Selection and Characteristics of Fermented Salted Seafood (jeotgal)-Originated Strains with Excellent S-adenosyl-L-methionine (SAM) Production and Probiotics Efficacy

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Abstract
This study is executed to develop probiotics which produce S-adenosyl-L-methionine (SAM), a methyl group donor of the 5-methyltetrahydrofolate methylation reaction within the animal cell. SAM is an essential substance for the synthesis, activation, and metabolism of hormones, neurotransmitters, nucleic acids, phospholipids, and cell membranes of animals. The SAM is also known as a nutritional supplement to improve brain functions of the human. In this study, the SAM-producing strains are identified in 18 types of salted fish, and then, the strains with excellent SAM productions are being identified, with 1 strain in the Enterococcus genus and 9 strains in the Bacillus genus. Strains with a large amount of SAM production include the lactic acid bacteria such as En. faecium and En. durans, En. sanguinicola, as well as various strains in the Bacillus genus. The SAM-overproducing strains show antibacterial activities with certain harmful microbes in addition to the weak acid resistances and strong bile resistances, indicating characteristics of probiotics. It is possible that the jeotgal-originated beneficial strains with overproducing SAM can be commercially utilized in order to manufacture SAM enriched foods.

Key words: S-adenosyl-L-methionine, fermented salted seafood probiotics, jeotgal

Introduction
S-adenosyl-L-methionine (SAM), first discovered by Cantoni in 1952, is a substance primarily present in tissues and body fluids of an organism, and it plays an important role in the 5-methyltetrahydrofolate (5-MTHF) methylation reaction as a methyl group donor (Shelly, 2000). SAM is an important bio-regulator made from essential amino acids, L-methionine, and ATP by methionine adenosyltransferase (Wang et al., 2001). It is mostly synthesized in the liver at a rate of about 8 g a day and is involved in many biochemical metabolism processes (Horikawa et al., 1990).

Through many studies, it has been confirmed that SAM content varies depending on the content of L-methionine in foods (Kim et al., 2008). Its functionality has been recognized in western societies including Europe and the USA, and SAM has been reported as an essential substance in processes including in polyamine synthesis in cells and the synthesis, activation, and metabolism of hormones, neurotransmitters, nucleic acids, phospholipids, and cell membranes (Santi et al., 1983). Also, it is required in the production of important brain compounds such as neurotransmitters and phospholipids, including phosphatidylcholine and phosphatidylserine, and is known to be effective in improving brain functions (Mato et al., 1990).

The metabolic reactions of SAM in the body include transmethylation, transsulfuration, and polyamine (Mato et al., 1999). In the methylation reaction, the methyl group of SAM is donated to various acceptor substrates such as DNA, phospholipids, and proteins; in the transsulfuration reaction, SAM is converted to taurine, a major antioxidant in cells, and cysteine, a precursor of glutathione, through several enzymatic reactions (Porter et al., 1986). Finally, SAM is used in the synthesis of polyamines that are essential to the growth of normal cells (Cooney, 1993). It also donates the methyl group to the propolyamine group in the synthesis of spermine and spermidine (Lee et al., 2006). It has been reported that SAM has pharmacological effects in the restoration of hepatic functions in alcoholic liver disease, in reducing muscle fatigue and
rigidity in patients with fibromyalgia syndrome, and in improvement in patients with depression with about a 66% clinical improvement (Koning, 1987; Lieber, 1999).

Up to now, studies on SAM reported its effects in the treatment of depression, and in arthritis and hepatic cirrhosis, and it has been recognized as a health-functional food with a recommended daily intake of 400 mg (Lee et al., 2008). A food containing SAM includes Jeotgal (fermented salted seafood), one of our traditional fermented foods. Thus, this study was performed to isolate jeotgal-originated strains that produce physiologically functional material, SAM, select and identify strains that produce large amounts of SAM in foods, and find strains producing SAM with probiotics through antibacterial activity to function against harmful microbes such as food poisoning bacteria.

