Effects of Plant Oils and Minerals for the Inhibition of Lipase Activity of *Staphylococcus aureus* Isolated from Fermented Pork Meat

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Abstract

Staphylococcus aureus lipase is regarded as a virulence factor. The response of lipase activity to various factors can provide important insights concerning the prevention of *S. aureus* during meat fermentation. This study was conducted to evaluate the main effects of nutrients used in culture media, and their combined effects on the inhibition of lipase activity and cell growth of pathogenic *S. aureus* SK1593 isolated from fermented pork meat. A Plackett-Burman design was used to evaluate the main effects of variables, including olive oil, soybean oil, grapeseed oil, sesame oil, CuSO₄, MgCl₂, KNO₃, CaCl₂, and KCl. Significant negative effects on lipase activity were detected with soybean oil, grapeseed oil, KNO₃, and CaCl₂. Additionally, these nutrients were further selected as variables for the investigation of their combined effect on lipase activity, via response surface methodology. In order to confirm the regression model, a situation that only inhibits lipase activity was simulated. The predicted lipase activity and cell growth of the simulated situation were 14.0 U/mL and 9.6 log₁₀ (CFU/mL), respectively, and the estimated value of those in the same medium showed 15.14 U/mL and 9.4 log₁₀ (CFU/mL) respectively. The lipase activity of the simulated medium was inhibited approximately 5-fold as compared to the basal medium, but no significant differences in cell counts were noted to exist between the basal and simulated media. These results suggest that soybean oil, grapeseed oil, KNO₃, and CaCl₂ can be used to inhibit the growth of pathogenic *S. aureus* during the process of meat fermentation.

Key words: *Staphylococcus aureus* SK1593, lipase activity, Plackett-Burman design, response surface model

Introduction

Meat products often contain pathogenic bacteria. For the effective preservation of sausage, fermentation processes have traditionally been used in its manufacture. Low-acid-fermentation following spontaneous fermentation without starter culture is preferred by the customers because of a dislike of an acidic taste. However, the risk of pathogenic bacteria is greater in low-acid-fermented than in high-acid-fermented sausage (Ananou *et al.*, 2010).

Staphylococcus aureus is a representative meat-borne pathogen that is most frequently found in chopped meat mixtures or fermented sausage. This organism is tolerant to salt and nitrite, and is also able to grow under environ-

mental conditions present in raw meat and in processing equipment (Ananou *et al.*, 2010; González-Fandos *et al.*, 1999). To control or prevent the contamination of sausage by pathogenic bacteria, various additives such as sulfite, nitrite, alcohol and antibiotics, have been added during its manufacture (Jofré *et al.*, 2008). However, the recent increasing preference for natural animal products by consumers has resulted in the use of restricted chemical additives. Hence, the use of bacteriocin which is produced from lactic acid bacteria (LAB) is now the preferred preservative (Ananou *et al.*, 2005). However, it is difficult to add LAB to low-acid-fermented meat or ready-to-eat sausage. The effective control of staphylococcal contamination is very important during meat processing.

The enzyme lipase of pathogenic bacteria is regarded as a control factor for the prevention of its colonization. Lipase has been shown to improve the adherence and colonization of pathogenic bacteria by degrading lipids on the external surface of the host, after that the liberated

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free fatty acids function as nutrients for the persistence of colonized bacteria (Grippa *et al.*, 1999; Longshaw *et al.*, 2000; Rosenstein and Götz, 2000). The lipase of *S. aureus* is also regarded as a virulence factor (Kuroda *et al.*, 2007; Pereira *et al.*, 2009). For these reasons, the response of lipase activity to various factors during bacterial growth can provide important insights concerning the control and prevention of pathogenic bacteria.

Most bacterial lipases are extracellular enzymes, and their activities are influenced by nutrients such as carbon, nitrogen, and mineral sources (Ebrahimpour *et al.*, 2008; Joseph *et al.*, 2006; Lakshmi *et al.*, 1999; Rath *et al.*, 2002). Few studies of the inhibition of lipase activity using nutrient components of *S. aureus* have been conducted.

