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Communication

Making an Ancient Fermented Bread from Wheat Flour and Water Dough

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Abstract

Ancient leavened bread is said to have been developed in Egypt. We hypothesized that microorganisms inherent in wheat flour were involved in fermentation of bread. The dough, made only with wheat flour and water, was kept warm for a day before being baked. The dough began to ferment after 12 hours, and when baked after 24 hours, it yielded bread with a specific volume of 1.7–2.0 cm³/g. Microorganisms were isolated from the dough before baking and identified using a rapid microbial identification mass spectrometry system. In spelt flour, *Kosakonia cowanii* was the dominant species. Numerous *Pantoea agglomerans* were isolated from strong flour B, followed by the detection of *Moraxella osloensis*. A considerable amount of the Gram-positive bacterium *Bacillus cereus* was detected from medium flour. These bacteria can be harmful to the human body. However, the high temperatures involved in the bread-baking process can potentially reduce the number of live bacteria.

Keywords: wheat flour; dough; specific volume; *Kosakonia cowanii*; *Pantoea agglomerans*

1. Introduction

The history of bread is ancient, dating back to the Mesopotamian civilization when wheat cultivation began. As wheat began to be cultivated in river basins, so-called unleavened bread, made simply by kneading wheat with water and baking it [1]. Fermented bread is thought to have evolved from unleavened bread. A comparison of the various forms of bread made in Egypt revealed that there were edible breads and beer-brewing breads, and the triangular and round breads used for beer had three lines drawn on them [1]. They made beer by dissolving the marked bread in water and allowing it to ferment. Why was it necessary to distinguish between food bread and beer bread? It seems that the dough was devised from the dough stage so that the edible bread would rise well, and the beer bread would ferment well in a liquid state. It was found that when dough containing malt flour is baked at around 120°C, the surface is slightly scorched, but the inside is maintained at 70–80°C and saccharification proceeds [2,3]. It is said that the yeast originating from the dough and the environment was involved in the fermentation of edible bread. This is what is known as the theory of spontaneous generation. Ishida made bread using barley malt and durum wheat flour. He then soaked the bread in water and tried to make beer through natural fermentation. However, yeast fermentation did not occur; only lactic acid fermentation and acetic acid fermentation took place [3,4]. It was unclear whether the world's first microorganism involved in bread fermentation was the baker's yeast (*Saccharomyces cerevisiae*) that is widely used today.

It has been reported that bread can be made using bacteria. The use of vegetable seeds or fruit soaking water is a traditional method. Nagano et al. prepared a starter using apple soaking water and isolated and identified the main fermentative bacterium, *Enterobacter cloacae* GAO [5]. *Enterobacter cloacae* GAO can be used to make steamed bread. Musattie et al. studied alternative to

leavening with baker's yeast [6]. Bellis et al. tried to select bacteria strains suitable for the production of a "yeast-free" bread [7]. The strains *Leuconostoc citreum* C2.27 and *Weissella confusa* C5.7 were selected for their leavening and acidification capabilities and individually used as starters in bread-making. Chen et al. fermented *Chenopodium formosanum* (djulis), a plant native to Taiwan, using 23 different strains of lactic acid bacteria [8]. They identified *Lactobacillus casei* BCRC10697 as the optimal strain for fermentation. The fermented djulis was further processed into bread to improve its nutritional content and texture.

A study of the microbial community in Brazilian wheat flour reported that *Escherichia hermannii* (relative abundance 43.56%) was dominant, followed by *Kosakonia cowanii* and *Pantoea ananatis* [9]. The subdominant microbial communities changed with temperature variations. A temperature of 21 °C was suitable for sourdough growth. However, a temperature of 30 °C was favorable for the persistence of Enterobacteriaceae (*Kosakonia cowanii*, *Pantoea ananatis*, and *Pseudomonas species*) [9]. Generally speaking, bacteria can grow even above 30 °C. The high temperatures in Egypt are suitable for bacterial fermentation, suggesting that bacteria other than yeast were likely involved in fermentation. We hypothesized that microorganisms specific to wheat flour were involved in the fermentation of ancient bread. To prove this hypothesis, we decided to make unleavened bread dough using several types of flour, including spelt flour, and water, and then let it ferment for one day.

