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Causative Microorganisms on Swelling Incident of Gochujang

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Gochujang is a typical fermented food of Korea, and similar as Japanese Miso in manufacturing process. Recent favorite of consumers is preservative additive-free gochujang, and this sometimes has brought swelling incidents. The previous studies reported that salt tolerant yeast or lactic acid bacteria caused the incidents. This study sought out acid and salt tolerant and gas-generating microorganisms from an incident product of gochujang through some kinds of microbiological tests, whereas they could not reveal any salt tolerant yeast nor lactic acid bacteria. DNA sequence data of 16S rRNA gene concluded acid and salt tolerant and gas-generating microorganisms isolated should be two species of *Bacillus* or their relatives: one was *Bacillus amyloliquefaciens* and another was *B. licheniformis*. The former generated ca.70% more gas than the later in intentional contamination of fresh gochujang. This could infer that the gas-generating *Bacillus* strains, *B. amyloliquefaciens* and/or *B. licheniformis* relatives might be the causative microorganisms of swelling incident of gochujang.

Keywords: Gochujang, Swelling, Gas evolution, *Bacillus*

1. Introduction

Gochujang is a typical fermented food of Korea, and similar as Japanese Miso in manufacturing process. Korean food public code defines the gochujang as follows, and registered in Codex; the addition of more than 6% of red pepper powder and salt is required, after the fermentation of main raw materials of legumes and grains with *Aspergillus* (Koji) [1].

Recently Korean consumers prefer preservative additive-free products [2, 3]. This has enhanced the distribution of gochujang without preservation additives and caused sometimes swelling incidents. The previous studies about gochujang swelling concluded that salt-resistant yeast was the causative microbe [4–7]. Similar results have been reported for the swelling of Japanese miso [8–12] and soy sauce [13, 14], while there was a report focused on lactic acid bacteria [15]. The purpose of this study is the followings;

- To isolation of candidates of causative microbe through microbial test (selective media and gas evolution test in stab culture).
- To estimate the microbial species from the sequence information of 16S rRNA gene of candidates.
- To reproduce the swelling phenomenon by intentional contamination.

2. Experiments

2.1 Sources

There was a returned product of the swelling incident gochujang (HANJU Co. Ltd, Kwangju, Korea). One source of microorganisms was this incident gochujang (A), while the other source was prepared from a storage that was produced in the same process (B). The process involved ca. 30 days fermentation at 18°C on average, ca. 30 days maturation at 23°C, and pasteurization at 75°C for 30 min just before bottling. Hereinafter, the sources used are abbreviated to A and B, and the isolates are named as series A and B.

2.2 Microbiological Tests

2.2.1 Salt-tolerant yeast

The concentration of NaCl in final gochujang product is 7%, while it was 10% before adding starch syrup and red pepper powder. Since YM media has been used for yeast culture [4–7, 16], YM agar plate including 7% of NaCl was used for the selection of salt tolerant yeast, and antibiotics of 100 µg/mL of chloramphenicol and/or kanamycin were added to prohibit the growth of bacteria following the previous studies [5, 6, 16, 17]. The value of pH was 5.7 to keep from deactivation of antibiotics although pH of the products was ca 4.5. Stepwise dilutions of homogenized gochujangs of A and B were cultivated on YM agar plates at 37°C for 3 days.

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2.2.2 Salt-tolerant lactic acid bacteria

Difco Lactobacilli MRS agar [18–23] was used in the selection for lactic acid bacteria. The concentration of NaCl was adjusted to 10%. Four levels in pH (4.5, 5.0, 5.5 and 6.3) were prepared with 0.1 N HCl solution.

Homogenized gochujang samples were diluted stepwise with 10% NaCl solution. The mixtures of 100 μ L of these dilutions and 15 mL of MRS agar were poured into petri dishes aseptically. After solidified, they were overlaid with 5 mL of the same pH of the MRS agar media, and cultivated at 37°C for 3 days.

2.2.3 Bacterial isolates and their screening

As would be described later (3.1 and 3.2), there was neither salt-tolerant yeast nor lactic acid bacteria appeared, but a lot of bacterial colonies were found in YM agar plates without antibiotics. These bacterial microbes were undergone the following screening protocols. Dilutions of homogenized gochujangs of A and B were cultivated on YM agar media (7% NaCl) of which pH values were adjusted to 4.3, 4.5, 4.7, 4.9, 5.1, 5.3, 5.5 and 5.7. The colonies appeared on pH 4.5, 4.7 and 4.9 plates were streaked on YM agar (7% NaCl, pH 4.9) plates for single colony isolation. As was mentioned earlier, pasteurization at 75°C for 30min was employed in manufacturing process before bottling, therefore a screening criterion of survivability to the pasteurization condition was applied. YM agar (7% NaCl, pH 4.9) plates after the inoculation of single colony isolates were exposed to the pasteurization condition and incubated at 37°C for 3 days. The survival isolates were subsequently examined their gas evolution capability in stab culture (YM agar, 7% NaCl, pH 4.9) at 37°C for 3 days.

