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Investigation of reduction and tolerance capability of lactic acid bacteria isolated from *kimchi* against nitrate and nitrite in fermented sausage condition

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ABSTRACT

Lactobacillus brevis KGR3111, *Lactobacillus curvatus* KGR 2103, *Lactobacillus plantarum* KGR 5105, and *Lactobacillus sakei* KGR 4108 isolated from *kimchi* were investigated for their potential to be used as starter culture for fermented sausages with the capability to reduce and tolerate nitrate/nitrite. The reduction capability of tested strains for nitrate was not dramatic. All tested strains, however, showed the capability to produce nitrite reductase with the reduction amount of 58.46–75.80 mg/l of NO_2^-. *L. brevis* and *L. plantarum* showed nitrate tolerance with the highest number of 8.71 log cfu/ml and 8.81 log cfu/ml, and *L. brevis* and *L. sakei* exhibited nitrite tolerance with the highest number of 8.24 log cfu/ml and 8.25 log cfu/ml, respectively. As a result, *L. brevis*, *L. plantarum*, and *L. sakei* isolated from *kimchi* showed a tolerance against nitrate or nitrite with a good nitrite reduction capability, indicating the satisfaction of one of the selection criteria to be used as starter culture for fermented sausages.

1. Introduction

Fermented sausages are produced from raw meat and a few additives, such as spices, starter culture, and nitrite curing salt. Unlike milk and malt, raw meats cannot be pasteurized and are therefore highly susceptible to the growth of undesirable microorganisms. Moreover, fermented sausages are generally manufactured without any heat treatment throughout the process of fermentation, ripening, and drying where the typical chemical, biochemical, physical and microbiological characteristics are developed (Flores & Bermell, 1996; Kunz, 1994; Luecke, 1997). Considering the general tendency that most fermented sausages are not heated prior to consumption and are usually stored without refrigeration, the selection of favorable conditions that encourage the specific growth and development of desirable and safe microflora and strict control of the growth of spoiling bacteria, including pathogenic strains, are essential for the microbial stability and shelf-life extension of fermented sausages (Bacus & Brown, 1981; Leistner, 1985; Luecke, 1997). Leistner (1984) suggested the sequence hurdle technology model to meet the safety requirements of fermented sausage with consideration of its typical characteristics of additives and processing. Among the hurdles, preservatives such as nitrite curing salt take the first hurdle before other critical hurdles, such as competitive flora, reduced pH as well as water activity are developed.

Nitrite and also nitrate are used as necessary curing agents in the production of fermented sausages by their antimicrobial effects (Hausschild, Hilsheimer, Jarvis, & Raymond, 1982), especially against *Clostridium botulinum* (Christiansen, Tomkin, Shaparis, Johnston, & Kautter, 1975; Collins-Thompson, Chang, Davidson, Larmond, & Pivnick, 1974) and *Staphylococcus aureus* (Labots, 1976) even their health risks with mutagenic effect forming nitrosamines (Lundberg, Weitzberg, Cole, & Benjamin, 2004). In Europe, for fermented sausages and ham curing agents are still used either solely as saltpeter (potassium nitrate, E 251) or sodium nitrate (E 252) or in combination with potassium nitrite (E 249) as regulated in Directive 2006/52/EC (Hammes, 2012). For such an antimicrobial effect, nitrate should first reduce into nitrite by the incorporation with microbial reductase. Nitrite can be reduced and can release nitrogen monoxide (NO) that might act as a bactericidal agent by blocking sulphydryl groups having active center of nonheme iron–sulphur proteins that are essential for electron transport, enzyme activity, and energy production as proposed by Tompkin, Christiansen, and Shaparis (1978). NO produced from nitrate and nitrite by reduction has also an important role as curing color agent by formation of pink nitrosomyoglobin as well as antioxidant (Giddings, 1977; Hammes, 2012). The most efficient nitrate reducing organisms are *staphylococci* and *micrococci* (Gøtterup et al., 2008). Some meat lactic acid bacteria

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(LAB) have also been reported to possess nitrate reductases and heme-dependent or heme-independent nitrite reductases (Hammes, Bantleon, & Min, 1990; Wolf, Arendt, Pfaehler, & Hammes, 1990). Such a capacity of LAB to possess both nitrate and nitrite reductase activities is regarded as one of the selection criteria for LAB to be used starter culture in fermented sausage production (Ammor & Mayo, 2007).

In the previous studies, *kimchi* (a common term of Korean traditional foods produced with vegetables by LAB fermentation) microorganisms were investigated for their potential utility as a substitute for starter culture for fermented sausages (Lee & Kunz, 2005, 2006). The LAB integrated via the addition of *kimchi* into meat mixture indicated the potential utility to be substituted for starter culture by showing good adaptation to the new habitat of fermented sausage condition, reaching maximum numbers of 8.65–8.80 log cfu/g after 1–2 days of fermentation (Park & Lee, 2012). Lee, Kim, and Kunz (2006) isolated the majority of LAB from *kimchi* during its fermentation and then they were identified as *Lactobacillus brevis* KGR 3111, *Lactobacillus curvatus* KGR 2103, *Lactobacillus plantarum* KGR 5105, and *Lactobacillus sakei* KGR 4108. These LAB were then investigated for their suitable properties, such as adaptability, growth as well as acidity profile, for use as starter culture in fermented sausage conditions. Among the tested LAB, *L. curvatus*, *L. plantarum*, and *L. sakei* showed relatively good potential to be used as starter culture in sausage production by showing good growth as well as souring properties.

