl-	1084	RI, MS	134	934-80-5	0.16	0.15	0.22	0.27	0.61	0.8
Benzene, 1-ethyl-2,3-dimethyl-	1086	RI, MS	134	933-98-2	0.00	0.00	0.00	0.15	0.00	0.00
Benzene, 1,2,4,5-tetramethyl-	1114	RI, MS	134	95-93-2	0.27	0.2	0.34	0.67	0.66	0.00
Benzene, 1,2,3,4-tetramethyl-	1145	RI, MS	134	488-23-3	0.00	0.00	0.04	0.00	0.00	0.02
Phenols										
2-Indanol	1057	RI, MS	134	4254-29-9	0.15	0.00	0.00	0.00	0.00	0.00
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	1149	RI, MS	154	28564-83-2	0.00	0.00	0.3	0.04	0.77	0.00
Phenol, 4-(2-propenyl)-	1360	MS	134	501-92-8	0.31	0.00	0.00	0.00	0.00	0.00
3-Allyl-6-methoxyphenol	1370	MS	164	501-19-9	0.00	0.00	0.24	0.05	0.00	0.00
Eugenol	1374	RI, MS	164	97-53-0	0.00	0.00	0.93	0.00	0.00	0.00
o-Eugenol	1390	MS	164	579-60-2	0.00	0.00	0.21	0.00	0.00	0.00
trans-Isoeugenol	1543	MS	164	5932-68-3	0.02	0.00	0.00	0.00	0.00	0.00
Phenylpropanoids										
Estragole	1203	RI, MS	148	140-67-0	0.00	0.00	0.24	1.82	0.00	0.00

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Volatile compounds	LRI	Identification method[a]	MW	CAS number	Relative peak area (%)					
					TSA	DSA	TSB	DSB	TSC	DSC
Anethole	1307	RI, MS	148	4180-23-8	0.00	0.00	5.63	5.9	0.00	0.00
Safrole	1293	MS	162	94-59-7	0.00	0.00	2.85	0.00	0.00	0.00
Myristicin	1550	MS	192	607-91-0	0.00	0.00	0.14	0.25	0.00	0.00
Other hydrocarbons (lactones, ketones, furans, aromatic hydrocarbons)										
Mesitylene	993	RI, MS	120	108-67-8	1.1	1.24	0.84	2.24	4.37	2.44
Benzene, 1,3-dichloro-	1013	RI, MS	147	541-73-1	0.45	1.3	1.3	1.41	1.67	4.58
Indane	1036	RI, MS	118	496-11-7	0.00	0.00	0.04	0.19	0.16	0.07
3-Methyl-2-(2-methyl-2-butenyl)-furan	1098	MS	150	15186-51-3	0.03	0.00	0.00	0.00	0.00	0.00
Azulene	1184	MS	128	275-51-4	1,00	0.00	0.06	0.17	0.00	0.00
2-Undecanone	1293	RI, MS	170	112-12-9	0.15	0.35	0.00	0.00	0.00	0.00
δ-Octalactone	1298	RI, MS	142	698-76-0	0.00	0.00	0.00	1.04	0.00	0.43
Unknown compounds										
Unknown 1	1102	-	146	-	2,00	2.27	0.00	0.00	0.00	0.00
Unknown 2	1279	-	152	-	10.58	6.19	1.95	0.00	0.00	0.93
Unknown 3	1288	-	152	-	0.00	0.28	0.03	0.00	0.73	0.00
Unknown 4	1285	-	152	-	0.00	0.00	0.00	0.00	0.00	1.84
Unknown 5	1328	-	178	-	0.3	0.00	0.51	0.00	0.00	0.00
Unknown 6	1286	-	152	-	0.14	0.00	0.00	0.00	0.00	0.00
Unknown 7	1387	-	146	-	0.00	0.13	0.00	0.00	0.00	0.00
Unknown 8	1669	-	234	-	0.00	0.06	0.00	0.00	0.00	0.00
Unknown 9	1089	-	136	-	0.00	0.62	0.71	0.00	0.00	0.00
Unknown 10	1115	-	134	-	0.00	0.00	0.25	0.52	0.48	0.00
Unknown 11	1291	-	148	-	0.00	0.00	13.19	4.63	0.00	0.00
Unknown 12	1261	-	148	-	0.00	0.00	0.03	0.00	0.00	0.00
Unknown 13	1022	-	120	-	0.00	0.00	0.00	0.77	0.00	0.00
Unknown 14	1056	-	156	-	0.00	0.00	0.00	0.00	0.33	0.00