In the current study, we isolated *S. aureus* SK1953 from fermented pork meat and evaluated the effects of certain nutrients on its lipase activity. Two statistical methods-Plackett-Burman design and response surface methodology were used for the first time to determine the main effects of nutrients and their combined effects to inhibit the lipase activity of *S. aureus* SK1953. We found that some components, such as minerals and plant oils, had negative effects at lipase activity.

Materials and Methods

Isolation of lipolytic strain

Cooked pork meat was spontaneously fermented at room temperature for three days. It was suspended in sterilized 0.8% (w/v) NaCl solution and homogenized with a blender (Philips, Holland). The suspended solution was subsequently diluted with the same buffer and spread onto LB (Difco, USA) agar plates. It was incubated overnight at 30°C. Morphologically different colonies were then selected and streaked onto a lipolytic strain screening agar plate, then incubated overnight at 30°C. The screening media was prepared via the addition of 1 mL of tributyrin to 100 mL of LB agar media, and was homogenized to minimize tributyrin droplets and increase turbidity. The lipolytic colony showing a large clear zone was isolated, then grown for 24 h in LB broth at 30°C for the preparation of the stock cultures. One hundred microliters of dimethyl sulfoxide (DMSO) were added to 0.9 mL of grown culture, and stored at below -70°C.

Identification of isolated strain

For the identification of the isolated strain, the 16S rRNA gene sequence of the isolated strain was amplified

using two universal primers (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492R: 5'-GGT TAC CTT GTT ACG ACT T-3'). Next, the amplified sequence was analyzed with an ABI PRISM 3730XL DNA analyzer. All analyses were conducted at Solgent Co. Ltd (Korea, Daejeon). Homology of the sequence of the isolated strain was searched in the GeneBank database, using the BLAST program. Multiple alignments were conducted using CLUSTAL_W software (Thompson *et al.*, 1994) with 16S rRNA gene sequences of type strains involved in the same genus of the isolated strain as a result of the homology search. The construction of a phylogenetic tree to determine evolutionary distance was conducted via the Maximum Likelihood method in MEGA 4 (Tamura *et al.*, 2007; Tamura *et al.*, 2004).

Medium preparation and culture conditions

The inoculum was prepared via the streaking of a piece of frozen stock culture on a LB agar plate, followed by incubation at 30°C until colonies became visible. Then, a single colony was transferred to 5 mL of LB broth at 30°C for 24 h with shaking (150 rpm) and used as an inoculum for the test culture. The basal medium employed for the screening of effective nutrients and their interaction contained (g/L): beef extract, 10; yeast extract, 5 and NaCl, 5. After the addition of all of the screening nutrients into basal medium in accordance with the experimental design to prepare the experimental medium, the pH of each medium was adjusted to 7.0 with 10 N NaOH and incubated for 24 h at 30°C with shaking (150 rpm). Two hundred microliters of inoculum were transferred to 20 mL of experimental medium in 100 mL Erlenmeyer flasks. The media containing oil were homogenized (Ultra Turrax, Janke & Kunkel, Germany) to minimize oil droplets prior to autoclaving.

The assay of lipase activity

Lipase activity was determined via a spectrophotometric assay using *p*-nitrophenyl butyrate (*p*NPB) as previously described with slight modification (Ryu *et al.*, 2006). In brief, a reaction mixture composed of 10 µL of 50 mM *p*NPB in acetonitrile, 40 µL of ethanol, 950 µL of 50 mM Tris-HCl (pH 8.0) and 50 µL of culture supernatant was incubated at 37°C and the optical density at 405 nm was estimated at 1 min intervals until it reached a level of 0.5. The concentration of liberated *p*-nitrophenyl was then calculated via regression of a standard *p*-nitrophenyl solution, and one unit was defined as one nM of *p*-nitrophenyl per min.