2. Materials and Methods

2.1. Experimental Materials

Strong flour A (Nippon Co., Ltd.; carbohydrate 72%, protein 12%, lipid 1.5%), strong flour B (Nippon Co., Ltd.; carbohydrate 71%, protein 13%, lipid 1.1%), strong flour C (Nissin Flour Milling Co., Ltd.; carbohydrate 72%, protein 12.5%, lipid 1.4%) were used. Spelt A (Tokura Trading Co., Ltd., German-made, carbohydrate 72%, protein 13%, lipid 0.7%), Spelt B (Tomizawa Shoten Co., Ltd., Made in Japan, Whole Wheat Flour, protein 17%), Medium strong flour (Nippon Co., Ltd.; carbohydrate 75.2%, protein 9.6%, lipid 1.7%), Cake four (Nippon Co., Ltd.; carbohydrate 75.0%, protein 9.0%, lipid 1.5), and aged Strong flour (Nissin Flour Milling Co., Ltd.; carbohydrate 72%, protein 12.5%, lipid 1.4%) were also used in this research.

2.2. Flour Water Dough Fermentation

Twenty grams of flour (spelt, medium strong flour, and strong flours B), and 13 ml of water were used (Table 1). These were thoroughly mixed and packed into a 200ml graduated cylinder. The surface of the dough was flattened, and the upper part of the graduated cylinder was covered with plastic wrap. This graduated cylinder was placed in an incubator (MI-100G, Sansyo Co., Ltd.) set at 30 °C. The upper surface after fermentation was read on the scale and used as the dough volume.

Table 1. Composition used in dough fermentation tests and prototype bread making.

Ingredients	Dough fermentation	Prototype bread
Flour	20 g	200 g
Water	13 ml	130 ml

2.3. Prototype Bread Making

The dough was made using 200g of wheat flour and 130ml of tap water (Table 1). The dough was mixed and kneaded using the bread program (room temperature) of a rice cake making machine (PFC-20FK, Toshiba). About 90 g of the dough was taken and made into a ball. The dough ball was placed on paper cup (paper, 13.4 cm in diameter). The top of the cup was covered with plastic wrap. The dough was fermented for 20–24 hours in an incubator (MI-100G, Sansyo Co., Ltd.) set at 30°C.

Finally, the fermented dough was baked at 200 °C in an Electric oven (ER-C7 microwave oven, Toshiba) for 15 minutes. The prototype bread was allowed to cool naturally for one and a half hours, and after the internal temperature had dropped to almost room temperature, it was placed in a polyethylene bag (Asahi Kasei Home Products Co., Ltd., Ziploc) [10]. After storing at room temperature for 24 hours, the weight and volume of the bread were measured. The volume was calculated using the rapeseed replacement method, and the specific volume of the bread (cm³/g) was calculated (n = 3) [11,12].

The cross-section of the bread was photographed with a digital camera (Cyber-shot WX350, SONY).

2.4. Measurement of Microbial Count

One gram of each flour water dough was placed aseptically into a sterile tube and homogenized in 10 ml of 0.9% saline using a vortex mixer. The homogenized samples were serially diluted by tenfold, and 1 ml aliquots were spread plated on the general-purpose nutrient agar medium (Eiken Chemical, Japan) plates. Yeast was cultured on a plate medium prepared by adding 10 mg/100 ml of chloramphenicol (Daiichi Sankyo Healthcare Co., Ltd.) to PDA medium (Eiken Chemical, Japan). Triplicate plates for each dilution were incubated at 30°C for 3 days. The total number of bacteria and yeasts was calculated from the colonies that appeared on the agar plates.

2.5. Bacterial Identification

Ten to twenty strains were isolated from colonies that appeared on general-purpose nutrient agar medium plates used to measure the viable bacterial counts of spelt flour, medium strong flour, and strong flour B. These isolates were identified using a rapid microbial identification mass spectrometry system (MALDI BioTyperR, Bruker) [12,13]. This microbial identification system, based on MALDI-TOF mass spectrometry, enables identification of microorganisms down to the species level. The accuracy of the identification was determined by the BioTyperR score.

2.6. statistical Analysis

The differences between mean values of bread specific volume were analyzed using one-way ANOVA and Tukey's multiple comparison test in Excel 2017 (for Windows). Significant difference testing was performed with a p-value of 0.05.

3. Results

3.1. Wheat Flour Water Dough

When water was added to wheat flour to make dough and kept warm, the dough expanded after 12 hours (Fig. 1). The dough made with strong flour A showed a slow volume change. The doughs made with spelt and medium strong flour followed similar expansion curves, but showed a faster volume change than the dough made with strong flour A.

Table 2 summarizes the results of examining the volume changes in dough prepared using strong flour, medium flour, cake flour, spelt flour, and aged flours. Volume changes in the dough due to fermentation were observed with many types of flour. In particular, it was found that dough made with cake flour started fermenting faster than dough made with other types of flour. However, no such volume expansion was observed with spelt flour and strong flour that were more than two years past their expiration date. It was thought that adding water would activate the microorganisms in the flour, and that older flour would have fewer of these microorganisms.

