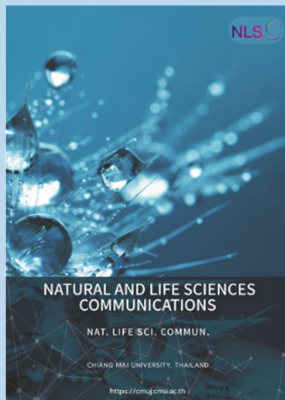


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Metabolite Profiling, *In vitro* Evaluation of Cytotoxic, and Anti-atherosclerosis Effects of Fermented Sambal Lalapan Extract

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ABSTRACT

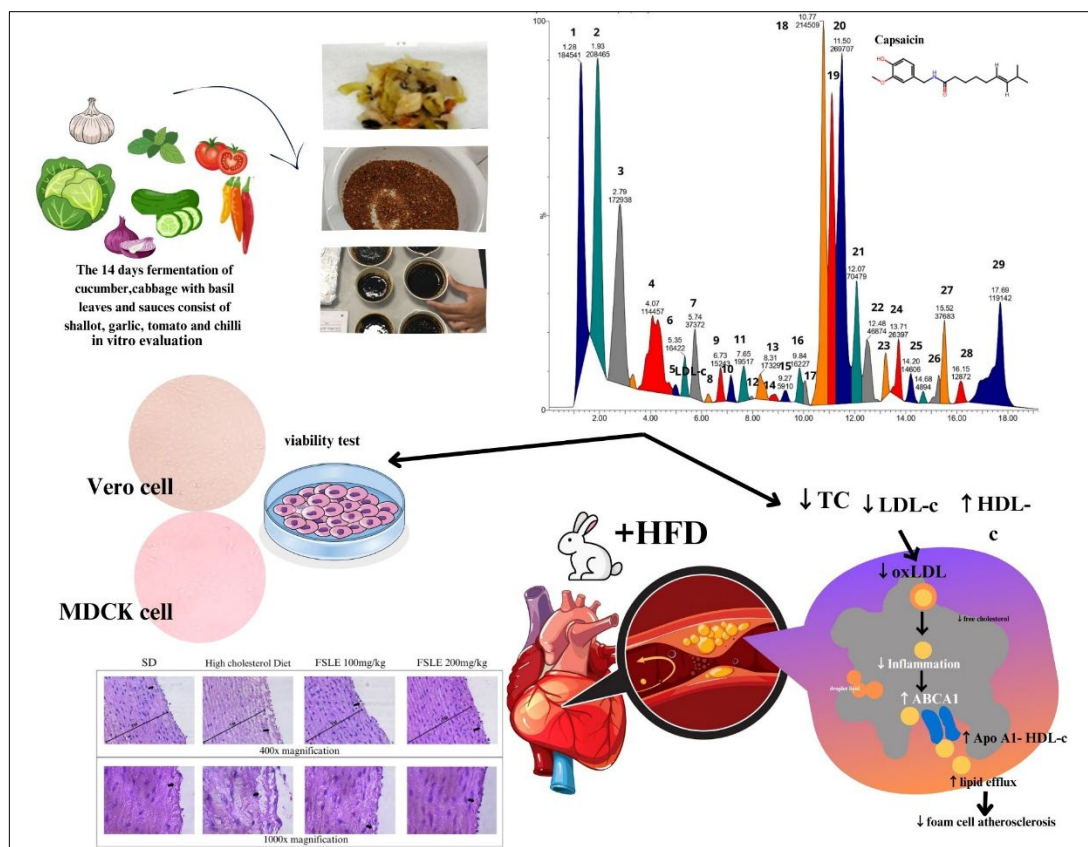
The increasing incidence of coronary heart disease (CHD) worldwide, along with concerns over the long-term use of statins and low adherence to a healthy diet, highlights the potential of natural product-based alternatives. This study aimed to investigate the metabolite profiling, cytotoxicity, and anti-atherosclerotic effects of fermented sambal lalapan extract (FSLE). FSLE was derived from an ethanolic extract of fermented powder that underwent spontaneous fermentation for 14 days at 20°C. Results from LC-MS/MS demonstrated that capsaicin was the predominant among 29 metabolites determined in FSLE. The percentage of cell viability in Vero and MDCK cells treated with FSLE at 5 µg/mL was 90.14 ± 5.35% and 88.27 ± 0.97%, respectively. At the highest concentration of 1,280 µg/mL, cell viability of 99.79 ± 1.87% and 99.85 ± 9.19% was obtained in Vero and MDCK cells. The effect of FSLE on atherosclerosis was evaluated using an atherosclerosis animal model. The oral administration of FSLE at 200 mg/kg body weight resulted in lower total cholesterol (TC) (154.40 ± 34.13 mg/dL), lower low-density lipoprotein (LDL)-c (130.59 ± 15.68 mg/dL) ($P = 0.001 < 0.05$), and higher high-density lipoprotein (HDL)-c (33.73 ± 1.67 mg/dL) compared to the HFD group ($P = 0.001 < 0.05$). Observation using HE staining revealed a lower number of foam cells in the aortic tissue of the FSLE compared to the HFD group ($P = 0.045 < 0.05$), which may be attributed to increased ATP Binding Cassette A (ABCA)1 expression, as determined by real-time PCR and ELISA. In conclusion, FSLE showed promise against CHD by attenuating atherosclerosis.

Keywords: Fermentation, Vegetable, Hyperlipidemia, Foam cell atherosclerosis, ABCA1

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Graphical Abstract:



INTRODUCTION

Atherosclerosis is the underlying mechanism of coronary heart disease (CHD), which is the leading cause of mortality worldwide (Jebari-Benslaïman et al., 2022; Tsao et al., 2022). High levels of low-density lipoprotein cholesterol (LDL-c) in the circulation provoke atherosclerosis due to its ability to penetrate the endothelial cells of the vascular wall, which is oxidized into ox-LDL and phagocytosed by subintimal macrophages. The accumulation of ox-LDL-c leads to foam cell formation, an early-stage lesion of atherosclerosis, and drives the progression of plaque development. A deficiency of ATP-binding cassette transporter A1 (ABCA1) is a hallmark of foam cell formation. This membrane receptor plays a critical role in the efflux of free cholesterol (FC) and phospholipids (PL) from macrophages through interacting with lipid-poor apolipoprotein A-I (ApoA-I), the major component of high-density lipoprotein (HDL), and is involved in reverse cholesterol transport (RCT). However, the accumulation of ox-LDL triggers macrophage inflammation and reduces ABCA1 level which will further lower the efflux cholesterol (Yu et al., 2013; Mizuno et al., 2015; Chistiakov et al., 2017; Jebari-Benslaïman et al., 2022).