Materials and Methods

Sample preparation

Naturally existing strains in 18 kinds of jeotgal were isolated and their colony was analyzed to select strains that improve SAM production. Samples used in the study included 18 types of fermented salted seafood (toha shrimp, whitesaddled reeffish, baby octopus, herring roe, scallop, clam, pen-shell, hairtail, gizzard shad, hairtail guts, sand lance sauce, large-eyed herring, branchia, shrimp, anchovy, squid, small octopus, pollack roe) purchased in Gangeo, Chungcheongnam-do (April 18, 2012).

Characteristics of selected strains

Samples were diluted 10 times (0.85% NaCl 225 mL + sample 25 g) under a sterilized environment and then homogenized using a stomacher (speed level 5, 1 min). Then, the homogenate was smeared on each selective medium and incubated to separate total microbes, lactic acid bacteria, anaerobic bacteria, yeast and mold. Microbial separation and collection were performed depending on the colony types of microbes produced. Microbes grown in different selective media were identified. The separation of microbes used tryptic soy agar (TSA) for analysis of the number of total microbes. In addition, for the analysis of total lactic acid bacteria, MRS agar with adjusted pH 5.5 was used. For a detailed analysis of the lactic acid bacteria, m-LBS (Lactobacillus species), KF-Streptococcus (Enterococcus, Pediococcus species) and PES (Leuconostoc species) were used. Also, potato dextrose agar (PDA) with an adjusted pH using tartaric acid was used for the analysis of the yeast and mold (Table 1).

| Medium type                  | Target bacteria                           |
|------------------------------|-------------------------------------------|
| TSA¹ (Merck)                 | Total microbes                            |
| MRS³ (adjust pH 5.5)         | Total LAB                                 |
| KF-Streptococcus             | Enterococcus/Pediococcus                  |
| m-LBS⁴                      | Lactobacillus                             |
| PES⁵                        | Leuconostoc                               |
| PDA⁶ (Difco, adjust pH using tartaric acid solution) | Yeast and mold |

¹TSA: Tryptic soy agar  
²MRS: Lactobacilli MRS agar  
³KF: KF- streptococcus  
⁴m-LBS: modified Lactobacillus selection agar  
⁵PES: phenylethyl alcohol with 2% sucrose agar  
⁶PDA: Potato dextrose agar

Culture of strains isolated from jeotgal

To examine SAM production ability, strains were inoculated onto a nutrient medium TSB, incubated at 35°C for 24-48 h to maintain 10⁸-10⁹ CFU/mL, and centrifuged (4,000 rpm, 4°C, 10 min) to eliminate microbial cells and collect the supernatant for analysis.

SAM production

The production of SAM was analyzed using high-performance liquid chromatography (HPLC) under the conditions listed in the table (Guattari, 1991; Katie et al., 2006). Prepared samples were filtered using a 0.45 μm syringe filter and stored at -20°C for analysis. The standard for SAM was purchased from Sigma-Aldrich, and the ammonium acetate and methanol used in the analysis and all other reagents were purchased for HPLC use.

Analysis of sugar fermentation by isolated strains

Among the isolated strains, the Enterococcus genus strains used an API 20 Strept system kit (bioMérieux, France) and the Bacillus genus strains used an API 50 CHB system kit (bioMérieux, France) for the analysis of the sugar fermentation of the strains. According to the API kit manual, diluted strain samples were transferred to each selective medium and incubated at 37°C for 24-48 h to identify the presence/absence of various sugar fermentation.

Acid resistance and bile resistance of strains

For the resistance to pH, strains were activated in TSB (10⁸-10⁹ CFU/mL), washed with PBS or 0.85% NaCl, and then centrifuged for sample use (centrifuge use). After TSA medium preparation with pH levels of 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5 (control pH 7.0 - medium pH), samples were spotted on TSA and changes of the colony...
Identification of isolated strains by 16S rRNA sequence analysis

Strains with a higher production of SAM were identified by Macrogen Inc. (Korea). Genomic DNA was extracted from each strain and was used for the PCR amplification of 16S rRNA gene.