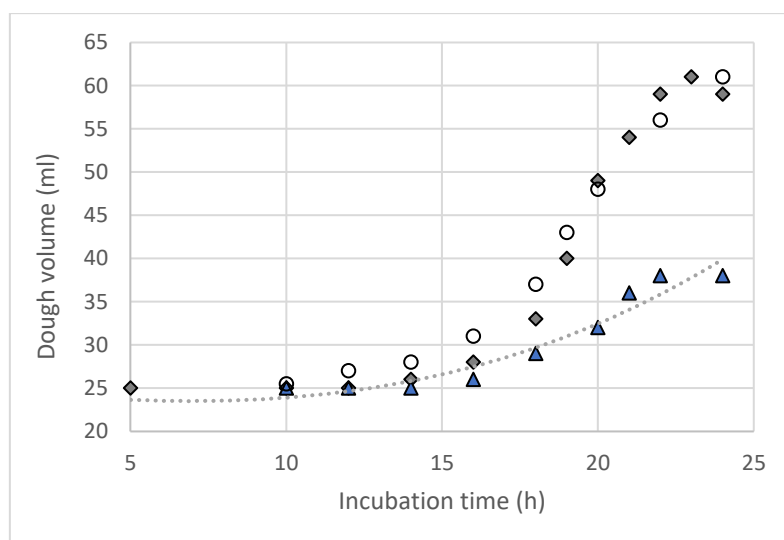


Figure 1. Fermentation test of wheat flour and water dough. ◆ : spelt flour, ○ : medium flour, ▲ : strong flour
A. Twenty grams of flour and 13 ml of water dough was incubated at 30 °C.

Table 2. The start time of fermentation for wheat flour dough.

Wheat flour ¹	Protein content (%)	Start time of fermentation (h)
Strong A	12.0	15
Strong B	13.0	12
Strong C	12.5	12
Medium strong	9.6	12
Cake flour	9.0	8
Spelt A (German-made)	13.0	12
Aged spelt A (German-made) ²	13.0	No raising
Spelt B (Whole meal, Japan)	17.0	12
Aged strong flour ³	12.5	No raising

¹ mainly from the United States and Canada. ² Two years and ten months have passed since expiration date. ³ Two years and eight months have passed since the expiration date.

When dough made from three types of wheat flour was cultured for 24 hours, microorganisms proliferated to more than 10^9 cfu/g (Table 3). Yeast levels were approximately 10^3 cfu/g in spelt flour and medium flour, while slightly higher at 10^4 cfu/g was detected in strong flour B. When a dough of wheat flour and water was kept warm at 30°C for 24 hours, bacteria were thought to be the dominant microorganism, with a low proportion of yeast.

Table 3. Number of bacteria and yeast in flour water dough¹.

Microbes	Spelt (cfu/g)	Medium strong flour (cfu/g)	Strong flour B (cfu/g)
Bacteria	$4.4 \pm 1.2 \times 10^9$	$3.4 \pm 2.1 \times 10^9$	$2.3 \pm 1.6 \times 10^9$
Yeast	$1.7 \pm 0.8 \times 10^3$	$6.2 \pm 0.7 \times 10^3$	$2.3 \pm 0.3 \times 10^4$

¹ The dough made from wheat flour and water was incubated at 30°C for 24 hours. n=3.

Table 4 shows the results of identifying bacteria in the three types of wheat flour dough used in Table 3. In spelt flour, *Kosakonia cowanii* was considered the dominant species. In strong flour B, *Pantoea agglomerans* was isolated in large numbers followed by *Moraxella osloensis*. In the dough made with medium strong flour, *Bacillus cereus* was mainly detected, followed by *Kosakonia cowanii* and *Pantoea agglomerans*.