In this study, *L. brevis* KGR 3111, *L. curvatus* KGR 2103, *L. plantarum* KGR 5105, and *L. sakei* KGR 4108 isolated from *kimchi* were investigated for their potential to be used as starter culture of fermented sausages by having the capability to reduce nitrate and nitrite. Nitrite and the released product such as NO are well known as the agents that interfere with the bacterial growth because of its mutagenic effects. Nitrous acids remove amino acids of DNA bases such as adenine, guanine and cytosine, causing their false match and replication. Therefore, one of the desirable characteristics of a starter used in the sausage production is the tolerance to the presence of at least 100 mg/kg of sodium nitrite (Roca & Incze, 1990). Accordingly, the selected strains were evaluated for their reduction and tolerate of nitrate and nitrite in fermented sausage condition. For this study, model media designed as like the fermented sausage and added with potassium nitrate or sodium nitrite were used to exclude the influence of other contaminants.

2. Materials and methods

2.1. Keeping and preparation of LAB strains isolated from kimchi for the tests

Each strain of four LAB species, *L. brevis* KGR 3111, *L. curvatus* KGR 2103, *L. plantarum* KGR 5105, *L. sakei* KGR 4108, isolated from *kimchi* and identified in the previous study (Lee et al., 2006) was used for the tests. The strains of four LAB species were kept at −72 °C in a Microbank (Microbank™ Mast Diagnostica, Laboratorium-Praeparate GmbH, UK), a sterile vial containing porous beads which serve as carriers for microorganisms. For the tests, the beads of each strain of Microbank were incubated for 2 days at 30 °C in the test tubes containing MRS broth (Merck, Germany). After the second inoculation, 50 ml of the suspension was centrifuged at 3000 ×*g* (4 °C) for 20 min. The collected cells were washed in a sterile physiological saline solution (0.9% NaCl solution) twice. These harvested cells were re-suspended in saline solution. Then the absorbance was measured at 550 nm and the solution was diluted to 10^6 log cfu/ml medium with the help of the corresponding standard curves as shown in Fig. 1.

2.2. Preparation of model-media and inoculation

The basic model-medium used in this study was composed to simulate the substantial conditions of meat mixtures employed for the sausage production (Table 2). The basic composition of model-medium was made of meat extract (Fluka, Germany) and as fermenting sugar, D(+)-glucose (Merck, Germany) was added. To investigate the influence of nitrate and nitrite on the growth of LAB from *kimchi* and their capability of reducing nitrate and nitrite, 0.6 g/l of potassium nitrate (Merck, Germany) and 0.15 g/l of sodium nitrite (Merck, Germany) were added into the basic model-medium, respectively. The start pH condition of model-media was adjusted by the addition of 0.5 N HCl into 5.8 (Hechelmann, 1985; Koch, 1982). The model-media were autoclaved for 20 min at 121 °C and 1.2 bar. To avoid Maillard reactions owing to heat treatment, glucose was sterilized separately and added aseptically to the medium after cooling. After cooling, the media were inoculated with approximately 10^6 log cfu/ml of each strain and the inoculates were distributed evenly.

2.3. Fermentation of model-media and sampling procedure

The model-media formulated in 250 ml or 500 ml Erlenmeyer flasks and inoculated with each LAB of four references (Table 1) were fermented at 25 °C for 120 h. During the fermentation for 120 h, the sampling was performed in duplicate after 0, 4, 8, 12, 16, 24, 36, 48 and then every 24 h. The medium was shortly mixed with a sterile magnetic stirrer and then 1 ml for the determination of cfu and 1 ml for the enzymatic tests for nitrate and nitrite reduction.

2.4. Determination of nitrate and nitrite reduction by reference LAB

The capability of reference LAB isolated from *kimchi* to reduce nitrate or nitrite was evaluated by determining their residue amount in each model-medium. The quantitative evaluation was carried out with the cuvette tests of the Boehringer Mannheim Company (Germany) according to the enclosed instructions. A test-tube containing 1 ml sample solution from each model-medium was first led in water bath at 80 °C for 15 min to stop enzymatic processes and then centrifuged (Biofuge 17RS, Heraeus Sepatech, USA) at 10,000 *g* for at least 1 min at room temperature (25 °C). The supernatant was filtered with filter-paper No. 595 1/2 (Whatman, Germany) in a funnel. The supernatant was kept at −72 °C in Safe-Lock tubes (Eppendorf-Netheler-Hanz GmbH, Germany) until used for the different enzymatic analysis. The test for nitrate was based on the photometric measurement of NADPH. In the presence of the enzyme nitrate reductase, nitrate is reduced to nitrite by NADPH. The amount of NADPH oxidized during the reaction is linearly proportional to the amount of nitrate. The amount of reduced nitrate was determined by measuring the decrease in NADPH by means of its light absorbance at 340 nm. The determination of nitrite is based on measuring the light absorbance of the red-violet diazo dye. Nitrite reacts with sulphanilamide and N-(1-naphthyl)-ethylene-diamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range at 540 nm. The reduction of nitrate/nitrite was calculated by percentage of their start amount, 100.02 mg/l for nitrate (calculated as mass percentage of 61.328% of NO_3^- in KNO_3) and 367.97 mg/l for nitrite (calculated as mass percentage of 66.679% of NO_2^- in $NaNO_2$), respectively.

2.5. Determination of nitrate and nitrite tolerance of reference LAB

The tolerance of reference LAB (Table 1) against nitrate and nitrite was estimated by determining their evolution in viable cell counts during the fermentation. The changes of viable cell number of test LAB were evaluated every 0, 4, 8, 12, 16, 24, 48 and then every 24 h.

To evaluate the growth rate of the corresponding LAB, the generation time (n) was calculated. The generation time means the time it takes a population to double during exponential growth