Results and Discussion

Isolation of jeotgal-originated colonies

The microbes isolated using various selective medium were sorted by the morphology of colony. The colony was analyzed using 18 kinds of jeotgal as shown in the Table below. Microbes were grouped by shape, color, and characteristics, and a total of different 169 colony types were classified (Table 3).

SAM production

The amount of SAM production was measured in 169 strains isolated from 18 kinds of jeotgal as shown in Table 3. Among 169 strains, 10 strains with excellent SAM production were selected from strains that had originated from each jeotgal. And then, the strains were identified by 16S rRNA sequencing at Macrogen Co. and the population size were detected (Table 4). As a result, 9 strains of the Bacillus genus and 1 strain of the Enterococcus genus were identified.

The Bacillus genus, which is known as the major fermentation strain in jeotgal, used a method for all the microbes using a TSA medium; although yeast and molds, in addition to lactic acid bacteria, were separated using a selective medium in order to discover and utilize strains with various ecosystems in jeotgal, most of the dominant microbes were in the Bacillus genus and the Enterococcus genus (Hur, 1996; Lee, 1969; Sands and Crisan, 1974).

The strain with the maximum production of SAM was a J6-9 strain isolated from salted clam, which produced 1.41
mM of SAM. In addition, a total of 11 isolated strains including salted toha shrimp (J1-9), salted herring roe (J4-3), salted scallop (J5-9), salted pen-shell (J7-10), salted hairtail (J8-3, J8-4), salted gizzard shad (J9-8), and salted branchia (J13-4) that produced about 1.00 mM SAM were identified. Among 169 strains, 10 strains with excellent SAM production were selected from strains that had originated from each *jeotgal*. The results were similarly shown in a previous study as SAM product strains from *Kimchi*. The strains isolated from the fermented kimchi products, produced the amount of SAM 1.22-1.58 mM (Lee et al., 2008). To utilize these strains in future studies, it was considered necessary to examine the characteristics and identification of these 10 strains.

Sugar fermentation of strains

For the 9 strains identified as *Bacillus* genus, an experiment was performed using an API 50 CHB system kit (Table 6) and for the J6-9 strain identified as *Enterococcus durans* genus, the sugar fermentation was measured using an API 20 Strep kit (Table 5). In the Delgado and Mayo’s study, the differences *Enterococcus* sp. between strains were observed for glycerol, L-arabinose, D-xylose, mannitol, sorbitol, N-methyl-D-glucoside, amygdaline, mellibiose, sucrose, trehalose, melezitose, starch, β-gentibiose, tagatose, and gluconate (Delgado and Mayo, 2004). As a result, the *Enterococcus durans* genus strain showed sugar fermentation ability with ribose, lactose, trehalose, and raffinose, among 10 carbohydrates in the API 20 Strep kit. Each of the remaining 9 *jeotgal*-originated strains in the *Bacillus* genus had different substrates for fermentation depending on the strain, but they also showed strong fermentation in substrates such as glucose, fructose, mannose, glycerol, sucrose, trehalose, maltose, and N-acetylglucosamine. Although the difference among fermentable substrates was not great for each strain, it is considered that these strains seem to have enzymes to use many sugars and can ferment various sugars available in the body through food intake.

