Studies on the color improvement technique & mechanism with high ZnPP-forming bacteria in dry-cured meat products

（乾塩漬食肉製品における高 **ZnPP** 形成細菌による色調改善技術と
メカニズムに関する研究）

Hokkaido University　Graduate School of Agriculture
Division of Frontiers in Bioscience　Doctor Course

Md Asaduzzaman

Table of contents

Chapter 1

General introduction

The color of meat and meat products is of utmost importance and one of the main focusing viewpoints in the meat industry. It is the dominant visual factor affecting their quality and an imperative selection criterion by consumers' purchase decisions. Myoglobin is a sarcoplasmic protein, which is the major pigment responsible for the red color of meat (Sakata, 2000). In meat, myoglobin can exist in several redox states (Mancini & Hunt 2005), namely deoxymyoglobin, oxymyoglobin, and metmyoglobin. Deoxymyoglobin and oxymyoglobin are in a ferrous state and oxymyoglobin provides bright cherry-red color in meat. Undesirable discoloration of meat is due to the oxidation of myoglobin and the resulting accumulation of brown metmyoglobin (Renerre, 1990). In the meat products, curing has been performed from ancient times to protect them from spoilage as well as the formation of undesirable color. Generally, nitrite/nitrates are added to cured meat products as a curing agent. The characteristic red color of cured meat products is typically due to nitrosylmyoglobin, which is formed by the reaction of nitric oxide derived from nitrate/nitrite with myoglobin in the presence of endogenous or added reductants (Honikel, 2008). Moreover, nitrite also helps to improve the antimicrobial (especially *Clostridium botulinum*) and antioxidant properties of the cured meat products (Bedale *et al*., 2016; Pearson & Gillett, 1996). Due to these various beneficial effects, nitrite/nitrate has become a popular curing agent to improve the color in cured meat products.

Instead of the the beneficial effects, the undesirable impact on the health of nitrites/nitrates was exposed in the 1960s, and debate on the safety of cured meat products was initiated. The major concern of nitrates/nitrites in cured meat products is the involvement in the potential ability of nitrites to form carcinogenic N-nitroso compounds (De Mey *et al*., 2014). Generally, N-nitroso compounds include nitrosamines and nitrosamides, which are formed by the reaction of amines and amides, respectively, with nitrosating agents derived from nitrite (Berleur & Cordier, 1995; Dietrich *et al*., 2005; Inskip *et al*., 1995; Preston-Martin *et al*., 2006). Nitrosamides are direct alkylating agents that do not require metabolic activation (Dietrich *et al*., 2005; Lijinsky, 1987) and are potent neuro-carcinogens, especially through transplacental or perinatal exposure in animal models (Berleur *et al*., 1995; Dietrich *et al*., 2005; Inskip *et al*., 1995; Preston-Martin *et al*., 2006; Ohgaki, 2009; Wrensch *et al*., 2002). N-nitrosamines and biogenic amines are generated from residual nitrite in cured meat products and have harmful effects on health after consumption (Skibsted, 2011). The formation of N-nitrosamines is mainly associated with nitrite-containing foods, especially nitrite-containing cured meat products. Fiddler *et al*. (1978) revealed that bacon and its cookout on frying contained considerable amounts of nitrosopyrrolidine. The formation of nitrosamines are commonly occured in heated cured meat products. Reduction of added nitrite is already a *matte*r of awareness for a few decades, whereby color formation in meat products without the use of nitrate/nitrite or other artificial coloring agents is one of the challenges. For this safety reason, some consumers desire that meat products should be free from nitrate/nitrite.

On the other hand, zinc protoporphyrin IX (ZnPP) is a bright red pigment produced in the meat and meat product without the addition of nitrite/nitrate. The occurrence of ZnPP as a red-colored pigment was first reported in Parma ham (*Prosciutto di Parma*)

(Wakamatsu *et al*., 2004a), which is produced in the Parma region of northern Italy. Parma ham is a traditional Italian dry-cured ham, and the color is very aesthetically pleasing despite no addition of nitrite/nitrate. It is produced only with fattened pig thigh and sea salts. Despite the addition of no color former, Parma ham shows a stable, bright red color. Wakamatsu *et al*. (2004a) also confirmed that ZnPP having divalent zinc coordinated to protoporphyrin IX (PPIX). This pigment increases through the maturation period of Parma ham (Adamsen *et al*., 2006b). The amount of ZnPP in Parma ham was larger than that of heme, and ZnPP was considered for 60–70% of all porphyrins (Bou *et al*., 2018; Wakamatsu *et al*., 2009b) . The formation of ZnPP was observed not only in Parma ham but also in the other nitrite-free ham (Wakamatsu *et al*., 2009a). ZnPP formation was also confirmed in several other studies focusing on the production of nitrite-free meat products, such as dry-cured Iberian hams and dry-fermented sausages (Adamsen *et al*., 2006a; De Maere *et al*., 2016). Moreover, it was observed that nitric oxide (NO) originated from nitrite inhibited the formation of ZnPP (Wakamatsu *et al*., 2010). Thus, ZnPP might be useful as a potential pigment for the improvement of meat products exhibiting a preferable color without any color former.

Bacteria play an important role in the production of fermented meat products. Uses of bacteria for the preparation of dry-fermented sausages has been practiced for a long time, where they are not only characterized by distinctive aroma and flavor but also extended shelf life by preventing the growth of pathogenic bacteria as well. Besides the enormous beneficial properties, it has also been reported that bacteria isolated from meat products convert myoglobin to the desirable red myoglobin derivatives (Arihara *et al*., 1993, 1994; Faustman *et al*., 1990; Morita *et al*., 1994). Staphylococci, pediococci and lactobacilli are used as starter cultures for dry-cured meat products and also observed their

positive effects on color formation due to nitrate reduction or decline in pH. *Pseudomonas* strain was found to convert brown color of metmyoglobin to red color in media; however, the red myoglobin derivative was not identified (Faustman *et al*., 1990). These bacteria contribute to the indirect color formation by nitrate reduction or decrease in pH. Accordingly, along with other beneficial properties, the addition of bacteria have also importance to improve the color in meat and meat products.

The formation of ZnPP in dry-cured meat products by the contribution of bacteria has been observed in some studies (Khozroughi *et al*., 2018; Morita *et al*., 1996; Sakata, 2000; Wakamatsu *et al*., 2020). The formation of ZnPP with the involvement of bacteria in Parma ham remains unclear. Since there are few microorganisms present inside it (Toldra, 1998), it is thought that microorganisms are not involved in the formation of ZnPP in Parma ham. Thus, endogenous enzymes in meat, such as ferrochelatase (FECH), rather than microorganisms, were attributed to promote ZnPP formation in Parma ham (Becker *et al*., 2012; Chau *et al*., 2011). However, *Staphylococcus epidermidis*, *Staph. warneri* and *Staph. lentus* from Parma ham were participated in the formation of ZnPP (Morita *et al*., 1996). Moreover, it has also been shown that the amount of ZnPP increased in pork homogenate without the addition of antibiotics (Wakamatsu *et al*., 2004b), suggesting that the presence of microorganisms promoted ZnPP formation. So, the involvement of ZnPP formation in meat considered endogenous enzyme, as well as bacterial contribution. Recently, a several bacterial strains capable of forming ZnPP in meat were identified (Kawazoe, 2013; Ohya, 2017; Wakamatsu *et al*., 2020), but these are not suitable for meat products either their lower ZnPP-forming ability or pathogenicity. Therefore, suitable high ZnPP-forming bacteria help to improve the color of fermented meat products without nitrite/nitrate is awaited.

ZnPP is a rare metabolic compound formed in the heme biosynthesis pathway in the living body. It is generally found in red blood cells when heme production is inhibited by lead and/or during the shortage of iron or reduced iron utilization in the red blood cells (Labbé *et al*., 1999). The biosynthesis pathway of heme in the animal body involves a series of several enzymatic steps that occurs partly in the cytoplasm and mitochondrial compartments. The heme biosynthesis pathway is initiated by the synthesis of aminolevulinic acid (ALA) from the glycine and succinyl-CoA by the ALA synthetase in mitochondria (Franken *et al*., 2011) and then subsequently form protoporphyrinogen III and PPIX. The final reaction in the heme synthesis is the chelation of divalent iron with PPIX by the enzyme FECH (Ajioka *et al*., 2006; Moretti *et al*.,2000). During the periods of iron insufficiency or impaired iron utilization in the heme biosynthesis pathway, zinc becomes an alternative metal substrate to increase the formation of ZnPP in the living body (Jacobs *et al*., 1998; Labbé *et al*., 1999). The reaction to insert a Zn^{2+} into PPIX is also catalyzed by FECH (Labbé *et al*., 1999). However, it is uncertain whether ZnPP is formed in dry-cured meat products by a similar mechanism as in the living body.

The formation mechanism of ZnPP in meat and meat products by high ZnPP-forming bacteria is not elucidated yet. Several theories of the ZnPP formation were proposed by meat scientists such as i) a non-enzymatic reaction in which ZnPP is formed under anaerobic conditions; ii) an enzyme-induced substitution of Fe^{2+} from heme with Zn^{2+} by endogenous FECH, and iii) bacterial enzymatic reactions (Taketani *et al*., 2007; Wakamatsu *et al*., 2004a, 2004b). Moreover, Becker *et al*. (2012) reported that ZnPP formation occurred both enzymatically and nonenzymatically. Grossi *et al*. (2014) showed that ZnPP was formed by partial degradation of myoglobin in post mortem muscle and supported the theory of ZnPP formation from heme in myoglobin. However,

it was observed that ZnPP was not formed by Fe^{2+}-Zn^{2+} substitution, rather the insertion of Zn^{2+} into PPIX (Wakamatsu *et al*., 2007b). On the other hand, Chau *et al*. (2011) explained that ZnPP was formed from heme in the presence of recombinant yeast FECH was added. Chau *et al*. (2011) also reported that incubation of yeast FECH with myoglobin in the presence of ascorbic acid and cysteine resulted in the efficient conversion of myoglobin-heme to ZnPP. Consequently, bacterial metabolites such as aldehydes, ketones, esters, alcohols, organic acids, amines, and sulfur compounds etc. (Ercolini *et al*., 2009; Samelis, 2006) might have a contribution to the formation of ZnPP. It was also proposed that the formation of ZnPP in porcine muscle is associated with both a meat-inherent and a bacterial enzymatic reaction during meat storage (Fig. 1) (Khozroughi *et al*., 2018). Since, it is considered that FECH possessed by microorganisms may be involved in the ZnPP formation mechanism. Thus, it is important to clarify the contribution of bacteria for a proper understanding of the mechanism of bacterial ZnPP formation. Elucidation of the mechanism of ZnPP formation by bacteria will lead to establish a technology for the improvement of the color meat products with the formation of a higher amount of ZnPP.

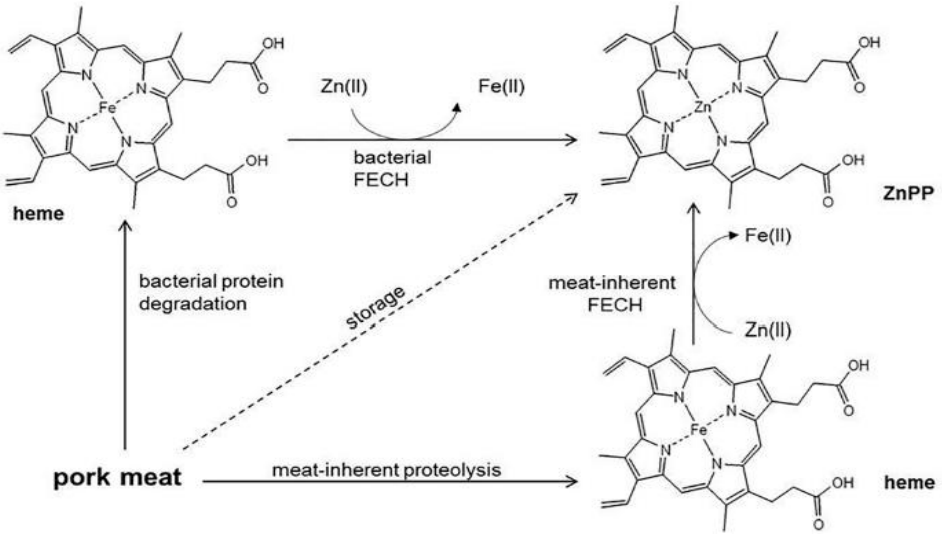


Fig. 1. Two pathways of ZnPP formation in stored pork meat by the conversion of heme from meat proteins (Khozroughi *et al*., 2018).

Since many kinds of edible bacteria have been isolated from diverse environmental sources, it is likely to obtain strains responsible for the improvement of the color of meat products with its ZnPP-forming capability. In the next chapter, high ZnPP-forming bacteria were screened from various sources to improve the color of meat products as an alternative to nitrite/nitrate. High ZnPP-forming bacteria were screened from various sources to improve the color of meat products as an alternative to nitrite/nitrate. Accordingly, the promotion mechanism of the ZnPP formation by selected bacteria was investigated in chapter 3.

Chapter 2

Isolation and identification of high ZnPP-forming edible bacteria to improve the color of dry-cured meat products

2.1. Introduction

Meat and meat products are not processed aseptically. There might have many different kinds of microorganisms in meat and meat products. Similarly, Parma ham is processed not in aseptic conditions but also there are few microorganisms present in the ham (Toldra, 1998). The involvement of ZnPP formation by microorganisms in Parma ham may have important roles. Besides, Morita *et al*. (1996) reported that Parma ham-specific red pigment i.e., ZnPP, is formed by different species of staphylococci. Khozroughi *et al*., (2018) reported that microorganisms are involved in the formation of red pigments in cured meat products. It was also found that the amount of ZnPP increased in pork homogenate without the addition of antibiotics (Wakamatsu *et al*., 2004b), suggesting that the presence of microorganisms promotes ZnPP formation. So, it can be concluded that the presence of bacteria in meat products would contribute to the formation of ZnPP for improving the color in nitrite/nitrate-free dry-cured meat products.

Bacteria are an integral component in the manufacturing of dry-cured meat products. Dry-cured sausages produced by using suitable bacteria in which they are not only characterized by distinctive aroma and flavor, but also by extended shelf life as well. Selected species of bacteria have long been used as starter culture in the dry-cured and products such as Italian salami, Vienna sausages, and chicken hamburger which includes *Lactobacillus, Pediococcus, Bifidibacterium, Bacillus,* and *Enterococcus* (Bomdespacho *et al*., 2014; Jafari *et al*., 2017; Ruiz-Moyano *et al*., 2011; Sparo *et al*., 2013). In the

production of dry-cured sausages, the main role of bacteria is the acidification of mixture realized through the production of lactic acid that occurs desirable changes in the final product. Suitable meat starter cultures assist in the improvement of color, flavor and texture of cured meat products (Kamarudheen *et al*., 2014). Moreover, incorporating these bacteria in meat products ensures microbiological safety and reduces or inhibits foodborne pathogens and spoilage microorganisms as well (Arief *et al*., 2014; Marianthi *et al*., 2014). Thus, the favorable quality of the starter culture bacteria can improve the color and help in the bio-preservation process for dry-cured meat products.

The meat scientists have been trying to improve the color of dry-cured meat products by using high ZnPP-forming bacteria. In our laboratory, Ohya (2017) searched for high ZnPP-forming from selected bacterial strains that are generally used in livestock processed products. In her study, *Lactobacillus plantarum* was isolated as a ZnPP-forming bacteria in dry-cured sausages, but the capability of ZnPP formation of the strain was not so high. In a recent study, two contaminating bacterial species, *Carnobacterium divergens* and *Serratia liquefaciens* were identified which significantly promoted ZnPP formation and capable of improving the color of the inoculated meat (Wakamatsu *et al*., 2020). However, *C. divergens* is responsible for the spoilage of raw meat and seafood (Laursen *et al*., 2005) and *S. liquefaciens* is a causative agent of many diseases, numerous outbreaks, and opportunistic microorganisms (Mahlen, 2011). These identified bacteria will not be used in dry-cered meat products. Thus, high ZnPP-forming suitable bacteria are expected to improve the color of dry-cured meat products. Besides, the ZnPP-forming bacteria should have the edible and biopreservative characteristics for application to nitrite-free dry-cured meat products. However, high ZnPP-forming bacteria suitable for dry-cured meat products are not recognized yet.

In this chapter, it was hypothesized that edible bacteria from any sources of the environment might be useful for improving the color of meat products by forming ZnPP. The edible bacteria should have adequate salt tolerance because salt is an integral part of meat products. Therefore, the objectives of this chapter's study was to screen and identify edible bacteria from various sources of the environment for the application to improve the color of dry-cured meat products without nitrite/nitrate by the formation of ZnPP.

2.2. Materials and methods

2.2.1. Meat sample

Porcine *Longissimus thoracis et lumborum* (LTL) muscle was used for the meat homogenate model experiment and salted minced meat system. The pork meat was used for the preparation of dry-cured sausages. Meat samples were obtained from common domestic crossbred pork from the local market in Hokkaido, Japan. For preparing the aseptic meat homogenate and minced meat, portions (250-300 g) of the muscles were trimmed from LTL, then the muscles were vacuum-packed and stored at -30°C until use**.**

2.2.2. Preparation of reagents

2.2.2.1. Preparation of sterile physiological saline

Physiological saline was prepared by dissolving 9 g/L of NaCl (Kanto Chemical Co. Inc., Tokyo, Japan) in ultra-pure water and sterilized by autoclaving. The autoclaving was performed at 121℃ for 15 min.

2.2.2.2. Preparation of nutrient broth (NB)

For the preparation of NB medium, 2.5 g/L of yeast extract (Difco Laboratories, New Jersey, USA), 5 g/L of tryptone (Difco Laboratories) and 1 g/L of glucose (Kanto Chemical Co. Inc.) was dissolved in ultra-pure water and sterilized by autoclaving. The autoclaving was performed at 121°C for 15 min. The pH of the medium was 7.0.

2.2.2.3. Preparation of antibiotic solutions

The antibiotic solution was prepared by dissolving penicillin G potassium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and streptomycin sulfate (Wako Pure

Chemical Industries, Ltd.) in ultra-pure water so as to be 7 mg/mL and 25 mg/mL respectively, then filtered through a sterile syringe filter (Minisart® syringe filter, 0.2 μm, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and stored frozen (-20℃) until use. Similarly, Gentamicin sulfate (Wako Pure Chemical Industries, Ltd.) was also dissolved in ultra-pure water so as to be 10 mg/mL after the sterile filtration through a syringe filter and stored frozen (-20℃) until use.

2.2.2.4. Preparation of sodium nitrite solution

The sodium nitrite solution was prepared and sterilized by passing it through a sterile syringe filter. The solution was prepared just before use.

2.2.3. Isolation and identification of bacteria

2.2.3.1. Isolation of bacteria

The bacterial isolates were collected from various environmental sources (soil, water, refuse, fruits, probiotics, etc. obtained from around Hokkaido University, Japan). Initially, the environmental bacterial isolates were collected by swabbing the source with sterile cotton and dispersed in the sterile physiological saline, following which they were inoculated in the modified standard plate count (SPC) agar supplemented with 3% NaCl, and adjusted to pH 5.5 (mSPC agar). The isolates were then incubated anaerobically at 25°C. Next, each isolate was transferred to the modified NB medium containing 3% NaCl, which had been adjusted to pH 5.5 (mNB), and the isolates were then incubated anaerobically at 25°C. The edibility of the high ZnPP-forming isolates was confirmed according to 16S rRNA gene sequencing analysis, as shown below.

2.2.3.2. Identification of bacteria using 16S rRNA gene sequencing

2.2.3.2.1. Extraction of DNA from isolated bacteria

At first, 1 mL of freshly cultured broth of bacteria was taken in a microtube and centrifuged at 16,000 × g for 3 min at 4°C (CT15RE, Hitachi Koki, Tokyo, Japan). Then 50 µL of PrepMan^{TM} Ultra Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and 3 ceramics balls (YTZ ball φ 1.8 mm, Nikkato, Osaka, Japan) were added and vigorously stirred for 5 min at room temperature using a microtube mixer (MT-360, Tommy Seiko Co. Ltd., Tokyo Japan). Thereafter microtubes were heated in a dry thermo bath at 105°C for 10 min and allowed to cool at room temperature and centrifuged for 15 min at 16,000 × g for 3 min at 4°C (CT15RE, Hitachi Koki). Three µl of ice-cold 3 M sodium acetate solution and 75 µl of 100% ice-cold ethanol was added to 30 µl of the above supernatant and mixed well. The mixture was allowed to stand for 10 min at room temperature and centrifuged at 16,000 × g for 30 min at 4°C (CT15RE, Hitachi Koki) and gently removed the supernatant with a pipette so as not to destroy the precipitate. In the precipitate, 100 µl of 70% ice-cold ethanol was added and mixed by inverting the microtubes, followed by centrifugation at 16,000 × g (CT15RE, Hitachi Koki) for 10 min at 4°C (CT15RE, Hitachi Koki). Again the supernatant was removed gently with a pipette so as not to destroy the precipitate. The remaining ethanol was dried from the precipitate at room temperature for 15 min. Finally, 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added for dissolution of the DNA and used as a template for PCR.