LRI = linear retention index; MW = molecular weight; CAS = Chemical Abstracts Service

[a]Compounds identification method (RI = retention index; MS = ion fragments (*m/z*) match with NIST 17.0)

greater than 1 indicates a strong influence of the X variables on the Y variables (Vieira et al. 2023). The positive correlated compounds showed in S-plot (Figure 4(B)) suggested that a higher concentration of these compounds will enhance the antimicrobial activity of rendang seasoning against *C. sporogenes*. Conversely, the compounds positioned in the lower quadrant of S-plot are hypothesized as the non-active compounds which have minimal contribution in inhibiting *C. sporogenes*. However, the additional studies are needed.

The variable importance in projection (VIP) is utilized to spot how much the compounds contribute to antimicrobial activity. OPLS S-plot also showed the others compounds in rendang seasoning that had the VIP values exceeding 1 and also positive correlation as antimicrobial agents against *C. sporogenes* (Table 3). These compounds have been reported of their antimicrobial ability against spore-forming bacteria. According to Table 3, the great galangal and garlic are the key spices in rendang seasoning that play a role for antimicrobial activity. This is attributed to their compounds which have the higher VIP values in the OPLS analysis. OPLS analysis also identified two unknown compounds which do not match with the database but that may contribute as antimicrobial agent in rendang seasoning.

This result was also supported with X-variable plot feature in OPLS analysis that aims to show the distribution of the antimicrobial compounds in each sample group (Yuliana et al. 2020). The X-variable plot (Figure 5) demonstrated the relative concentration of four compounds, i.e., unknown 2 (A), 2-vinyl-1,3-dithiane (B), 2-vinyl-4H-1,3-dithiine (C), and (S)-4-(1-acetoxyallyl) phenyl acetate, were predominant in TSA. Among the samples, formula B contained the lowest concentration of 2-vinyl-4H-1,3-dithiine (Figure 5(C)), whereas formula C did not show the presence of 2-vinyl-1,3-dithiane (Figure 5(B)). The presence of (S)-4-(1-acetoxyallyl) phenyl acetate or galangal acetate (Figure 5(D)) was uniquely identified in formula A (TSA and DSA) and absolutely absent in formula B and C.

The quantities of spices in rendang seasoning formula may influence the antimicrobial compound concentration against *C. sporogenes*. As shown in Figure 5, formula A utilized more garlic compared to other formula that affected the abundance of sulfur-containing compound linked to antimicrobial activity. Both 2-vinyl-4H-1,3-dithiine and 2-vinyl-1,3-dithiane are derived from allicin during thermal processing. Allicin is a major compound found in garlic that acts as antimicrobial agent. The previous studies have demonstrated that the frying process leads to the degradation of allicin, resulting in the formation of vinyl dithiins, sulfur-containing compounds with antimicrobial properties (Ramirez, Altamirano & Camargo 2021; Varga-Visi et al. 2019). The use of great galangal was also very significant between each formula. Formula A was rich in (S)-4-(1-acetoxyallyl)phenyl acetate, as presented in X-variable plot (Figure 5(D)), due to its higher amounts of great galangal compared to formula B and C. A study showed that (S)-4-(1-acetoxyallyl)phenyl acetate or galangal acetate is one of major compound found in great galangal that offers the antimicrobial activity (Zhang et al. 2021). This finding interprets that increasing the amount of great galangal and garlic in rendang seasoning formula may increase the concentration of these compounds which may provide an antimicrobial property against *C. sporogenes*. In addition, this analysis also showed the presence of unknown 2, an antimicrobial associated compound, which was characterized by ion fragments at *m/z* 69, 84, 109, 137, and 152 (Table 3). The compound might be produced by transformation during cooking and interactions between ingredients components. A more in-depth study is needed to investigate this unknown compound correlated with antimicrobial activity in rendang seasoning.