Acid resistance & bile resistance

Among the probiotic characteristics of the 10 strains isolated from *jeotgal*, acid resistance and bile resistance were examined. The gastric pH in healthy humans could be as low as about 2-2.5 (Fernandez et al., 2003) or high as pH 6 or above after food intake (Erikkila and Petaja, 2000). It was difficult to screen strains with strong acid resistance because strains could not grow below pH 5.0, but grew above pH 5.0. Most strains showed resistance to weak acids; particularly, J1-9 and J7-10 strains grew similarly at pH 5.0 compared to the control group at a neutral pH, and the remaining strains could grow at pH 5.5. The strains showed weak resistance to strong acid but some resistance to a weaker acid of about pH 5.5 (Table 6). As for bile resistance, all 10 strains showed similar growth compared to the control group, suggesting that these strains have strong bile resistance. The structure of bacterial membrane can be disorganized by bile salt. So, bile salt tolerance is one of the essential properties for probiotic bacteria to survive (Lee and Salminen, 1995; Succi et al., 2005).

Antibacterial activity

The antibacterial activity was measured for five indicators such as *Escherichia coli* O157:H7, *Enterococcus faecalis*, *Salmonella choleraesuis*, *Staphylococcus aureus*, and *Listeria monocytogenes* using *jeotgal*-originated strains, which showed that the antibacterial activity was not observed by paper disc method. Instead, the antibacterial activity for the five indicators was partially identified in 21 strains with the exception of two strains, J7-1 and J8-3, through a soft agar method (Table 7). In particular, it is considered that J4-3 has strong antibacterial activity for *En. faecalis* because it had a relatively wide,

### Table 4. SAM production and identification of strains isolated from various *jeotgal*

| Name | Isolation medium | SAM Production (mM) | Population size (CFU/mL) | Identification result | Identity (%) |
|------|------------------|---------------------|--------------------------|-----------------------|--------------|
| J6-9 | MRS              | 1.41                | 5.7×10⁷                  | Enterococcus durans   | 99           |
| J7-1 | TSA              | 1.37                | 3.1×10⁴                  | Bacillus sp.          | 99           |
| J7-5 | TSA              | 1.33                | 9.1×10⁴                  | Bacillus subtilis     | 99           |
| J13-4| TSA              | 1.29                | 1.3×10⁴                  | Bacillus pumilis      | 99           |
| J8-4 | TSA              | 1.27                | 9.7×10⁴                  | Bacillus amylofaciens | 99           |
| J7-10| mLBS             | 1.23                | 2.9×10⁵                  | Bacillus amylofaciens | 99           |
| J5-9 | mLBS             | 1.16                | 7.4×10⁵                  | Bacillus sp.          | 99           |
| J8-3 | TSA              | 1.07                | 2.1×10⁵                  | Bacillus methylotrophicus | 99         |
| J4-3 | TSA              | 1.05                | 1.8×10⁵                  | Bacillus sp.          | 99           |
| J1-9 | MRS              | 1.03                | 2.1×10⁴                  | Bacillus sp.          | 99           |
Table 5. Sugar fermentation characteristics of strains with excellent SAM production (*Bacillus* spp.)