2.2.3.2.2. PCR

PCR was carried out using 10F (5´-GTTTGATCCTGGCTCA-3´) and 1500R (5´-TACCTTGTTACGACTT-3´) as primers and KOD-Plus-Neo (Toyobo Co. Ltd. Osaka, Japan) PCR kit, targeting the full-length (~1.5 kbp) sequence of the eubacteria 16S rRNA

gene. The reaction conditions were 94°C, 120 sec → (94°C, 15 sec → 55°C, 30 sec → 68°C, 90 sec) × 45 cycles. The PCR product was purified using a Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co. Ltd., Tokyo, Japan). The partial sequence of the 16S rRNA gene was analyzed using 357F (5´-CTCCTACGGGAGGCAGCAG-3´) as a primer, and DNA sequencing was entrusted to Eurofin Genomics Co. Ltd., Japan. The results of the sequences were compared with GenBank data using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

2.2.3.3. Storage of bacteria

Identified bacteria were stored as stock in an equal volume of broth and 40% glycerol (finally 20% glycerol) at -20°C until use. The starter culture was prepared by using the stock bacteria.

2.2.4. ZnPP formation model experiment

2.2.4.1. Preparation of bacterial culture

The required bacteria were cultured in the mNB and allowed to grow for 24-48 hr anaerobically at 25°C. Before using the bacteria in the meat homogenate model experiment and salted minced meat system, the number of bacteria was counted using a hemocytometer under a phase-contrast microscopy, which was then adjusted to the appropriate number using sterilized physiological saline.

2.2.4.2. Preparation of aseptic model sample

Aseptic 30% meat homogenate was prepared using a pre-sterilized cup and a homogenizer (CELL MASTER CM-100, ASONE Co. Tokyo, Japan) at 10,000 rpm for

90 sec. Then, 0.9 mL of 30% meat homogenate was dispensed into a sterilized test tube with 0.45 mL of sterilized 10% NaCl solution and 0.15 mL of bacterial culture (final meat homogenate concentration of 20%, NaCl 3%, and bacteria 2.0×10^6 CFU/mL). Then, the test tubes were capped and placed in gas-impermeable bags and incubated anaerobically at 25°C for 7 days. The anaerobic conditions were maintained by using an oxygen absorber (A-500HS, I. S. O. Inc., Yokohama, Japan). To check the aseptic preparation of the non-inoculated group (control), antibiotics were added to the antibiotics group at a final concentration of 70 μg/mL penicillin G potassium, 250 μg/mL streptomicin sulfate, and 50 μg/mL gentamicin sulfate.

2.2.4.3. Fluorescence analysis of the sample

The amount of ZnPP in the meat homogenate was measured using the 75% acetone extraction method as described by Wakamatsu *et al*. (2007a). The incubated solution was mixed with three times the volume of cold acetone (HPLC grade, Wako Pure Chemical Corporation) and then allowed to stand at 4°C in the dark for 30 min to extract ZnPP. After filtration through filter paper (No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), ZnPP was measured using a fluorescence spectrophotometer (RF-5300PC, Shimadzu Corporation, Kyoto, Japan). Fluorescence intensity at 590 nm for excitation at 420 nm was regarded as the amount of ZnPP formed.

2.2.5. Manufacturing of dry-cured sausages

2.2.5.1. Preparation of starter culture for sausage

The high ZnPP-forming edible bacteria were used as a starter culture isolated and identified from various sources. The high ZnPP-forming bacteria were inoculated into

500 mL of De Man, Rogosa and Sharpe (MRS) broth **(**Oxoid Ltd., Basingstoke, Hampshire, UK) separately and allowed to grow at 30°C for 24 hr. The concentration of the cell in the broth was measured using a hemocytometer under a phase-contrast microscopy. The incubated broth was centrifuged at 10,000 × g, 15 min, and the pellets (containing crude cells) were washed twice with sterile physiological saline solution, using the same centrifugation technique (10,000 × g, 15 min) to collect the cells. The appropriate concentration of the cells was maintained by dilution of the cells with a sterile physiological saline solution. The freshly prepared (within 4-6 hr) cells were inoculated for the preparation of dry-fermented sausages.

2.2.5.2. Preparation of sausages

The dry-cured sausages were manufactured at the pilot food plant in Hokkaido University Agri-Food Center (AFC). The sausages were prepared from boneless pork, which was chopped to 4 to 5 cm square, ground with a chopper (3.2 mm hole size). Then 3% salt, 1% glucose and 0.3% polyphosphate were added to raw meat at a temperature of 1 to 3 ℃ and mixed well. High ZnPP-forming bacteria were added to 6 log CFU/g of raw meat in each individual group. Sterile ultra-pure water was used instead of bacteria in the non-inoculated group. In the nitrite-added group, sodium nitrite was added so as to be 0.02% with respect to the total amount of sodium nitrite. The sausages were stuffed into a cellophane casing (Tohcello bista SP-S 450 sheet type, Shikoku Tohcello Co. Ltd., Tokushima, Japan) to be 150-200 g per sausage, hung in a thermal-hygrostat (QBX-132 HRST 1, Fukushima Galilei Co. Ltd. Osaka, Japan) and dry ripening started. The manufactured sausages were kept at 1^{o}C with 90% humidity for 7 days for conditioning. Then fermentation was performed at 25°C for 24 hr and then the sausages were kept at

7°C and increased 1°C for every two days with 90% humidity. Finally, the sausages were kept at 14°C with 85% up to the completion of drying and ripening.

2.2.6. Analysis of sausages

2.2.6.1. Measurement of pH

The pH of sausage in each sampling day was measured with a digital pH meter (F-55, Horiba Ltd., Kyoto, Japan). Ten grams of fermented sausage samples taken from the core of the sausages were homogenized with 90 mL distilled water to determine the pH.

2.2.6.2. Measurement of microbial count

For microbiological analysis, samples were collected aseptically, which was maintained by wiping the sausages, knife and cutting board with 70% alcohol. Three grams of sausage samples were collected from the core of the sausage and taken in a sterile nylon bag with 27 mL of sterile physiological saline and properly mixed with a stomacher (Exnizer 400, Organo, Tokyo, Japan) for 3 min at 500 rpm. Serial 10-fold dilutions were carried out and transferred on the appropriate agar medium. Lactic acid bacteria (LAB) was enumerated on MRS agar (Oxoid Ltd.) in anaerobic conditions after 48 hr at 30°C, and *Enterobacteriaceae* was on VRB (violet red bile) agar (Oxoid Ltd.) after 24 hr at 37°C.

2.2.6.3. Measurement of water activity (a_w)

For the measuring of water activity (a_w), the sausage sample was chopped into small pieces and adjusted at a temperature at 25°C. The a_w of sausage samples was measured with a digital Lab Master meter (Novasina, Lachen, Switzerland). The device was calibrated with the standard before use.

2.2.6.4. Measurement of dryness and moisture content

Weight losses/dryness (%) were calculated as a reduction of weight of the sausages between day 0 and day 28. The moisture percentage of the manufactured sausage was calculated by weight loss of the sample (5-10 g) maintained in a hot air oven (DOV-300A, AS ONE Co.) at 105°C until the constant weight of the sample according to the ISO recommended method (ISO, 1973). After drying, cooling was performed inside the desiccator at room temperature for 30 min. The moisture contents of the sample were determined by weighing from the weights before and after drying.

2.2.7. Color and ZnPP autofluorescence analysis

2.2.7.1. Preparation of salted minced meat

Partial modification of the method used by Wakamatsu *et al.* (2020) was applied to observe the surface autofluorescence intensity and visual color in salted minced meat. In this method, a vacuum packaging bag (Hiryu N-1, Asahi Kasei Pax Corp., Tokyo, Japan) was sterilized under UV light overnight and each bag was prepared with 9 g of aseptic minced meat with 3% NaCl and 1 mL of bacterial culture (final concentration 2.0×10^6 CFU/g) as described in section 2.2.4.1. Sterilized water instead of microorganisms was added to the control group. Additionally, the sterilized sodium nitrite solution was added at a final concentration of 200 ppm to the nitrite group. After vacuum packaging, bags were incubated at 18°C in the dark for 14 days. Thereafter, the incubated minced meat bag was heated in a hot water bath at 75°C for 15 min.

2.2.7.2. Measurement of ZnPP from the dry-cured sausages

For analysis of fluorescence intensity of ZnPP, 20 g of sausage samples were cut into small pieces and taken in a homogenizer, then soaked for 1 hr in 80 mL of distilled water and then homogenized (CELL MASTER CM–100, ASONE Co.) at 10,000 rpm for 90 sec. The ZnPP of the dry-cured sausages was measured as shown section 2.2.4.3.

2.2.7.3. Observation of visual images and ZnPP autofluorescence

The visual images of salted minced meat and dry-cured sausages were taken with a digital camera (Nikon D3300, Nikon Corporation, Tokyo, Japan). The ZnPP autofluorescence of salted minced meat and dry-cured sausages was carried out by the purple LED lighting method used by Wakamatsu *et al*. (2020) with slight modification. The autofluorescence of ZnPP was emitted with two purple LED lighting devices fitted with a sheet-type bandpass filter that transmits around 420 nm (Fujifilm BPB 42, Fujifilm Corporation, Tokyo, Japan) and was taken with a digital camera through a sheet-type bandpass filter that transmits around 600 nm (Fujifilm BPB 60). The ZnPP autofluorescence of the samples was taken in dark conditions.

2.2.7.4. Autofluorescence intensity analysis

The fluorescence intensity of incubated minced pork was measured for each bag using a fluorescence spectrophotometer (RF-5300PC, Shimadzu Corporation) equipped with a solid sample holder. The fluorescence spectrophotometer was set similarly to the fluorescence analysis of the homogenate sample, as described in section 2.2.4.3. Fluorescence intensity at 590 nm for excitation at 420 nm was regarded as the amount of ZnPP formed.

2.2.7.5. Measurement of CIE L*a*b* color system

The color of the unheated and heated minced meat samples and dry-cured sausages were measured through vacuum packaging on white paper with a colorimeter (CM-700d; Konica Minolta, Inc., Tokyo, Japan) using an 8 mm port size filter, with illuminant D65 and a 10° standard observer. For sausages, samples were sliced with 4-5 mm thickness and put on a white paper, and the measurements were carried out. The colorimeter was calibrated by the white standard plate fitted with its cover before using it. The measurements were expressed as L* (lightness), a* (redness), and b* (yellowness) and were obtained at 25°C. Hue angle and C* (chroma) were also calculated from the b* and a* values.

2.2.8. Statistical analysis

Values were expressed as mean ± standard error. Statistical analyses were performed using Microsoft Excel 2016 (Microsoft corp., Redmond, WA, USA) with Ekuseru-Toukei 2012 (Social Survey Research Information Co. Ltd., Tokyo, Japan) for add-in software. One-way ANOVA with Dunnett's comparison test was performed to select the high ZnPP-forming bacteria. Color parameters (L*, a*, b*, C* and hue angle) and ZnPP formation in minced meat and sausages were evaluated by one-way ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

2.3. Results and discussion

2.3.1. Separation and identification of high ZnPP-forming edible bacteria from various sources

Selected species of bacteria have long been used as a starter culture for meat and meat products. It is reported that some strains bacteria have the ability to form ZnPP in meat and meat products (Ohya, 2017; Wakamatsu, 2020). Until now, the identified high ZnPP-forming bacteria are not suitable for using meat and products because of their pathogenicity. Therefore, in order to gather high ZnPP-forming edible bacteria for improving the color of meat products, bacteria from various environmental and probiotics sources were collected. The collected bacteria were evaluated for their ability to form ZnPP, and then high ZnPP-forming bacteria were identified.

First, in order to search for high ZnPP-forming bacteria, the bacterial isolates were picked up from various environmental and probiotic sources (soil, water, refuse, fruits, probiotics, etc.) by swabbing the sources with sterile cotton and were dispersed in sterile physiological saline. Then the isolates were inoculated in the mSPC agar containing 3% NaCl, which was adjusted to pH 5.5 and incubated anaerobically at 25°C. Since the pH of commercial SPC agar was around 7.0, the salt content and pH were modified, and bacteria were cultured anaerobically because of the application of screened bacteria to meat products. The bacteria grown in this way were transferred to the mNB medium, and the colonies were then incubated anaerobically at 25°C for growth. A large number of sources were used to collect isolate from the environment and probiotics, however, bacterial isolates could be collected from only 35 sources (Table 1). Due to the higher salt

Table 1. High ZnPP-forming bacteria screened from various environmental and probiotics sources

Name of sources	Number		
	Isolated colonies	High ZnPP-forming colonies	Identified bacteria
Pond Water	5	1	1
Grass	1	0	-
Refuse	3	2	1
Rat body	3	2	2
Salami	3	0	-
Kimuchi	2	0	-
Cheddar cheese	4	0	-
Tap water	3	3	0
Plant leaves	3	3	0
Human skin	3	0	-
Raw milk	5	2	2
Pasteurized milk	1	0	-
Gouda cheese	3	0	-
Yoghurt	2	0	-
Poultry wings	2	1	0
Poultry cages	3	2	0
Poultry feces	2	2	1
Pig feces	4	2	0
Fermented Mackerel	6	1	1
Dry shrimp	4	1	1
Fermented Saury	9	1	1
Orange	2	0	-
Fermented meat	2	2	1
Biofermin probiotics	3	3	1
Grape	5	5	2
Parma ham	6	6	1
Fermented milk	4	3	0
Natto	2	0	-
Sausage1	8	0	-
Sausage2	2	0	-
Sausage3	3	0	-
Sausage4	4	0	-
Sausage5	5	0	-
Miso	2	0	-
Cow dung	7	2	1
Total	126	44	16

content and lower pH of the agar, the growth of salt-sensitive and neutral to alkaline bacteria were suppressed. A total of 126 isolates (Table 1) were separated from environmental and probiotic sources and evaluated the ZnPP-forming capability for the identification of the bacteria.

In order to find out the ZnPP-forming capability, the separated isolates were evaluated using the aseptic meat homogenate model system. After incubation of 7 days at 25°C in anaerobic conditions, the fluorescence intensity of ZnPP was measured. From the result, it was observed that some bacteria could form a significantly higher amount of ZnPP compared to the control group meaning that the higher ZnPP was derived from high ZnPP-forming bacteria (in addition to an example shown in Fig. 2, all separations are attached in appendix Fig. 1-8). Since the formation of ZnPP in the control group was significantly suppressed, as in the antibiotics group, an aseptic condition during model preparation was confirmed. In total, 44 isolates possessed high ZnPP-forming ability could be separated (Table 1).

Then, high ZnPP-forming bacteria were identified by 16S rRNA partial gene sequencing. Consequently, only 16 species of bacteria were discovered due to duplication (Table 2). Although, 13 of these species were not edible and, only three species, *Lactococcus lactis, Leuconostoc mesenteroides*, and *E. faecium,* were found to be edible. *Lactococcus lactis* is the dominant acidifying mesophilic species used in the fermentation of various dairy products (Cavanagh *et al*., 2015; Leroy *et al*., 2004). *Leuconostoc mesenteroides* is also a LAB that produces characteristic flavors and is used in the production of fermented milk beverages with a single or mixed culture (Farnworth, 2005*).* Since *E. faecium,* which is generally considered an inedible bacterium, was isolated from

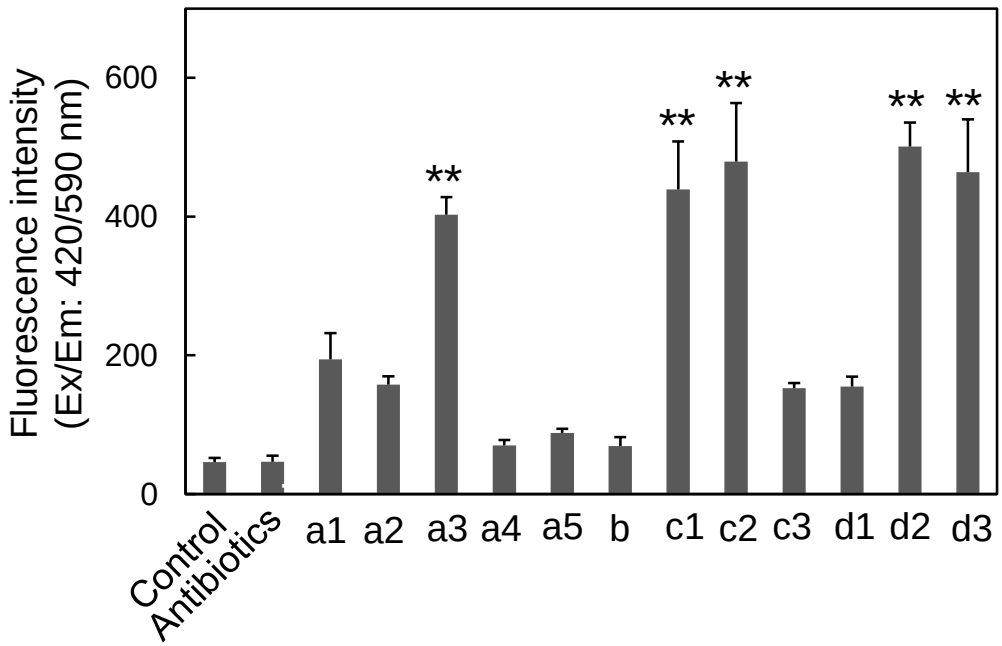


Fig. 2. ZnPP forming abilities of the bacteria isolated from various environmental sources (an example)

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with the isolated bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). Isolates from different sources are a1-a5: pond water, b: grass, c1-c3: refuse and d1-d3: rat body**.** Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control.

Table 2. Identification of bacteria isolated from environmental and probiotics sources with high ZnPP-forming ability in meat

Candidate (Closest strain)	Identity (%)	Accession No.
Enterococcus faecalis	95.10	MH285991.1
Enterococcus hirae	99.15	MK194297.1
Serratia liquefaciens	99.80	MG132665.1
Citrobacter amalonaticus	98.61	MH085466.1
Escherichia coli	99.59	KM870900.1
Erwinia toletana	99.79	JX134630.1
Pluralibacter gergoviae	99.69	CP009450.1
Leclercia adecarboxylata	99.68	MH071328.1
Pantoea eucalypti	99.49	NR_116112.1
Pantoea agglomerans	99.69	MG639900.1
Citrobacter murliniae	99.69	HQ407238.1
Vagococcus fluvialis	99.69	MK396597.1
Pseudomonas putida	99.90	MH379791.1
Lactococcus lactis	99.69	LT853603.1
Leuconostoc mesenteroides	99.17	LT853608.1
Enterococcus faecium	99.35	MK418596.1

commercial probiotics (Biofermin tablets, Taisho Pharmaceutical Co. Ltd., Tokyo, Japan), it was regarded as edible. According to the datasheet, Biofermin tablets contain *Enterococcus faecalis*, but it was identified in the present study as *E. faecium* through gene sequencing. However, *Enterococcus* species are commonly found in traditional fermented foods (Giraffa, 2003) due to their incredible capacity to resist extreme temperatures and high salinity (Franz *et al*., 2011). *E. faecium*, isolated from a Tunisian dried cured meat product "Dried Ossban," can be used as a probiotic (Zommiti *et al*., 2018). Therefore, these three edible bacteria could be the candidate to apply for meat products.

2.3.2. ZnPP-forming ability of the identified edible bacteria in the salted meat system

In order to use the screened edible bacteria identified in section 2.3.1 to dry-cured meat products, the ZnPP-forming ability of the bacteria was evaluated using salted minced meat. The salted minced meat experiment was closer to dry-cured meat products. In this experiment, nitrite and *S. liquefaciens* were used along with the edible bacteria as a positive control because *S. liquefaciens* have high ZnPP-forming ability in the meat system (Wakamatsu *et al*., 2020). Heat treatment in meat and meat products significantly changes their natural color. Nevertheless, in nitrite-free Parma ham, the bright red-colored pigment that is formed is heat stable (Adamsen *et al*., 2004). Thus, the heating effect of the red-colored pigment formed by the bacteria was evaluated.