Currently, statins, which are known as hypolipidemic drugs, are used to prevent atherosclerosis besides healthy diet consumption. Long-term statins consumption is associated with resistance and adverse effects, including hepatotoxicity, reduced cognitive function, and rhabdomyolysis (Newman et al., 2019). As a result, natural products have emerged as a potential alternative or complementary approach to atherosclerosis prevention. Several reports have demonstrated that combining vegetables with herbs and spices can enhance antioxidant activity (Radha Krishnan et al., 2014; Hill et al., 2017; Poelman et al., 2019; van Stokkom et al., 2019), which may provide protective effects against atherosclerosis (Voidarou et al., 2021).

Sambal lalapan is a traditional Indonesian condiment composed of sambal (a chili-based sauce) and fresh raw vegetables known as lalapan. As a culinary staple, sambal has played a significant role in the Indonesian diet for generations, reflecting both cultural heritage and dietary preferences (Rahayu et al., 2024). Typically, raw vegetables such as white cabbage and cucumber are rich in bioactive compounds from fresh, plant-based ingredients, each offering distinct health benefits (Nawirska-Olszańska et al., 2021). Cabbage provides glucosinolates and kaempferol. Cucumber's main active ingredients include flavonoids, cucurbitacins, and phenolic acids (Fiume et al., 2014). On the other side, sambal consists of blended garlic, shallots, tomatoes, chili, and basil leaves. High concentrations of quercetin, sulfur compounds are found in shallots and garlic (Batiha, Beshbishy, et al., 2020; Major et al., 2022). Tomatoes offer lycopene and quercetin, while chili peppers bring capsaicinoids (Batiha, Alqahtani, et al., 2020). Basil adds essential oils and various flavonoids. Previous research has elucidated the cardiovascular protective effect of sambal lalapan based on bioinformatic analysis (Rachmawati et al., 2023).

Accordingly, the present study aimed to investigate the cytotoxicity of this extract in normal cells *in vitro*. Although sambal lalapan is a traditional food that is generally consumed safely, the original preparation in this present study underwent a fermentation process, which could alter the chemical composition and biological activity of natural products, including metabolites with potential therapeutic as well as cytotoxic effects. Additionally, this study also measures the administration of FSLE on lipid profile and atherosclerotic plaque formation using an *in vivo* model. Moreover, all the parameter evaluations lay the groundwork for its prospective development as a functional food or herbal therapeutic agent.

MATERIALS AND METHODS

Materials

The study utilized organic vegetables, herbs, and spices sourced from a 950-meter altitude farm in Bumiaji, East Java, Indonesia. Materia Medica Batu authenticated the botanical identification with the registration number and key determination archived in the supplementary file.

Spontaneous fermentation and extraction process

As detailed in the previous study (Rachmawati et al., 2023), the cabbage was salted overnight. On the following day, the spicy sauces were prepared. The salted cabbage, cucumber, and sambal sauce were mixed (Figure 1). The spontaneous fermentation occurred in sealed jars for 14 days (Cho et al., 2017) at 20°C.

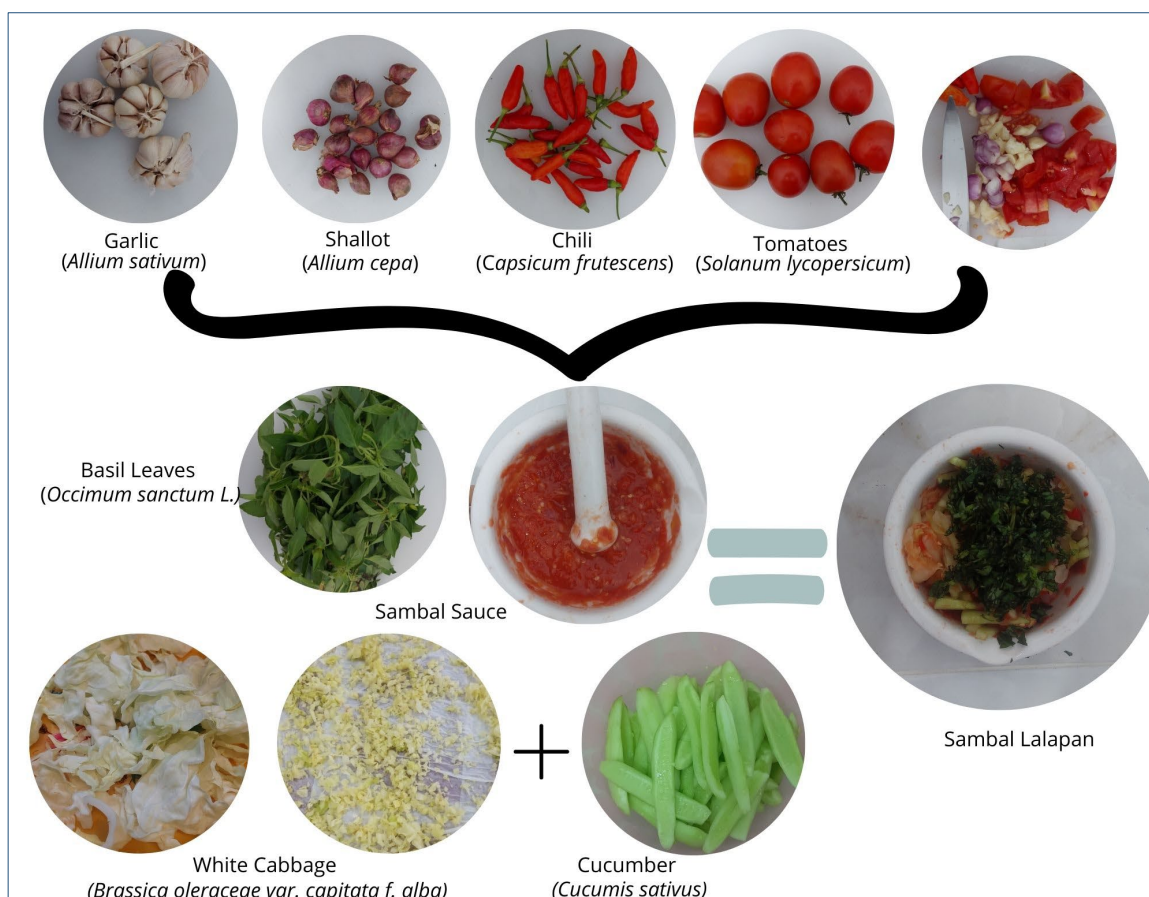


Figure 1. Ingredients of sambal lalapan. Sambal consists of garlic, shallots, chili, tomatoes with addition basil leaves. Lalapan was comprised of white cabbage and cucumber.