Table 1. Variables showing nutrients and their high and low concentrations used in Plackett-Burman design

Variables	Nutrients	+1 values (g/L)	-1 values (g/L)
X ₁	Olive oil	5	0.5
X ₂	Soybean oil	5	0.5
X ₃	Grapeseed oil	5	0.5
X ₄	Sesame oil	5	0.5
X ₅	CuSO ₄	1	0.05
X ₆	MgCl ₂	1	0.05
X ₇	KNO ₃	1	0.05
X ₈	CaCl ₂	1	0.05
X ₉	KCl	1	0.05

Determination of bacterial cell count

The cell count in culture was determined via serial dilution, as previously described (Lappe *et al.*, 2009) and was estimated as colony forming units (CFU/mL). Culture suspensions were diluted to 10⁻⁷ in sterilized 0.8% (w/v) NaCl solution and spread onto LB agar plates. The plates were then incubated for 24 h at 30°C, and 30 to 100 colonies were counted. The cell counts were used as the logarithm of CFU/mL to base 10 when evaluating the effects of the experiments.

Screening of effective nutrients on lipase activity

A Plackett-Burman design (Plackett and Burman, 1946) was used in this study to screen the important nutrients with regard to the major negative effects on lipase activity. A total of nine components (variable $k = 9$) were employed, and two concentration levels were selected (Table 1). High and low levels of concentration were represented as sign (+1) and sign (-1), respectively. The Plackett-Burman design matrix with nine variables is

shown in Table 2. Columns and rows represent variables and runs, respectively. In each of the runs, one of two signs of a variable was assigned and the number of high signs per run is $(k + 1)/2$, and that of the low sign is $(k - 1)/2$. The effects of variables, their probability, and analysis of variance were calculated and the variables showing negative effect with 95% confidence level were selected for further experiment.

Screening of interaction between selected nutrients

Response surface methodology (RSM) was employed to assess the combined effects on selected nutrients that showed negative values in effect within a 95% confidence level from the results of the Plackett-Burman design experiment. A total of four nutrients were selected as variables and their three levels of concentration were assigned to each run. Twenty seven runs of a Box-Behnken (Box and Behnken, 1960) design with three replicates at the center point were employed. The coded and actual values of the variables assigned to each run are provided in Table 4. A quadratic equation was employed to explain the response of the system:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_i X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is the predicted response, β_0 is the intercept, and β_i and β_{ij} are the regression coefficients. X_i and X_j are the independent variables ($i \neq j$). Solution of the quadratic equation and analysis of variance were conducted via the use of response surface methodology.

Statistical analysis

Normality test of the observed results of lipase activity and cell counts assigned to each runs in Plackett-Burman

Table 2. Plackett-Burman design matrix of 12 runs with 9 variables and related responses

Runs	Variables									Lipase activity ¹⁾ , U/mL	Cell count, log ₁₀ (CFU/mL)
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	X ₉		
1	1	-1	1	-1	-1	-1	1	1	1	7.35	6.50
2	1	1	-1	1	-1	-1	-1	1	1	27.01	6.99
3	-1	1	1	-1	1	-1	-1	-1	1	16.33	5.30
4	1	-1	1	1	-1	1	-1	-1	-1	24.60	7.26
5	1	1	-1	1	1	-1	1	-1	-1	26.74	5.54
6	1	1	1	-1	1	1	-1	1	-1	15.30	4.26
7	-1	1	1	1	-1	1	1	-1	1	24.57	6.45
8	-1	-1	1	1	1	-1	1	1	-1	19.95	4.22
9	-1	-1	-1	1	1	1	-1	1	1	44.76	4.12
10	1	-1	-1	-1	1	1	1	-1	1	30.43	3.92
11	-1	1	-1	-1	-1	1	1	1	-1	6.99	5.40
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	7.17	9.47

¹⁾Lipase activity was defined as nanomole of *p*-nitrophenyl liberated from *p*-nitrophenyl-butyrate per one min.