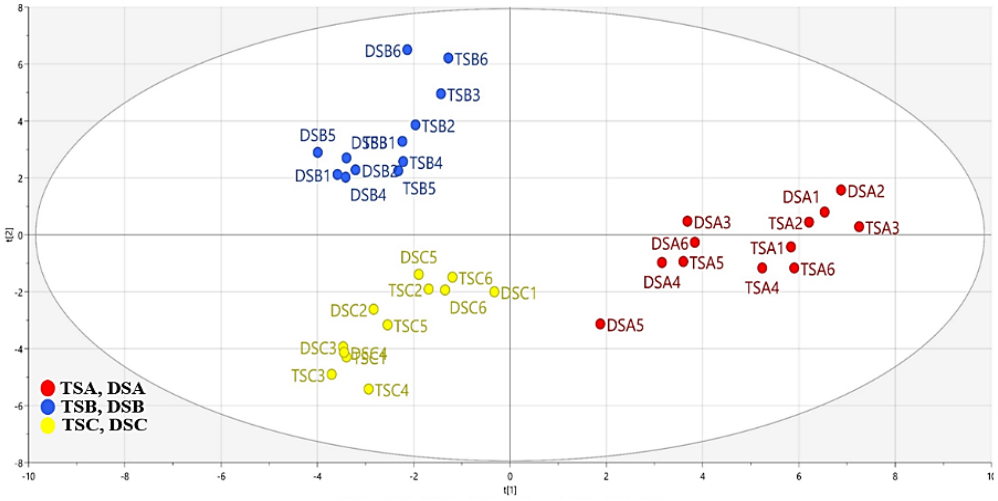

FIGURE 3. PCA score plot of the rendang seasoning compounds

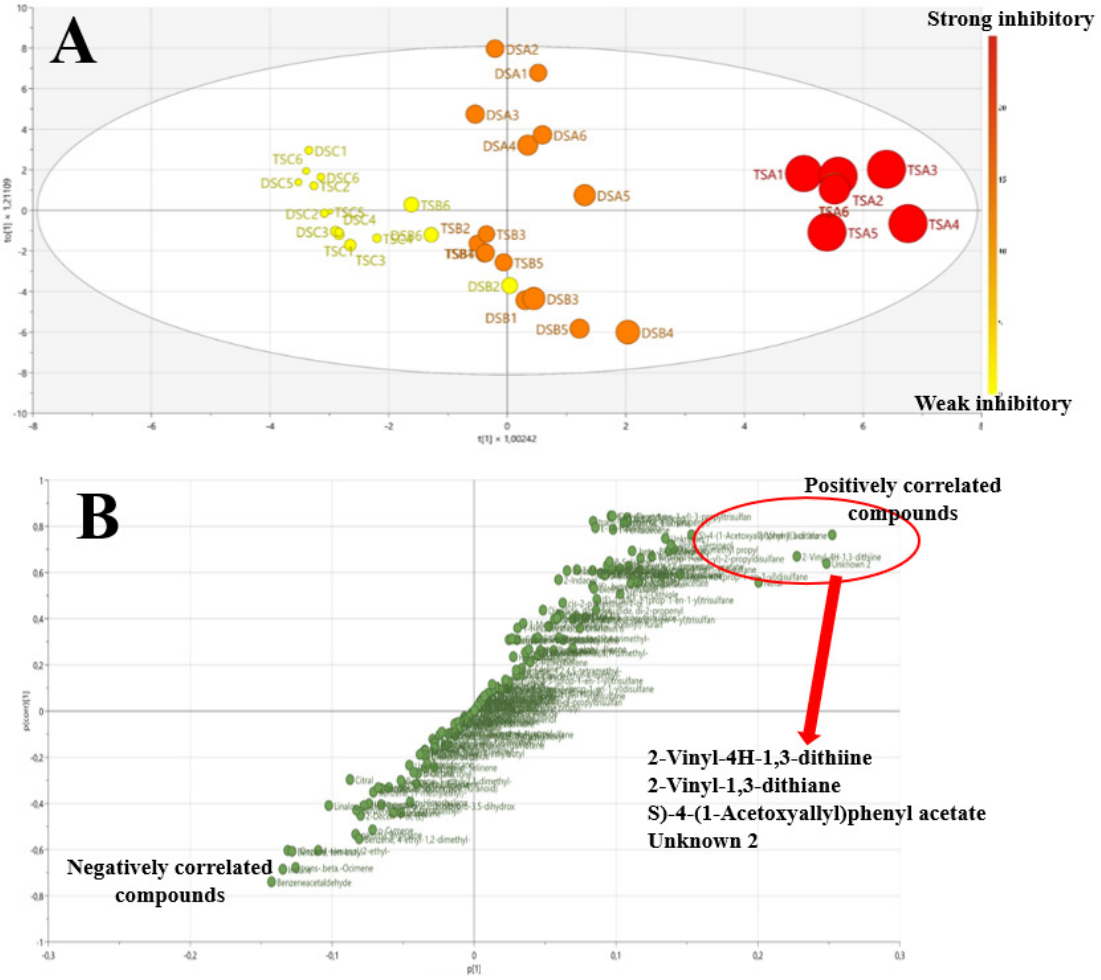

FIGURE 4. (A) OPLS score plot and (B) S-plot of rendang seasoning compounds