A GC-MS based metabolic profiling of fermented sausage supplemented with pineapple

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Abstract A GC-MS based metabolomic study was performed to understand metabolic changes during sausage fermentation and to investigate how the incorporation of pineapple affects the metabolic profiles of fermented sausages. Principal component analysis models showed clear metabolic differences among the fermented sausages according to the fermentation periods and the pineapple addition. Increased amounts of amino acids and organic acids except for citric acid, along with decreased levels of sugars were observed after fermentation. Higher levels of sugars and citric acid in the pineapple supplemented sausages dramatically decreased during the early stage of fermentation. The contents of lactic acid, phosphoric acid, succinic acid, ribonic acid, valine, leucine, isoleucine, glycine, threonine, glutamic acid, glucose, and sucrose were significantly increased in the 2% pineapple addition sausages. GC-MS and PCA analytical methods provide a new approach to understand of the metabolic changes in fermented sausages during fermentation.

Keywords: fermented sausage, pineapple, fermentation, metabolomics, GC-MS

Introduction

Fermented sausages are produced through fermentation of a meat mixture composed of fat and lean meat, with the addition of sugars, salts, nitrites, and spices as non-meat ingredients, which is stuffed into casings, subjected to fermentation and drying (1). The need for safe and standardized products has led to the use of microorganisms as starter cultures for the sausage fermentation by restraining the development of undesired microorganisms (2). Among the starter options, lactic acid bacteria (LAB) are the most important strains for the fermented sausages production because these organisms have hygienic advantages. LAB cultures used in sausage fermentation inhibit the growth of spoilage bacteria through the production of antimicrobial agents and acidification (3). LAB, such as *Lactobacillus plantarum*, *L. sakei*, *L. curvatus*, *Pediococcus acidilactici*, and *P. pentosaceus* have been ordinarily used for the production of fermented sausages (4). Other studies have reported that the addition of food ingredients such as kimchi (5) and wine (6) to the sausage mixture prior to fermentation for improved quality of fermented sausages. The addition of food ingredients will generate synergistic effects on the taste, aroma, and biological activity of fermented sausages. For example, several sausage producers in Italy use wine in their products to enhance aroma profiles (6).

Pineapple (*Ananas comosus* L., Merr.) is a tropical fruit, which has

a high nutritional value including sugar, organic acids, vitamins, and minerals (7). In our previous study, we reported that pineapple could be used as nutritional supplements for active fermentation (8). Moreover, we empirically found that pineapple addition could enhance the quality of fermented sausages.

Metabolites such as free sugars, amino acids, organic acids, and other flavoring compounds are important determinants of fermented sausage taste. However, many compounds in fermented sausages are not simultaneously detected by classical analysis. Thus, powerful analytical methods to determine fermented sausage metabolites are needed.

Metabolomics study deals with the simultaneous characterization of metabolome such as carbohydrates, amino acids, peptides, nucleic acids, polyphenols, vitamins, alkaloids, organic acids, and minerals (9). Metabolomics has been recently applied in food science to monitor the quality, safety, and processing (10). The study of metabolomics has helped unravel the unknown food metabolites. In metabolomics, principal component analysis (PCA) has been used to discriminate significant patterns from complex data sets obtained from global metabolites profiling. Multivariate analytical approaches have also provided potential means and biomarkers (11).

Many studies applied metabolomics approach coupled with multivariate statistical analysis to investigate the metabolite profiles of various fermented foods such as fermented bean paste (12), beer

(13), wine (14), and kimchi (15). However, little is known about the metabolic changes during the entire process of sausage fermentation and aging. In this first attempt, we applied a metabolomics approach using GC-MS data sets combined with PCA statistical method to understand metabolic changes during sausage fermentation. Further, we investigated how the incorporation of pineapple affects the metabolic profiles of fermented sausages.

Materials and Methods

Fermented sausage production Three batches of fermented sausages, 20 kg each, were processed. The ingredients used were 38% (w/w) pork (3×5cm), 32% (w/w) beef (3 mm), 24% (w/w) lean pork, and 6% (w/w) lean beef. The beef and pork were purchased from a local retailer in Jeju (Korea) and connective tissues and all visible fat were trimmed. Garlic (1 g/kg), NaNO₃ (50 mg/kg), starter culture (*L. plantarum* MLK 14-2), and fresh pineapple juice was added to the meat mixture production batches. Pineapples were purchased at a fully ripe stage from a local market in Jeju. Fresh pineapple was grounded using a food blender and then sieved with #10 mesh (2 mm). The pH, titratable acidity, and total soluble solids of pineapple juice were 3.44, 4.26%, and 14.8°Bx, respectively.

