

## Isolation of *Hafnia* Species from *Kimchi*

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Eight commercially packaged *kimchi* products were examined over 15 days of storage at 4°C to evaluate the occurrence of *Hafnia alvei* (*H. alvei*). Additionally, 7 *saeujeot* products, as a possible ingredient source, were analyzed to examine the bacteria's origin. Over the storage period, *kimchi* samples had decreasing pH levels, which stabilized at pH 4.2; acidity increased to 0.9±0.1%. Lactose-nonfermenting bacteria, which *H. alvei* belongs to, gradually reduced in numbers over the *kimchi* storage. However, the relative frequency of *H. alvei* to lactose-nonfermenting bacteria tended to increase. From the *kimchi* samples, 58 *H. alvei*-presumptive colonies were selected. Forty three colonies turned out to be *H. alvei* and 15 colonies were identified as other strains or uncertain identifications when the API 20E system was used. From further test, 3 of the 43 colonies were *H. alvei* (–) against the phage test. Finally, *H. alvei* was isolated from *saeujeot*, indicating that this ingredient can be an originating source of *H. alvei* in *kimchi*.

**Keywords:** *Kimchi*, *Hafnia alvei*, *saeujeot*

*Kimchi* is a traditional Korean fermented food prepared from vegetables, including Asian cabbage (*baechu*), radish, red pepper, garlic, green onions, and ginger, along with additions of salt and various *jeotgals* [3]. Studies on *kimchi* include reports of its functionality, component changes, industrial status, consumption patterns, and microbial content [8, 11, 12, 14, 17]. There is a report on the microorganism called *Hafnia alvei* (*H. alvei*) in commercially packaged *kimchi* products [10]. *H. alvei* belongs to the *Enterobacteriaceae* family and is the third most commonly identified enteric species, following *Escherichia coli* and *Enterobacter cloacae* (*E. cloacae*), respectively [4, 7]. It is reported that *H. alvei* is primarily found in milk products, meat, and fish [5, 13, 15, 20]. *H.*

*alvei* decarboxylates histidine, lysine, and ornithine to biogenic amines [16]. During the storage of foods, biogenic amines cause food spoilage or ageing [18]. Although it is known that *H. alvei* is possibly associated with gastroenteritis [7], very little is known about the *Hafnia* genus in regard to its role as both a human and veterinary pathogens, and there are a limited amounts of data available on disease states associated with *H. alvei* [7, 19].

The objective of this study was to examine the distribution and frequency of *H. alvei* in *kimchi* products during storage, as well as to confirm *saeujeot* as a potential ingredient source. In addition, the results of *H. alvei* identification obtained from biochemical and phage tests were compared.

### MATERIALS AND METHODS

#### Materials

Eight commercially packaged *baechu kimchi* products were purchased at local supermarkets in Daegu-si and through the internet. They were stored at 4°C and examined every 3 days over a total of 15 days. Seven *saeujeot* products were also purchased for microbial analysis from local markets in the Daegu-si and Gyeongsangbuk-do areas.

#### Preparation of Samples

To prepare the samples, *kimchi* was aseptically taken and homogenized with a blender for 1 min. The homogenized samples were filtered through sterile gauze, and the filtrate was used for chemical and microbial analyses [10]. For the *saeujeot* products, each product was homogenized in a blender. When it was necessary, the samples were pretreated to adjust osmolality. The osmolality was measured using a vapor pressure osmometer 5520 (Wescor, Logan, UT, U.S.A.).

#### pH and Acidity

The pH of samples was measured with a pH meter (Thermo Electron Co., Beverly, MA, U.S.A.). To measure acidity, 20 ml of the *kimchi* sample filtrate was titrated with 0.1 N NaOH to pH 8.1±0.2. The acidity was calculated on the basis of lactic acid [2].

$$\text{Acidity} = \frac{0.009 \times \text{ml of 0.1 N NaOH} \times F \times 100}{\text{Sample (ml)}}$$

(%, as lactic acid)

F: factor of 0.1 N NaOH

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**Microbial Analysis for *H. alvei***