2.3.2.1. Color and ZnPP-forming ability of the bacteria in the uncooked salted meat system

The effect of the edible screened bacteria on color and ZnPP formation was examined using the salted meat system. The color and ZnPP autofluorescence of salted minced meat

were assessed after 14 days of incubation at 18°C (Fig. 3A). In the visual images, the bright red color was observed in the nitrite group. Among the bacteria-inoculated groups, the color of minced meat inoculated with *Lactococcus lactis* and *Leuconostoc mesenteroides* were almost similar to the *S. liquefaciens*-inoculated and nitrite groups. Regarding ZnPP autofluorescence, strong autofluorescence was observed in all the inoculated groups, compared to the control and nitrite groups (Fig. 3A). The fluorescence intensity of ZnPP on the surface of salted minced pork was significantly higher in the *Lactococcus lactis*-, *Leuconostoc mesenteroides*-, *E. faecium*- and *Leuconostoc mesenteroides*-inoculated groups compared to the control group (Fig. 3B). However, among the edible groups, the fluorescence intensity of ZnPP of *E. faecium*-inoculated group was significantly lower than those of *Lactococcus lactis*- and *Leuconostoc mesenteroides*-inoculated groups.

For comparing the color of salted minced meat, CIE L*a*b* color parameters were evaluated (Fig. 4). The L* values of the *S. liquefaciens*-inoculated group were significantly lower than that of the control group, but there was no significant difference between all bacteria-inoculated groups and the nitrite group. The a* value of the inoculated groups except for the *E. faecium*-inoculated group was significantly higher than that of the control group. The b* value was significantly lower in the nitrite and some bacteria-inoculated groups compared to the control group. The hue angle of the nitrite group was the closest to the *Lactococcus lactis*- and *S. liquefaciens*-inoculated group. However, the C* values were not significant among all groups.

The visual color, autofluorescence, and fluorescence intensity were closely related to the ZnPP formation of the bacteria in the inoculated minced meat. The bacteria that resulted in brighter color corresponded to those which produced a large amount of ZnPP.

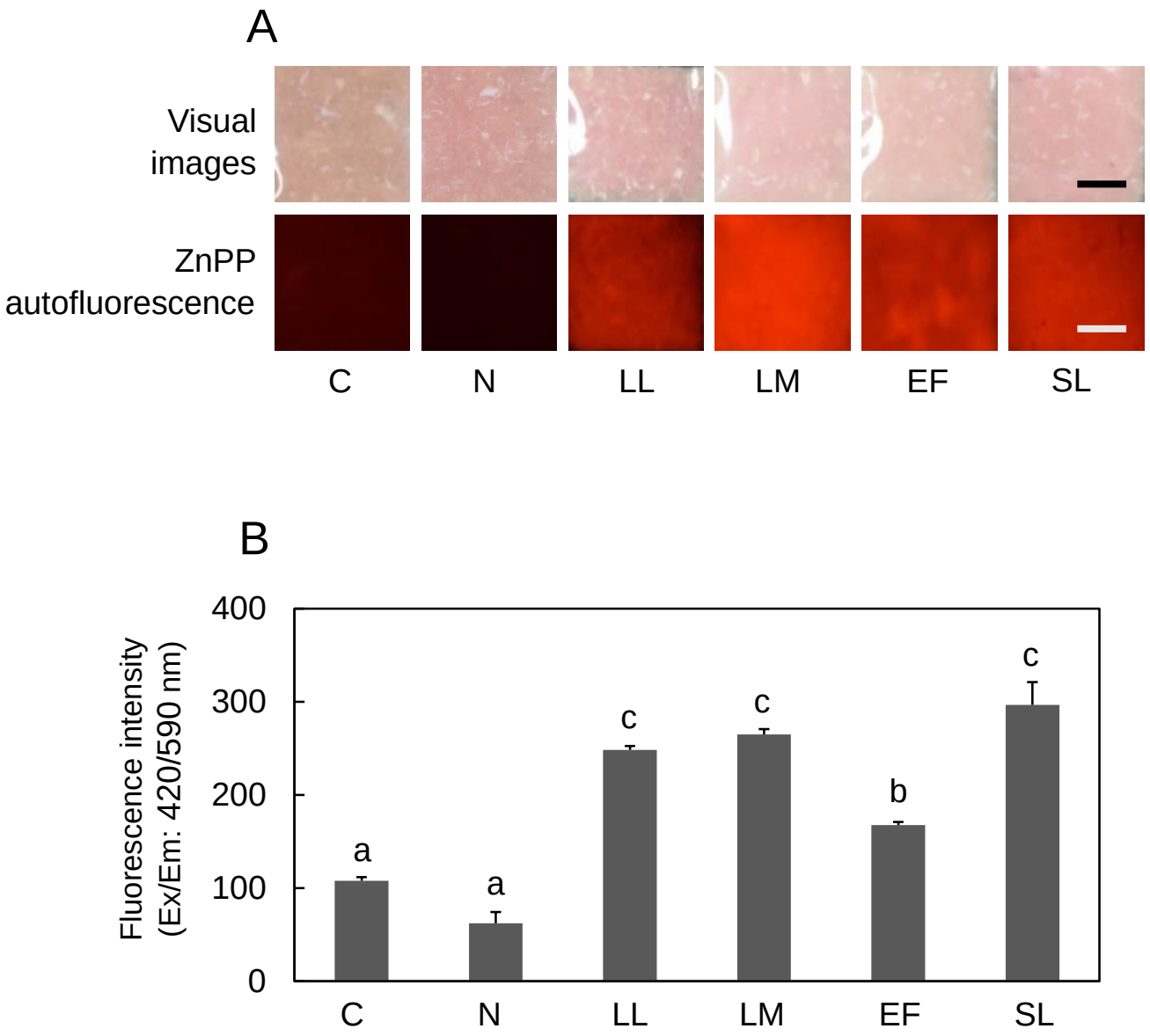


Fig. 3. Visual images, ZnPP autofluorescence images and fluorescence intensity of ZnPP of salted minced meat after 14 days of incubation

Aseptic salted minced meat was prepared with LTL muscle of pork and inoculated with ZnPP-forming bacteria. After vacuum packaging, bags were incubated at 18°C for 14 days. (A) Visual images and ZnPP autofluorescence images and (B) the fluorescence intensity of ZnPP (Ex/Em: 420/590 nm) of salted minced meat. C: Control, N: nitrite, LL: *Lactococcus lactis*, LM: *Leuconostoc mesenteroides*, EF: *Enterococcus faecium* and SL: *Serratia liquefaciens*. Data expressed as means ± SE (n = 3). abc: Significant differences among groups are indicated by different letters ($P < 0.05$). Scale bars: 2 cm.

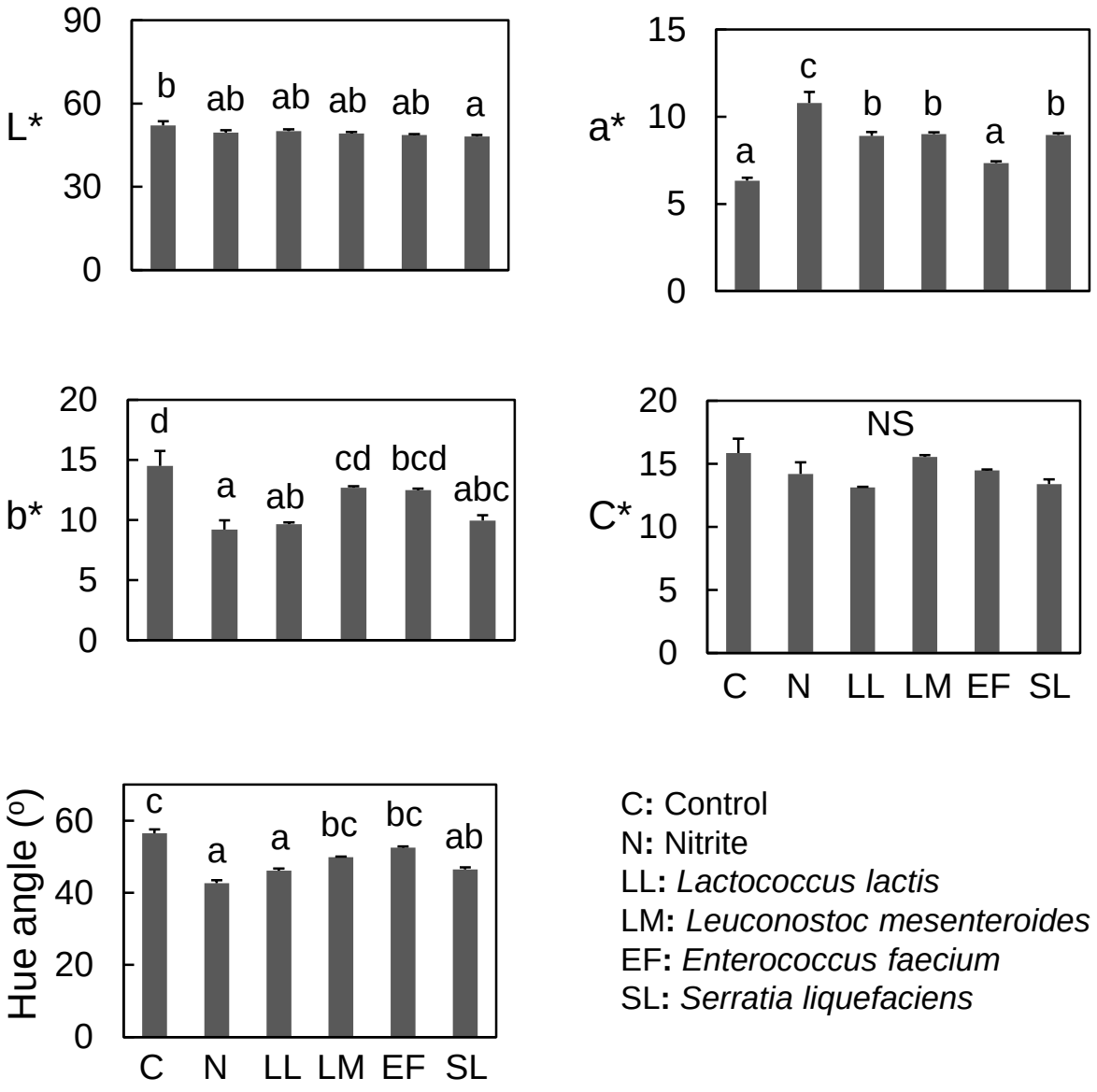


Fig. 4. The effect of high ZnPP-forming edible bacteria on CIE L*a*b*, hue angle, and C* of salted minced meat after incubation

CIE L*a* b* values were taken from salted minced meat that was prepared as shown in Fig. 3. Data expressed as means ± SE (n = 3). abcd: Significant differences among groups are indicated by different letters ($P < 0.05$). NS: Non-significant.

The fluorescence intensity of ZnPP of *Lactococcus lactis*- and *Leuconostoc mesenteroides*- and *S. liquefaciens*-inoculated groups were significantly higher compared to the control, nitrite and *E. faecium*-inoculated groups. These results were consistent with the autofluorescence of ZnPP (Fig. 3A) of the minced meat. When salted minced meat was incubated with the high ZnPP-forming bacteria, the formation of ZnPP was increased, and the color intensity was differentiated according to their ZnPP-forming ability. In contrast, the red color could be attributed to the formation of nitrosomyoglobin in the nitrite group (Adamsen *et al*., 2006b). On the other hand, very low fluorescence intensity and negligible ZnPP autofluorescence were observed in the nitrite group. This result was consistent with nitric oxide derived from nitrite/nitrate inhibiting the formation of ZnPP (Wakamatsu *et al*., 2010). In the control group, very weak fluorescence intensity and ZnPP autofluorescence were observed, suggesting that ZnPP is formed by mechanisms inherent to meat (Khozroughi *et al*., 2018) or contaminated bacteria. Comparing the color parameters, among the edible groups *Leuconostoc mesenteroides*- and *Lactococcus lactis*- showed close results compared to *S. liquefaciens*-inoculated and nitrite group. Thus, *Lactococcus lactis* and *Leuconostoc mesenteroides* will be suitable for color and ZnPP formation in dry-cured sausages. Moreover, in minced meat, fermentation was performed at 18°C with 3% salt, and these conditions did not affect the color and ZnPP-forming ability of the screened bacteria. The above three bacteria species showed color-improving activity at low temperatures despite the presence of salt in minced meat. Therefore, the color of the nitrite/nitrate-free meat products could be developed using the identified edible bacteria.

2.3.2.2. The heating effect of red pigments formed by bacteria in the salted meat system

To examine the effect of heating on the red pigments formed by the bacteria, the salted minced was cooked at 75°C for 15 min after incubation. In the visual images, a noticeable red color was observed in the nitrite group (Fig. 5A). Among the inoculated groups, the color of *Lactococcus lactis*-, *Leuconostoc mesenteroides*- and *S. liquefaciens*-inoculated groups were brighter than that of the control and *E. faecium*-inoculated groups. Intense ZnPP autofluorescence was observed in all bacteria-inoculated groups compared to the control and nitrite groups. The fluorescence intensity on the surface of the bacteria-inoculated groups was significantly higher compared to the control and nitrite groups (Fig. 5B). However, the fluorescence intensity of *Leuconostoc mesenteroides*- and *S. liquefaciens*-inoculated groups were significantly higher compared to the other inoculated groups.

The CIE L*a*b* color parameters of incubated minced meat were also measured after heating (Fig. 6). The L* value was significantly lower in the *Leuconostoc mesenteroides*-inoculated group compared to the control and the other treated groups. The a* values of the most of bacteria-inoculated groups was significantly higher than that of the control group but significantly lower than that of the nitrite group. These results were consistent with the visual images of heated minced meat (Fig. 5A), indicating that the redness of the inoculated groups was higher than that of the control group but remarkably lower than that of the nitrite group (Fig. 5B). The b* value was significantly lower in the nitrite group than the others. The hue angle of the bacteria-inoculated groups except *E. faecium*-inoculated group was significantly smaller than that of the control group.

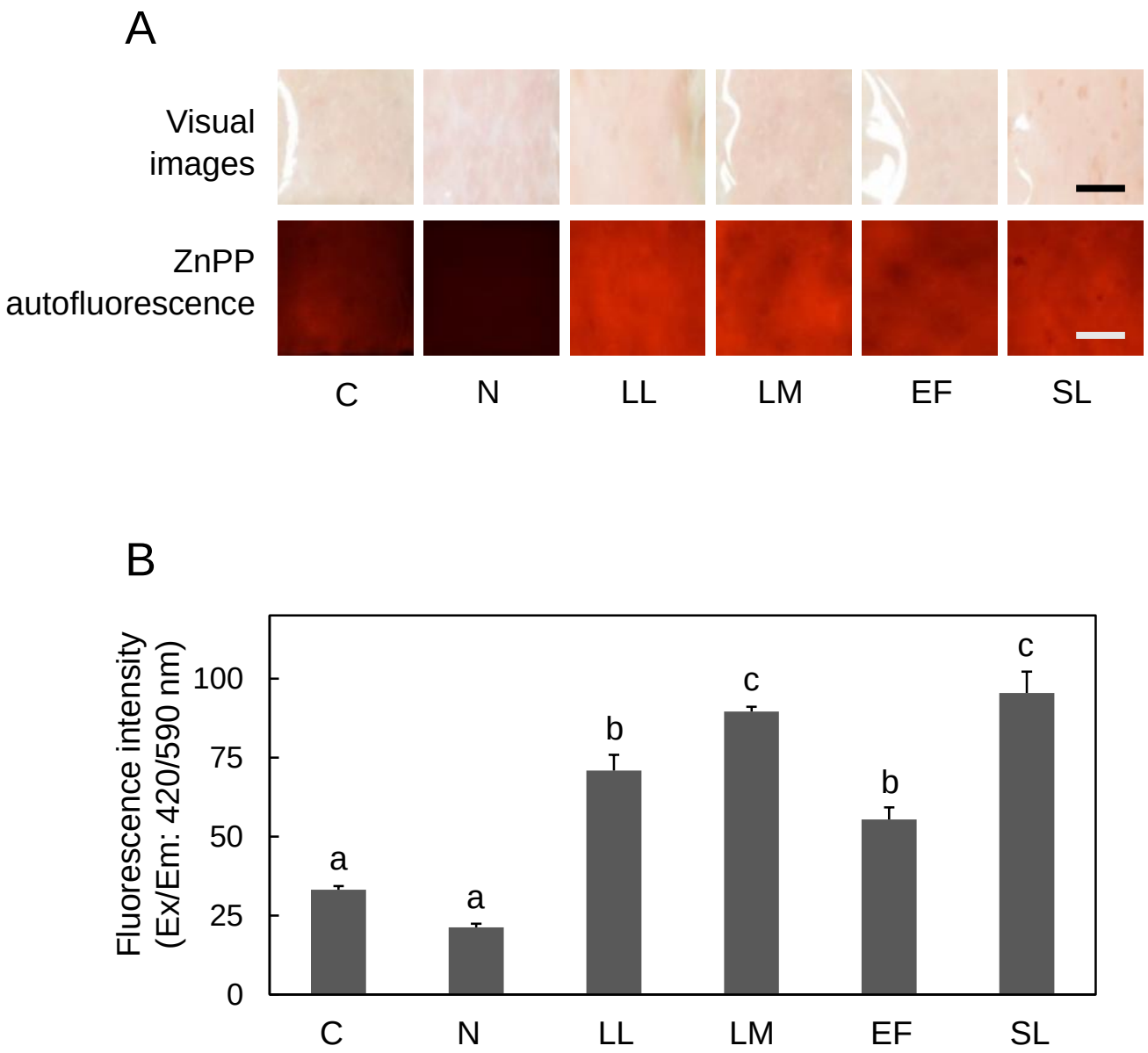


Fig. 5. Visual images, ZnPP autofluorescence images and fluorescence intensity of ZnPP of salted minced meat heated after 14 days of incubation

The salted minced meat bags were heated in a hot water bath at 75°C for 15 min. (A) Visual images and ZnPP autofluorescence images and (B) the fluorescence intensity of ZnPP (Ex/Em: 420/590 nm) of heated salted minced meat. C: Control, N: nitrite, LL: *Lactococcus lactis*, LM: *Leuconostoc mesenteroides*, EF: *Enterococcus faecium* and SL: *Serratia liquefaciens*. Data expressed as means ± SE (n = 3). abc: Significant differences among groups are indicated by different letters ($P < 0.05$). Scale bars: 2 cm.

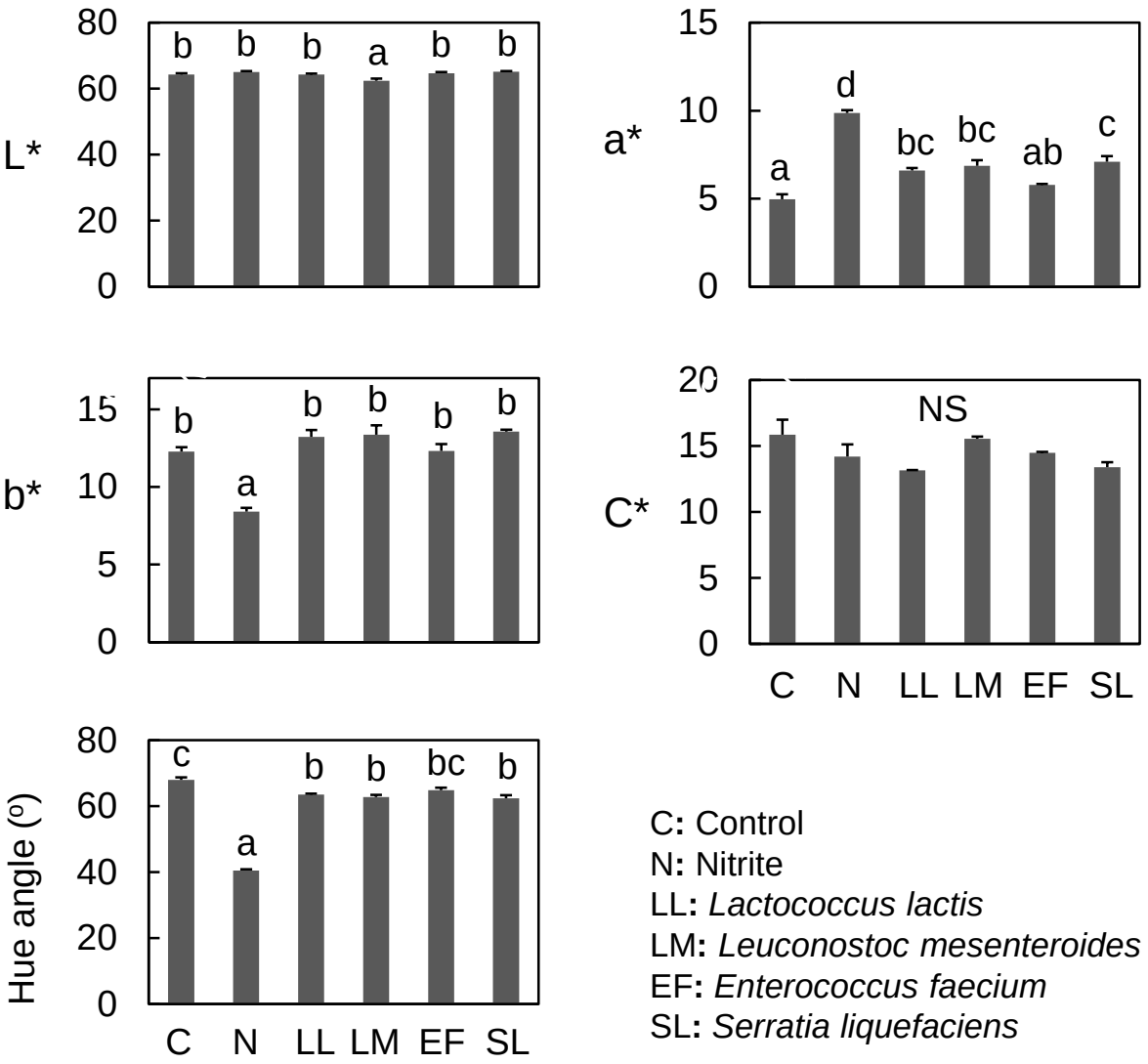


Fig. 6. Effect of high ZnPP-forming bacteria on CIE L*a*b*, hue angle, and C* of salted minced pork heated after 14 days of incubation

CIE L*a*b* values were taken from heated salted minced meat that was prepared as shown in Fig. 5. Data expressed as means ± SE (n = 3). abc: Significant differences among groups are indicated by different letters ($P < 0.05$). NS: Non-significant.