2.3 16S rRNA gene sequencing of the isolates

The isolates which displayed gas evolution were cultivated with YM broth (7% NaCl, pH 4.9), and their DNA were extracted using Wizard genomic DNA purification kit (Promega Co., WI, USA). Amplicons of 16S rRNA gene were obtained using primers of 8F [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1541R [5'-AAGGAGGTGATCCAGCCGCA-3']. DNA sequences of V3 region were determined using primers of 341F [5'-CCTACGGGAGGCAGCAG-3'] and 534R [5'-ATTACCGCGGCTGCTGG-3'] with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem: Thermo Fisher Scientific, MA, USA) and ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems: Thermo Fisher Scientific, MA, USA) [25–27]. BLAST search [28, 29] assigned bacterial species

similar to isolates, and a dendrogram was obtained through Clustal W [30].

2.4 Intentional Contamination and Gas Evolution

Two representative strains in the dendrogram described later (3.4) were cultured in 10 mL YM broth (7% NaCl, pH 4.5). The proliferated cells were resuspended in a 2 mL of 7% NaCl solution and added to 100 g of fresh gochujang in glass bottles. These bottles were immersed in a water bath at 37°C, and gas volumes generated from these bottles were measured by manometers (Dwyer Instruments Inc., IN, USA) [31]. As a blank and a control, fresh gochujang without inoculation and autoclaved gochujang after intentionally contaminated were also tested.

3. Results and Discussion

3.1 Salt tolerant yeast was not observed

Some reports concluded salt tolerant yeast should be responsible for incidental swelling [4–12]. Using YM agar added NaCl (7%) and antibiotics (100 μ g/mL of chloramphenicol and/or kanamycin), screening of salt tolerant yeast was trialed. The results are shown in Table 1. In the plates containing 100 μ g/mL of antibiotics, no colony emerged from the swelling incident gochujang. It is unlikely that the swelling incident handled in this study should not be caused by salt tolerant yeast.

On the contrary, around 10^7 of colonies were observed in YM agar plates without antibiotics. It could be inferred that the microbes responsible to swelling incident might be within these colonies. The pursuit of acid and salt tolerant microbe was followed.

3.2 No microorganism growing in MRS agar media at pH less or equal to 5

A study reported lactic acid bacteria should be a causative microorganism in swelling of packaged Japanese miso [15]. We attempted to find lactic acid bacteria selected by Difco Lactobacilli MRS agar from the returned goods by swelling incident (A), and the storage

Table 1 Screening of salt tolerant yeast on YM agar plate.

Source		A			B
Antibiotics added	CM	CM+KM	KM	None	None
Colony numbers	ND	ND	ND	8.7×10^6	1.4×10^7

CM: chloramphenicol, KM: kanamycin. ND: Not Detected.

(B), although some reports emphasized there was no lactic acid bacteria in gochujang which was produced neither in traditional manner nor home-made [19–22]. The results are shown in Table 2.

No colony emerged from A and B in Difco Lactobacilli MRS agar plate, of which pH value were 4.5 and/or 5.0. In the MRS agar plates of which pH value were more than 5.5, some organisms grew, and their number were the level of 10^4 in 1 g of gochujang sample.

These results indicate the MRS agar plate-selected microbes in gochujang are salt tolerant but acid intolerant. In addition, they were minority in gochujang because more than 10^7 microbes/1 g of gochujang were found in YM medium as mentioned earlier.

3.3 Causative microbe candidates

In YM agar medium adjusted pH at 4.3, 4.5, 4.7, 4.9, 5.1, 5.3, 5.5 and 5.7, the diluted suspensions of A and B were incubated at 37 °C for 3 days, respectively. The results are shown in Table 3. In the plates of pH 4.3, neither the source of A nor B yielded any colonies. From the plates of which pH were 4.5, 4.7 and 4.9, 131 of colonies were selected arbitrarily, and all of them were treated with single colony isolation procedure. As to their replica plates of YM agar (7% NaCl, pH 4.9) by toothpick, survivability in pasteurization condition (at 75°C for 30min) was examined. In consequence, 40 isolates survived. The survivors were subsequently inoculated to YM agar stab culture (7% NaCl, pH 4.9). Nine and 7 isolates from the sources of A and B exhibited crack in stab culture by gas evolution, respectively.

Table 2 Screening of salt tolerant lactic acid bacteria on MRS agar plate.

Source	A	B
pH 6.3	1.3×10^5	1.5×10^5
pH 5.5	8.5×10^3	2.9×10^3
pH 5.0	ND	ND
pH 4.5	ND	ND

ND: Not Detected.

These 16 isolated strains were suspected as candidates of causative microorganisms.