MacConkey agar (Becton, Dickinson and Company, Sparks, MD, U.S.A.) plates were utilized for the isolation of *H. alvei*, on which *H. alvei* forms colorless colonies due to the lactose-nonfermenting property [7]. Each sample of *kimchi* filtrate was serially diluted with 0.85% sterilized saline. Diluted samples of 0.1 ml were spread on MacConkey agar plates and incubated for 24 h at 35°C. Out of the 30–300 colonies formed on the MacConkey agar plates, 20 colonies were randomly picked and transferred to triple sugar iron agar (TSI; Becton, Dickinson and Company) slants and incubated for 24 h at 35°C. The TSI agar slant cultures showing red slants and yellow butts with gas were inoculated onto nutrient agar (NA; Becton, Dickinson and Company) plates and incubated for 24 h at 35°C. The colonies grown on these plates were identified using the API 20E system (bio-Merieux, Marcy-l’Etoile, France). Colonies identified as *H. alvei* from the API 20E system were further subjected to phage 1672 testing.

For the analysis of *H. alvei* in the saeujeot products, 10 ml of homogenized sample was aseptically taken, mixed with 90 ml of peptone water (peptone 10 g, NaCl 5 g, DW 1 l), and incubated for 18 h at 35°C. Following incubation, one loopful of each broth was spread onto MacConkey agar plates and incubated for 24 h at 35°C. The lactose-nonfermenting colonies on MacConkey agar plates were transferred to TSI agar slants and incubated for 24 h at 35°C. The cultures showing red slants and yellow butts with gas were inoculated onto NA plates. The colonies grown on the NA plate were identified using the API 20E system. Colonies identified as *H. alvei* were further subjected to phage 1672 testing.

**Phage 1672 Test**

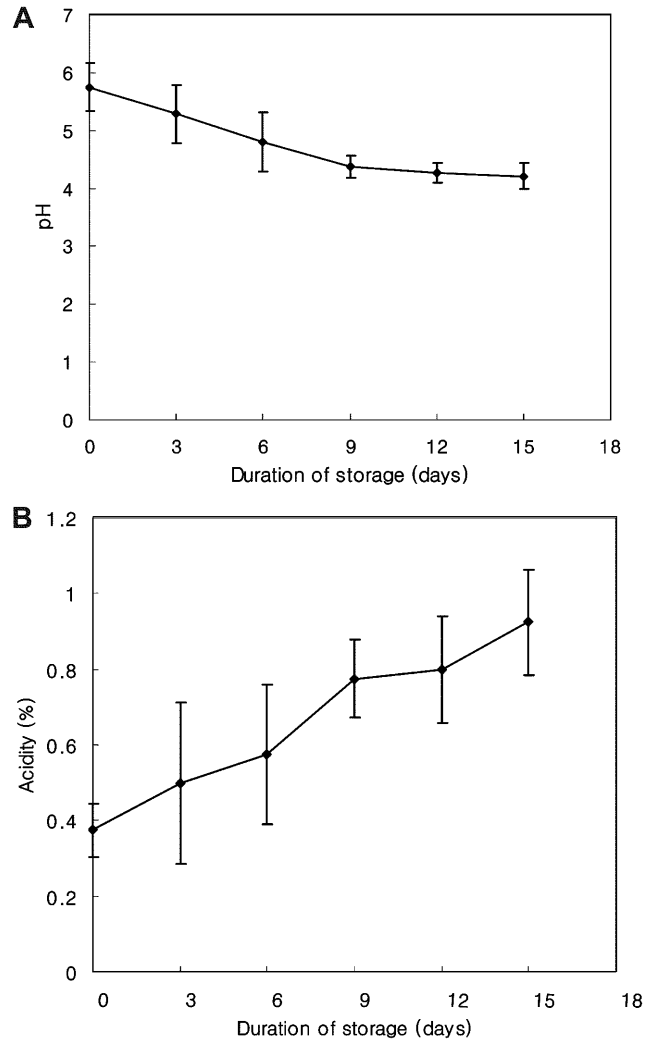
Phage 1672, a *Hafnia*-specific bacteriophage ATCC 51873-B1, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Following the ATCC’s method, phage 1672 was propagated with a host strain, *H. alvei* ATCC 51873. That is, the freeze-dried phage was recovered by the addition of 1.0 ml of nutrient broth (NB). One drop of the rehydrated phage was spotted on the surface of NA plates, on which 0.1 ml of the freshly grown host strain was overlaid and incubated for 24 h at 35°C. After incubation, the lytic zones were scraped off and the scrapes were transferred to 1 ml of NB and centrifuged at 1,000 rpm for 25 min. The supernatants were used for the phage 1672 test.

