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ARTICLE

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-methyltetrahydrofloat (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

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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 Ganggyeong, 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).

Table 1. Selective medium type and isolated colony

Medium type	Target bacteria
TSA[1)] (Merck)	Total microbes
MRS[2)] (adjust pH 5.5)	Total LAB
KF-Streptococcus[3)]	*Enterococcus/Pediococcus*
m-LBS[4)]	*Lactobacillus*
PES[5)]	*Leuconostoc*
PDA[6)] (Difco, adjust pH using tartaric acid solution)	Yeast and mold

[1)]TSA: Tryptic soy agar
[2)]MRS: *Lactobacilli* MRS agar
[3)]KF: KF- streptococcus
[4)]m-LBS: modified *Lactobacillus* selection agar
[5)]PES: phenylethyl alcohol with 2% sucrose agar
[6)]PDA: 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 10^8-10^9 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 Strep system kit (bioMereux, France) and the *Bacillus* genus strains used an API 50 CHB system kit (bioMereux, 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 (10^8-10^9 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

Table 2. HPLC conditions for SAM analysis

Item	Condition
HPLC	Jasco Co., Japan Pump : PU-980 Detector : UV-975 Column oven : CO-965 Auto sampler : AS-2057
Column	Shiseido C18 (4.6×205 mm, 5 μm)
Mobile phase	A: 100% Methanol B: 0.25M Ammonium acetate (pH 5.5) with acetic acid
Temperature	40°C
Velocity	1.5 mL/min
Detector	UV detector
Wavelength	254 nm

after 18-24 h were measured. Similarly, for the bile resistance of strains, the resistance of each strain was measured in the medium with or without oxgall. After strain activation on TSB (10^8-10^9 CFU/mL), samples were washed with PBS or 0.85% NaCl (centrifuge use), 1% of the washed strain was inoculated onto the medium with or without 0.3% oxgall and spotted on TSA, and the changes of the colony were measured after 18-24 h.

Antibacterial activity of strains

The activity of strains that inhibited five of the indicators including *Escherichia coli* O157:H7, *Enterococcus faecalis, Salmonella choleraesuis, Staphylococcus aureus,* and *Listeria monocytogenes* were measured. Similarly in the soft agar method, strains were activated and spotted on TSA and incubated for 12 h in an incubator. Then, 10 mL of soft agar with 0.75% agar concentration was prepared and indicators were added to the agar at 50°C to reach 10^5 CFU/mL before it was hardened. It was evenly spotted onto TSA with grown strains, and the clear zone of circle around the colony was observed every 4 h.

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

Table 3. *Jeotgal*-originated strains isolated by colony type

No.	Source name	Number of isolated strains*	No.	Source name	Number of isolated strains
1	salted toha shrimp	14	10	salted hairtail guts	9
2	salted white saddled reeffish	4	11	salted sand lance sauce	2
3	salted baby octopus	9	12	salted large-eyed herring	13
4	salted herring roe	14	13	salted branchia	9
5	salted scallop	9	14	salted shrimp	4
6	salted clam	9	15	salted anchovy	3
7	salted pen-shell	14	16	salted squid	15
8	salted hairtail	5	17	salted small octopus	10
9	salted gizzard shad	19	18	salted pollack roe	7

*The strains isolated using various selective medium were sorted by the morphology of colony.

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	5.7×10^5	*Enterococcus durans*	99
J7-1	TSA	1.37	3.1×10^5	*Bacillus* sp.	99
J7-5	TSA	1.33	9.1×10^4	*Bacillus subtilis*	99
J13-4	TSA	1.29	1.3×10^4	*Bacillus pumilus*	99
J8-4	TSA	1.27	9.7×10^4	*Bacillus amyloliquefaciens*	99
J7-10	mLBS	1.23	2.9×10^5	*Bacillus amyloliquefaciens*	99
J5-9	mLBS	1.16	7.4×10^5	*Bacillus* sp.	99
J8-3	TSA	1.07	2.1×10^5	*Bacillus methylotrophicus*	99
J4-3	TSA	1.05	1.8×10^5	*Bacillus* sp.	99
J1-9	MRS	1.03	2.1×10^4	*Bacillus* sp.	99

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 showed 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* 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, melliobiose, 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 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	+			+	++	–	+	–	–
Mannotol	–	–	–	–	++	++	++	++	++
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	–	–	+	–	–	–	–	–	–
Gentiobiose	+	–	–	+	–	–	+	+	++
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-

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

Strain	Acid resistance (pH)					Bile resistance
	pH 4.0	4.5	5.0	5.5	7.0 (con)	oxgall
J1-9	−*	−	+	+	+	+
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. coli*[1)]	*En. Faecalis*[2)]	*Sal. Choleraesuis*[3)]	*Sta. aureus*[4)]	*L. monocytogenes*[5)]
J1-9	+*	−	+	+	+
J4-3	+	++	−	−	−
J5-9	+	−	−	+	−
J6-9	−	−	−	+	+
J7-1	−	−	−	−	−
J7-5	−	−	++	+	+
J7-10	+	+	−	+	+
J8-3	−	−	−	−	−
J8-4	−	−	+	−	−
J13-4	+	+	−	−	−

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

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.

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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