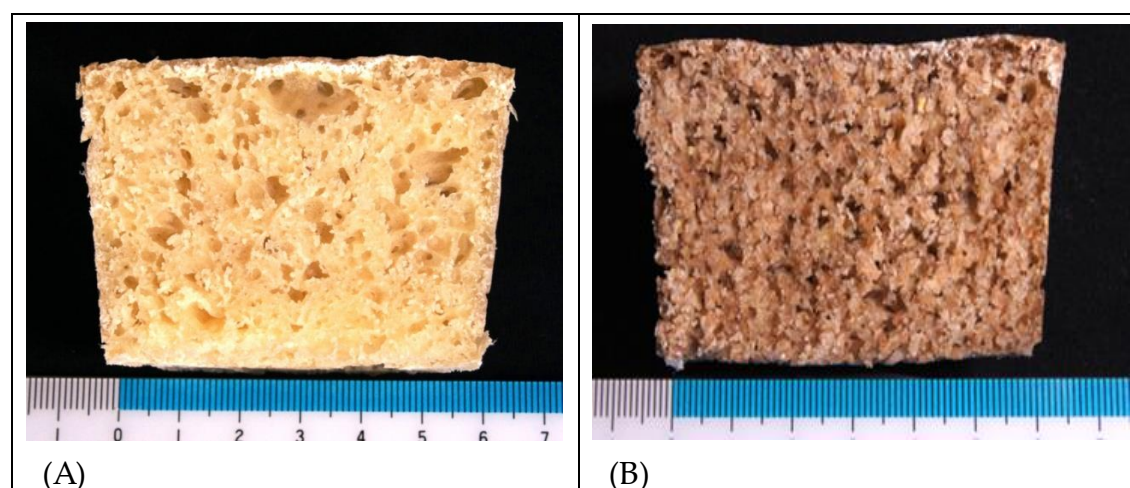
Table 4. Number of bacteria identified from flour and water dough ¹.

Bacteria name ²	Spelt	Medium strong flour	Strong flour B
<i>Kosakonia cowanii</i>	10	4	
<i>Pantoea agglomerans</i>		4	10
<i>Erwinia tasmaniensis</i>		1 *	
<i>Bacillus cereus</i>		10	1
<i>Bacillus amyloliquefaciens</i>		1 *	
<i>Moraxella osloensis</i>			5

¹ incubated at 30°C for 24 hours. ² Identified using a rapid microbial identification mass spectrometry system (MALDI BioTyperR, Bruker) * The identification was of moderate accuracy. Score value was 1.75-1.8.

3.2. Prototype Bread

Breads were obtained by baking dough with a simple composition of wheat flour and water. Figure 2 shows cross-sectional photographs of bread made with spelt flours, medium strong flour, and bread flour B, and Table 5 shows their specific volumes. As can be seen from the diagram, the bubbles were uneven and had a diameter of 5 mm or more. The crumb color was generally dull, and the whole wheat spelt flour was particularly brown. Medium strong flour had the largest specific volume at 2.02 ± 0.09 cm³/g (Table 5). The specific volumes of spelt flour, whole wheat spelt flour, and strong flour were all around from 1.7 to 1.8 cm³/g. A statistically significant difference ($p < 0.05\%$) was observed between medium strong flour and the other three types of flour.



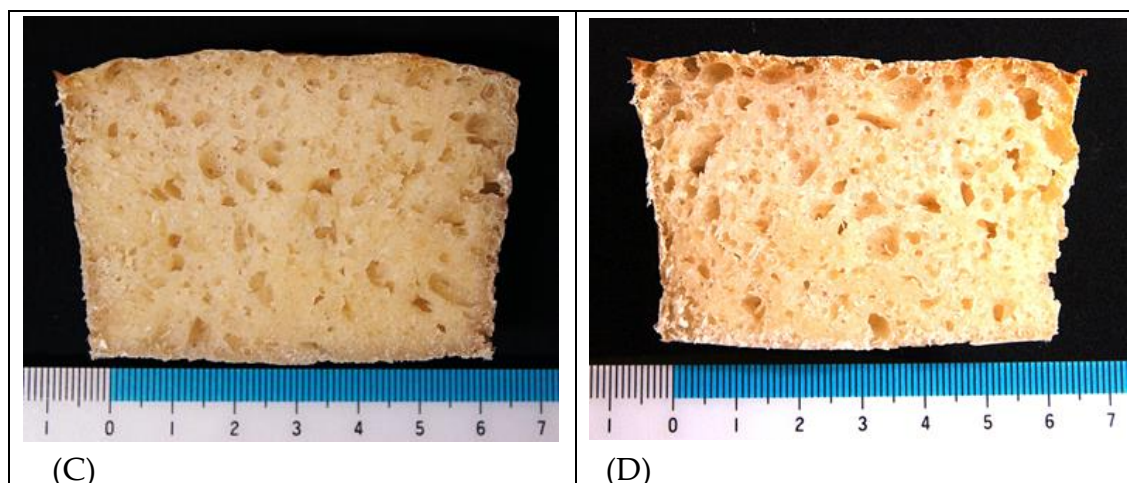


Figure 2. Cross-sectional photographs of bread made from flour water dough. (A): Spelt flour, (B): Spelt flour (Whole meal), (C): Medium strong flour, (D): Strong flour B.

Table 5. Specific volume of bread made from flour water dough.