However, the hue angle of the nitrite group was significantly far from those of the bacteria-inoculated and control groups. The C* values were insignificant among all the groups.

Heat treatment in meat and meat products significantly changes their natural color due to the denaturation of myoglobin (Sakata, 2000). After heating, the color of minced meat became fade. However, the red color was clearly noticed in the inoculated groups compared with the control group. This result was consistent with the autofluorescence image of ZnPP of heated minced meat (Fig. 5A). The higher fluorescence intensity of ZnPP of *Leuconostoc mesenteroides*- and *S. liquefaciens*-inoculated groups indicated that ZnPP was persisted after heating. These results were consistent with the Adamsen *et al*. (2004) and Morita *et al*. (1996) who have also observed that the Parma ham pigment was stable in heat. In the nitrite group of heated minced meat, the formation of nitrosylhemochromogen (Honikel, 2008; Sakata, 2000) showed a bright red color. Although the fluorescence intensity of ZnPP was different among the inoculated groups, the visual images after cooking were almost similar among them, indicating that the intensity of the visual images of the inoculated groups was lower than that of the nitrite group. During heating, the heme in myoglobin was more exposed and more likely to oxidize to form a dull-brown color (King & Whyte, 2006) in the inoculated and control groups. The redness of the *Lactococcus lactis*-, *Leuconostoc mesenteroides*- and *S. liquefaciens*-inoculated groups was significantly higher than that of the control group due to the stability of the color formed by the bacteria. However, hue angles of the inoculated groups were far from the nitrite group, meaning that the color of the nitrite and inoculated groups were different. Unlike the raw salted minced meat, the color of inoculated groups was not closer to that of the control group after heating. Consequently, the identified

edible bacteria are suitable for improving the color of dry-fermented raw meat products but less suitable for maintaining the color of cooked meat products.

2.3.3. Preparation of dry-cured sausages using high ZnPP-forming bacteria

In section 2.3.2, the prospective bacteria with high ZnPP-forming abilities were identified. The identified bacteria might be a potential culture for dry-cured sausages. Thus, dry-cured sausages were prepared to evaluate the color and ZnPP-forming ability of the bacteria in actual meat products. Sausages including control, nitrite, and inoculated groups were manufactured and ripened for 28 days. Nitrite was used as a positive control and sausage group without inoculated bacteria treated as a negative control.

2.3.3.1. Moisture, water activity (a_w) and pH changes in the dry-cured sausages

In order to observe the suitability of the bacteria for the quality of dry-cured sausages, the physical and chemical parameters of the fermented sausages were measured during 28 days of ripening. The initial moisture content of the sausages was around 70-71%, and finally, after 28 days of ripening, the moisture content was 35-37% (Table 3). After 28 days of ripening, there is no significant difference of moisture content among the inoculated and control groups. The drying of the sausages during ripening is closely related to the water activity (a_w). The initial a_w of the sausages was around 0.99 and after 28 days, it was reduced about 0.85. Acton & Dick (1976) reported that the moisture content of the complete fermented sausage is about 35%, and the fermented sausages prepared in this study attained within the range. The measurement of a_w and moisture is very important in dry-cured meat products since these parameters are directly influencing the chemical reaction and microbial growth in the final products. Most of the microorganisms cannot grow in the dry-cured sausages below a_w 0.91 with a few

Table 3. Moisture content, water activity (a_w) and pH of the sausages inoculated with different starter cultures

Treatments	Moisture content (%)		a_w		pH	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day 28
Control	70.37±0.02	36.09±0.57	0.994±0.000	0.849±0.004^{ab}	6.02±0.00	6.08±0.01^{c}
Nitrite	70.72±0.16	36.96±0.92	0.992±0.001	0.853±0.001^{b}	6.06±0.01	6.06±0.01^{c}
Lactococcus lactis	70.54±0.04	35.97±0.53	0.991±0.001	0.841±0.000^{a}	6.03±0.01	6.03±0.01^{c}
Leuconostoc mesenteroides	70.68±0.13	35.86±0.76	0.994±0.002	0.843±0.001^{a}	6.00±0.02	5.65±0.03^{a}
Enterococcus faecium	70.80±0.13	36.17±0.70	0.994±0.000	0.847±0.002^{ab}	6.02±0.02	5.96±0.00^{b}

Data expressed means ± SE ($P < 0.05$, n = 3). abc: Meaning at the same time at the same column with different letters differ significantly.

exceptions, notably *Staphylococcus aureus*, which remains active until a_w 0.86 (Troller & Christian, 1978). The growth of molds can be controlled by low moisture levels as well. The activity of most spoilage and pathogenic bacteria ceases when a_w of 0.89 is reached (Lücke, 1997). Accordingly, the a_w of the prepared inoculated sausages were considered acceptable in terms of food quality.

The pH value of the manufactured dry-cured sausage was measured at the beginning and the end of the ripening process (Table 3). Initially, the pH value was around 6.0 in all the treated groups and control. At the day 28, the pH value was significantly decreased in the *Leuconostoc mesenteroides* and *E. faecium*-inoculated groups compared to the other groups. The lowest pH was observed in the *Leuconostoc mesenteroides*-inoculated group. It has been reported that the pH decreases when lactic acid is produced by LAB in dry-cured sausages (Ammor & Mayo, 2007). On the contrary, the pH value of the *Lactococcus lactis*-inoculated group and the control group did not decrease much that might be the polyphosphate content of the sausages that neutralized the acidity (Young *et al*., 2005), produced by autochthonous bacteria in the control and nitrite group or starter culture bacteria inoculated group. However, the inoculated bacteria, especially *Leuconostoc mesenteroides* and *E. faecium* were suitable for lowering the pH of the manufactured sausages. As a result, the physical and chemical parameters of the inoculated sausages were considered well for food safety, and the inoculated bacteria especially *Leuconostoc mesenteroides* was the most suitable for dry-cured sausages.

2.3.3.2. Selective microbial profile of the dry-cured sausages

Lactic acid bacteria (LAB) and Enterobacteriaceae are important for dry-cured sausages because they maintain the acceptability of the sausages. In order to monitor the

microbial quality of the dry-cured sausages, the viable counts of LAB and Enterobacteriaceae during the processing of the fermented sausages were enumerated at the beginning and the end of the ripening process (Table 4). Initially, the LAB count was 4.12 log CFU/g for control sausages and 6.5 log CFU/g for the inoculated ones. After 28 days of ripening, the number LAB was significantly higher in the inoculated groups compared to the control and nitrite groups. The highest LAB was observed in the *Leuconostoc mesenteroides*-group followed by the *Lactococcus lactis*- and *E. faecium*-inoculated groups. The lowest LAB was found in the nitrite-added group. LAB was the dominant microflora at the end of the ripening of the inoculated sausages. Lowest LAB growth was observed in the nitrite added group because the growth of bacteria might be affected by nitrite (Bedale *et al*., 2016). However, LAB growth was significantly higher in the inoculated groups than that of the control and nitrite groups of sausage, meaning that the good adaptation of LAB to the meat environment and during fermentation and maturation of sausages. The LAB is also effective for the control of pathogenic and spoilage bacteria through the antimicrobial properties by producing their metabolites during fermentation (Ammor & Mayo, 2007). Initial counts of Enterobacteriaceae were almost similar in all the inoculated and control groups (around 4 log CFU/g). After 28 days of ripening, the number of Enterobacteriaceae was significantly decreased in the inoculated groups (2.7-3.0 log CFU/g) compared to the control group (around 4.2 log CFU/g). However, the lowest Enterobacteriaceae was observed in the nitrite group (around 2.4 log CFU/g). The number of Enterobacteriaceae bacteria depends on not only the hygienic quality of the raw materials but also the handling and fermentation conditions during processing (Casquete *et al*., 2012). They are considered as the indicators of contaminated bacteria for post-processing contamination. The reduction of this group through ripening is due to their sensitivity to the acidic environment. The acidifying

Table 4. Lactic acid bacteria (LAB) and Enterobacteriaceae content of sausages inoculated with different starter cultures

Treatments	LAB counts (log CFU/g)		Enterobacteriaceae counts (log CFU/g)	
	Day 0	Day 28	Day 0	Day 28
Control	4.12±0.12[a]	6.02±0.13[a]	3.78±0.10	4.19±0.08[c]
Nitrite	3.83±0.07[a]	5.82±0.09[a]	3.53±0.03	2.36±0.11[a]
Lactococcus lactis	6.41±0.06[b]	7.32±0.29[c]	3.66±0.05	2.77±0.11[b]
Leuconostoc mesenteroides	6.53±0.06[b]	7.92±0.05[c]	3.70±0.08	2.79±0.06[b]
Enterococcus faecium	6.49±0.07[b]	6.85±0.02[b]	3.78±0.04	2.96±0.07[b]

Data expressed means ± SE. ($P < 0.05$, n = 3). abc: Meaning at the same time at the same column with different letters differ significantly.

activity of *Leuconostoc mesenteroides*- and *E. faecium*-inoculated group (Table 3) could be associated with the reduction of Enterobacteriaceae. Casaburi *et al*. (2008) reported that the final count of Enterobacteriaceae was more important in the sausages inoculated with particular lactic acid strains compared to the control group. In this regard, the inoculated starter culture bacteria, especially *Leuconostoc mesenteroides* considered favorable for food safety through controlling the growth of pathogenic bacteria.

2.3.3.3. Visual images and ZnPP formation evaluation of dry-cured sausages

In order to observe the color formation by the bacteria in the dry-cured sausages, visual images, ZnPP formation, and color parameters were evaluated of the sausages incubated by high ZnPP-forming bacteria. The color of fermented sausages and the ZnPP autofluorescence was assessed after 28 days of ripening (Fig. 7A). As compared with the visual images, the brightest red color was observed in the nitrite group. Among the bacteria-inoculated groups, the inoculation of *Leuconostoc mesenteroides, Lactococcus lactis*, followed by *E. faecium* showed a bright red color compared to the control group and showed an almost similar color to the nitrite group. Regarding ZnPP autofluorescence on the cross-section of the fermented sausages (Fig. 7A), higher autofluorescence was observed in the *Lactococcus lactis*-, *Leuconostoc mesenteroides*-, and *E. faecium*-inoculated groups, compared to the control and nitrite groups. Similar to visual images, the highest autofluorescence was observed in the *Leuconostoc mesenteroides*-inoculated group.

The CIE L*a*b* color parameters of dry-cured sausages were measured after 28 days of fermentation (Fig. 7B). The L* values of the fermented sausages were non-significant among the groups. The a* value of the nitrite and *Lactococcus lactis*-inoculated groups

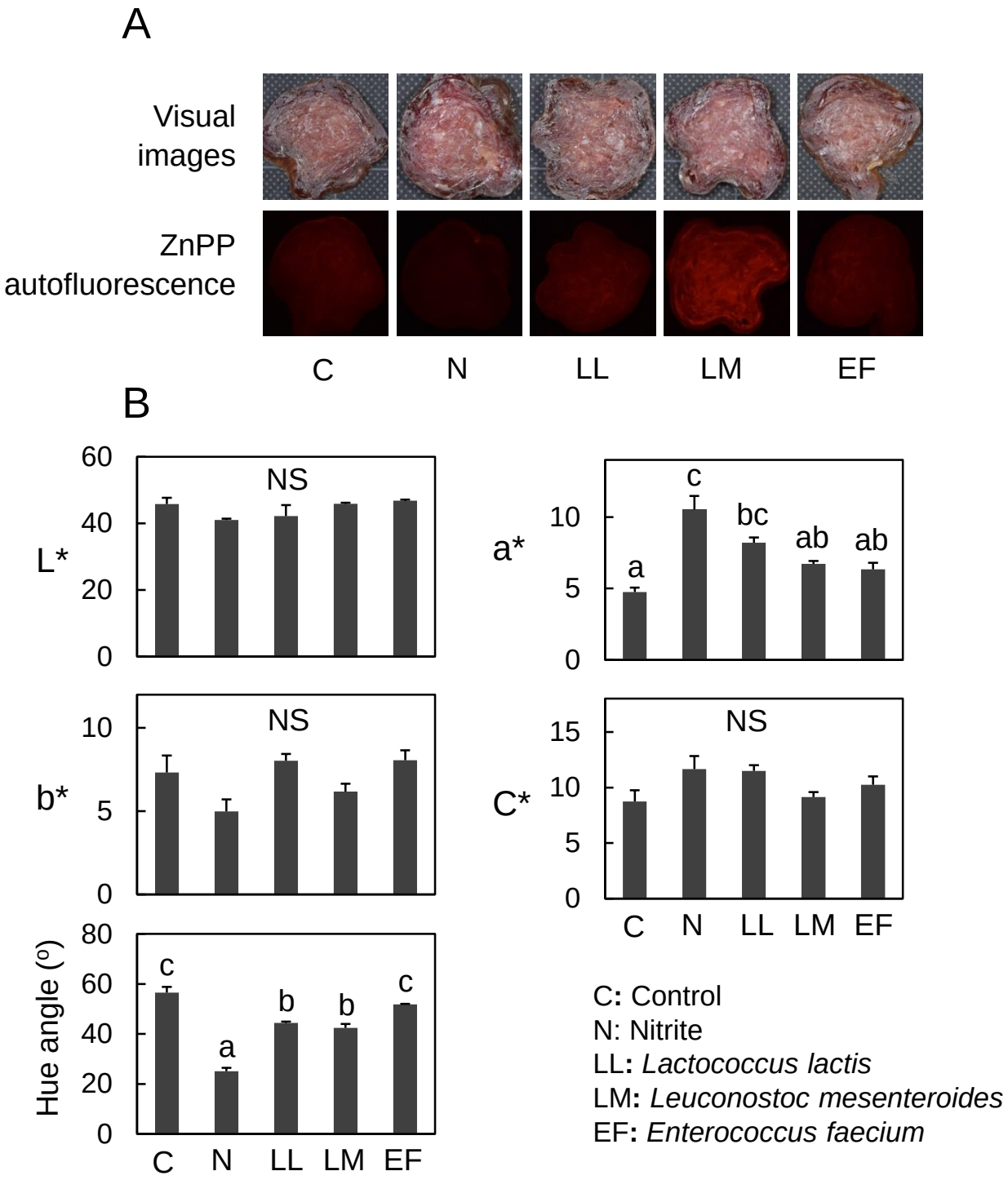


Fig. 7. Visual images, ZnPP autofluorescence images and CIE L*a*b*, hue angle and C* of dry-cured sausages after 28 days of processing

Dry-cured sausages were prepared with high ZnPP-forming bacteria and ripened up to 28 days. (A) Visual images and ZnPP autofluorescence images and (B) CIE L*a* b* values of sausages after 28 days of processing. Data expressed as means ± SE (n = 3). abc: Significant differences among groups are indicated by different letters ($P < 0.05$). NS: Non-significant.

were significantly higher than that of the control group. The a* value of the *Leuconostoc mesenteroides*- and *E. faecium*-inoculated groups showed a higher tendency than that of the control group, though it was not significant. The formation of nitrosylmyoglobin increases the a* value in the nitrite group (Adamsen *et al*., 2006a), but the higher a* of the bacteria-inoculated groups than that of the control group was due to the formation of ZnPP (Morita *et al*., 1998). The b* value of the nitrite and bacteria-inoculated groups were non-significant, and the hue angles in the nitrite group were also closer to that of bacteria-inoculated groups except for *E. faecium*-inoculated group than that of the control group. However, the C* values were not significant among all groups.

The fluorescence intensity of ZnPP of the fermented sausages was significantly higher in the *Lactococcus lactis*-, *Leuconostoc mesenteroides*- and *E. faecium*-inoculated groups compared to the control groups (Fig. 8A). However, among the inoculated groups, the highest fluorescence was observed in the *Leuconostoc mesenteroides*-inoculated group. Similar results were also observed in the ZnPP spectrum analysis of the sausages, indicating that the highest intensity of the ZnPP spectrum (590 nm) was observed in the *Leuconostoc mesenteroides-inoculated* group followed by the *E. faecium*- and *Lactococcus lactis*- group (Fig. 8B).

Regarding the ZnPP formation of the sausages, it was observed that the inoculated bacteria formed ZnPP in the manufactured sausages and ZnPP formation was depended on the ZnPP-forming capability of the specific bacteria. The bacteria that made the color brighter redder corresponded to those which produced a large amount of ZnPP in the sausages. In the manufactured sausages, bright red color was observed exclusively in the internal part of the sausages. The outside color of the sausages was dark color. These results clearly demonstrated that the inoculated bacteria could form ZnPP only in

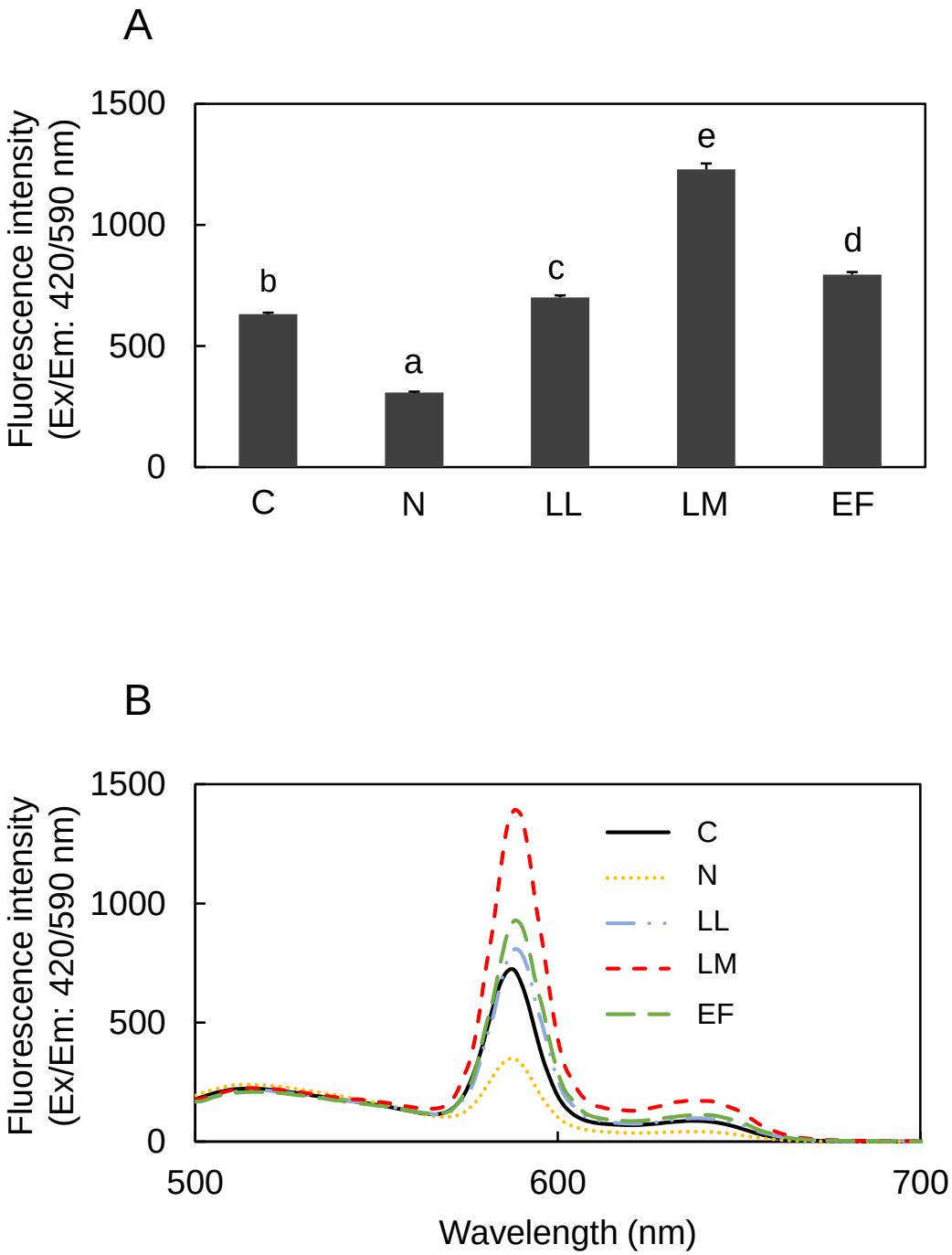


Fig. 8. Fluorescence intensity of ZnPP and ZnPP spectrum of the inoculated dry-cured sausages after 28 days of processing

Sausages homogenate (20%) was prepared with each inoculated sausage after 28 days processing. ZnPP was extracted with 75% acetone and then the fluorescence intensity of ZnPP (A) and spectrum of ZnPP (B) was measured. C: Control, N: nitrite, LL: *Lactococcus lactis*, LM: *Leuconostoc mesenteroides* and EF: *Enterococcus faecium*. Data expressed as means ± SE (n = 3). abcde: Significant differences among groups are indicated by different letters ($P < 0.05$).

anaerobic conditions and it was consistent with the report of Wakamatsu *et al.* (2019) showing that ZnPP formation was suppressed in the aerobic condition. As concern with the fluorescence intensity of ZnPP, the extent of fluorescence intensity of the three inoculated samples was correlated with the height of the peak in the fluorescence spectrum of ZnPP, which implied the fluorescence was derived from ZnPP. This result was consistent with the visual images and autofluorescence of ZnPP (Fig. 7A). However, similar to visual color, the autofluorescence of sausages was also observed internally that formed a ring over the circumference of the sausages. It is assumed that this ZnPP formation in the inoculated groups was due to the proteolytic breakdown of myoglobin (Grossi *et al*., 2014) or longer ripening period (De Maere *et al*. 2016). pH also plays an important role in the formation of ZnPP (Wakamatsu *et al.,* 2019). As expected, bright red color, very low fluorescence intensity and almost no ZnPP autofluorescence was observed in the nitrite group. This result was consistent with the findings that nitric oxide derived from nitrite/nitrate inhibited the formation of ZnPP (Wakamatsu *et al.*, 2010). In the control group, lower fluorescence intensity and ZnPP autofluorescence were observed, suggesting that ZnPP was formed by meat-inherent mechanisms (Khozroughi *et al*., 2018) or contaminated bacteria present in the meat. The above three bacteria species increased the fluorescence intensity of ZnPP and improved color in the dry-cured sausage. Among the inoculated bacteria, *Leuconostoc mesenteroides* formed the highest amount of ZnPP in the sausages. Therefore, it was suggested that *Leuconostoc mesenteroides* is suitable for ZnPP formation and color improvement of dry-cured sausage.