and antimicrobial activity correlation

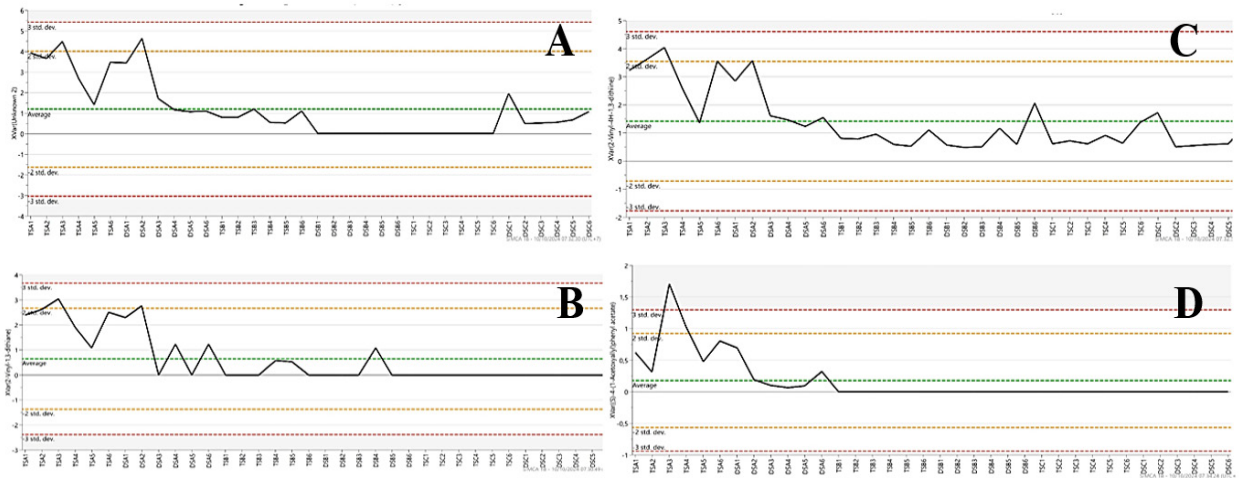

FIGURE 5. X-variable plot of (A) Unknown 2 (B) 2-vinyl-1,3-dithiane (C) 2-vinyl-4H-1,3-dithiine (D) (S)-4-(1-acetoxyallyl)phenyl acetate