The simplicial powder, which was obtained after drying in a hot oven at 50°C for 5 days (Fabricio et al., 2022), was then subjected to ultrasound-assisted extraction (UAE) for 30 minutes at 50°C using a 1:10 powder to 70% ethanol ratio (Nascimento et al., 2021). The resulting fermented sambal lalapan extract (FSLE) was concentrated at 50°C in a rotary evaporator for 2 days.

LC-MS/MS experiment

The determination of bioactive compounds was carried out using liquid chromatography-tandem mass spectrophotometry (LC-MS/MS) systems with quadrupole time of flight as the analyzer and positive electrospray ionization (ESI) as the ionization source with the acuity C18 column 1.8 μm ; 2.1 mm $\times$ 150 mm. A combination of (a) Water (HPLC [High Performance Liquid Chromatography] grade)/formic acid (Merck, Darmstadt, Germany) 99.9/0.1 (v/v); (b) Acetonitrile (Merck, Darmstadt, Germany)/formic acid 99.9/0.1 (v/v) was applied as the eluent. The source and desolvation temperatures were at 100 and 350°C, respectively. Briefly, 10 mg of extract was dissolved in a 10 mL volumetric flask containing absolute methanol. Subsequently, 5 μL of the mixture was then introduced into the LC-MS/MS apparatus. MassLynx 4.1 software (Waters, Massachusetts, USA) and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) were used to process the chromatogram and then identify the compound. The MS/MS matching and an error of <5 ppm was used to verify a compound's accuracy (Mutiah et al., 2019).

***In vitro* viability test**

The extract of FSLE was tested for *in vitro* cytotoxicity, using Vero and MDCK cells by CCK8 assay (Baloyi et al., 2023; Chen et al., 2024). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Cat. No 11995073, Thermofisher, USA) supplemented with 10% Hyclone Characterized Fetal Bovine Serum (FBS) (SH30071.03, Cytiva, USA) and incubated at 37°C in a humidified 5% CO₂ incubator. For the assay, the cells were plated at a density of 5,000 cells/well into 96-well plates. The next day, extracts in 0.5% Dimethyl sulfoxide (DMSO) (Sigma Aldrich, CAS 67-68-5) were prepared in two-fold dilutions i.e. from 5, 10, 20, 40, 80, 160, 320, 640, 1,280 µg/ml and 10 µL was added to each well with 3 replicates (Rachmawati et al., 2022). The cells were incubated at 37°C in a humidified 5% CO₂ incubator for 24 hours. Subsequently, 10 µL of CCK-8 reagent (Dojindo Laboratories, Japan) was added to each well, and the cells incubated for another 1 hour until purple precipitates became clearly visible. The absorbance for each well was measured at 540 nm in a micro-titre plate reader, and the percentage cell viability was calculated manually using the formula: Viability (%) = Absorbance Sample-Absorbance blank/ Absorbance Control-Absorbance blank x 100%. In addition, the IC₅₀ of FSLE in MDCK and Vero cells was also determined.

***In vivo* study**

Animal experiment

All experiment processes followed the 3R principles of Replacement, Reduction, and Refinement. Brawijaya Lab-Animal in Indonesia supplied a total of 20 male, aged 5 months, New Zealand White (NZW) rabbits weighing between 2.2 to 2.5 kg. The rabbits were housed in 50x70x70 cm³ cages that were kept at 20°C with a 12-hour cycle of darkness and light. Following a 14-day acclimatization period, the rabbits were randomly assigned into 4 groups (n=5 per group) as follow: (1) Standard Diet (SD) - positive control: Rabbits in this group were fed a regular diet; (2) High Fat Diet (HFD) group - negative control: Rabbit received a standard diet + 1% cholesterol from mashed-up fresh cow brain (Rachmawati and Muhammad, 2021); (3) The interventional group received HFD supplemented with FSLE at 100 and 200 mg/kg BW/day, respectively (Kieczykowska and Musik, 2020; Rachmawati et al., 2025). The determination of sample size in this study used the resource equation approach: $n \geq k(10/k+1)$ (Arifin and Zahiruddin, 2017). The FSLE concentration in the intervention group was determined based on evidence obtained from previous studies.

The fermented extract was dissolved in a 0.5% Na-CMC solution prepared by adding 5 mg of Na- Na-Carboxymethyl Cellulose (CMC) (Brataco Chemical, Surabaya) to 100 ml of distilled water and stirring until a clear gel formed. The FSLE stock solution was prepared using a 1:20 ratio of extract to Na-CMC for dose 1, and a ratio of 1:10 for dose 2. Fresh food was delivered every day, and leftovers from the previous day were thrown away after being documented. The rabbits had unrestricted access to food and water throughout the 60-day treatment period. All experimental protocols for *in vivo* study had been approved by the ethics committee of the Faculty of Medicine at Brawijaya University, with registration number 104-KEP-UB-2022.

Animal termination and sample collection

After eight weeks (60 days) of intervention, the rabbits were fasted for 12 hours, given anesthesia with ketamine (20 mg/kg BW) and diazepam (0.5 mg/kg BW). Blood was collected using a heart puncture while the animal was in the sedation phase, and the serum was isolated for lipid profile examination. The aorta was isolated and washed in cold Normal Saline (NS) at 4°C. Before the histopathological assessment, the aortic arch and nearby segment (6 mm) were submerged in a 10% neutral-buffered formaldehyde (NBF) (Sigma Aldrich, HT501320). The tissues were soaked in RNA lather (Sigma-Aldrich R0901) for mRNA measurement and immediately kept at -80°C (both mRNA and protein).

Lipid profile assessment

A colorimetric assay was used to quantify the TC, HDL-c, and LDL-c (E-BC-K109-S, E-BC-K206-S, and E-BC-K221, Elab Science, USA) (Ruanpang et al., 2018). The optical density was assessed using the spectrophotometer Sentra BD Biowave DNA #9IS80-3004-70 with a wavelength of 510 nm for TC, 600 nm for LDL. The calculation of TC, HDL and LDL concentrations (mg/dl) followed the manufacturer's instructions.

Foam cell determination

Immerse aortic tissue was dehydrated and embedded in a paraffin block using a tissue embedding console. The specimen was sectioned until a thickness of 5 μ m. Subsequently, the section was deparaffinized using xylol and tissue hydration with graded concentrations of alcohol (100, 90, and 80%). The tissue was stained with a Mayer Hematoxylin (Sigma Aldrich 51275)-eosin (Merck, CAS No 17372-87-1) stain and observed under Nikon Eclipse E-200 binocular microscope and quantified using ImageJ (Ganesan et al., 2018; Weng et al., 2023).