2.4. Conclusions

In this chapter, for improving the color of dry-cured meat products, three high ZnPP-forming bacterial strains were identified. The high ZnPP-forming bacteria can improve the color of salted minced meat; however, the red color formed by the bacteria showed a minimal degree of stability upon heating. The high ZnPP-forming bacteria isolated in this study are LAB and are suitable for fermented meat products. In dry-cured sausages, the color and fluorescence intensity of ZnPP was higher in the inoculated sausages than non-inoculated control. These results also expressed that the formation of ZnPP was greatly suppressed by nitrite. The inoculated bacteria also have the capability to improve microbiological characteristics by reducing the pathogenic bacterial count and improve the hygienic quality of dry-cured sausages. Moreover, the inoculated bacteria affect the physical characteristics of the sausages positively. Among the isolated bacteria, *Leuconostoc mesenteroides* showed the highest ZnPP-forming capability and it will be the potential candidate as a starter culture to enhance the color by producing ZnPP and other quality characteristics in the dry-cured sausages. However, these bacteria are only able to form ZnPP in anaerobically and can form red color only inside the product. Thus, searching for more efficient bacteria should be continued that can form ZnPP both aerobically and anaerobically.

Chapter 3

Mechanism of bacteria-assisted ZnPP formation in dry-cured meat products

3.1. Introduction

ZnPP is formed naturally during the storage of meat (Adamsen *et al*., 2006a), but the inoculation of suitable starter culture bacteria increases the formation of ZnPP in dry-cured meat products. The formation of ZnPP by bacteria is highly dependent on time, temperature, and environmental conditions (Khozroughi *et al*., 2018; Ohya, 2017), meaning that bacterial enzymes or metabolic end products of the ZnPP-forming bacteria might contribute to the ZnPP formation. Though ZnPP can be formed without the addition of bacteria in meat and meat products (Wakamatsu *et al*., 2004b), the contribution of bacteria in the ZnPP formation is either together with meat components or parallel. Thus, it is important to elucidate the bacteria-assisted ZnPP formation mechanism for establishing a suitable technology for improving the color of dry-cured meat products.

Regarding the ZnPP formation in meat and meat products, it is proposed the contribution of an enzyme-induced substitution of Fe^{2+} from heme with Zn^{2+} by endogenous as well as bacteria-induced ferrochelatase (FECH) (Paganelli *et al*., 2016; Wakamatsu *et al*., 2004a & 2004b). It was reported that both cell-extracted and bacterial FECH have been demonstrated to be able to replace Fe^{2+} in heme by Zn^{2+} and thereby lead to the formation of ZnPP (Chau *et al*., 2011). The formation of ZnPP in porcine muscle is associated with both a meat-inherent and a bacterial enzymatic reaction during meat storage (Khozroughi *et al*., 2018). It was presumed that ZnPP was formed by

Pseudomonas fluorescens as a part of a competitive reaction, in which some part of heme did not react with heme oxygenase, but was transformed by bacterial FECH to ZnPP. Therefore, FECH from bacteria might play a vital role in the bacteria-assisted ZnPP formation mechanism.

In the mechanism of ZnPP formation in meat products, heme, protoporphyrin IX (PPIX), FECH and Zn play a vital role as like proposed meat-inherent ZnPP formation mechanism (Fig. 1). Heme source as myoglobin, is known as a major heme protein in skeletal muscle *i.e*. meat and has been considered as a heme donor in the formation of ZnPP. PPIX is a chief precursor to biologically important prosthetic groups such as heme. FECH is an enzyme located at the inner and outer membrane of the mitochondria in mammalian cells of bacteria or higher eucaryotes plays an important role in the heme biosynthesis pathway (Taketani, 1993). FECH catalyzes the insertion of Fe^{2+} into PPIX in a living body but inserts other divalent metals such as zinc into porphyrins (Taketani *et al*., 2007; Chau *et al*., 2010). FECH was observed in mitochondria of Parma ham and other manufactured ham, and its localization almost coincided with that of ZnPP (Shimoda, 2014). The formation of ZnPP in the fermented meat products involves many complex steps; accordingly, the interaction of bacteria with the components will be helpful to elucidate how high ZnPP-forming bacteria assist the meat-inherent ZnPP formation mechanism.

In the present study, it was hypothesized that ZnPP-forming bacteria promoted the endogenous formation of ZnPP. Therefore, the objectives of this chapter were to find out the bacteria or meat components by which the ZnPP-forming bacteria promoted to form ZnPP. This chapter will be focused on the bacterial pathway of ZnPP formation in order to clarify the actual impact of ZnPP-forming bacteria in meat and meat products. In this

research, *S. liquefaciens*, which is already known as high ZnPP-forming bacteria by Wakamatsu *et al*. (2020) and *Leuconostoc mesenteroides* whch is screened in chapter 2 as high ZnPP-forming bacteria are used to elucidate the bacteria-assisted ZnPP formation mechanism in meat and meat products.

3.2. Materials and methods

3.2.1. Materials

3.2.1.1. Sampling

Porcine *Longissimus thoracis et lumborum* (LTL) muscle and liver were obtained from common domestic crossbred pork, which were purchased from the local market in Hokkaido, Japan. For preparing the aseptic meat homogenate, portions (250–300 g) of the muscles were dipped in 70% alcohol for one min and trimmed out the surface from LTL, only inside portions of muscle were taken and stored at -30°C in vacuum-packed until use. Similarly, the aseptic liver homogenate was prepared with the inside portions of the liver which was taken after swabbing with 70% alcohol and trimmed out the surface of the liver. The aseptic samples were vacuum-packed and stored at -30°C until use.

3.2.1.2. Preparation of buffer

Mitochondria separation (MS) buffer was prepared with 0.25 M glucose (Kanto Chemical Co. Inc.) and 10 mM Tris-HCl (Nacalai Tesque Inc.) adjusted at pH 7.6. Mitochondrial membrane separation (MMS) buffer was prepared with a final concentration of 3 mM HEPES-KOH (Dojindo Laboratories, Kyushu, Japan), 210 mM mannitol (Wako Pure Chemical Industries, Ltd), 70 mM glucose, 0.2 mM EGTA (Dojindo Laboratories) Complete Mini EDTA-free (protease inhibitor cocktail- one tablet for 50 mL of buffer) (Roche Diagnostics Gmbh, Mannheim, Germany) and 0.5 mg/mL digitonin (Wako Pure Chemical Industries, Ltd). Buffer was heated at 60℃ to improve the solubility of digitonin. After the preparation of all buffers, they were placed in a cool (4℃) condition before use.

3.2.1.3. Other reagents

The chemicals and reagents used in this study are acetone (HPLC grade, Wako Pure Chemical Industries, Ltd.), N-methyl mesoporphyrin IX (N-MMP) (Frontier Scientific, Logan, USA), sodium dodecyl sulfate (SDS) (Nacalai Tesque, Inc.), CBB R-250 (Wako Pure Chemical Corporation), acrylamide (Nacalai Tesque, Inc.), sodium nitrite (Wako Pure Chemical Industries, Ltd.), digitonin, myoglobin and hemoglobin (Nacalai Tesque Inc.).

3.2.1.4. Culture of ZnPP-forming bacteria

S. liquefaciens and *Leuconostoc mesenteroides* were grown in a NB broth at 25°C for 24-48 hr. For the separation of culture fluid and bacteria cell, the inoculated broth was centrifuged at 10,000 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd., Tokyo, Japan). Culture fluid was sterilized by passing through a sterile syringe filter (Minisart® syringe filter, 0.2 µm; Sartorius Stedim Biotech). Bacteria cells were diluted in 0.9% NaCl (physiological saline) solution and the desired number of bacteria (2.0×10^6 CFU/mL) was enumerated by a phase-contrast microscopy using a hemocytometer. Freshly prepared bacterial culture and culture fluid were used in the experiment.

3.2.2. Methods

3.2.2.1. ZnPP formation of bacteria in commercial media

For the observation of ZnPP-forming ability of bacteria in a medium, commercial culture media were used for this purpose. NB broth and commercial cooked meat medium (CMM) (Oxoid Ltd., Hampshire, England) were prepared and autoclaved at 121°C for 15 min. ZnPP-forming bacteria were added and incubated anaerobically at 25°C for 7 days. ZnPP formation was observed with or without the addition of myoglobin (0.1 mg/mL).

3.2.2.2. Fractionation of muscle

Porcine aseptic LTL muscle homogenate was prepared as section described in 3.2.1.1. Then the homogenate was centrifuged at 40,000 × g for 20 min at 4°C (himac CR20F, Hitachi Koki, Tokyo, Japan). The supernatant was filtered through a filter paper (No. 2, 90 mm, Toyo Roshi Ltd.), which was sterilized by heating at 180°C for one hr in the heating chamber and used for the filtration of the "the soluble fraction". The precipitate was diluted with sterile ultra-pure water up to the initial volume and then homogenized and centrifuged in the same manner as before for twice to completely remove the water-soluble compounds. The precipitate was homogenized with ultra-pure water up to the initial volume to yield "the insoluble fraction". Moreover, the soluble fraction was dispensed into an ultrafiltration spin column (VIVASPIN 20, 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd.), which was sterilized by keeping it under UV ray overnight inside the clean bench. It was then separated by centrifugation at 8,000 × g for 90 min at 4°C, (himac CR20F, Hitachi Koki) and the filtrate was regarded as "the <10 kDa soluble fraction" and the retentate was diluted with sterile ultra-pure water up to the initial volume and regarded as "the >10 kDa soluble fraction".

3.2.2.3. Separation of mitochondria from the liver

Mitochondria was separated from pork liver according to the method described by Okabe (1996) with slight modifications. Liver homogenate (10%) was prepared by adding 2 g of the liver with 18 mL of MS buffer. Homogenization was performed using glass homogenizer and pestles. During homogenization, the homogenizer was placed in the ice container and gently homogenized the liver tissue for ten times up and down with the pestle. After homogenization, the homogenate was centrifuged at 600 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd.) to remove the nucleus and other organelles as

pellets. Then the supernatant was again centrifuged at 8,000 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd.) and the pellets were collected as mitochondria and diluted as the original volume.

3.2.2.4. Separation of the outer membrane (OM), inner membrane (IM) and mitoplast (MT) from liver mitochondria

The outer membrane (OM), inner membrane (IM) and mitoplast (MT) of mitochondria were separated as described by Nishimura and Yano, (2014) with slight modification. The mitochondria separated in section 3.2.2.3 was taken a pellet and the mitochondria fractions were diluted with MMS buffer. Then intensive mixing was performed using vortex mixture at maximum speed for 15 min to solubilize the OM of mitochondria. OM was separated as supernatant after centrifugation at 10,000 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd.). Thereafter, the pellet containing IM and MT was again diluted with MMS buffer and sonicated for 15 min in a sonication bath (Hitachi Koki). After centrifugation at 100,000 × g for 30 min at 4°C (himac CS120GXII, Hitachi Koki) the pellet and supernatant were recovered as IM and MT, respectively.

3.2.2.5. ZnPP formation model experiment

Meat homogenate was prepared according to section 2.2.4.2. in chapter 2 and then the homogenate was pre-heated to a different temperature (40, 50, 60, 70, and 80°C). The ZnPP-forming bacteria were inoculated and incubated anaerobically at 25°C for 7 days. A similar experiment also performed with ZnPP-forming bacteria and N-MMP. Fluorescence analysis of ZnPP was performed as shown in section 2.2.4.3. The separated meat fractions described in section 3.2.2.2 were applied to the ZnPP formation model experiment according to Table 5, 6 & 7. The mitochondria of liver described in section

Table 5. Experimental design: Effect of different concentration of <10 kDa fraction on ZnPP formation

Group	**<10 kDa (mL)**	>10 kDa (mL)	Insoluble fraction (mL)	Bacteria/ antibiotics (mL)	Ultrapure water (mL)	Total volume (mL)
Control	**0.5**	0.5	0.25	-	0.45	1.7
Antibiotics	**0.5**	0.5	0.25	0.03	0.42	1.7
Bacteria	**0.5**	0.5	0.25	0.15	0.30	1.7
Control	**0.6**	0.5	0.25	-	0.35	1.7
Antibiotics	**0.6**	0.5	0.25	0.03	0.32	1.7
Bacteria	**0.6**	0.5	0.25	0.15	0.20	1.7
Control	**0.7**	0.5	0.25	-	0.25	1.7
Antibiotics	**0.7**	0.5	0.25	0.03	0.22	1.7
Bacteria	**0.7**	0.5	0.25	0.15	0.10	1.7
Control	**0.8**	0.5	0.25	-	0.15	1.7
Antibiotics	**0.8**	0.5	0.25	0.03	0.12	1.7
Bacteria	**0.8**	0.5	0.25	0.15	-	1.7

Table 6. Experimental design: Effect of different concentration of >10 kDa fraction on ZnPP formation

Group	<10 kDa (mL)	**>10 kDa (mL)**	Insoluble fraction (mL)	Bacteria/ antibiotics (mL)	Ultrapure water (mL)	Total volume (mL)
Control	0.5	**0.5**	0.25	-	0.45	1.7
Antibiotics	0.5	**0.5**	0.25	0.03	0.42	1.7
Bacteria	0.5	**0.5**	0.25	0.15	0.30	1.7
Control	0.5	**0.6**	0.25	-	0.35	1.7
Antibiotics	0.5	**0.6**	0.25	0.03	0.32	1.7
Bacteria	0.5	**0.6**	0.25	0.15	0.20	1.7
Control	0.5	**0.7**	0.25	-	0.25	1.7
Antibiotics	0.5	**0.7**	0.25	0.03	0.22	1.7
Bacteria	0.5	**0.7**	0.25	0.15	0.10	1.7
Control	0.5	**0.8**	0.25	-	0.15	1.7
Antibiotics	0.5	**0.8**	0.25	0.03	0.12	1.7
Bacteria	0.5	**0.8**	0.25	0.15	-	1.7

Table 7. Experimental design: Effect of different concentration of insoluble fraction on ZnPP formation

Group	<10 kDa (mL)	>10 kDa (mL)	**Insoluble fraction (mL)**	Bacteria/ antibiotics (mL)	Ultrapure water (mL)	Total volume (mL)
Control	0.5	0.5	**0.25**	-	0.45	1.7
Antibiotics	0.5	0.5	**0.25**	0.03	0.42	1.7
Bacteria	0.5	0.5	**0.25**	0.15	0.30	1.7
Control	0.5	0.5	**0.35**	-	0.35	1.7
Antibiotics	0.5	0.5	**0.35**	0.03	0.32	1.7
Bacteria	0.5	0.5	**0.35**	0.15	0.20	1.7
Control	0.5	0.5	**0.45**	-	0.25	1.7
Antibiotics	0.5	0.5	**0.45**	0.03	0.22	1.7
Bacteria	0.5	0.5	**0.45**	0.15	0.10	1.7
Control	0.5	0.5	**0.55**	-	0.15	1.7
Antibiotics	0.5	0.5	**0.55**	0.03	0.12	1.7
Bacteria	0.5	0.5	**0.55**	0.15	-	1.7

3.2.2.3 were applied to the ZnPP formation model experiment as Table 8. The fractions of mitochondria described in section 3.2.2.4 were added in the ZnPP formation model experiment as Table 9.

3.2.2.6. SDS-PAGE

SDS-PAGE was performed by the conventional method, as described by Laemmli (1970). An equal volume of SDS treatment solution composed of 100 mM Tris-HCl (pH 6.8), 2% SDS, 2% β-mercaptoethanol (β-ME), 40% glycerol and 0.1% bromophenol blue were added to sample. The mixture was heated for 5 min at 100°C and stored at -20°C until use. The gel was composed of 4.5% acrylamide in the stacking gel and 15% acrylamide in the separating gel, both of which contained 0.1% SDS. The completely polymerized gel plate was applied to the electrophoresis apparatus (AE-6530P, ATTO) with SDS-PAGE electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The electrophoresis conditions were 10 mA for 30 min, followed by 20 mA for 90 min. After applying the sample, electrophoresis was done and then stained overnight with the conventional CBB G-250 method. The gel was photographed with the ChemiDoc XRS Plus System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

3.2.2.7. Dot Blotting

The samples were directly added to the PVDF membrane, which was pre-treated with methanol for 5 min. The membrane was blocked with 2% skim milk and then reacted with Anti-FECH, Rabbit-Poly (2,000-fold dilution) (Aviva Systems Biology, San Diego, USA) for 12 hr at 4 ℃. Horse raddish peroxidase-conjugated Anti-IgG (H+L), Rabbit, Goat-Poly (10,000-fold dilution) (KPL, Gaithersburg, USA) was used to probe the corresponding primary antibody for 2 hr at 4 ℃. After reacting with chemiluminescence

Table 8. Experimental design: Effect of different concentration of mitochondria liver fraction on ZnPP formation

Group	Mitochondria fraction (mL)	Soluble fraction (mL)	Bacteria/ antibiotics (mL)	Ultrapure water (mL)	Total volume (mL)
Control	0	1.00	-	0.5	1.5
Antibiotics	0	1.00	0.03	0.47	1.5
Bacteria	0	1.00	0.15	0.35	1.5
Control	0.1	1.00	-	0.4	1.5
Antibiotics	0.1	1.00	0.03	0.37	1.5
Bacteria	0.1	1.00	0.15	0.25	1.5
Control	0.2	1.00	-	0.3	1.5
Antibiotics	0.2	1.00	0.03	0.27	1.5
Bacteria	0.2	1.00	0.15	0.15	1.5
Control	0.3	1.00	-	0.2	1.5
Antibiotics	0.3	1.00	0.03	0.17	1.5
Bacteria	0.3	1.00	0.15	0.05	1.5

Table 9. Experimental design: Effect different concentration of mitochondrial fraction on ZnPP formation

Group	OM/IM/MT (mL)	Soluble fraction (mL)	Bacteria/ antibiotics (mL)	Ultrapure water (mL)	Total volume (mL)
Control	0	1.00	-	0.65	1.65
Antibiotics	0	1.00	0.03	0.62	1.65
Bacteria	0	1.00	0.15	0.5	1.65
Control	0.1	1.00	-	0.55	1.65
Antibiotics	0.1	1.00	0.03	0.52	1.65
Bacteria	0.1	1.00	0.15	0.4	1.65
Control	0.2	1.00	-	0.45	1.65
Antibiotics	0.2	1.00	0.03	0.42	1.65
Bacteria	0.2	1.00	0.15	0.3	1.65
Control	0.3	1.00	-	0.35	1.65
Antibiotics	0.3	1.00	0.03	0.32	1.65
Bacteria	0.3	1.00	0.15	0.2	1.65
Control	0.4	1.00	-	0.25	1.65
Antibiotics	0.4	1.00	0.03	0.22	1.65
Bacteria	0.4	1.00	0.15	0.1	1.65
Control	0.5	1.00	-	0.15	1.65
Antibiotics	0.5	1.00	0.03	0.12	1.65
Bacteria	0.5	1.00	0.15	-	1.65

OM: Outer membrane, IM: Inner membrane and MT: Mitoplast of mitochondria

reagent (Chemi-Lumi One L, Nacalai Tesque, Kyoto, Japan), the proteins were detected with ChemiDoc XRS Plus System (Bio-Rad Laboratories Inc.).