Fig. 1. Phylogenetic tree based on the 16S rRNA sequence of *Staphylococcus aureus* SK1593 and other type strains of staphylococci. Bootstrap values (based on 1,000 trials and only values >50%) are shown at the nodes. Bar, 0.002 substitutions per nucleotide position.

design and Box-Benken design were employed with Anderson-Darling method prior to performing the analysis of variance. MINITAB[®] program (version 14.0, Minitab Inc., USA) was used for the calculation of normality test of responses of each experiment, the effects of variables, their probability and analysis of variance in Plackett-Burman design experiment, a response surface methodology and a multiple response optimizer with the responses of Box-Benken design experiment. The significance levels of the differences between cell count and lipase activity of basal medium and those of simulated medium in the experiment of prediction and validation of model were determined using Student *t*-test.

Results and Discussion

Screening and isolation of a lipolytic strain from fermented pork meat

Three colonies showing a clear zone surrounding the growth on LB-tributyrin were detected and the colony with the largest clear zone among them was selected. With the 16S rRNA gene sequence of the isolate, the homology was searched in the GenBank database, and a high level of identity with *Staphylococcus aureus* was assessed. Then, a multiple alignment with 11 species of staphylococcal-type strains was conducted in order to analyze the evolutionary distance and to construct a phylogenetic tree for identification. Finally, the isolated strain was determined to be closely related to *Staphylococcus aureus* subsp. *aureus* ATCC 12600^T, with an identity of 99.7% (Fig. 1), and was named *Staphylococcus*

aureus SK1593 (GenBank accession no. GQ389795)

Screening of effective nutrients to inhibit lipase activity

Prior to evaluating the effect of nutrients on the lipase activity of *Staphylococcus aureus* SK1593, a lipase assay was conducted with buffer solution at various pH values in order to optimize the assay conditions. The pH of buffer solution varied from 3.0 to 9.0, and the overnight culture supernatants of *S. aureus* SK1593 cultivated in basal medium were employed for the assay. The highest level of activity was detected at a pH of 8.0, and subsequent analyses were conducted at pH 8.0. The Plackett-Burman design is a convenient and accurate statistical method that involves relatively small numbers of experiments (*runs*) when only the main effects of variables, rather than their combined effects, were considered (Chauhan *et al.*, 2007; Plackett and Burman, 1946). The nutrients were used as independent variables which are represented with their respective high and low concentrations, as is shown in Table 1. The Plackett-Burman design matrix with 9 nutrients as variables and their related responses (lipase activity and cell count) is shown in Table 2. In normality test of responses assigned to each runs, the *p* value of lipase activity and cell count were 0.432 and 0.330, respectively. The results of the experiment with regard to lipase activity, the effect, standard error, *t* value, *p* value, and analysis of variance are presented in Table 3. All of the nutrients showed significance at a confidence level in excess of 95%. The *p* value of the main effect in analysis of variance was 0.002, which

Table 3. ANOVA (Analysis of variance) of experiment and effects of media components on the lipase activity of *S. aureus* SK1593 as determined by 12 runs based on the Plackett-Burman design

Items	DF ¹⁾	SS ²⁾	MS ³⁾	F	P > F
Main effect	9	1376.8	153.0	629.6	0.002
Residual error	2	0.5	0.2		
Total	11	1377.2			

Variables	Effect	Coefficient		t value	p value
		Effect	SE ⁴⁾		
Constant		20.935	0.1423	147.13	<0.001
Olive oil	1.944	0.972	0.1423	6.83	0.021
Soybean oil	-2.885	-1.443	0.1423	-10.14	0.010
Grapeseed oil	-5.837	-2.918	0.1423	-20.51	0.002
Sesame oil	14.008	7.004	0.1423	49.22	<0.001
CuSO ₄	9.302	4.651	0.1423	32.69	0.001
MgCl ₂	7.018	3.509	0.1423	24.66	0.002
KNO ₃	-3.190	-1.595	0.1423	-11.21	0.008
CaCl ₂	-1.413	-0.707	0.1423	-4.97	0.038
KCl	8.280	4.140	0.1423	29.10	0.001

¹⁾DF, degree of freedom.

²⁾SS, sum of square.

³⁾MS, mean of square.