ABCA1 gene expression

RNA isolation was carried out using the Trizol™ (Thermofisher, Cat. No. 15596026, USA). The total RNA obtained was first checked for purity and concentration using NanoDrop™ 2000/2000c Spectrophotometers ND2000CLAPTOP. Later, the cDNA synthesis was performed using the steps described in the manual kit from Promega GoScript™ Reverse Transcriptase A5000. The cDNA was stored at -20°C until used for Real Time Polymerase Chain Reaction (RT-PCR). PCR was performed using GoTaq® Green Master Mix (M1722 LOT0000404274, Promega, USA) was prepared. The primers used for PCR were as follows:

GADPH: Antisense 5'-CCA GTG AGT TTC CCG TTC -3', Sense 5'-GGA GCC AAA AGG GTC ATC -3'; ABCA1: Antisense 5'-TTG GTC CTT GGC AAA GTT CAC -3', Sense 5'- GTT CAG GTG CCT TGG CAG TG-3'.

GoTag® Green Master Mix, primers, cDNA template, and nuclease free water (NFW) were mixed in a 200 μ l PCR tube. Subsequently, the thermal cycler Select BioProduct Select Cyclor II was set for temperature and time according to PCR guideline as follows: (1) 2 minutes initial denaturation at 95°C; (2) The PCR ran 35 cycles with each cycle consisting of: denaturation for 15 seconds, annealing at 60°C for 1 minute; extension with optimal temperature at 72°C for 1 minute. The PCR results were then calculated for the Ct value based on the software analysis. The relative expression was obtained using the Livak formula $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen, 2001).

ABCA1 protein

The isolation of the aortic protein was carried out by RIPA Buffer (Cat. No. 89901, Thermofisher, USA), and the determination of ABCA1 protein was tested by commercially available ELISA kits (Rabbit ABCA1 Cat MDBE0014Rb, Medikbio, Malang, Indonesia) in strict compliance with the manufacturer's guidelines. The optical density which was proportional to the protein concentration, was measured using an ELISA reader at 450 nm. The protein concentration (ng/mL) was determined by plotting a standard curve.

Statistical analysis

The profile of bioactive compounds was presented using descriptive statistics. One-way ANOVA was performed to evaluate the significant differences among the groups, followed by Tukey's post-hoc test for pairwise comparison. All values were represented as mean $\pm$ standard error (SE), and a *P*-value $\leq$ 0.05 was considered

statistically significant. All statistical analysis were carried out using SPSS software version 26.0 (IBM Corp., New York, USA).

RESULTS

Determination of active ingredients in FSLE

Figure 2 demonstrated the LC-MS/MS chromatogram of the crude extract from the fermentation of sambal lalapan.

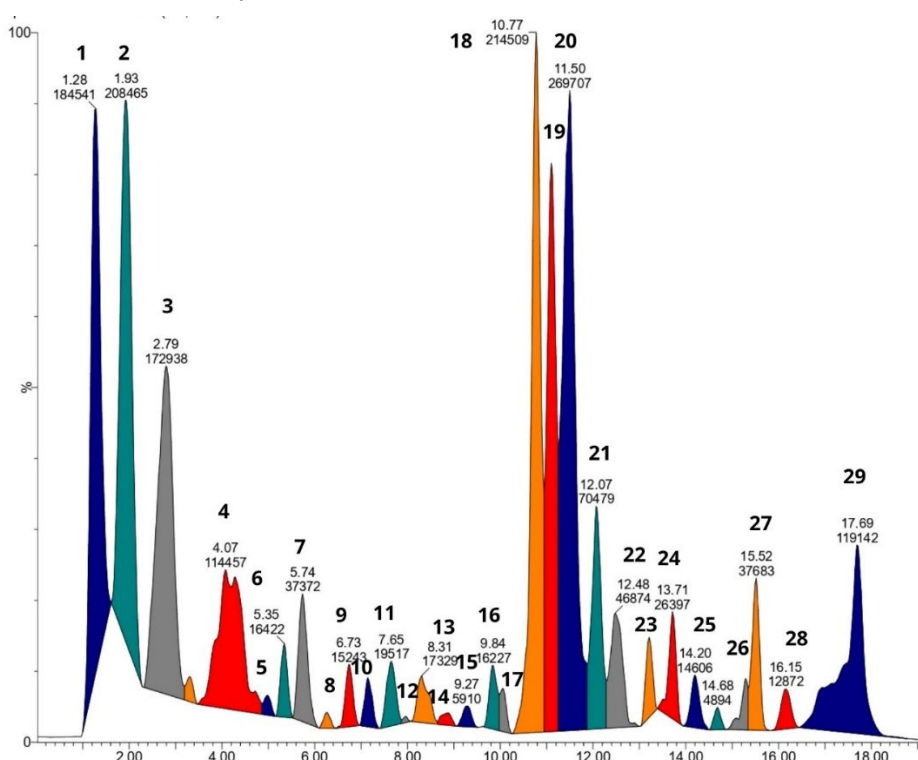


Figure 2. Chromatogram of FSLE. A total of 29 peaks identified in the chromatogram. Each peak in the chromatogram corresponded to a specific bioactive compound.

29 bioactive compounds were described in Table 1. The bioactive compounds were categorized as fatty acids/lipids, peptides, monosaccharides, flavonoids, phenols, saponins, amines, amides, and their derivatives. Notably, the primary component of this extract was capsaicinoid, including capsaicin, dihydrocapsaicin, nordihydrocapsaicin, and capsiamide.

Table 1. The detailed phytoconstituent identified in FSLE based on LC-MS/MS analysis.