3.2.2.8. Observation of degradability of myoglobin and hemoglobin by ZnPP-forming bacteria

The degradation of myoglobin and hemoglobin was observed by ZnPP-forming bacteria in NB. Separately myoglobin and hemoglobin were added in the broth at a concentration of 0.1 mg/mL and 0.3 mg/mL, respectively and incubated the medium in anaerobic condition at 25°C. Samples were collected everyday and continued up to 7 days. Finally, SDS-PAGE was performed to check the degradability of hemoglobin and myoglobin.

3.2.2.9. Statistical analysis

Data are expressed as means ± standard error. Statistical analyses were performed using Microsoft Excel 2016 (Microsoft corp., Redmond) with Ekuseru-Toukei 2012 (Social Survey Research Information Co. Ltd., Tokyo, Japan) for add-in software. Differences among individuals were evaluated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. $P < 0.05$ was considered statistically significant.

3.3. Results and discussion

3.3.1. ZnPP formation capability of the high ZnPP-forming bacteria in commercial culture medium with myoglobin

The formation of ZnPP was significantly increased in meat when ZnPP-forming bacteria were added. Hence, it is important to clarify how the bacteria contribute to form ZnPP in meat and meat products. Firstly, it was analyzed whether ZnPP-forming bacteria can form ZnPP without meat. In this section, the two commercial media were used to check the ZnPP-forming ability of the bacteria. One is a simple medium and another one is the medium that contains meat as a main component. Moreover, it was suggested that the bright red color in Parma ham is caused by ZnPP in which the iron in the heme of myoglobin has been replaced by zinc (Grossi *et al*., 2014) since the structure of heme and ZnPP are similar. In this context, exogenous uncooked myoglobin (0.1 mg/mL) was also added in the media to check the ZnPP-forming ability of the bacteria from myoglobin.

First, in order to check the ZnPP-forming ability of the bacteria without meat components, the nutrient broth (NB) medium was used as a simple medium, because NB is a universal medium for bacteria of its suitability for almost all kinds of bacterial growth (Atlas & Snyder, 2014). The high ZnPP-forming bacteria *S. liquefaciens* and *Leuconostoc mesenteroides* were inoculated into the medium and incubated for 7 days in anaerobic conditions at 25°C. After extraction with 75% acetone, the fluorescence spectrum was measured. From the result, no ZnPP fluorescence peak at 590 nm was observed in the control and inoculated groups (Fig. 9 A, B & C). A similar experiment was performed with the addition of uncooked myoglobin (0.1 mg/mL) in the medium, and ZnPP

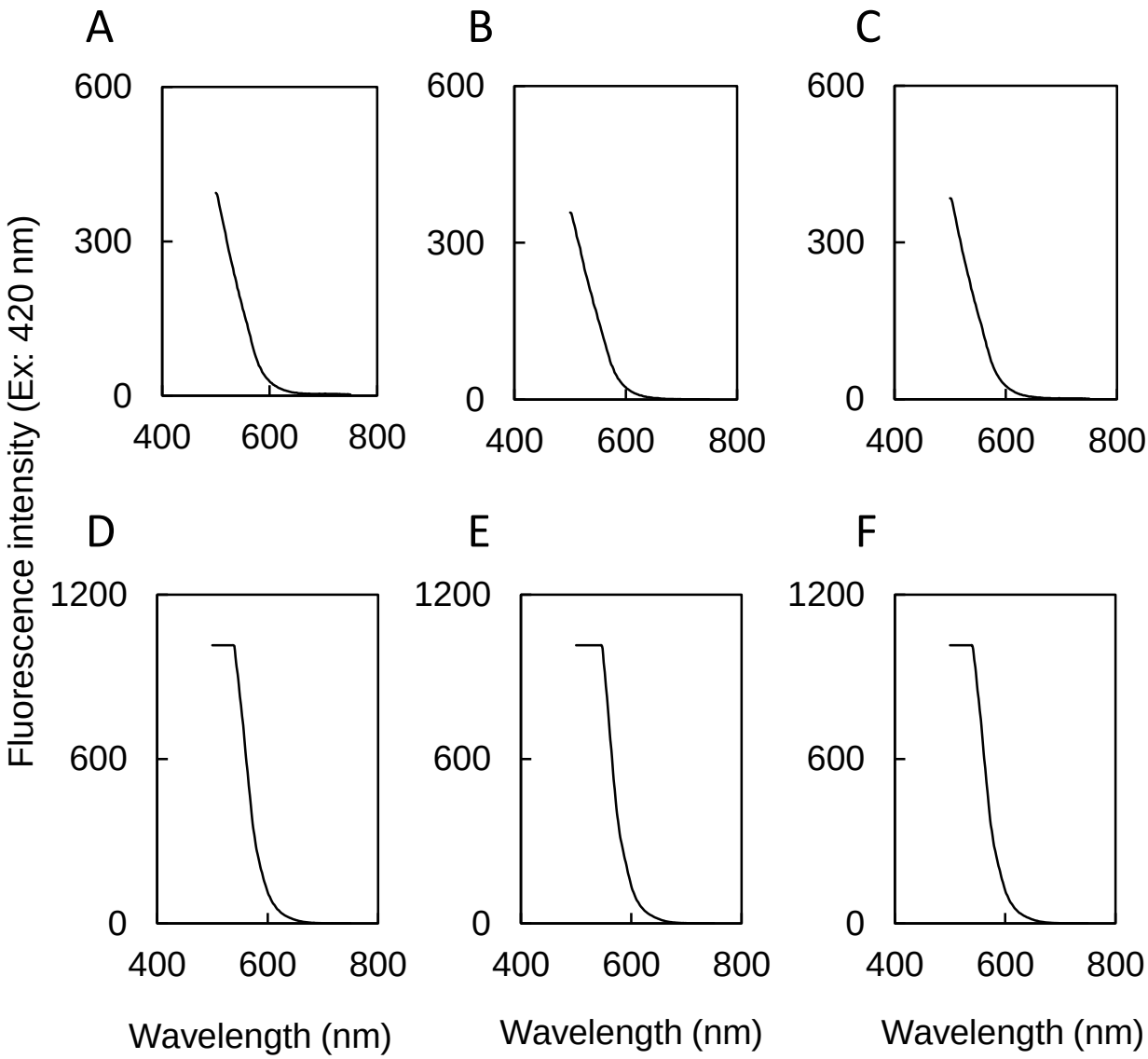


Fig. 9. ZnPP formation by ZnPP-forming bacteria in nutrient broth (NB) medium

NB medium was inoculated with ZnPP-forming bacteria and then incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence spectrum was measured (Ex: 420 nm). A: Control, B: *Serratia liquefaciens*, C: *Leuconostoc mesenteroides*, D: control with 0.1 mg/mL myoglobin, E: *Serratia liquefaciens* with 0.1 mg/mL myoglobin and F: *Leuconostoc mesenteroides* with 0.1 mg/mL myoglobin.

formation was observed. From the result, no fluorescence peak of ZnPP at 90 nm was observed in the inoculated groups as well as the control group (Fig. 9 D, E & F).

Then, cooked meat medium (CMM) was used to check whether raw meat components are essential or not for the bacteria-assisted ZnPP formation. CMM is also a non-selective medium used for the growth of a large spectrum of aerobic and anaerobic bacteria (Atlas & Snyder, 2014). After the inoculation of bacteria into the CMM, the medium was incubated, and ZnPP formation was observed. After extraction of ZnPP from the medium, no ZnPP peak at 590 nm was observed in the inoculated and control groups (Fig. 10 A, B & C). Then uncooked myoglobin (0.1 mg/mL) was added in the CMM as heme source and incubated in the same way. After extraction of ZnPP, no fluorescence peak of ZnPP at 590 nm was observed in the control group and as well as in the inoculated groups (Fig. 10 D, E & F). This result was consistent with the NB medium.

Nevertheless, the bacteria used in the current research can form plenty of ZnPP in meat and meat products as shown in chapter 2, no ZnPP formation was observed in the two types of bacterial growth media irrespective of unheated myoglobin. Akter (2019) reported that heme, PPIX, FECH, and Zn^{2+} are the essential components for ZnPP formation where FECH helps in the removal of Fe^{2+} from heme and finally insertion of Zn^{2+} into PPIX to form ZnPP. In the NB, the particular nutrients were peptone, yeast extract, and glucose present for bacterial growth. The components required for ZnPP formation was not present in the NB, and bacteria could not supplement the components. CMM contains the components present in meat. However, all proteins including the enzymes in the medium were denatured due to autoclaving. Since CMM is prepared from meat, it contains a sufficient amount of Zn. Exogenous myoglobin was added in both media as a heme source, but ZnPP was not observed in any of the media. These results

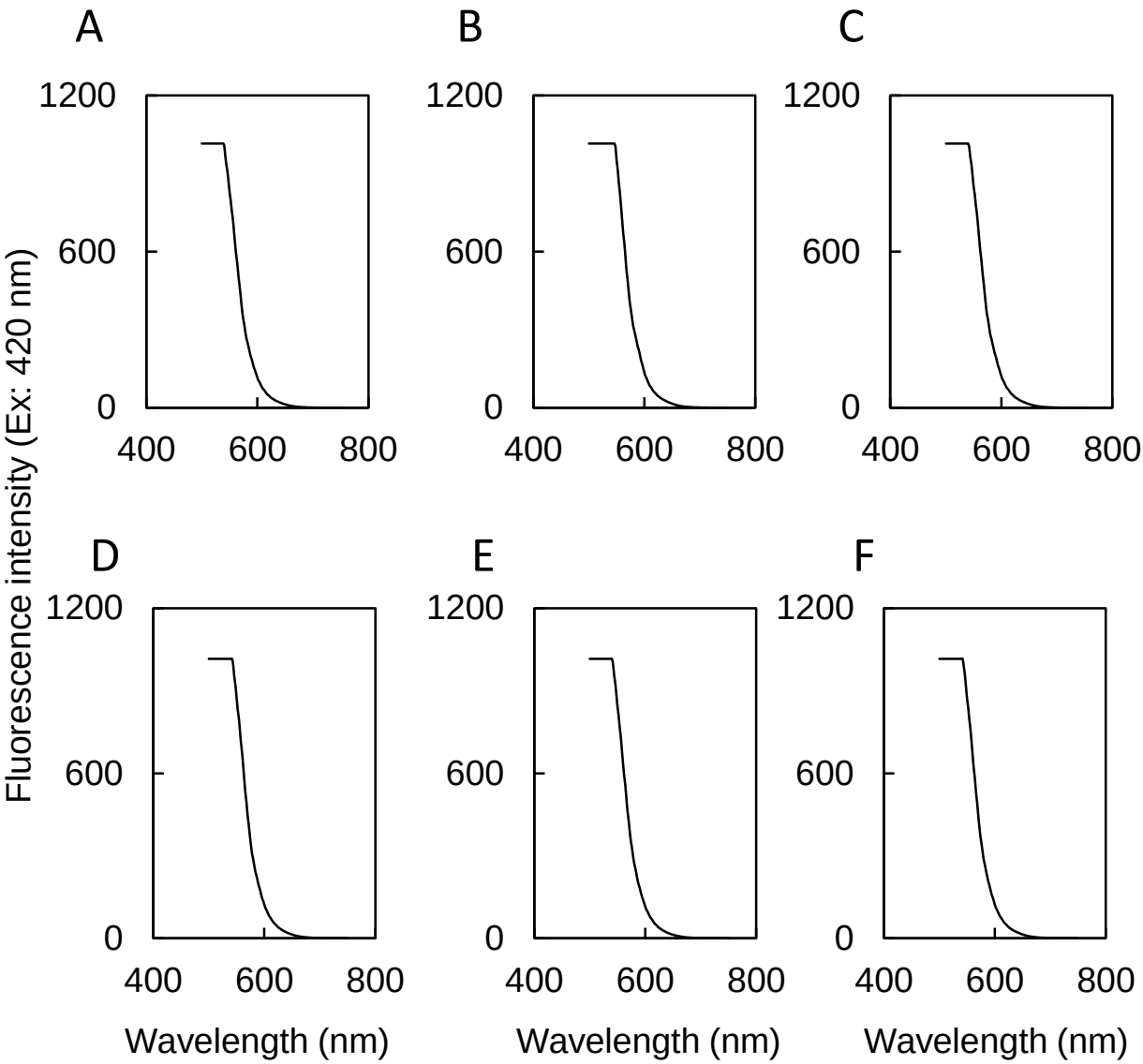


Fig. 10. ZnPP formation by ZnPP-forming bacteria in cooked meat medium (CMM)

CMM was crashed, autoclaved and inoculated with ZnPP-forming bacteria and then was incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and fluorescence spectrum was measured (Ex: 420 nm). A: Control, B: *Serratia liquefaciens*, C: *Leuconostoc mesenteroides*, D: control with 0.1 mg/mL myoglobin, E: *Serratia liquefaciens* with 0.1 mg/mL myoglobin and F: *Leuconostoc mesenteroides* with 0.1 mg/mL myoglobin.

explained that the ZnPP was not formed in the medium due to the absence of some precursors or activated contributors needed for ZnPP formation. In addition, ZnPP-forming bacteria could not form the precursor from myoglobin. Consequently, the present study suggested that raw meat components are essential for ZnPP formation, and ZnPP-forming bacteria itself has no capability to form ZnPP from myoglobin.

3.3.2. Effect of heat, FECH inhibitor and bacterial metabolites on bacteria-assisted ZnPP formation

The formation of ZnPP in meat and meat products depends on several factors. It was reported that FECH was one of the main enzymes that contributed to ZnPP in the meat-inherent mechanism (Chau *et al*., 2011). In fact, N-methyl mesoporphyrin (N-MMP), a potent inhibitor for FECH (Tephly *et al.*, 1979) suppresses the ZnPP formation in meat (Dailey & Fleming, 1983). Moreover, not only meat-inherent FECH but also bacterial FECH is possible to contribute to the formation of ZnPP. In order to clarify the bacteria-assisted ZnPP formation mechanism by separately considering the contribution of meat-inherent and bacterial FECH, the effects of pre-heating temperature of meat and FECH inhibitor (N-MMP) were investigated using the meat homogenate model system with ZnPP-forming bacteria. In addition, the effect of bacteria secreted metabolite on ZnPP formation was also investigated.

3.3.2.1. Effect of heat and FECH inhibitor on bacteria-assisted ZnPP formation

First, in order to examine the heat-stability of the meat derived components in ZnPP formation by bacteria, high ZnPP-forming bacteria were inoculated to the meat homogenate model solution which was pre-heated at different temperature (unheated, 40, 50, 60, 70 and 80°C) and incubated at 25°C for 7 days under anaerobic condition. The

formation of ZnPP in the control and antibiotics groups were considerably decreased at 50°C of pre-heating and then gradually decreased with increasing the pre-heating temperature, and the ZnPP formation was almost suppressed at 70°C or higher temperature (Fig. 11). However, the formation of ZnPP was not suppressed in the both inoculated groups at 50°C of pre-heating. The ZnPP formation in the *S. liquefaciens*-inoculated group was maintained highly at 60°C of pre-heating, whereas the ZnPP formation in the *Leuconostoc mesenteroides*-inoculated group was partially suppressed. Thus, at least two components with different heat susceptibility (40-60 and >60°C) were suggested to be involved in ZnPP formation in meat. High ZnPP-forming bacteria seems to compensate the activity of the endogenous compounds denatured at 50°C.

In order to confirm whether the heat-inactivated component in meat is the FECH, the effect of N-MMP on ZnPP formation was investigated. Since inhibition of ZnPP formation by N-MMP occurred at 5 μM (Akter, 2019) the same concentration was applied to this system. Thus, N-MMP solution was added to the different pre-heated meat homogenate (unheated, 50 and 60°C) and ZnPP-forming bacteria was inoculated. After incubation, ZnPP formation was suppressed in all the N-MMP treated groups compared to the respective N-MMP free treatments (Fig. 12). But the formation of ZnPP in the inoculated groups, especially, *S. liquefaciens,* was significantly higher than that in the control and antibiotics groups within the N-MMP treated group. However, the formation of ZnPP in the pre-heated (up to 60°C) inoculated group was compensated by both ZnPP-forming bacteria. Though FECH is involved in the formation of ZnPP (Chau *et al*., 2011), it is considered that bacterial FECH contributes to the ZnPP formation in the pre-heated inoculated meat. Therefore, it is necessary to have two components in the meat, one deactivates at 50°C and another deactivates at 70°C, and the component that deactivates

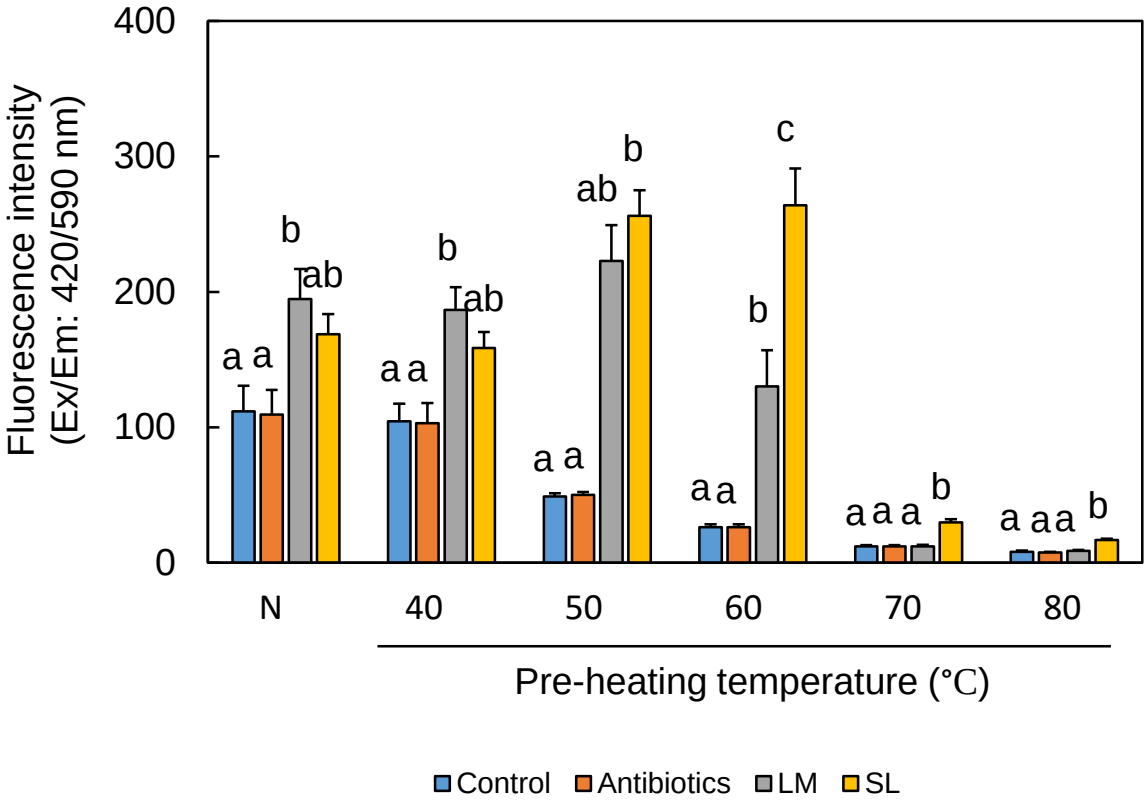


Fig. 11. Effect of pre-heating on ZnPP formation by ZnPP-forming bacteria in the model experiment

LTL muscle homogenate (20%) was prepared and heated with different temperature (40, 50, 60, 70 and 80°C). ZnPP-forming bacteria were added in the different heat treated groups. The samples were incubated at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens*, LM: *Leuconostoc mesenteroides* and N: not heated. Data expressed as means ± SE (n = 3). abc: Values of different columns with different letter differ significantly within the group.