Batches were named according to the additive: (i) Control, without any pineapple; (ii) P1, with 1% pineapple (w/w); and (iii) P2, with 2% pineapple (w/w). The mixtures were stuffed into casings, approximately 300 g each ($n=3$). The sausages were processed at 20°C and 87% relative humidity (RH) for 5 days; then 17°C and 73% RH for 5 days, and finally 20°C and 85% RH. Samples were taken on the 1, 3, 6, 9, and 30 days of fermentation for the metabolic analyses.

Lactic acid bacteria analysis Ten grams of sausages were homogenized with 90 mL of sterilized saline water (0.85% NaCl) and diluted serially (1:10). LAB count was determined by growing LAB in de Man-Rogosa-Sharpe (MRS) agar (Difco, Sparks, MD, USA) and incubating at 37°C for 48 h. Tests were carried out in triplicate, and the results were indicated as log CFU/g. pH was determined using a pH meter (pH-250L; ISTEK, Seoul, Korea) and the mean of three measurements were recorded.

GC-MS analysis The samples were derivatized using methoxyamine hydrochloride (MEOX) in pyridine and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) containing trimethylchlorosilane (1%, v/v). Samples were analyzed by 6890N gas chromatography (Agilent Technologies, Santa Clara, CA, USA) equipped with a 5973N mass selective detector. Separation was achieved using a DB-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness). The GC-MS was operated as previously described (15). Mass spectra (m/z scanning range of 50–550) were recorded at a 2 scans/s with the electron impact ionization at 70 eV. Ribitol was used as an internal standard to test batch reproducibility and to correct experimental errors

originated from sample preparation and analysis. The mass spectra acquired from MSD ChemStation software (Agilent Technologies) were compared with spectra in the Wiley and NIST library for identification.

Data analysis Metabolite quantification was performed by calculating of the metabolites's peak area and the peak area of ribitol on the same chromatograph. The normalized metabolite content (variables) was imported into SIMCA-P version 14.0 program (Umetrics, Umea, Sweden) for PCA analysis. Hotelling's T² region defines the 95% confidence interval of the modeled variation (16). The quality of PCA models was expressed by R^2 (the variance in the data) and Q^2 values (the prediction of model) (17).

Statistical analysis The SPSS version 22.0 statistical package (SPSS Inc., Chicago, IL, USA) was used for data analysis including analysis of variance (ANOVA) and Duncan multiple-range test. The intensity of individual metabolites identified by GC-MS was used for statistical comparison.

Results and Discussion

pH and LAB count changes during fermentation Changes in pH and the LAB counts during the ripening of fermented sausages with pineapple are presented in Fig. 1. Typically, the LAB populations increase sharply together with the decrease of pH at the early stages of fermentation. The usual trend of microbial changes was observed

Fig. 1. Changes in pH (A) and viability (log CFU/g) of lactic acid bacteria (B) during sausage fermentation. Data are given as mean±SD ($n=3$).

Table 1. Identification of metabolites by GC-MS and their changes during sausage fermentation

Peak	Rt (min)	Chemical formula	Parent Mass	Quantification ions	Identification	Change during fermentation
1	8.2	C ₃ H ₆ O ₃	90.07	117.1	Lactic acid	↑*** ¹⁾
2	9.73	C ₃ H ₇ NO ₂	89.09	116.1	Alanine	↑***
3	12.94	C ₅ H ₁₁ NO ₂	117.15	218.1	Valine	↑***
4	13.59	CH ₄ N ₂ O	60.06	189.1	Urea	↑***
5	14.52	C ₆ H ₁₃ NO ₂	131.18	158.2	Leucine	↑***
6	14.64	H ₃ O ₄ P	97.99	299.1	Phosphoric acid	↑**
7	14.73	C ₃ H ₈ O ₃	92.09	205.2	Glycerol	↑***
8	15.10	C ₆ H ₁₃ NO ₂	131.18	158.2	Isoleucine	↑***
9	15.39	C ₂ H ₅ NO ₂	75.07	174.1	Glycine	↑***
10	15.56	C ₄ H ₆ O ₄	118.09	247.1	Succinic acid	↑*
11	17.72	C ₄ H ₉ NO ₃	119.12	218.2	Threonine	↑***
12	21.70	C ₄ H ₇ N ₃ O	113.12	329.2	Creatinine	↑***
13	23.21	C ₅ H ₉ NO ₄	147.13	246.2	Glutamic acid	↑***
14	25.69	C ₅ H ₁₀ N ₂ O ₃	146.15	245.2	Glutamine	↑***
15	25.82	C ₅ H ₁₀ O ₆	166.129	292.1	Ribonic acid	-
16	26.03	C ₅ H ₄ N ₄ O	136.112	265.1	Hypoxanthine	↑***
17	26.43	C ₆ H ₈ O ₇	192.12	273.1	Citric acid	↓*
18	27.22	C ₆ H ₁₂ O ₆	180.16	307.2	Fructose	↓*
19	27.53	C ₆ H ₁₂ O ₆	180.16	319.2	Glucose	↓***
20	28.72	C ₁₆ H ₃₂ O ₂	256.43	313.3	Palmitic acid	-
21	29.53	C ₆ H ₁₂ O ₆	180.16	305.1	Myo-inositol	↑***
22	34.74	C ₁₂ H ₂₂ O ₁₁	342.30	361.2	Sucrose	↓↑