| Carbohydrate                        | J1-9 | J4-3 | J5-9 | J7-1 | J7-5 | J7-10 | J8-3 | J8-4 | J13-4 |
|-------------------------------------|------|------|------|------|------|-------|------|------|-------|
| Control                             | -*   | -    | -    | -    | -    | -     | -    | -    | -     |
| Glycerol                            | ++   | -    | +    | ++   | ++   | ++    | +    | +    | +     |
| Erythritol                          | -    | -    | -    | +    | -    | -     | -    | -    | -     |
| D-arabinose                         | -    | -    | -    | -    | +    | -     | -    | -    | -     |
| L-arabinose                         | -    | -    | -    | -    | -    | ++    | +    | -    | +     |
| Ribose                              | -    | -    | +    | -    | +    | -     | -    | +    | ++    |
| D-xylose                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| L-xylose                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Adonitol                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Methyl-B-xylopyranoside             | -    | -    | -    | -    | -    | +     | -    | -    | -     |
| Galactose                           | -    | -    | +    | -    | -    | +     | -    | -    | -     |
| Glucose                             | ++   | -    | ++   | ++   | ++   | ++    | ++   | ++   | ++    |
| Fructose                            | ++   | -    | ++   | ++   | ++   | ++    | ++   | ++   | ++    |
| Mannose                             | ++   | -    | ++   | ++   | ++   | ++    | ++   | ++   | ++    |
| Sorbose                             | -    | -    | -    | -    | +    | -     | -    | -    | -     |
| Rhamnose                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Dulcitol                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Inocitol                            | +    | -    | -    | +    | +    | -     | -    | -    | -     |
| Mannitol                            | -    | -    | -    | -    | ++   | ++    | ++   | ++   | ++    |
| Sorbitol                            | -    | -    | -    | -    | ++   | ++    | ++   | ++   | ++    |
| Methyl-α-D-mannopyranoside          | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Methyl-α-D-glucoside                | -    | -    | -    | -    | ++   | ++    | ++   | ++   | ++    |
| N-acetyl-glucosamine                | +    | -    | +    | +    | ++   | +     | +    | +    | +     |
| Amygdalin                           | +    | -    | +    | +    | -    | ++    | +    | +    | +     |
| Arbutin                             | -    | -    | -    | -    | -    | ++    | +    | +    | +     |
| Esculin                             | ++   | -    | ++   | ++   | -    | ++    | ++   | ++   | ++    |
| Salicin                             | +    | -    | -    | +    | -    | ++    | +    | +    | +     |
| Cellobiose                          | ++   | -    | ++   | ++   | -    | ++    | ++   | ++   | ++    |
| Maltose                             | +    | -    | +    | +    | ++   | ++    | ++   | ++   | ++    |
| Lactose                             | +    | -    | -    | +    | -    | ++    | ++   | ++   | ++    |
| Melibiose                           | -    | -    | +    | -    | +    | -     | +    | +    | +     |
| Sucrose                             | ++   | -    | ++   | ++   | ++   | ++    | ++   | ++   | ++    |
| Trehalose                           | +    | -    | ++   | ++   | ++   | ++    | ++   | ++   | ++    |
| Inulin                              | -    | -    | -    | -    | -    | +     | -    | -    | -     |
| Melezitose                          | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Raffinose                           | +    | -    | -    | +    | -    | -     | -    | +    | +     |
| Starch                              | -    | -    | -    | -    | -    | -     | -    | +    | +     |
| Glycogen                            | -    | -    | -    | -    | -    | -     | -    | ++   | +     |
| Xylitol                             | -    | -    | +    | -    | -    | -     | -    | -    | -     |
| Gentiofbose                          | +    | -    | -    | +    | -    | -     | +    | +    | +     |
| D-turanose                          | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| D-lyxose                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| D-tagatose                          | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| D-fucose                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| L-fucose                            | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| D-arabitol                          | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| L-arabitol                          | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| Gluconate                           | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| 2-keto-gluconate                    | -    | -    | -    | -    | -    | -     | -    | -    | -     |
| 5-keto-gluconate                    | -    | -    | -    | -    | -    | -     | -    | -    | -     |

*++: strong, +: positive, -: negative

clear zone around the colony, and the J7-5 strain strongly inhibited *Sal. choleraesuis*. There was no strain that inhibited all five indicators, but two strains (J1-9, J7-10) inhibited at least four indicator strains, showing bacteria-
inhibiting ability for several indicators. J1-9 and J7-10 were identified Bacillus sp. and Bacillus amyloliquefaciens, respectively. Strains of Bacillus sp. have been studied as antibacterial activity of plant pathogens (Cook et al., 1995; Mari et al., 1996). Among the strains with acid resistance mentioned above, J1-9 and J7-10 could grow at the lowest pH and showed a strong inhibition to indicators, suggesting that these may be considered the most beneficial strains with a strong efficacy of probiotics.