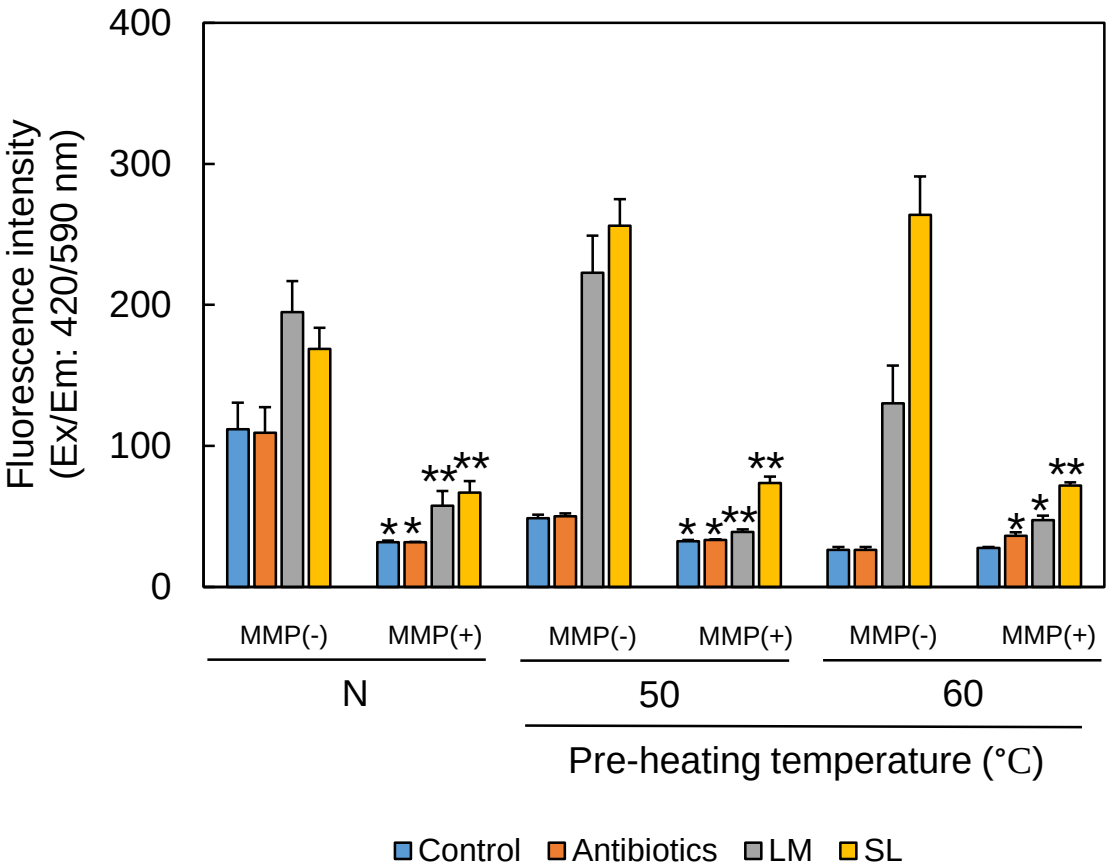


Fig. 12. Effect of FECH inhibitor on ZnPP formation in pre-heated model solution by ZnPP-forming bacteria

After pre-heating (50 and 60°C) of meat homogenate, N-MMP (5 μM) and ZnPP-forming bacteria were added and incubated at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens*, LM: *Leuconostoc mesenteroides* and N: not heated. Data expressed as means ± SE (n = 3). **($P < 0.01$) and *($P < 0.05$) are significantly differed with the corresponding group.

at 50°C was supplemented by the addition of high-ZnPP-forming bacteria, suggesting to be FECH.

3.3.2.2. Effect of bacterial metabolites on bacteria-assisted ZnPP formation

Since N-MMP was not completely suppressed the ZnPP formation of high ZnPP-forming bacteria, it was assumed that factors other than FECH, *i.e.* bacterial metabolites play an important role in the ZnPP formation. In order to observe the effect of bacterial metabolites on ZnPP formation, culture fluid including bacterial metabolites was added in the model experiment and the sample was incubated anaerobically for 7 days at 25°C. After incubation, metabolites from both bacteria significantly increased ZnPP formation compared to the control and antibiotic groups (Fig. 13). However, the ZnPP-forming ability of metabolites collected from *S. liquefaciens* was significantly higher than that of the metabolites collected from *Leuconostoc mesenteroides*.

Though, the ZnPP formation by high ZnPP-forming bacteria in the pre-heated meat homogenate at 50 and 60°C was considerably suppressed by the addition of a FECH inhibitor. N-MMP did not completely suppress the ZnPP formation in the heated inoculated group compared to the non-inoculated group (Fig. 12). These results are agreement with Adamsen *et al*. (2006a); Parolari *et al*. (2008); Taketani & Tokunaga (1981) reported that several compounds such as salt, ascorbic acid, and dithiothreitol could increase the formation of ZnPP to some extent. Bacterial metabolites usually consist of aldehydes, ketones, esters, alcohols, organic acids, amines, and sulfur compounds etc. (Ercolini *et al.*, 2009; Samelis, 2006). Previously, it was reported that exogenous gluconic acid significantly promoted the ZnPP formation in the model solution (Kawazoe, 2013). The metabolic activity of bacteria is species or strain-specific (Doulgeraki *et al*., 2011;

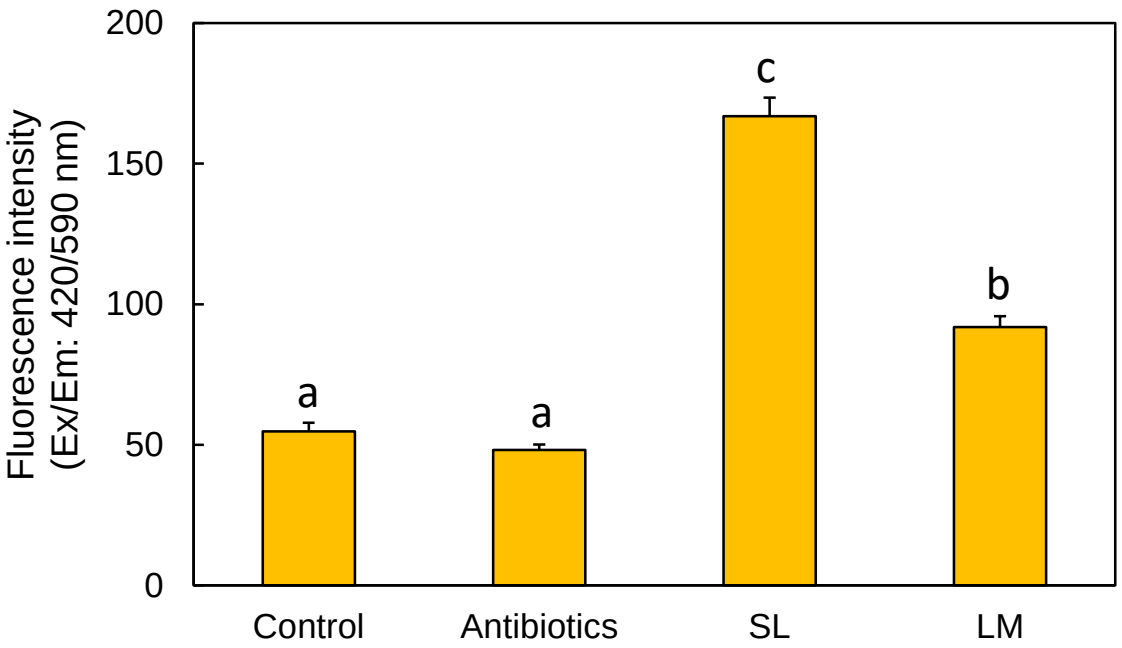


Fig. 13. Effect of culture fluid of ZnPP-forming bacteria on ZnPP formation in the model experiment

The culture fluid of high ZnPP-forming bacteria were added to the meat homogenate and the samples were incubated at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens* and LM: *Leuconostoc mesenteroides.* Data expressed as means ± SE (n = 3). abc: Values of different columns with different letter differ significantly within the group.

Ercolini *et al.*, 2010), and in the current research, *S. liquefaciens* showed more higher metabolic activity than *Leuconostoc mesenteroides.* Therefore, metabolites secreted from high ZnPP-forming bacteria were suggested to have a promotion effect of ZnPP formation. But the promotion effect is likely to be dependent on bacterial species or strains and the metabolites were not identified.

3.3.3. Myoglobin and hemoglobin degradation by ZnPP-forming bacteria

Myoglobin is a major sarcoplasmic heme protein present in the muscle, and hemoglobin is also a heme protein present in the muscle with a minute amount. It was reported that the degradation of myoglobin during muscle salting and ham maturation is necessary for the formation of ZnPP (Grossi *et al*., 2014). Since, there is a possibility to degrade proteins by high ZnPP-forming bacteria and form the precursors of ZnPP. The degradability of myoglobin and hemoglobin by high ZnPP-forming bacteria was investigated using SDS-PAGE.

The degradability of myoglobin and hemoglobin in the NB medium was examined by using the two-representative high ZnPP-forming bacteria *Leuconostoc mesenteroides* and *S. liquefaciens.* Myoglobin and hemoglobin were added separately in the broth (0.1 mg/mL and 0.3 mg/mL, respectively) and after the inoculation of ZnPP-forming bacteria, the medium was incubated for 7 days at 25°C under anaerobic conditions. Samples were collected every day and then the SDS-PAGE was performed. The band thickness of myoglobin in the both inoculated groups was constant throughout the 7-days incubation as well as the non-inoculated control group and no degradation of myoglobin was observed (Fig. 14A & B Arrow). Similarly, there were no change on bands of hemoglobin obtained from SDS-PAGE throughout the incubation (Fig. 15A & B Arrow). Iron is an

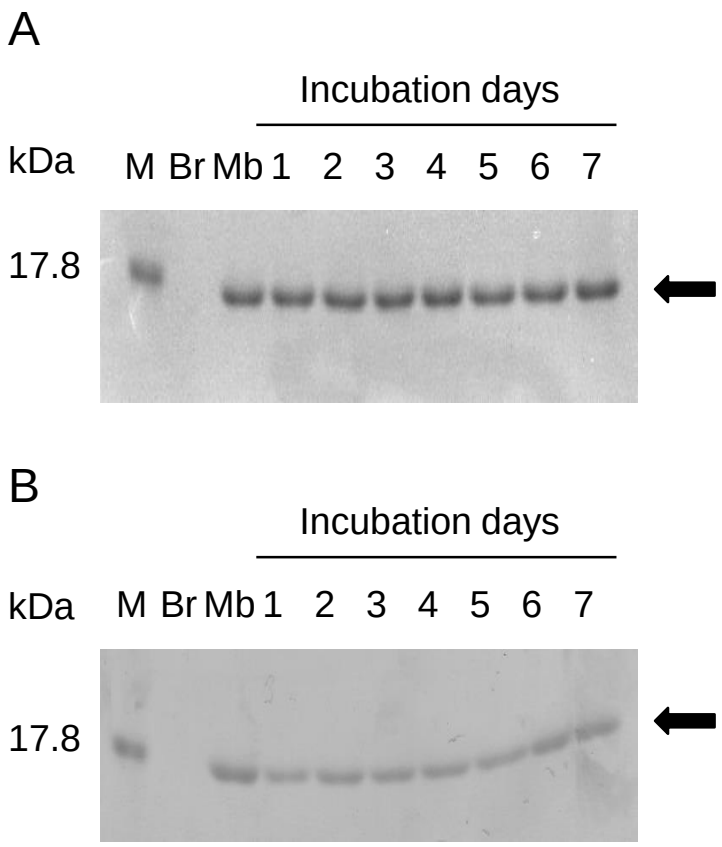


Fig. 14. Degradation of myoglobin in NB medium inoculated with with *Serratia liquefaciens* and *Leuconostoc mesenteroides*

Myoglobin (0.1 mg/mL) was added to NB medium and inoculated with *Serratia liquefaciens* (A) and *Leuconostoc mesenteroides* (B) and incubated anaerobically at 25°C for 1 to 7 days. SDS-PAGE of incubated broth was performed and the gel was stained with Coomassie brilliant blue. Arrows indicate the band of myoglobin, M: marker, Mb: myoglobin added broth without bacteria and Br.: only broth.

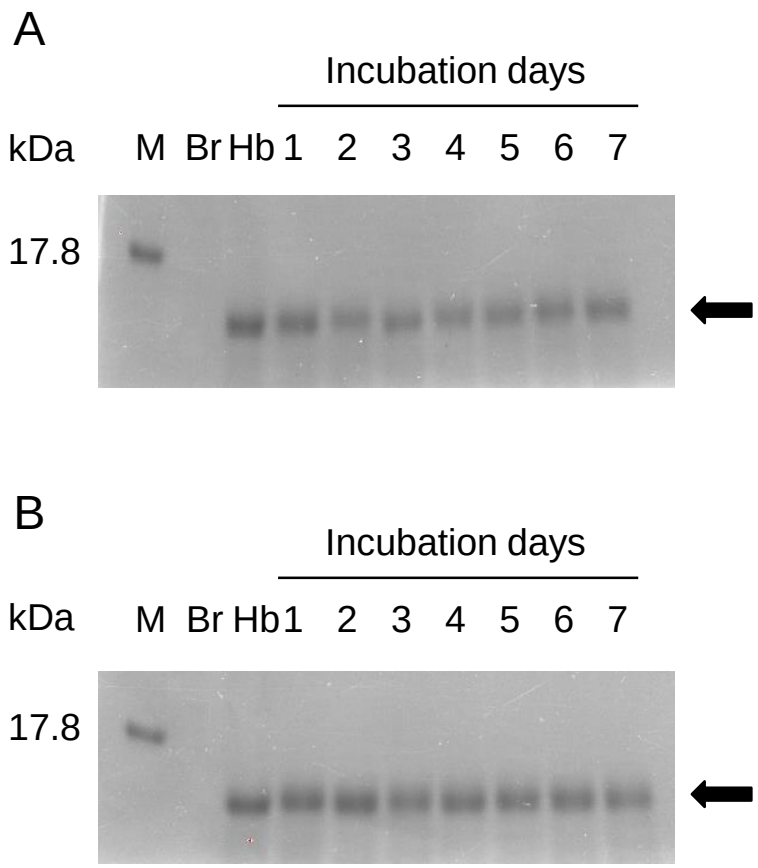


Fig. 15. Degradation of hemoglobin in NB medium inoculated with *Serratia liquefaciens* and *Leuconostoc mesenteroides*

Hemoglobin (0.3 mg/mL) was added to NB medium and inoculated with *Serratia liquefaciens* (A) and *Leuconostoc mesenteroides* (B) and incubated anaerobically at 25°C for 1 to 7 days. SDS-PAGE of incubated broth was performed and the gel was stained with Coomassie brilliant blue. Arrow indicate the band of hemoglobin, M: marker, Hb: hemoglobin added broth without bacteria and Br: only broth.

essential nutrient for the survival of bacteria, and many bacteria utilized iron from myoglobin and hemoglobin for their growth (Contreras *et al*., 2014). However, the bacteria used in this study was not degraded the myoglobin and hemoglobin. Khozroughi *et al*. (2017) also reported that ZnPP was formed by a Fe^{2+}-Zn^{2+} substitution in heme in myoglobin, where accompanying myoglobin degradation was not essential, and other proteins might had been involved in the formation of the ZnPP as well. In conclusion, high ZnPP-forming bacteria do not degrade myoglobin and hemoglobin during incubation, resulting in not assisting the release of heme.

3.3.4. Effect of the different fraction of meat homogenate on ZnPP formation with the association of ZnPP-forming bacteria in the model experiment

As indicated in section 3.3.1, that high ZnPP-forming bacteria itself could not form ZnPP, and raw meat components are necessary to form ZnPP. Hence, it is essential to clarify the individual raw meat components for promoting ZnPP with the high ZnPP-forming bacteria. Akter *et al*. (2019) fractionated the meat homogenate into the < 10 kDa and >10 kDa soluble fractions and the insoluble fraction and confirmed that all three fractions are essential for ZnPP formation. Thus, meat homogenate was fractionated according to the procedures of Akter (2019) and dose-dependent experiments of each fraction of meat homogenate were performed with the high ZnPP-forming bacteria.

First, in order to demonstrate which fraction includes rate-limiting components, different concentrations of the <10 kDa soluble fractions were used along with a constant concentration of the >10 kDa soluble fraction and the insoluble fraction (Table 5). After the addition of high ZnPP-forming bacteria in the inoculated group, the solution was incubated and then ZnPP was measured. It was observed that the <10 kDa fractions

showed no dose-dependence regarding ZnPP formation (Fig. 16). However, the ZnPP in the inoculated groups was higher than that of the non-inoculated groups.

Next, different concentrations of the >10 kDa soluble fractions were used along with the constant concentration of the <10 kDa soluble fractions, and the insoluble fraction (Table 6). After the addition of high ZnPP-forming bacteria in the inoculated group, the solution was incubated and then ZnPP was measured. It was observed that the addition of a higher concentration of the >10 kDa fraction did not affect the ZnPP formation in all the groups (Fig. 17).

Finally, different concentrations of the insoluble fractions were used along with the >10 kDa soluble and the <10 kDa soluble fraction (Table 7). After the addition of high ZnPP-forming bacteria in the inoculated group, the solution was incubated and then ZnPP was measured. As the amount of the insoluble fraction increased in the model experiment, ZnPP formation was correspondingly increased in all the groups (Fig. 18). This result demonstrated that the rate-limiting component for ZnPP formation is present in the insoluble fraction of meat.

Each meat fractions affected ZnPP formation with both ZnPP-forming bacteria. The <10 kDa soluble fractions of pork homogenate are mainly composed of many low molecular weight components such as amino acids, vitamin B_1, pantothenic acid, Zn^{2+}, and Fe^{2+}. Zn^{2+} is considered as an essential component for ZnPP formation. However, Zn^{2+} is not a rate-limiting compound to form ZnPP. Similarly, from the >10 kDa fraction, myoglobin other sarcoplasmic proteins were not influenced by the bacteria on the formation of ZnPP. This result was consistent with section 3.3.1, and section 3.3.3, where myoglobin was not involved in the bacteria-assisted ZnPP formation. On the other hand,

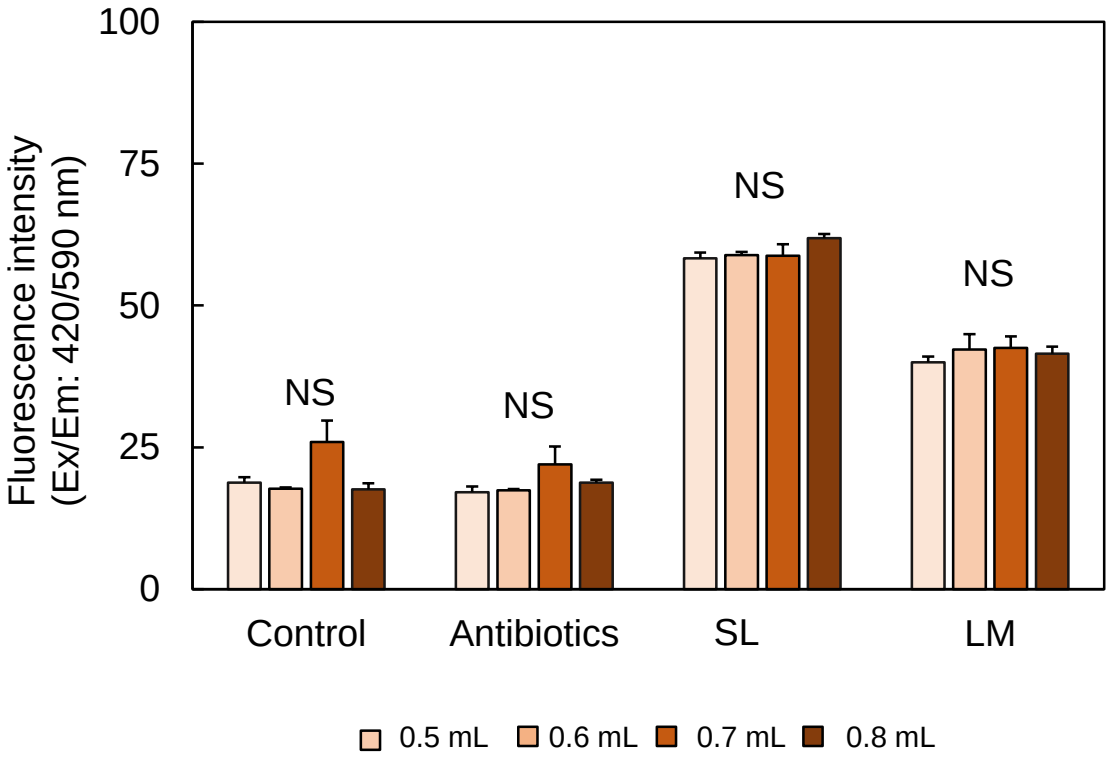


Fig. 16. Effect of different concentrations of <10 kDa fractions on ZnPP formation by ZnPP-forming bacteria in the model experiment

LTL muscle homogenate (20%) was separated into the water-soluble and insoluble fractions. The water-soluble fraction was separated into the >10 and <10 kDa soluble fractions. Different amount (0.5, 0.6, 0.7 and 0.8 mL) of <10 kDa fractions were added with the constant amount of >10 kDa and insoluble fractions in the model experiment. Then ZnPP-forming bacteria were added and the samples were incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens* and LM: *Leuconostoc mesenteroides.* Data expressed as means ± SE (n = 3). NS: Non-significant.