⁴⁾SE, standard error.

indicates that the result of the experiment reflected adequate for the screening of the main effects of variables. Olive oil, sesame oil, CuSO₄, MgCl₂, and KCl showed positive effects, thus demonstrating that these nutrients are capable of increasing lipase activity with increased concentration. Conversely, soybean oil, grapeseed oil, KNO₃, and CaCl₂ evidenced a negative effect, which indicates that an increase in these nutrients can reduce lipase activity. A variety of oil sources have been investigated in order to evaluate their effects on staphylococcal lipase activity, and they have been identified as excellent inducers of lipase activity (Gupta *et al.*, 2004; Hasanuzzaman *et al.*, 2004). A 1% supplementation of olive oil resulted in an increase of more than two-fold in the lipase activity of *Staphylococcus epidermidis* (Joseph *et al.*, 2006). However, the opposite results also have been noted in other studies (Al-Waili, 2005; Walavalkar and Bapat, 2002). In this study, olive and sesame oils exerted positive effects, whereas soybean and grapeseed oils exerted a negative effect on the lipase activity of *S. aureus* SK1593. In previous reports, calcium chloride has generally been regarded as an excellent lipase inducer, as it is capable of stabilizing the three-dimensional structure of the lipolytic enzyme during the reaction by binding to free fatty acid liberated during lipid hydrolysis (Gupta *et al.*, 2004;

Longshaw *et al.*, 2000). However, in this study, it was shown to exert a negative effect on lipase activity. The effects of nutrients on the growth of *Staphylococcus aureus* SK1593 were also evaluated. However, the *p* value of the main effect from analysis of variance was 0.145, which indicates that the evaluation of the main effect with this experiment was not proper. Therefore, the effects of nutrients on growth were not considered in the following experiments.

Interaction of selected nutrients

We determined that soybean oil, grapeseed oil, KNO₃, and CaCl₂ can inhibit lipase activity within a 95% confidence level, via an experiment based on a Plackett-Burman design. These nutrients were selected for subsequent experiments because the objective of this study was to determine the response of lipase to various nutrients and to identify negatively effective nutrients in order to reduce staphylococcal lipase activity. Furthermore, these nutrients were employed to evaluate their combined effects using RSM. Twenty seven runs of the Box-Behnken design with different combinations of the four selected nutrients were conducted. Table 4 shows the Box-Behnken design matrix including the selected variables, their coded values, assigned actual concentrations, and the results of estimated and predicted responses (lipase activity and cell growth). In normality test of responses assigned to each runs, the *p* value of lipase activity and cell count were 0.979 and 0.218, respectively. The responses were analyzed via quadratic multiple regressions, and the following equations were obtained for lipase activity and cell growth:

$$Y_{lipase} = 5.43 + 3.44X_1 + 7.01X_2 + 2.25X_3 + 35.50X_4 - 0.26X_1^2 - 0.99X_2^2 + 0.84X_3^2 - 28.00X_4^2 - 0.51X_1X_2 + 0.66X_1X_3 + 0.17X_1X_4 - 0.83X_2X_3 + 0.33X_2X_4 - 9.17X_3X_4$$

$$Y_{cellcount} = 8.34 - 0.13X_1 + 0.07X_2 + 1.39X_3 + 2.83X_4 + 0.02X_1^2 - 0.03X_2^2 - 1.23X_3^2 - 2.71X_4^2 - 0.005X_1X_2 + 0.03X_1X_3 + 0.03X_1X_4 - 0.13X_2X_3 + 0.13X_2X_4 + 0.06X_3X_4$$

where Y_{lipase} and $Y_{cellcount}$ are the predicted responses of lipase activity and cell growth, respectively. With regard to cell growth, the response was represented as the logarithm of CFU/mL to base 10. X_1 , X_2 , X_3 and X_4 are the uncoded values (g/L) of variables for soybean oil, grapeseed oil, KNO₃, and CaCl₂, respectively. The results were evaluated via analysis of variance in order to validate the

Table 4. Box-Behnken design configuration with coded and actual values for lipase activity and cell counts of *S. aureus* SK1593