No	Retention time	Area (%)	Graphical mass	m/z	Molecular formula	Name of compound	Classification of a compound
1	1.28	9.92	180.15	203.05	C ₆ H ₁₂ O ₆	Fructose	Monosaccharide
2	1.93	11.21	294.15	294.15	C ₁₂ H ₂₃ NO ₇	33-Methyl-2-[[[2,3,4-trihydroxy-5-(hydroxymethyl)oxolan-2-yl]methylamino]pentanoic acid	Amino Acid Derivative
3	2.79	9.30	328.13	328.14	C ₁₅ H ₂₁ NO ₇	N-Fructosyl phenylalanine	Monosaccharide Derivative
4	4.07	6.16	457.18	457.18	C ₁₆ H ₃₀ N ₆ O ₆ S	Glu-Met-Arg	Oligopeptide
5	4.97	0.25	182.22	183.09	C ₁₂ H ₁₀ N ₂	Azobenzene	Organic Chemicals
6	5.34	0.88	382.42	348.16	C ₁₆ H ₂₆ N ₆ O ₅	Ile-Asn-His	Oligopeptide
7	5.73	2.01	467.61	468.27	C ₂₄ H ₃₃ N ₇ O ₃	1-(5-{5-[(2S)-1-(1-Acetyl-4-piperidinyl)-2-pyrrolidinyl]-1,2,4-oxadiazol-3-yl}-2-pyridinyl)-4-piperidinecarboxamide	Amide Derivative
8	6.26	0.19	246.11	246.11	C ₁₄ H ₁₅ NO ₃	4-oxo-4-(5,6,7,8-tetrahydronaphthalen-1-ylamino)but-2-enoic acid	Tetralins
9	6.73	0.82	1,065.20	231.11	C ₅₁ H ₈₄ O ₂₃	Trigofenoside F	Saponin
10	7.14	0.65	1,101.20	1188.56	C ₅₀ H ₈₄ O ₂₆	Capsianoside III	Saponin
11	7.65	1.05	648.18	652.58	C ₄₂ H ₈₁ NO ₃	Ceramide	Lipid
12	7.96	0.05	300.26	301.07	C ₁₆ H ₁₂ O ₆	Isokaempferide	Flavonoids
13	8.31	0.93	1,085.20	1102.56	C ₅₀ H ₈₄ O ₂₅	Capsianside II	Saponin
14	8.88	0.23	289.40	289.40	C ₁₇ H ₂₃ NO ₃	Atropine	Endogenous Metabolites
15	9.27	0.32	330.46	353.23	C ₁₈ H ₃₄ O ₅	Pinellic acid	Fatty Acids, Unsaturated
16	9.84	0.87	344.31	345.09	C ₁₈ H ₁₆ O ₇	Pachypodol Quercetin 3,3',7'-trimethyl ether	Flavonoid
17	10.04	0.48	291.40	202.19	C ₁₇ H ₂₅ NO ₃	Norcapsaicin	Phenols
18	10.77	11.54	305.44	306.20	C ₁₈ H ₂₇ NO ₃	Capsaicin	Phenols
19	11.10	8.87	305.44	306.20	C ₁₈ H ₂₇ NO ₃	Capsaicin	Phenols
20	11.49	14.51	315.50	316.28	C ₁₈ H ₃₇ NO ₃	N,N-bis(2-hydroxyethyl)tetradecanamide Synonym: Myristic acid diethanolamide	Saturated Fatty Acid
21	12.06	3.79	317.29	318.30	C ₁₈ H ₃₉ NO ₃	2-Aminooctadecane-1,3,4-triol Synonym: Phytosphingosine	Organic Amino
22	12.48	2.52	303.52	304.30	C ₂₁ H ₃₇ N	2-Hexyl-3,5-dipentylpyridine	Pyridines Derivatives

No	Retention time	Area (%)	Graphical mass	m/z	Molecular formula	Name of compound	Classification of a compound
23	13.21	0.95	495.61	496.33	C ₂₂ H ₄₁ N ₉ O ₄	Arginyl-prolyl-lysyl-prolinamide	Peptide
24	13.71	1.42	331.60	332.33	C ₂₃ H ₄₁ N	Piptamine(antibiotic)	Aromatic Amine
25	14.19	0.79	383.60	384.34	C ₂₃ H ₄₅ NO ₃	Serinolamide A	Lipid, Fatty Amide
26	15.29	0.61	281.77	282.27	C ₁₈ H ₃₅ NO	Oleamide	Oleic Acids
27	15.51	2.03	269.50	270.27	C ₁₇ H ₃₅ NO	Capsiamide	Acetamides
28	16.15	0.69	493.90	494.56	C ₃₄ H ₇₁ N	Palmitylstearylamine	Fatty Amine
29	17.69	6.41	522.00	522.59	C ₃₆ H ₇₅ N	Tridodecylamine	Amines

Evaluation of cytotoxic profile

The MDCK and Vero cells were kidney cells, which are important in the metabolism and excretion of a nutraceutical product. Figure 3 displayed the percentage viability of MDCK and Vero Cells after 24 hours of incubation with the extract.

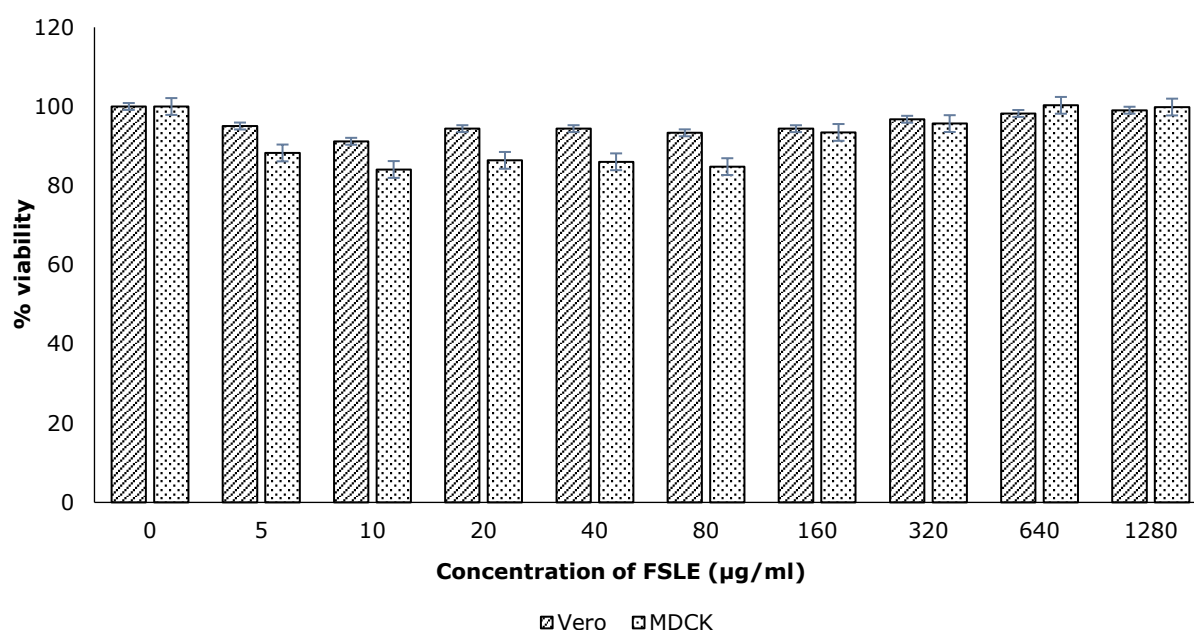


Figure 3. Cytotoxicity profile of FSLE on Vero and MDCK cells. Percentage of viable cells after 24 hours of incubation with FSLE. FSLE, fermented sambal lalapan extract. Cells grown in medium with 0.5% DMSO were controls. The experiment was performed in triplicate.