The *H. alvei*-presumptive cultures, presenting red slants and yellow butts with gas on TSI agar slants, were cultured with NA plates, followed by NB. For the phage 1672 test, 0.1 ml of the NB culture was spread onto the NA plates and allowed to dry at room temperature for 30 min. Next, 10 µl of undiluted phage 1672 was spotted onto the NA plates, allowed to dry, and incubated at 35°C for 24 h. As a result, if a spot made a visually clear lytic zone, the result was interpreted as *H. alvei* (+). *H. alvei* ATCC 51873 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) KCTC 2954 (Korean Collection for Type Cultures, Daejeon, Korea) served as positive and negative controls, respectively.

**RESULTS AND DISCUSSION**

**pH and Acidity of *Kimchi***

Fig. 1 shows the changes in pH and acidity in the *kimchi* samples over 15 days of storage at 4°C. During the storage

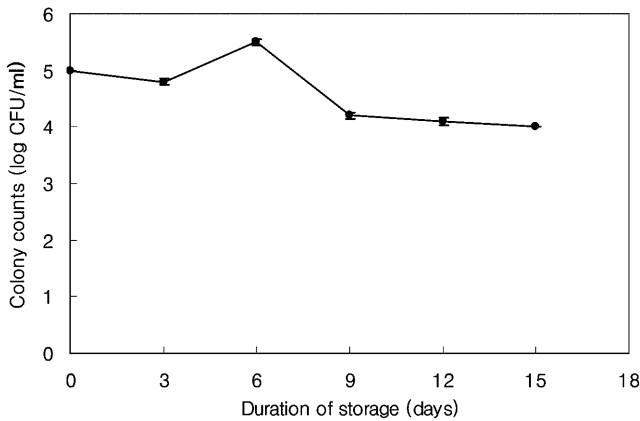


**Fig. 1.** pH (A) and acidity (B) changes of commercially packaged *kimchi* during storage at 4°C.

period, the pH decreased and stabilized at pH 4.2; the acidity increased to 0.9±0.1%. These pH and acidity patterns were similar to previously obtained results [10]. It seems the differences in pH and acidity between samples were attributed to the optional ingredients in the *kimchi* products. Similarly, Ko *et al.* [9] reported that pH and acidity changes in *kimchi* were different depending on the type of *jeotgal* in the product.

**Lactose-Nonfermenting Bacteria in *Kimchi***

Nearly 100% of *Hafnia* strains grow on MacConkey agar plates, where strains appear as colorless colonies because they do not use lactose as a carbon source [7]. Fig. 2 presents the distributions of lactose-nonfermenting colonies on the MacConkey agar plates during the storage. Lactose-nonfermenting bacteria were distributed in the range of 4.0–5.5 log CFU/ml. The number was gradually decreased over the storage days.