¹⁾The up (↑)/down (↓) arrow means increase/decrease of the metabolites during fermentation ($n=9$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

in this study, where the LAB count increases at the very early stage of fermentation leading to rapid pH decrease. The pH of fermented sausages on the first day of fermentation was in the range of 5.9–6.0, and the value sharply decreased. After 6 days of ripening, the pH of the fermented sausages preserved until the end of ripening. The decline of pH value during the first days of fermentation is important due to the control of undesired bacteria, formation of desired flavor,

and color conversion of fermented sausages (18). A large and rapid pH decrease was observed in the sausages with pineapple, suggesting that pineapple addition would give a positive effect on the safety by inhibiting the growth of undesirable bacteria.

The number of LAB rapidly increased from the initial count of log 6.1–6.4 CFU/g to log 7.0–7.3 CFU/g on 6th day, and then decreased to log 6.1–6.8 CFU/g. LAB counts decreased slightly throughout the

Fig. 2. PCA score plot derived from the GC-MS profile on each day of fermentation without pineapple. Each dot represents the replicate of samples in the plot.

Fig. 3. PCA score (A) and loading scatter (B) plots derived from the 22 identified metabolites of fermented sausages from before and after fermentation, demonstrating clear metabolic differences between the sausage samples on day 1 and day 30. The more distantly the compounds are from the origin in the loading plot, the more important they are for the differentiation pattern.

remaining fermentation period. The rapid increase of LAB at the early stage of fermentation leads to the decrease of pH of the fermented sausages, which was responsible for reducing spoilage flora, such as Enterobacteriaceae (19). Although the LAB counts were lower in the fermented sausage with 2% of pineapple during fermentation, we considered that pineapple addition might not strongly affect the LAB growth in the fermented sausages.

Metabolite changes during fermentation In order to investigate more details on the metabolite changes in fermented sausages according to the fermentation period and the pineapple addition, metabolites were analyzed with the metabolomics approach using GC-MS. Among the 55 metabolites detected by GC-MS, 22 metabolites were identified. Table 1 shows the identified metabolites and their changes during fermentation.

To overview the alteration of metabolites in fermented sausages during fermentation, the PCA scores plot was generated based on the first and second principle components (PC1 and PC2) with total variances of 75.7% (Fig. 2). The spectral properties at day 0 samples were very different from others GC profile and hence, were excluded to maximize the separation among samples. The generated PCA model showed high-fitting ($R^2=0.757$) and high-predicting ($Q^2=0.65$) quality. Five groups were separated clearly according to the fermentation time indicating distinct metabolite patterns during fermentation. Movements of the dots to the right from the left in the PCA score plot indicate continuous metabolic changes during fermentation. Larger variations were observed between the first three days of fermentation, compared to the following 30 days, indicating that fermented sausage metabolites rapidly changed during the early stages of fermentation.

Fig. 4. PCA score (A) and loading scatter (B) plots derived from the 20 identified metabolites of fermented sausages produced with pineapple, showing clear separation between the fermented sausages according to the pineapple addition.