Cell viability in either Vero or MDCK cells after FSLE administration of 5 to 1,280 µg/ml for 24 hours showed no difference compared with the controls. Treatment with FSLE at a concentration of 5 µg/mL resulted in cell viabilities of 100 ± 6.96% in Vero cells and 100 ± 15.66% in MDCK cells. These values did not significantly differ from those observed at the highest tested concentration of 1,280 mg/mL, which yielded viabilities of 99.79 ± 11.87% for Vero cells and 99.85 ± 9.19% for MDCK cells. There was no point in Figure 3 showing a decrease in viability of 50% or less. It was concluded that the IC₅₀ value was not reached in the concentration range tested.

These findings indicated that the FSLE extract was not toxic to Vero or MDCK cells in the range of doses.

The extract improved lipid profiles in an atherosclerosis model induced by HFD in rabbits

Since the development of atherosclerosis plaque is triggered by hyperlipidemia, this study tried to investigate the effect of FSLE on lipid profiles in rabbits with an atherosclerosis model.

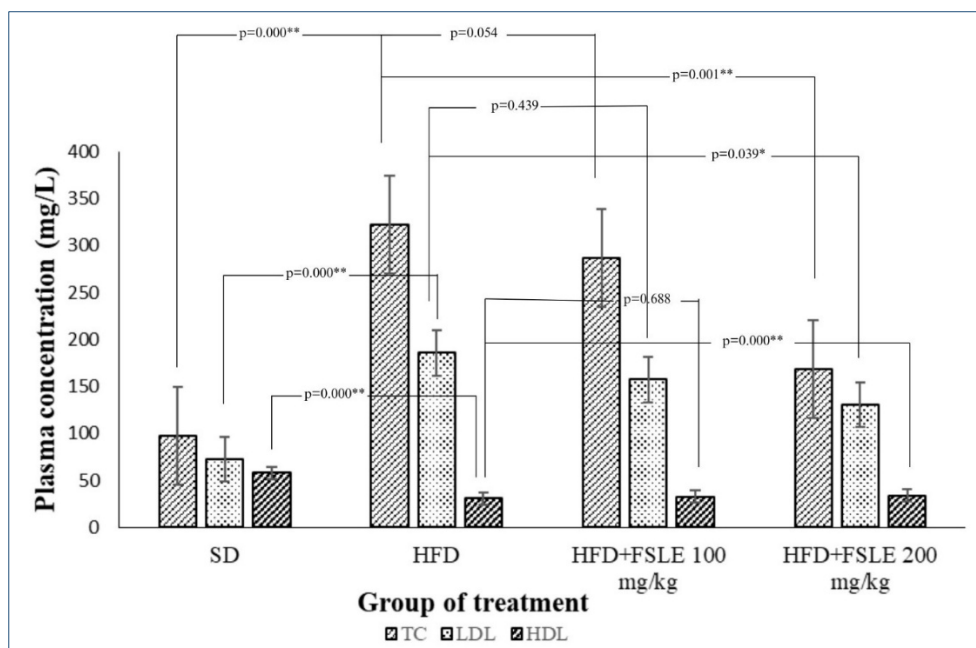


Figure 4. Effect of FSLE on TC, LDL-c, HDL-c level in high-fat diet-induced atherosclerosis in rabbits. The data in each group were expressed as mean $\pm$ SE ($n = 5$). The * and ** represent $P < 0.05$, < 0.001 , respectively. Standard Diet (SD), high fat diet (HFD), fermented sambal lalapan extract (FSLE).

According to Figure 4, the data showed that the administration of HFD increased the TC to 322.07 ± 25.70 from 97.39 ± 11.60 mg/dl in the SD group ($P = 0.000 < 0.001$). Furthermore, the administration of 200 mg/kg fermentation extract significantly inhibited the increase of the TC levels by 154.40 ± 34.13 mg/dL ($P = 0.001^{**}$). Consistent findings were obtained from LDL-c values after extract administration. The LDL-c value in the rabbit exposed to HFD was 185.93 ± 51.56 mg/L. The LDL-c concentration in FSLE 200 mg/kg was 130.59 ± 15.68 mg/dL, lower than the HFD group ($P = 0.000 < 0.01^{**}$). The data showed that the HDL-c level in the FSLE 200 mg/kg group was 33.73 ± 1.67 mg/dL, which was higher than the HFD group (30.61 ± 2.93 mg/dL) ($P < 0.05$).

Attenuation of foam cell formation in subendothelial space and enhancement of ABCA1 expression by administration of FSLE in rabbits fed a high-fat diet

Under the microscope, foam cells appear as round cells located in the tunica intima and media with white cytoplasm, where the nucleus lies at the edge of the cell under microscope. Figure 5 illustrated the gross histopathological aorta after HE staining.

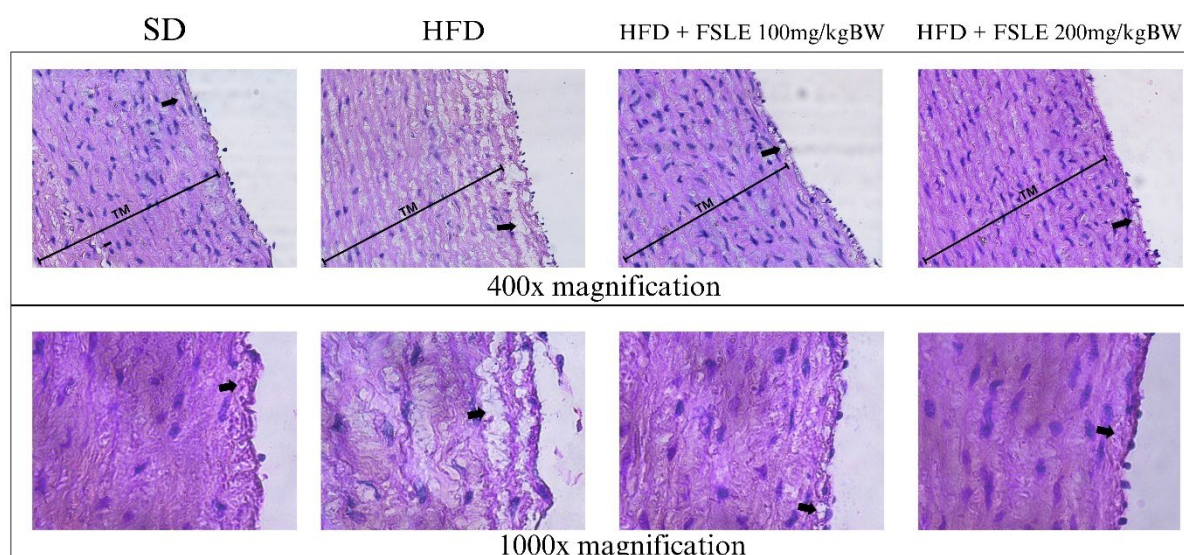


Figure 5. FSLE inhibited foam cell formation in HFD-induced atherosclerosis in rabbits. Histopathology of rabbit aortas that were stained with hematoxylin and eosin (H&E) in 4 groups (n=5). Foam cells are observed in the tunica intima as cells that have white cytoplasm, and the nucleus is observed at the edge. Thirteen random fields (400 and 1000x magnification) were analyzed for each group. SD, standard diet; HFD, high-fat diet; FSLE, fermented sambal lalapan extract.