Types of flour	Specific volume (cm ³ /g) ¹
Spelt	1.73 ± 0.07 a
whole meal spelt	1.83 ± 0.11 a
Medium strong	2.02 ± 0.09 b
Strong	1.77 ± 0.04 a

¹ The results are shown as the mean and standard deviation (n=3). A statistically significant difference (p<0.05%) was observed between medium strong flour and the other three types of flour.

4. Discussion

Fujimoto focused on the traditional sourdough starter production process, which is most frequently used in Japan [14]. Using rye flour and wheat flour from different milling facilities. As a result, regardless of the type of raw materials used, Gram-negative bacteria, such as *Pantoea* group and *Erwinia* group, grew on CVT medium on the first day of fermentation. While lactic acid bacteria detected on modified MRS medium became dominant on the second day of fermentation. An investigation of the microbial consortium in wheat flour was reported to be dominated by Enterobacteriaceae. Menezes et al. aimed to clarify how temperature changes during sourdough starter growth affect the dynamics of the bacterial ecosystem [9]. At 21°C, lactic acid bacteria were dominant at the end of the fermentation and transplantation stages. At 30°C, the persistence of bacteria such as *Pseudomonas* and Enterobacteriaceae was promoted. This study revealed that when dough made from spelt flour and strong flour B was kept warm at 30°C, Enterobacteriaceae (*Kosakonia cowanii* and *Pantoea agglomerans*) were frequently isolated (Table 4).

Kosakonia and *Pantoea* are a rod-shaped, Gram-negative, facultative anaerobic bacterium, commonly found in soil and water as well as internally in plants [15–17]. Due to its ability to live in different environments and conditions, the bacteria are highly competitive environmentally and has huge metabolic potential [18]. Several strains of *Kosakonia* and *Pantoea* can promote plant growth through direct or indirect mechanisms [19,20]. The main route for vertically inherited bacteria in plants is considered to be transmitted via the seed [19]. Plant-derived protein domains are abundant in the genus *Kosakonia*, suggesting that *Kosakonia* and plants interaction [15].

A study investigating the microbial distribution of Australian wheat flour reported that the number of *Bacillus* species ranged from 10² to 10⁴ cfu/g, and the number of *Bacillus cereus* species ranged from 0.1 to 1 MPN/g [21]. However, since *Bacillus cereus* was isolated fairly frequently from medium strong flour dough (Table 4), it is suggested that it is involved in dough fermentation. Some

strains of *Bacillus cereus* produce multiple bacterial enterotoxins (HBL, NHE, and CytK) that cause two types of food poisoning syndromes in humans and animals, including vomiting and diarrhea [19,22,23]. However, non-pathogenic strains of *Bacillus cereus* exhibit plant growth-promoting properties and can be used in agriculture as biofertilizers and biocontrol agents [19,20,23–27]. These microorganisms (*Kosakonia*, *Pantoea*, *Bacillus cereus*) can exhibit both beneficial and harmful behaviors. Fortunately, bread undergoes a baking process, and high temperatures can reduce the number of viable bacteria. However, low-temperature baking, such as at 120°C, may allow bacteria like spore forming *Bacillus cereus* to survive.

The history of bread is ancient, dating back to the Mesopotamian civilization when wheat cultivation began. As wheat began to be cultivated in river basins, so-called unleavened bread was made by simply kneading barley and wheat flour with water and baking it [1]. It is thought that this evolved to making a thick porridge with wheat flour, letting it stand at room temperature for a while, and then baking the porridge in an earthenware oven. This research traced the history of unleavened bread used in Mesopotamian civilization. It revealed that keeping wheat flour dough warm for a day allows bacteria such as *Kosakonia cowanii*, *Pantoea agglomerans*, *Bacillus cereus*, which originate from the wheat flour, to ferment, resulting in fermented bread. Further research is needed to understand how microorganisms involved in food poisoning are controlled. Possible solutions include the discovery of a sourdough starter [28], the use of salt [12], and controlling the baking temperature. It was thought that higher baking temperatures were necessary to safely consume bread fermented by bacteria, and this may be one reason why edible bread (high baking temperature) was distinguished from beer bread (low baking temperature).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Fermentation test of wheat flour and water dough; Table S2: Number of bacteria and yeast in flour water dough cultured for 24 hours; Table S3: Identification results of bacteria isolated from flour water dough; Table S4:

Author Contributions: The authors confirm contributions to the paper as follows: Conceptualization, N.O. and T.K.; methodology, T.C.; investigation, N.O. and T.C.; writing—original draft preparation, N.O. and T.K.; project administration, T.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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