TABLE 3. Volatile compounds in rendang seasoning associated with antimicrobial activity against C. sporogenes

Volatile compounds	Ion fragments	VIP	Antimicrobial activity	Spices origin	References
Unknown 2	69, 84, 109, 137, 152	3,13	-	-	-
2-Vinyl-1,3-dithiane	45, 73, 103, 146	2,95	-	Garlic	(Avgeri et al. 2020)
2-Vinyl-4H-1,3-dithiine	45, 72, 111, 144	2,78	*B. cereus*	Garlic	(Grinzeanu et al. 2024)
(S)-4-(1-Acetoxyallyl)phenyl acetate	55, 77, 121, 132, 150, 192	1,76	*S. aureus*	Great galangal	(Zhang et al. 2021)
Camphene	67, 93, 121, 136	1,68	*Clostridium* spp.	Ginger, great galangal, coriander, lemongrass, turmeric leaf	(de Paula Duarte Alves et al. 2023; Badrunanto et al. 2024; Ivanović, Makoter & Razboršek 2021; Moro et al. 2015; Plata-Rueda et al. 2020; Raissa et al. 2020; Wildani et al. 2021)
Isoborneol	57, 95, 110, 136	1,67	*S. aureus, B. cereus*	Ginger, great galangal, turmeric, turmeric leaf	(de Paula Duarte Alves et al. 2023; Guimarães et al. 2019; Ivanović, Makoter & Razboršek 2021; Zhu et al. 2013)
.alpha.-Terpineol	59, 93, 121, 154	1,66	*C. botulinum*	Great galangal, ginger, turmeric, cardamom, bay leaf, *ruku-ruku* leaf	(Ivanović, Makoter & Razboršek 2021; Pradhan, Paul & Singh 2024; Rahim et al. 2018; Smitha & Tripathy 2016)
Unknown 1	45, 81, 104, 146	1,61	-	-	-
(Z)-1-Methyl-2-(prop-1-en-1-yl)disulfane	45, 72, 120	1,54	-	Shallot	(González-de-Peredo et al. 2024)
Terpinen-4-ol	55, 71, 111, 136, 154	1,53	*B. cereus*	Star anise, nutmeg, coriander, cardamom, great galangal, ginger, turmeric	(Ivanović, Makoter & Razboršek 2021; Nie et al. 2021; Nikolic et al. 2021; Wildani et al. 2021; Zhao et al. 2021)
(E)-1-(Prop-1-en-1-yl)-2-propyldisulfane	45, 73, 106, 148	1,44	*S. aureus*	Shallot, garlic	(Mnayer et al. 2014; Wang et al. 2023)
(E)-1-Allyl-2-(prop-1-en-1-yl)disulfane	45, 73, 105, 146, 148	1,31	*B. cereus*	Garlic	(Zalepugin et al. 2013)
Trisulfide, di-2-propenyl	45, 73, 113, 137, 178	1,19	*B. cereus*	Garlic	(Grinzeanu et al. 2024)
Trisulfide, methyl 2-propenyl	45, 87, 111, 152	1,17	*Clostridium* spp.	Garlic	(Kirkpinar, Ünlü & Özdemir 2011)
α-Cubebene	55, 91, 105, 161, 204	1,15	*B. cereus*	Chili, bay leaf, *ruku-ruku* leaf	(Debnath, De & Das 2019; Milenković et al. 2023; Rahim et al. 2018; Smitha & Tripathy 2016)