It was shown that in the HFD group, there was an appearance of a layered foam cells layer (fatty streak). Moreover, the FSLE supplementation tend to suppress the formation of foam cells. Consistently, it was confirmed from image area quantification where foam cell area in FSLE100; 200 mg/kg BW ($13,545.03 \pm 3,913.8$; $10,046.71 \pm 3,734.93 \mu\text{m}^2$) was lower than in the HFD group ($19,471.86 \pm 6,861.47$) ($P = 0.045 < 0.05^*$).

Furthermore, this study tried to measure the aortic ABCA1 expression. Increased expression of mRNA ABCA1 and ABCA1 concentration were identified in FSLE group compared to the HFD group ($P < 0.05^*$) (Figure 6).

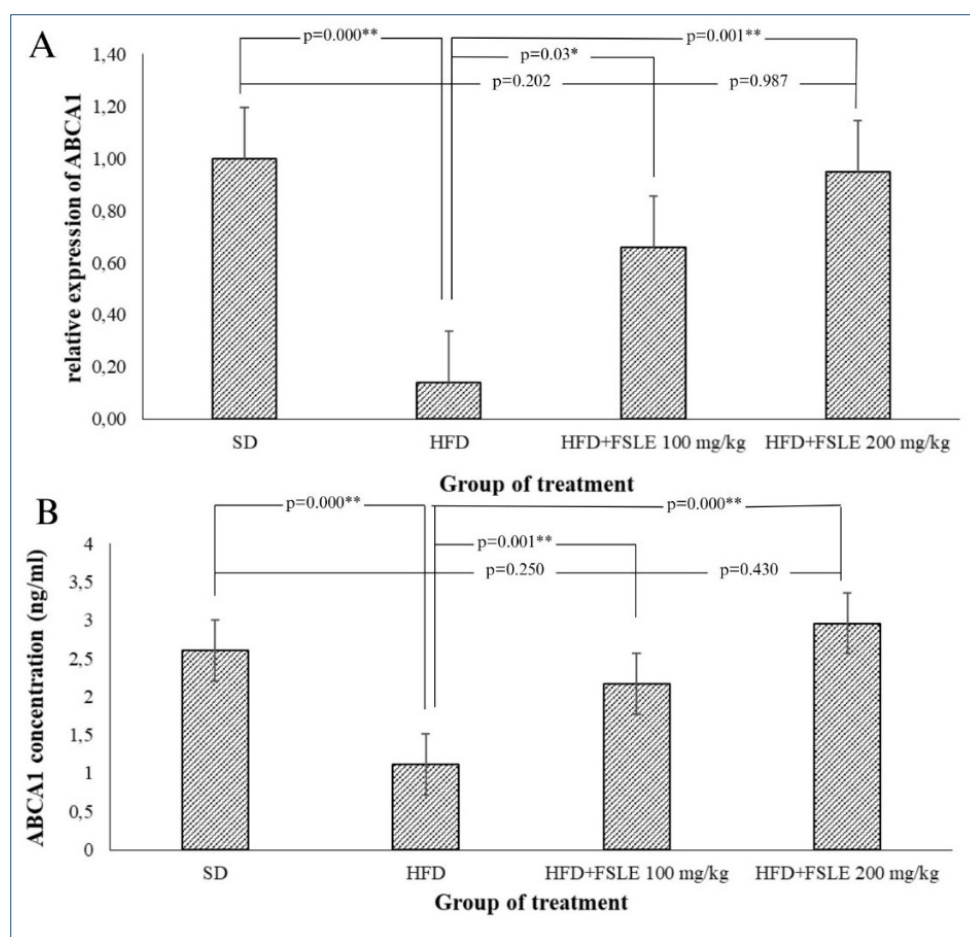


Figure 6. Effect of FSLE on ABCA1 expression in HFD-induced atherosclerosis in rabbits. (a) The mRNA ABCA1 relative expression, which was quantified using qPCR. (b) The concentration of ABCA1 protein from ELISA analysis. The data in each group ($n=5$) were expressed as mean $\pm$ SE ($n=5$). The * and ** represent $P < 0.05$, < 0.001 , respectively. Standard Diet (SD), high-fat diet (HFD), fermented sambal lalapan extract (FSLE).

DISCUSSION

Fermented natural products from vegetables have the advantage of improving health outcomes, including cardioprotective effects (Patra et al., 2016; Asgary et al., 2018; Fabricio et al., 2022; Pothirat et al., 2024). The fermentation condition stimulates the growth of lactic acid bacteria (LAB), which could produce new bioactive compounds, expose the trapped molecules in plant matrices, and modify the bioactive compounds through enzymatic reaction into several metabolites that all increase the number of constituents and enhance their antioxidant activity. Interestingly, the fermentation of sambal lalapan resulted in the identification of the predominant compounds: capsaicinoid, capsiamide (amide), and capsianoside II, III (alkaloid).

IC_{50} , or half-maximal inhibitory concentration, represents the concentration of a substance needed to cause 50% inhibition of cell viability or growth. Plant extracts with IC_{50} values above 100 $\mu\text{g/mL}$ in normal cells, including kidney cells, are generally considered to have low or negligible cytotoxicity and thus regarded as safe for further pharmacological evaluation (Lin et al., 2023). In our study, the extract showed IC_{50} values above this threshold, suggesting that the fermented extract is safe for further development of functional food or herbal medicine.