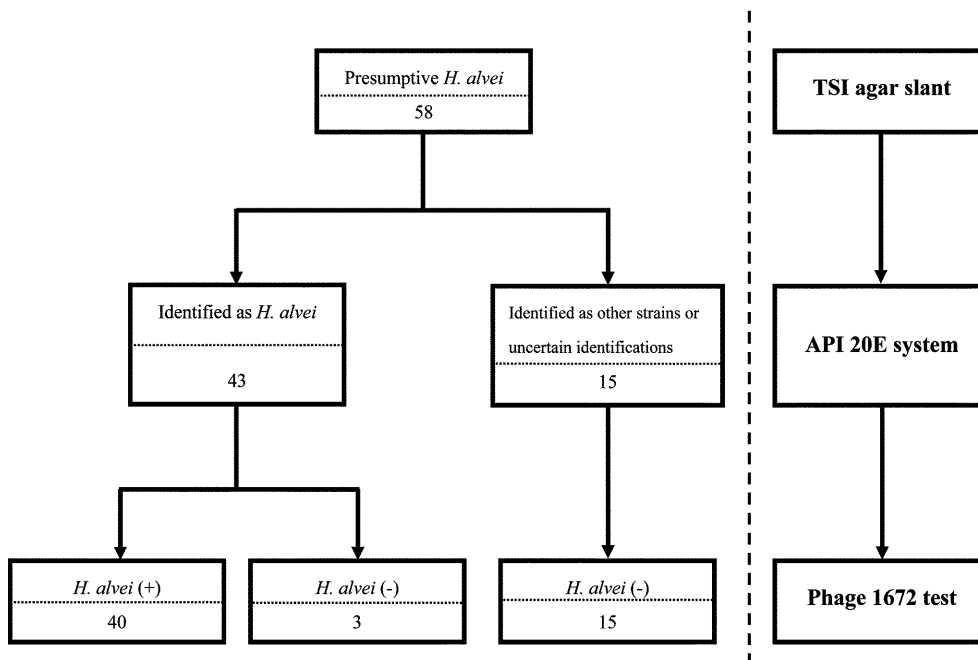
**Fig. 2.** Lactose-nonfermenting colonies on MacConkey agar plates during storage at 4°C.

**Frequency of *H. alvei* in Kimchi**

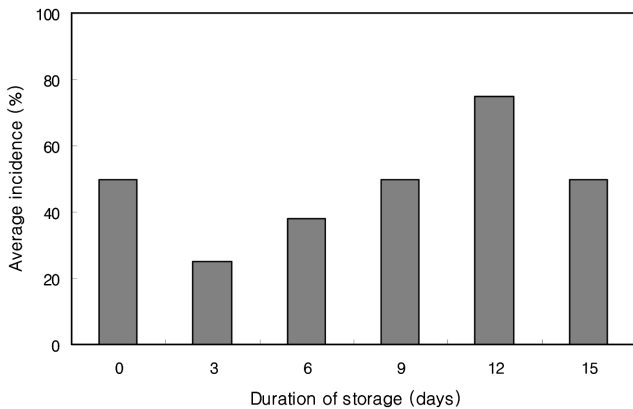
In order to determine the frequency of *H. alvei*, 20 lactose-nonfermenting colonies were randomly picked from the MacConkey agar plates every 3 days over the entire storage period and transferred to TSI agar slants for further identification of *H. alvei*. The detailed process and colony count results are shown in Fig. 3. Fifty eight colonies from samples showed typical characteristics of *H. alvei* on the TSI agar slants, presenting red slants and yellow butts with gas. These 58 colonies were further identified using the API 20E system. As the result, 43 among the 58 colonies were identified as *H. alvei* with confidence levels of 61.9-

99.9%. The remaining 15 colonies were identified as other strains, including *Enterobacter* spp., or were evaluated as uncertain identifications. Next, the same 43 colonies and 15 colonies were confirmed by phage 1672 testing. This test has been recognized as a suitable method for distinguishing *Hafnia* strains from other similar *Enterobacteriaceae* organisms such as *E. cloacae*, *Enterobacter aerogenes*, *Klebsiella* spp., *Serratia* spp., *Citrobacter* spp., *Salmonella* spp., and *Enterobacter liquefaciens* [6]. According to the results given in Fig. 3, the 15 colonies identified as non-*H. alvei* by the API 20E system were also negative against the phage 1672 test, indicating good agreement between the two testing methods. Among the 43 colonies identified as *H. alvei* by the API 20E system with confidence levels of 61.9–99.9%, 40 were positive against the phage 1672 test. However, 3 colonies were negative against the phage 1672 test, although the confidence levels were high (71.6%, 71.6%, and 91%). This result means that the API 20E system occasionally shows false-positive reactions. As mentioned in other reports, supplementary tests such as the phage 1672 test, glutamate decarboxylase test, or L-prolineaminopeptidase test [1, 6, 7] will be helpful for the definite *H. alvei* identification.

Fig. 4 depicts the frequency ratio of *H. alvei* over the storage period. Both the API 20E system and phage 1672 tests were used to monitor the presence of *H. alvei*. *H. alvei* was found throughout the entire storage period and its relative presence had a tendency to increase over the storage period. *Hafnia* grows in a pH range of 4.9–8.3 and at temperatures of 2.6–44°C [7, 20]. The conditions of



**Fig. 3.** Identification process and colony counts of *H. alvei*. Results are based on TSI agar slant culture, API 20E system, and phage 1672 tests.