3.4 16S rRNA gene sequence

As for all candidate strains, DNA sequences of V3 region in 16S rRNA gene were determined. The information of similarity from BLAST was entered into Clustal W, and a dendrogram shown as Fig. 1 was obtained.

All of candidates which exhibited gas evolution in stab culture belong to the genus of *Bacilli*, which is well-known as spore-forming microorganisms. Six strains from swelling incident product A are concordant with *Bacillus amyloliquefaciens*, while 3 strains are concordant with or highly similar to *Bacillus licheniformis*. All isolated strains from storage B are concordant with *B. amyloliquefaciens*. From these results, it is likely that some *B. amyloliquefaciens* strain should be habitual present in gochujang, and that *B. licheniformis* strain might be causative microbe. However, in Bergey's Manual of Systematic Bacteriology there are descriptions that both *B. licheniformis* and *B. amyloliquefaciens* produce acids without gas from glucose and other carbohydrates [32]. Then we designed the following intentional contamination test.

3.5 Gas evolution in intentional contamination

Strains of A2 and B3 (see Fig. 1) were selected as representatives of *B. licheniformis* and *B. amyloliquefaciens* relatives, respectively. Their cell suspensions were mixed with fresh gochujang, and intentional contamination was realized in test bottles.

Gas outlet was connected to a manometer. The level of manometer was recorded every 12 h. After measurement the level of manometer returned at balanced position by releasing the inner pressure. The cumulative amounts of gas evolution are shown in Fig. 2.

Without contamination by adding any cell suspension, gochujang did not generate gas at all. Another control bottle autoclaved also showed no gas evolution. From the intentionally contaminated gochujang with *B. licheniformis*

Table 3 Salt and acid tolerant, survivable through pasteurization, and gas generating isolates.

Source	Number of tested isolates from YM agar (pH4.5–pH4.9)	Survivors number through pasteurization	Number of gas generating isolates in stab culture	Abbreviation of isolates
A	44	18	9	A2, A7, A9, A103, A104, A147, A150, A245, A246
B	87	22	7	B3, B8, B9, B11, B103, B158, B185

Fig. 1 Dendrogram based on DNA sequences of V3 region in 16S rRNA gene. Strain A series came from the incidental product, while B series were from the storage. 0.01 of substitutions per nucleotide position is shown as bar.

Fig. 2 Gas generation from bottles of intentionally contaminated, autoclaved or fresh gochujang.

- ◆ : contaminated with *B. licheniformis* strain A2,
- : contaminated with *B. amyloliquefaciens* strain B3,
- : autoclaved gochujang, △ : fresh gochujang.

mis strain A2 and *B. amyloliquefaciens* strain B3, ca. 270 and 460 mL/week of gas were discharged, respectively.

As was mentioned earlier, *B. licheniformis* strain was isolated from the incidental goods (A), while from storage (B) only *B. amyloliquefaciens* strains were found. However, this might mislead the identification of causative microbe for swelling incident. It could not be ignored that *B. amyloliquefaciens* strain which might be natural habitants of gochujang produced 70% more amount of gas than *B. licheniformis* strain. At least, both strains of *B. amyloliquefaciens* and *B. licheniformis* isolated here from gochujang can produce gas and might cause swelling in the specific condition. More clear insight would be provided by such as the combination of

quantitative PCR and microbial profile analysis through Next Generation Sequencing.

3.6 Product pH value and permissively germination of *Bacillus* species

A review report [33] concluded that a lower pH than 4.5 prohibits germination of *Bacillus* species, although it reviewed the studies in soy sauce manufacture. From the manufacturing records, pH value of the product that resulted in swelling incident was 4.69, while the other products that received no swelling complaints showed pH between 4.34 and 4.49. These could imply that *Bacillus* species might sporulate through pasteurization operation, and that an incidentally higher pH than 4.5 in end product might allow germination, proliferation and consequent swelling. This speculation could suggest the control point must be the pH value of the final-end product.

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ゴチュジャン膨れ事故の原因微生物

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ゴチュジャン (gochujang) は韓国の代表的な発酵食品である。近年、消費者の嗜好から添加物不使用の製品となり、そのためガス発生による膨れ事故が発生するようになってきている。耐塩性酵母または乳酸菌が膨れ事故の原因とされてきた。本研究では、実際の膨れ事故を生じたゴチュジャンから、各種の微生物学的試験を通して耐塩性、耐酸性でガス発生微生物を単離したが、それらは酵母や乳酸菌ではなかった。単離し

た微生物の 16S rRNA 遺伝子 DNA 配列からの判定では *Bacillus amyloliquefaciens* および *B. licheniformis* の類縁菌であった。これらで新鮮なゴチュジャンを汚染させたところ、両者からガスの発生が観測され、*B. amyloliquefaciens* または (and/or) *B. licheniformis* の類縁菌が、ゴチュジャンの“膨れ”事故の原因微生物と推定できた。

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