CONCLUSIONS

This study found that rendang seasoning formula A and B were potentially effective as antimicrobial agents against *C. sporogenes*, whereas the formula C showed weak inhibition. Rendang seasoning TSA had the most antimicrobial activity as characterized strong inhibition to the spore-forming target bacterium. The sulfur-containing compounds, terpenes, and terpenoids were abundant in rendang seasoning according to GC-MS analysis. The PCA model successfully clustered the three rendang seasoning formulas, suggesting the different volatile compound profiles. The addition of coconut milk did not enhance the antimicrobial activity of rendang seasoning DSA, but moderately increased the effectiveness in rendang seasoning DSB. The OPLS analysis showed 15 compounds associated with the antimicrobial activity against *C. sporogenes*. Among these, the compounds with highest VIP values are 2-vinyl-1,3-dithiane, 2-vinyl-4H-1,3-dithiine, and (S)-4-(1-acetoxyallyl)phenyl acetate, which are primarily found in garlic and great galangal. These findings highlight that garlic and great galangal can be the leading spices in rendang seasoning which contribute to the antimicrobial properties against *C. sporogenes*. Even so, it raises concern that fat-rich food system, such as rendang seasoning containing coconut milk, may require higher concentration of active and stable spice-derived compounds with synergistic effects to maintain the effectiveness of antimicrobial.

In future study, it would be useful to evaluate the antimicrobial activity against *C. botulinum*, notably to highlight its relevance as a foodborne pathogen. Moreover, further analytical studies are also needed to characterize the unknown and polar compounds in rendang seasoning that may contribute to antimicrobial activity.

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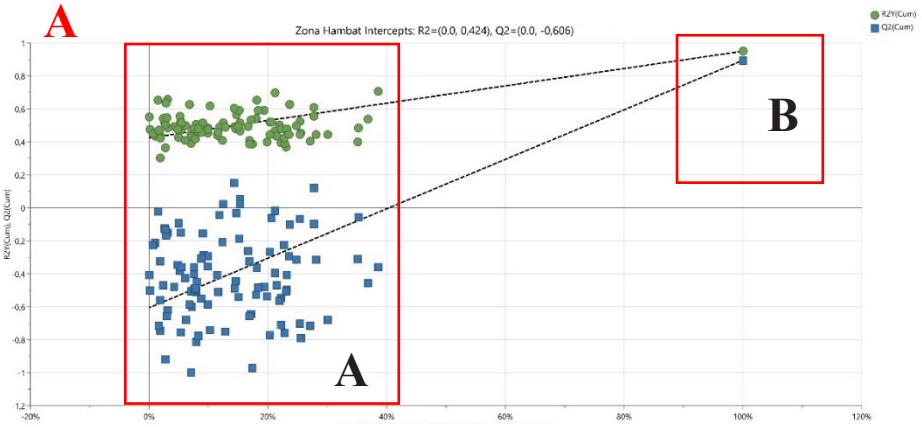

B

M3	SS	DF	MS	F	p	SD
Zona Hambat						
Total corr.	174,549	35	4,98712			2,23319
Regression	156,16	6	26,0267	41,0452	6,9973e-13	5,10164
Residual	18,3889	29	0,634099			0,796303

FIGURE S1. (A) Permutation plot and (B) CV-ANOVA for OPLS analysis validation

Permutation and CV-ANOVA are OPLS features that contribute in validating the OPLS result. The 100 random permutation was used in this study. This feature would randomize all variables 100 times and the result should be lower from the actual result. Supplementary Figure 1(A) showed that the randomized data (box A) were lower than the actual result (box B), indicating the OPLS model was satisfactory. Another validation, CV-ANOVA (Supplementary Figure 1(B)), also provided the significant p-value of $6{,}99 \times 10^{-13}$ (p-value < 0.05) which supported the results of OPLS analysis.