**Fig. 4.** Frequency of *H. alvei*.

The judgement of frequency was made from the results obtained from both the API 20E system and phage 1672 test. Frequency ratio is the percentage of the number of *H. alvei* when the 20 colonies obtained from MacConkey agar plate were subjected to API 20E system and phage 1672 tests.

when kimchi is stored in a refrigerator, temperature of 4°C and pH range of 4.2–5.8, seem to make a favorable environment for *H. alvei*.

***H. alvei* in Saeujeot**

*Kimchi* is made of a variety of ingredients, including garlic, green onion, ginger, pepper, radish, *jeotgal*, etc. Because of this complicated matrix, it is not easy to trace the source of *H. alvei* in kimchi. According to various reports, *H. alvei* is primarily isolated from animal foods [5, 13, 20, 21]. As saeujeot was raised as an originating source of *H. alvei* in a previous report [10], saeujeot products were analyzed in an attempt to isolate *H. alvei*. Prior to microbial analysis, the osmolalities of the saeujeot samples were adjusted to 686–888 mmol/kg, which is similar to the osmolality of *kimchi*. Among the 7 saeujeot samples tested, *H. alvei* was isolated from one sample. The isolate was confirmed as *H. alvei* by the phage 1672 test (Fig. 5). These results indicate that saeujeot is one of originating sources of *H. alvei* in *kimchi*.

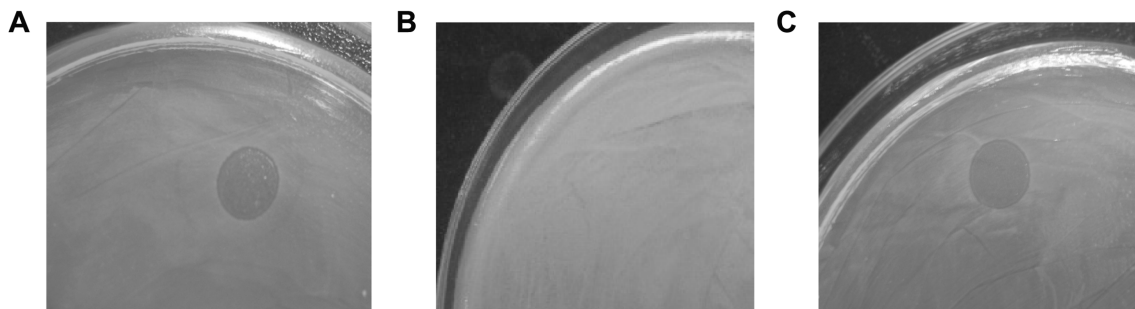
Since *kimchi* is a nonsterilized and fermented product, various microorganisms coexist during its ripening period. Therefore, dynamic changes occur in its composition of microbial flora. When *kimchi* is stored at 4°C, *H. alvei* becomes relatively dominant to lactose-nonfermenting bacteria. The isolation of *H. alvei* from saeujeot supports the presumption that *H. alvei* could originate from this *kimchi* ingredient. *H. alvei* can be misidentified occasionally by the API 20E system, regardless of the confidence percentage of the identification kit. Supplementary tests including the phage 1672 test seem to be beneficial for the definite identification of *H. alvei*.

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**REFERENCES**

1. Abbott, S. L., J. O'Connor, T. Robin, B. L. Zimmer, and J. M. Janda. 2003. Biochemical properties of a newly described *Escherichia* species, *Escherichia albertii*. *J. Clin. Microbiol.* **41**: 4852–4854.
2. AOAC. 2000. *Official Methods of Analysis of AOAC Intl.* 17th Ed. Method 942.15. Association of Official Analytical Communities, Gaithersburg, U.S.A.
3. Codex Alimentarius Commission (Codex). 2001. *Codex Standard for Kimchi*. Codex Stan 223. Food and Agriculture Organization of the United Nations, Rome, Italy.
4. Gordon, D. M. and F. FitzGibbon. 1999. The distribution of enteric bacteria from Australian mammals: Host and geographical effects. *Microbiology* **145**: 2663–2671.
5. Gran, H. M., A. Wetlesen, A. N. Mutukumira, G. Rukure, and J. A. Narvhus. 2003. Occurrence of pathogenic bacteria in raw milk, cultured pasteurized milk and naturally soured milk produced at small-scale dairies in Zimbabwe. *Food Control* **14**: 539–544.
6. Guinee, P. A. M. and J. J. Valkenburg. 1968. Diagnostic value of a *Hafnia*-specific bacteriophage. *J. Bacteriol.* **96**: 564.