Runs	Soybean oil, g/L		Grapeseed oil, g/L		KNO ₃ , g/L		CaCl ₂ , g/L		Lipase activity, U/mL		Cell count, log ₁₀ (CFU/mL)	
	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Actual value	Predicted value	Actual value	Predicted value
1	-1	0.50	-1	0.50	0	0.53	0	0.53	14.66	20.10	9.71	9.58
2	1	5.00	-1	0.50	0	0.53	0	0.53	28.14	29.91	9.55	9.59
3	-1	0.50	1	5.00	0	0.53	0	0.53	23.89	24.95	8.92	9.02
4	1	5.00	1	5.00	0	0.53	0	0.53	26.96	24.36	8.67	8.94
5	0	2.75	0	2.75	-1	0.05	-1	0.05	24.36	22.70	8.33	8.23
6	0	2.75	0	2.75	1	1.00	-1	0.05	22.47	24.79	7.80	8.05
7	0	2.75	0	2.75	-1	0.05	1	1.00	28.85	29.36	9.16	9.05
8	0	2.75	0	2.75	1	1.00	1	1.00	18.68	23.18	8.69	8.93
9	-1	0.50	0	2.75	-1	0.05	0	0.53	30.98	29.43	9.11	9.30
10	1	5.00	0	2.75	-1	0.05	0	0.53	34.05	32.63	9.00	9.21
11	-1	0.50	0	2.75	1	1.00	0	0.53	29.58	25.98	9.29	9.09
12	1	5.00	0	2.75	1	1.00	0	0.53	35.47	31.99	9.28	9.11
13	0	2.75	-1	0.50	0	0.53	-1	0.05	23.41	19.10	8.78	8.68
14	0	2.75	1	5.00	0	0.53	-1	0.05	19.86	18.04	7.85	7.80
15	0	2.75	-1	0.50	0	0.53	1	1.00	24.12	20.92	9.20	9.27
16	0	2.75	1	5.00	0	0.53	1	1.00	21.99	21.27	8.81	8.92
17	-1	0.50	0	2.75	0	0.53	-1	0.05	19.16	20.11	8.46	8.56
18	1	5.00	0	2.75	0	0.53	-1	0.05	19.86	24.37	8.57	8.47
19	-1	0.50	0	2.75	0	0.53	1	1.00	24.59	22.28	9.42	9.36
20	1	5.00	0	2.75	0	0.53	1	1.00	26.01	27.24	9.63	9.38
21	0	2.75	-1	0.50	-1	0.05	0	0.53	25.54	26.65	9.11	9.15
22	0	2.75	1	5.00	-1	0.05	0	0.53	25.07	28.07	9.04	8.81
23	0	2.75	-1	0.50	1	1.00	0	0.53	27.20	26.38	9.20	9.27
24	0	2.75	1	5.00	1	1.00	0	0.53	23.18	24.25	8.58	8.38
25	0	2.75	0	2.75	0	0.53	0	0.53	29.32	31.14	9.28	9.35
26	0	2.75	0	2.75	0	0.53	0	0.53	31.22	31.14	9.47	9.35
27	0	2.75	0	2.75	0	0.53	0	0.53	32.87	31.14	9.32	9.35

regression coefficient. In the RSM for lipase activity, the lack of fit, model F , and model $P > F$ were found to be 0.165, 2.27, and 0.08, respectively, and the determination coefficient (R^2) was 72.6%. In the RSM for cell growth, the lack of fit, model F , and model $P > F$ were found to be 0.150, 7.73 and 0.001, and the determination coefficient (R^2) was 90.0%. In this study, the model for cell growth exhibited better fit than that of lipase activity. Figs. 2 and 3 represent the interaction of each of the two nutrients on lipase activity and cell growth, respectively. The combined effects of the two variables were explained using three-dimensional plots, which were prepared with the regression model coefficients evaluated by the uncoded values of variables. When the combined effects of the two variables were calculated, the other remaining variables were fixed at their central point assigned to the coded value of 0, as shown in Table 4. In the lipase response, a dual quadratic interaction was found between grapeseed oil and CaCl₂ (Fig. 2E). The lowest level of

lipase inhibition was detected in the combination of grapeseed oil and CaCl₂, at approximately 2.5 g/L and 0.5 g/L, respectively. In regard to the response of cell growth, a dual quadratic interaction was derived between KNO₃ and CaCl₂ (Fig. 3F).