To investigate the metabolites responsible for the differentiation over fermentation period, PCA models between day 1 and day 30 were regenerated using 22 identified metabolites (Fig. 3). The PCA score plot showed clear metabolic differences between the sausage samples from day 1 and day 30, accounting for 86.3% of total variance of data set with high-predicting ($Q^2=0.770$) quality. The complementary PCA loading scatter plot provided the specific metabolites that contributed to the separation. The farther the compounds locate in positive or negative direction from the origin in the loading plot, the more important they are for the differentiation pattern. The differentiation of fermented sausages before and after fermentation was due to increases in amino acids and organic acids, along with a decrease of sugars. As expected, most metabolites were associated with the later fermentation, indicating the production of

metabolites during fermentation by LAB. Metabolic differences between fermented sausages according to fermentation time (between day 1 and day 30) were larger than metabolic differences due to pineapple addition.

Metabolite changes by pineapple addition To investigate the effects of the pineapple addition on the altered metabolites in fermented sausages, PCA models for fermented sausages after fermentation were applied on the 20 identified metabolites (Fig. 4). The PCA score plot which accounted for 78.3% of the total variance showed clear separation between the fermented sausages according to the pineapple addition, revealing good fitness and predictability of the PCA model with statistical values of R^2 (0.783) and Q^2 (0.499), respectively. These results indicate that metabolites in the fermented

Fig. 5. Time-course changes in sugars (A), organic acids (B), amino acids (C), and polyols (D) during sausage fermentation with pineapple addition. Data are given as mean±SD ($n=3$).

sausage with pineapple were significantly different from those of the fermented sausage without pineapple. The loading plot also indicated the metabolites responsible for the separation among fermented sausages according to pineapple addition. We found that the levels of lactic acid, phosphoric acid, succinic acid, ribonic acid, valine, leucine, isoleucine, glycine, threonine, glutamic acid, myo-inositol, urea, and sucrose were increased in the 2% pineapple addition sausages. The levels of glycerol, alanine, and glucose in the 1% pineapple addition sausages, and the levels of glutamine in control sausages had the highest ($p<0.05$). The levels of creatinine, hypoxanthine, citric acid, fructose, and palmitic acid were not significant in all sausage treatments and control.

Figure 5 shows the quantitative changes in main metabolites during sausage fermentation. Decreased levels of fructose, glucose, and sucrose, along with increased levels of the other identified metabolites, were observed. Higher levels of sugars were observed in the sausage with pineapple on the first day of fermentation,

indicating the sugars primarily originated from pineapple. Glucose and fructose were mostly exhausted by days 6 and 3 of fermentation, respectively. This indicates that glucose and fructose were mainly consumed by LAB during this period. Conversely, the level of sucrose changed little compared to the glucose and fructose, suggesting that sucrose is not an optimal fermentable sugar for LAB. Sucrose fermentation is initiated by the cleavage of sucrose hydrolase to glucose and fructose. The ability of LAB to ferment sucrose differs between different species and only a few LAB possess the ability to ferment sucrose (20). Therefore, a monosaccharide, such as glucose and fructose from pineapple, could be used as nutrients for LAB growth during sausage fermentation.

The organic acids (lactic, phosphoric, succinic, and glutamic acids) increased during the fermentation. Generally, organic acids in fermented sausages are caused by an accumulation of mainly lactic acids through carbohydrate breakdown by LAB (2). Therefore, lactic acid levels are the main factor contributed to the reduction of pH

Fig. 5. Continued.

(21). In this study, lactic acid levels increased considerably during early stage of fermentation. The lactic acid contents of the sausages with pineapple were higher than those of the control batches; this high value is consistent with low pH shown in Fig. 1. We hypothesize that pineapple addition may affect the lactic acid production in the fermented sausage.

In our previous study, most organic acids in fermented sausages increased during fermentation. However, in this study, citric acid in the pineapple supplemented sausages dramatically decreased

during the early stage of fermentation. This indicates that citric acid primarily originated from pineapple and was consumed by microorganisms, such as LAB. Palmitic acid was the predominant free fatty acid detected in dry fermented sausage (22). Although higher levels of palmitic acid were observed in the fermented sausages with pineapple during early stage of fermentation, its relationship is not clear.

The degradation of meat proteins produces small peptides and free amino acids. Many studies reported increases in total amino

acid content during sausage fermentation (9,23,24). In this study, most amino acids increased after fermentation, indicating production of proteins from meat by enzymatic degradation. It is also interesting that higher levels of amino acids were observed in sausages with pineapple after fermentation. Pineapple juice may be useful as a tenderizer and meat marinade because of bromelain, which is a mixture of proteolytic enzymes present in all parts of the pineapple plant (25). Further studies are needed to demonstrate the effect of proteolytic enzymes in pineapple on the hydrolysis of meat proteins in the fermented sausages.

Disclosure The authors declare no conflict of interest.

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