**Fig. 5.** Results of the phage 1672 test.

The bacteria strains overlaid are *H. alvei* ATCC 51873 as a positive control (A), *S. Typhimurium* KCTC 2954 as a negative control (B), and *H. alvei* isolated from saeujeot (C).

7. Janda, J. M. and S. L. Abbott. 2006. The genus *Hafnia*: From soup to nuts. *Clin. Microbiol. Rev.* **19**: 12–28.
8. Kim, J. and J. Yoon. 2005. Changes of index microorganisms and lactic acid bacteria of Korean fermented vegetables (*kimchi*) during the ripening and fermentation - part 1. *Kor. J. Env. Hlth.* **31**: 79–85.
9. Ko, Y., J. Hwang, and I. Baik. 2004. Effects of jeotkal addition on quality of *kimchi*. *Korean J. Food Sci. Technol.* **36**: 123–128.
10. Kwon, E. A. and M. Kim. 2007. Microbial evaluation of commercially packed *kimchi* products. *Food Sci. Biotechnol.* **16**: 615–620.
11. Lee, M., K. Rhee, J. Kim, S. Kim, J. Jeong, and D. Jang. 2007. A survey of research papers on Korean *kimchi* and R&D trends. *Korean J. Food Culture* **22**: 104–114.
12. Lee, M. and M. Jang. 2000. Physicochemical characteristics and intake rate of *kimchi* provided to the elementary school lunch program in Sung-nam area. *J. Korean Diet. Assoc.* **6**: 79–85.
13. Lindberg, A. M., A. Ljungh, S. Ahrne, S. Lofdahl, and G. Molin. 1998. *Enterobacteriaceae* found in high numbers in fish, minced meat and pasteurised milk or cream and the presence of toxin encoding genes. *Int. J. Food Microbiol.* **39**: 11–17.
14. Nha, Y. and J. Park. 2003. Effect of dried powders of pine needle, pine pollen, green tea and horseradish on preservation of *kimchi-yangnyum*. *Korean J. Culinary Res.* **9**: 179–190.
15. Oh, Y. and S. Lee. 2001. Hygienic quality of beef and distribution of pathogens during cut-meat processing. *J. Food Hyg. Safety* **16**: 96–102.
16. Özogul, F. and Y. Özogul. 2005. Formation of biogenic amines by Gram-negative rods isolated from fresh, spoiled, VP-packed and MAP-packed herring (*Clupea harengus*). *Eur. Food Res. Technol.* **221**: 575–581.
17. Park, M., Y. Jeon, and J. Han. 2001. Antioxidative activity of mustard leaf *kimchi* added green tea and pumpkin powder. *J. Korean Soc. Food Sci. Nutr.* **30**: 1053–1059.
18. Pircher, A., F. Bauer, and P. Paulsen. 2007. Formation of cadaverine, histamine, putrescine, and tyramine by bacteria isolated from heat, fermented sausages, and cheeses. *Eur. Food Res. Technol.* **226**: 225–231.
19. Ramos, A. and D. Damaso. 2000. Extraintestinal infection due to *Hafnia alvei*. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**: 708–710.
20. Ridell, J. and H. Korkeala. 1997. Minimum growth temperatures of *Hafnia alvei* and other *Enterobacteriaceae* isolated from refrigerated meat determined with a temperature gradient incubator. *Int. J. Food Microbiol.* **35**: 287–292.
21. Tornadijo, E., J. M. Fresno, J. Carballo, and R. Martin-Sarmiento. 1993. Study of *Enterobacteriaceae* throughout the manufacturing and ripening of hard goats' cheese. *J. Appl. Bacteriol.* **75**: 240–246.