Prediction and validation of the model

To validate the model, we simulated a situation - a combination of nutrients - in which lipase activity was inhibited without any inhibition of cell growth by using multiple response optimizer in MINITAB®. The simulated medium was composed of the following (g/L): soybean oil, 0.5; grapeseed oil, 0.5; KNO₃, 0.5; CaCl₂, 1.0 in basal medium. The predicted values of lipase activity (Y_{lipase}) and cell count ($Y_{cellcount}$) from the simulated medium were calculated as 14.0 U/mL and 9.6 log₁₀ (CFU/mL), respectively. The preparation and culture conditions for the simulated medium were conducted as described in the 'Materials and Methods' section. The

Fig. 2. Three-dimensional plot showing the effect of nutrients on lipase activity. (A) effects of soybean oil and grapesed oil at the central point of KNO_3 and CaCl_2 ; (B) effect of soybean oil and KNO_3 at central point of grapesed oil and CaCl_2 ; (C) effect of soybean oil and CaCl_2 at the central point of grapesed oil and KNO_3 ; (D) effect of grapesed oil and KNO_3 at the central point of soybean oil and CaCl_2 ; (E) effect of grapesed oil and CaCl_2 at the central point of soybean oil and KNO_3 ; (F) effect of KNO_3 and CaCl_2 at the central point of soybean and grapesed oils.

results of lipase activity and cell growths were compared with those of basal medium (Table 5), and their significances were determined via the Student's *t*-test. On basal medium, the cell count and lipase activity were measured at $9.75 \pm 0.08 \log_{10}(\text{CFU/mL})$ and $73.31 \pm 2.95 \text{ U/mL}$, respectively. The cell count (9.37 ± 0.18) of simulated medium was slightly lower than that of basal medium, but no significant differences were detected. However, the

lipase activity of simulated medium (15.14 ± 0.82) was significantly lower than that of the basal medium. These results were quite similar to the predicted value.

Our results showed that soybean oil, grapesed oil, KNO_3 , and CaCl_2 exerted negative effects on the lipase activity of *S. aureus* SK1953. These components can be sprayed on meat surfaces during the fermentation process or added directly to the meat mixture, including pork meat

Fig. 3. Three-dimensional plot showing the effect of nutrients on cell count. (A) effect of soybean oil and grapeseed oil at the central point of KNO_3 and CaCl_2 ; (B) effect of soybean oil and KNO_3 at the central point of grapeseed oil and CaCl_2 ; (C) effect of soybean oil and CaCl_2 at the central point of grapeseed oil and KNO_3 ; (D) effect of grapeseed oil and KNO_3 at the central point of soybean oil and CaCl_2 ; (E) effect of grapeseed oil and CaCl_2 at the central point of soybean oil and KNO_3 ; (F) effect of KNO_3 and CaCl_2 at central point of soybean and grapeseed oils.

Table 5. Lipase activity and cell growth of basal and simulated media

	Basal medium ¹⁾	Simulated medium ²⁾	<i>t</i> value	<i>p</i> value
Cell count, $\log_{10}(\text{CFU/mL})$	9.75±0.08 ³⁾	9.37±0.18	2.52	0.128
Lipase activity, U/mL	73.31±2.95	15.14±0.82	35.51	0.001

¹⁾Basal medium was consisted of (g/L) beef extract, 10; yeast extract, 5 and NaCl, 5. ²⁾Simulated medium was consisted of (g l⁻¹) beef extract, 10; yeast extract, 5; NaCl, 5; soybean oil, 0.5; grapeseed oil, 0.5; KNO_3 , 0.5 and CaCl_2 .

³⁾Mean±standard deviation from triplicates.

to inhibit the growth of pathogenic *S. aureus*. Artificial contamination experiments with *S. aureus* are needed to verify the growth-inhibiting effects of these components.

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