The ability of FSLE to lower TC and LDL-c can be explained by the presence of capsaicin. A study reported that capsaicin improves lipid profiles by increasing HDL-c and reducing triglyceride levels (Qin et al., 2017). This data was also supported by a report from Previous study demonstrated that capsaicin increased the levels of chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), β -muricholic acid (β -MCA), and tauro- β -muricholic acid sodium salt (T- β -MCA). This molecule, in turn, modulates the farnesoid X receptor (FXR) to inhibit Fgf15, increases CYP7A1 expression to lower TG and TC (Gong et al., 2022)

Our findings demonstrated that this extract could suppress the establishment of foam cells, which might be partially explained by stimulating the ABCA1 expression. ABCA1 function in the reverse cholesterol transport pathway (RCT) facilitates the export of cellular cholesterol to its extracellular acceptor protein apolipoprotein-A1 (apoA-1) (Zhang et al., 2020). Previous studies described that capsaicin increased ABCA1 by Nuclear factor kappa B (NFkB)/Mitogen Activated Protein Kinase (MAPK) inflammatory signaling inhibition and attenuation of oxidative stress (Yang et al., 2019). In addition, capsaicin induced the expression of the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor, which could stimulate the ABCA1 expression. Additionally, besides capsaicin, LC-MS/MS data indicated the presence of another compound, such as peptides and fatty acids. The peptides, consisting of 2-20 amino acids, which are inactive protein fragments in their precursor molecules, could be activated through microbial fermentation. Fatty acids such as oleamide and pinellic acid (Bartolomaeus et al., 2019; Sasaki et al., 2024) demonstrated its ability to transactivate the peroxisome proliferator-activated receptor (PPAR), and suppress inflammation that stimulates the ABCA1 expression (Cong et al., 2021; Guo et al., 2023).

Even though our research gave positive results, there are several limitations in conducting this research. Since this study aimed to explore the potential of FSLE as a preventive agent against atherosclerosis, despite the fact that its development is influenced by multiple risk factors beyond an HFD, further *in vitro* and *in vivo* investigations are warranted. These future studies should focus on elucidating the modulation of atherosclerosis-related signaling pathways by FSLE, using various models such as the low chronic inflammation state, hyperlipidemia, and hypertension. Inclusion of appropriate positive controls, such as anti-hypertensive and or hypolipidemic agents, will also be essential to validate the findings. (Karami, Akrami, et al., 2023; Karami, Mehrzad, et al., 2023) Although this work successfully identified the secondary metabolites of FSLE, the quantification of active compounds as a standard marker and their bioavailability should be considered to be assessed in the future studies.

CONCLUSION

In summary, fermented sambal lalapan inhibits atherogenesis by improving lipid profiles and reducing foam cell development. These results indicate that fermented sambal lalapan could be a potential nutraceutical agent for preventing CHD.

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AUTHOR CONTRIBUTIONS

Ermin Rachmawati: Conceptualization (Lead), Methodology (Lead), Formal Analysis (Lead), Validation (Lead), Data Curation (Lead), Writing – Original Draft (Lead), Writing – Review & Editing (Lead), Investigation (Lead), Supervision (Equal), Project Administration (Equal); **Larasati Sekar Kinasih:** Data Curation (Equal), Writing – Original Draft (Equal), Writing – Review & Editing (Equal), Investigation (Equal); **Tias Pramesti Griana:** Data Curation (Equal), Formal Analysis (Equal), Writing – Review & Editing (Equal), Investigation (Equal); **Kanokwan Poomputsa:** Resource (Equal), Data Curation (Equal), Formal Analysis (Equal), Writing – Original Draft (Equal), Writing – Review & Editing (Equal), Investigation (Lead), Supervision (Equal); **Dwiki Pramudika Abdul Azis:** Formal Analysis (Supporting), Visualization (Supporting), Writing – Review & Editing (Equal), Project administration (Supporting); **Sabila Rosyidah Wibawa Putri:** Formal Analysis (Supporting), Validation (Equal), Writing – Review & Editing (Equal), Visualization (Supporting), Project administration (Supporting); **Roihatul Mutiah:** Resource (Equal), Writing – Review & Editing (Equal), Investigation (Equal), Supervision (Equal); **Suharti Suharti:** Resource (Equal), Writing – Review & Editing (Equal), Investigation (Equal), Supervision (Equal); **Djangan Sargowo:** Resource (Equal), Writing – Review & Editing (Equal), Investigation (Equal), Supervision (Equal); **Yudi Her Oktaviono:** Resource (Equal), Writing – Review & Editing (Equal), Investigation (Equal), Supervision (Equal).

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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Supplementary

Table 1. Registration number of sambal lalapan ingredients.

	Registration number	Key Determination
1) Chili	067/2253/102.20/2023	1b-2b-3b-4b-6b-7b-9a-41b-42b-43b-54a-55b-57b-58a: Solanaceae-1b-3b-5b-6b-7a: Capsicum-1b: <i>C. Frutescens</i>
(2) Shallot	067/2250/102.20/2023	1b-2b-3b-4b-6b-7b-9b-10a-92b-100a-101b-102b: Amaryllidaceae-1a-2b-3a-4a: Allium-1a-2a-3b: <i>A. Cepa</i>
(3) Garlic	067/2251/102.20/2023	1b-2b-3b-4b-6b-7b-9b-10a-92b-100a-101b-102b: Amaryllidaceae-1a-2b-3a-4a: Allium-1b-4b-6b: <i>A. Sativum</i>
(4) Tomato	067/2249/102.20/2023	1b-2b-3b-4b-6b-7b-9a-41b-42b-43b-54a-55b-57b-58a: Solanaceae-1b-3b-5b-6b-7b: Solanum-6-1a: <i>S. Lycopersicum</i>
(5) Basil leaves	067/2252/102.20/2023	1b-2b-3b-4b-6b-7b-9b-10b-11b-12b-13b-14b-16a-239b-243b-244b-248b-249b-250b-266b-267b-273b-276b-278b-279b-282a: Labiatae-1a-2b-4b-6b-7b: Ocimum-8: <i>O. Basilicum</i>
(6) Cucumber	067/2248/102.20/2023	1b-2a-27a-28b-29b-30b-31b: Cucurbitaceae-1a-2b-3a: Cucumis-2: <i>C. Sativus</i>
(7) White cabbage	067/2247/102.20/2023	1b-2b-3b-4b-12b-13b-14b-17b-18b-19b-20b-21b-22b-23b-24b-25b-26b-27a-28b-29b-30b-31a-32a-33b-35a-36d-37b-38b-39b-41b-42b-44b-45b-46e-50b-51b-53b-54b-56b-57b-58b-59d-72b-73b-74a-75a-76a-77a-78b-103c-104b-106b-107a-108b-109a-110b-115b-119a-120b-122a: Brassicaceae-1b-6b-7b-10a: Brassica-3-1a: <i>B. oleracea</i>

Note: with registration number; and key determination; respectively.