These strains are considered as strains that can be involved in the regulation of the intestines. In terms of fermentation characteristics, the results of the substrate utilization of seven strains showed that fermentable substrates vary depending on the strains and thus, strains seem to have enzymes for using sugars and can ferment various sugars in the body through food intake. Finally, strains showed weak acid resistance but excellent bile resistance and antibacterial activity; thus, it is considered that in the future, various strains originating from jeotgal can be used as starters in fermented foods to provide SAM and probiotics in food. Also, it is expected that these strains can be used in the development of after-meal desserts, considering the characteristics of the stomach, which contains higher pH levels with a full stomach compared to an empty stomach, due to acid resistance to weak acids, and can be utilized in foods using strain coating such as capsules to supplement acid resistance.

### Table 6. Acid resistance and bile resistance of SAM-producing strains

| Strain | pH 4.0 | 4.5 | 5.0 | 5.5 | 7.0 (con) | Bile resistance
|--------|--------|-----|-----|-----|---------|----------------|
| J1-9   | −      | −   | +   | +   | −       | oxgall
| J4-3   | −      | −   | −   | +   | +       | −               |
| J5-9   | −      | −   | −   | +   | −       | −               |
| J6-9   | −      | −   | −   | +   | −       | −               |
| J7-1   | −      | −   | −   | +   | −       | −               |
| J7-5   | −      | −   | −   | +   | −       | −               |
| J7-10  | −      | −   | +   | +   | −       | −               |
| J8-3   | −      | −   | −   | +   | −       | −               |
| J8-4   | −      | −   | −   | +   | −       | −               |
| J13-4  | −      | −   | −   | +   | −       | −               |

*+: positive, −: negative

### Table 7. Antibacterial activity of strains with excellent SAM production

| Strain | E. coli¹ | En. Faecalis² | Sal. Choleraesuis³ | Sta. aureus⁴ | L. monocytogenes⁵ |
|--------|----------|--------------|-------------------|-------------|------------------|
| J1-9   | +        | −            | +                 | +           | +                |
| J4-3   | +        | ++           | −                 | −           | −                |
| J5-9   | +        | −            | −                 | +           | −                |
| J6-9   | −        | −            | −                 | +           | −                |
| J7-1   | −        | −            | −                 | −           | −                |
| J7-5   | −        | −            | +                 | +           | −                |
| J7-10  | +        | +            | −                 | +           | +                |
| J8-3   | −        | −            | −                 | −           | −                |
| J8-4   | −        | −            | +                 | −           | −                |
| J13-4  | +        | +            | −                 | −           | −                |

*++: strong, +: positive, ¹E. coli; Escherichia coli O157:H7, ²En. faecalis; Enterococcus faecalis, ³Sal. choleraesuis; Salmonella choleraesuis, ⁴Sta. aureus; Staphylococcus aureus, ⁵L. monocytogenes; Listeria monocytogenes

Conclusion

This study was performed to isolate fermented salted seafood-originated strains select and identify strains that produce larger amounts of SAM in food. The production of SAM was measured in 169 strains from 18 kinds of jeotgal and, after the identification of these strains, most belonged to the Bacillus genus. Particularly among strains isolated from salted pen-shell, 3 strains showed over 1.0 mM of SAM production. Among probiotic characteristics, acid resistance and bile resistance were examined in 10 strains with more than 1.0 mM of SAM production; most strains could grow in the weak acidic condition over pH 5.5, and only J1-9 and J7-10 strains could grow below pH 5.0, while no resistance was observed in any of the strains below pH 4.5. On the other hand, for bile resistance, all
strains showed similar growth compared to the control group, suggesting that these strains have bile resistance and, thus, probiotics characteristics. The results of antibacterial activity for five indicators such as *Escherichia coli* O157:H7, *Enterococcus faecalis*, *Salmonella choleraesuis*, *Staphylococcus aureus*, and *Listeria monocytogenes* in the strains originating from the *jeotgal* showed that the antibacterial activity was not observed in the paper disc method and that 2 out of 10 strains did not show the inhibitory activity in the soft agar method, but the remaining 8 strains showed strong or excellent antibacterial effects.

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