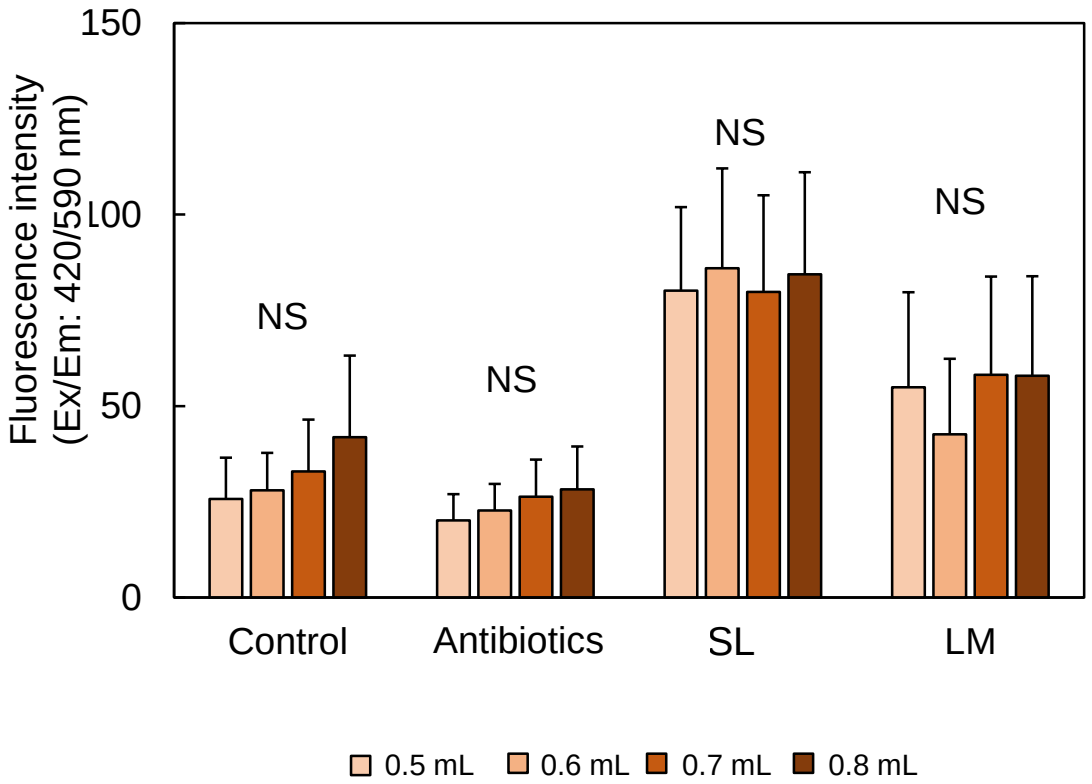


Fig. 17. Effect of different concentrations of >10 kDa fractions on ZnPP formation by ZnPP-forming bacteria in the model experiment

Different amount (0.5, 0.6, 0.7 and 0.8 mL) of >10 kDa fractions as prepared in Fig. 16 were added with the same amount of <10 kDa and insoluble fractions in the model experiment. Then ZnPP forming bacteria were added and the samples were incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens* and LM: *Leuconostoc mesenteroides.* Data expressed as means ± SE (n = 3). NS: Non-significant.

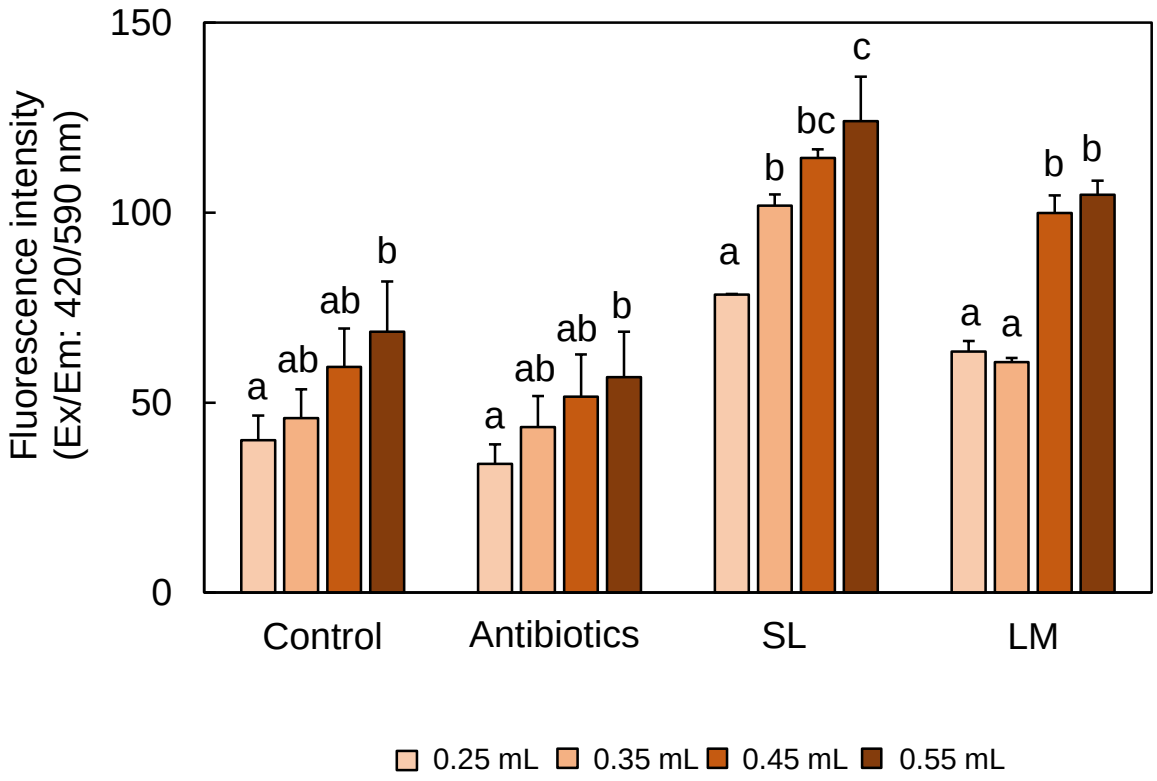


Fig. 18. Effect of different concentrations of insoluble fractions on ZnPP formation by ZnPP-forming bacteria in the model experiment

Different amount (0.25, 0.35, 0.45 and 0.55 mL) of insoluble fractions as prepared in Fig. 17 were added with the same amount of >10 kDa and <10 kDa fractions in the model experiment. Then the ZnPP-forming bacteria were added and the samples were incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens* and LM: *Leuconostoc mesenteroides.* Data expressed as means ± SE (n = 3). abc: Values of different columns with different letter differ significantly within the group.

the insoluble fraction contains mainly myofibril, connective tissue, and organelle, such as the nucleus, mitochondria. Ishikawa *et al*. (2007) suggested that mitochondria have the ability to form ZnPP from oxymyoglobin and are directly related to the release of Fe^{2+} from the porphyrin ring in myoglobin. The FECH enzyme is located in the membranes of mitochondria (Taketani *et al.,* 2007) and helps in the formation of ZnPP. These results indicated that FECH from mitochondria helps ZnPP formation and the formation was greatly increased when bacterial FECH added in the media.

3.3.5. Effect of mitochondria and its membranes on ZnPP formation with high ZnPP-forming bacteria

From section 3.3.4, it was observed that the higher concentration of the insoluble fraction significantly increases the ZnPP formation. The insoluble fraction is a rich source of mitochondria. It was reported that the terminal enzyme for ZnPP formation, FECH, was located in the membranes of mitochondria (Taketani *et al*., 2007). In this section, in order to confirm whether FECH is a rate-limiting factor in the formation of ZnPP with high ZnPP-forming bacteria, the formation of ZnPP with mitochondria and its membranes in association with high ZnPP-forming bacteria were investigated using the model experiment system. Due to the limited availability of mitochondria of the LTL muscle, porcine liver mitochondria was used instead.

Firstly, the effect of mitochondria on bacteria-assisted ZnPP formation was investigated using the ZnPP formation model experiment. In this model experiment, the soluble fraction from the meat homogenate and different concentrations of liver mitochondria were used instead of the insoluble fraction of meat homogenate (Table 8). After the addition of high ZnPP-forming bacteria in the inoculated groups, the model

solutions were incubated for 7 days at 25°C under anaerobic conditions. In all groups, the ZnPP formation was significantly increased as the amount of mitochondria fraction increased. Obviously, the ZnPP formation in the inoculated groups, especially *S. luquefaciens* extremely increased with increasing mitochondria (Fig. 19).

Then, in order to clarify the effect of the mitochondrial membranes on bacteria-assisted ZnPP formation, the outer membrane (OM), inner membrane (IM), and mitoplast (MT) were separated from porcine liver mitochondria and used in the ZnPP formation model experiment in the presence of high ZnPP-forming bacteria. In the model experiment, the soluble fraction of meat homogenate with different concentrations of mitochondrial membrane fractions were used (Table 9). The formation of ZnPP was increased in the OM and IM membrane fractions of mitochondria; as the dose-dependent manner while ZnPP formation was independent on the MT (Fig. 20). Similarly, in the *S. liquefaciens*- and *Leuconostoc mesenteroides*-inoculated groups (Fig. 21 & 22), the formation of ZnPP was significantly increased with increasing the OM and IM; but the ZnPP formation was not influenced by the addition of MT. FECH enzyme localizes in the mitochondrial membrane (Sakaino *et al*., 2009) and is considered to concern the final step of the heme biosynthetic pathway resulting in ZnPP formation (Becker *et al*., 2012; Benedini *et al*., 2008; Parolari *et al*., 2009; Wakamatsu *et al*., 2007b). Next, the distribution of FECH in the mitochondrial fractions was investigated using SDS-PAGE and the dot blotting. The OM, IM and MT of porcine liver mitochondria were separated using SDS-PAGE and the presence of a 50 kDa protein considered as FECH was observed on the SDS-PAGE of OM and IM (Fig. 23A). Moreover, the presence of FECH was confirmed by a dot blot analysis using anti-FECH antibody in both OM and IM of mitochondria (Fig. 23B).

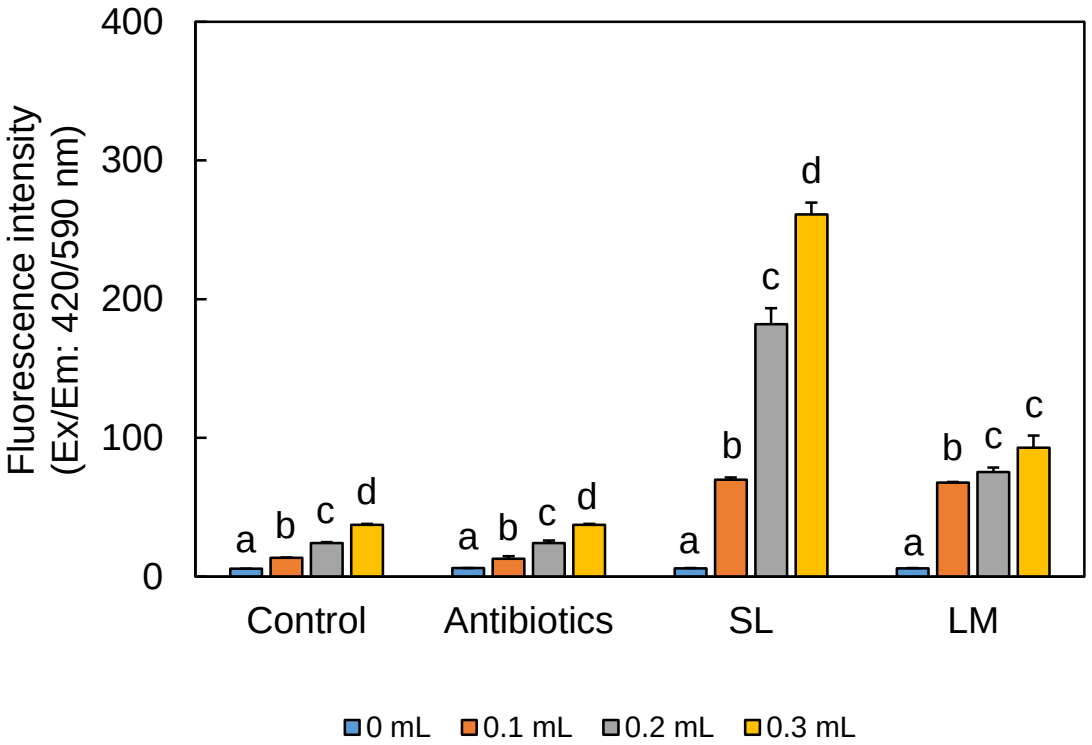


Fig. 19. Effect of different concentrations of mitochondria on ZnPP formation by ZnPP-forming bacteria in the model experiment

Instead of insoluble fractions different amount (0, 0.1, 0.2, and 0.3 mL) of liver mitochondria fractions were added with the soluble fraction of meat homogenate. Then ZnPP-forming bacteria were added and the samples were incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). SL: *Serratia liquefaciens* and LM: *Leuconostoc mesenteroides.* Data expressed as means ± SE (n = 3). abc: Values of different columns with different letter differ significantly within the group.

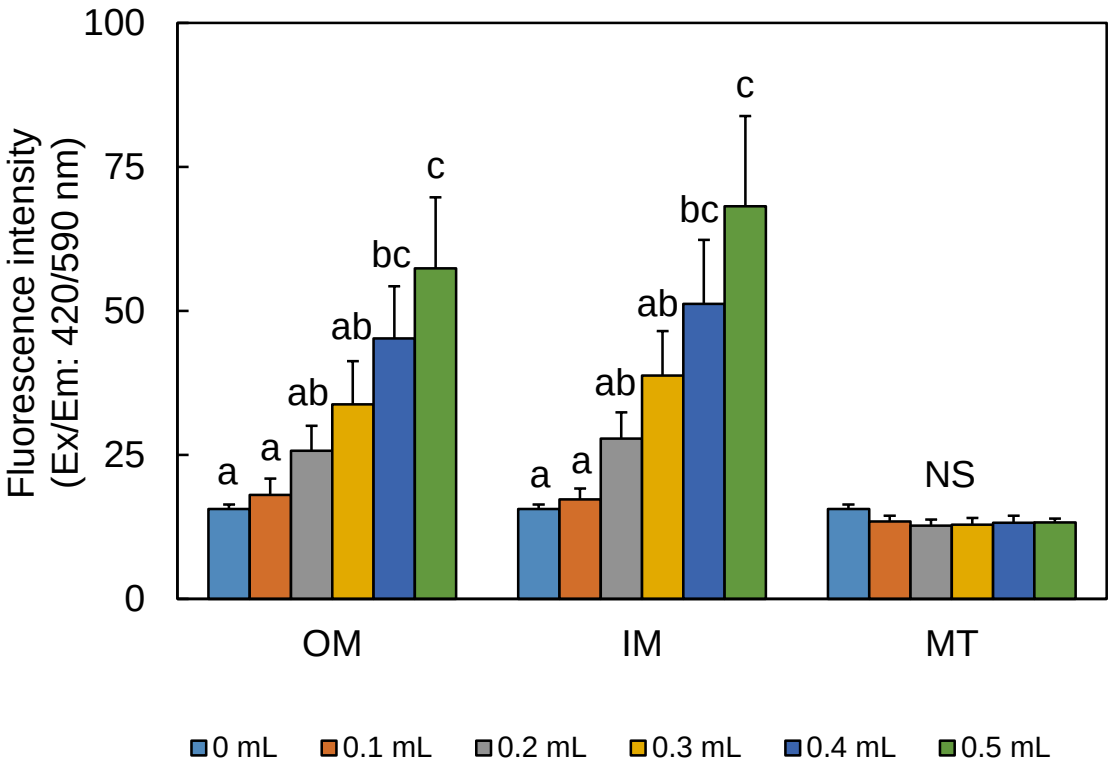


Fig. 20. Effect of different concentrations of mitochondrial fractions on ZnPP formation in the model experiment

Outer membrane (OM), inner membrane (IM) and mitoplast (MT) were separated from porcine liver mitochondria. Different amount (0.1, 0.2, 0.3, 0.4 and 0.5 mL) of mitochondrial fractions were added with the soluble fractions of meat homogenate. Then the samples were incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). Data expressed as means ± SE (n = 3). abc: Values of different columns with different letter differ significantly within the group. NS: Non-significant

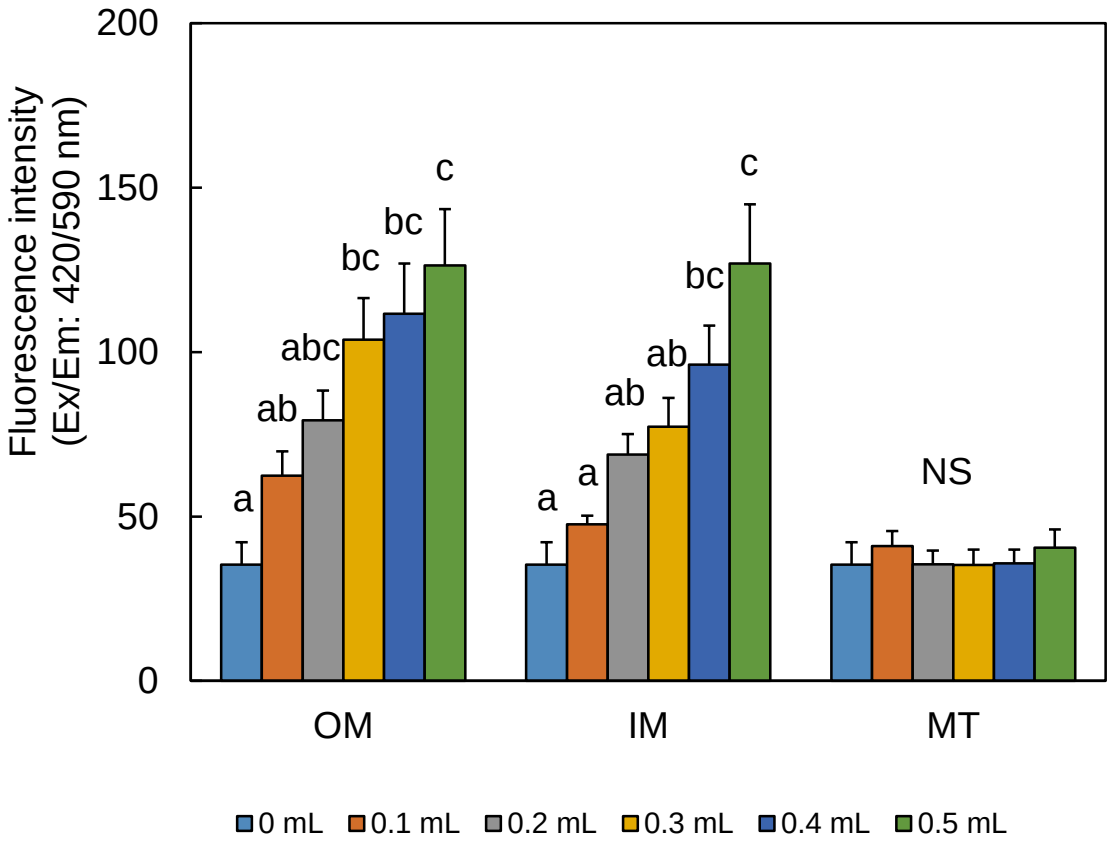


Fig. 21. Effect of different concentrations of mitochondrial fractions on ZnPP formation in the model experiment inoculated with *Serratia liquefaciens*

The sample was prepared as shown in Fig. 20 and after adding *S. liquefaciens* it was incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). OM: Outer membrane, IM: inner membrane and MT: mitoplast of mitochondria. Data expressed as means ± SE (n = 3). abc: Values of different columns with different letter differ significantly within the group. NS: Non-significant.

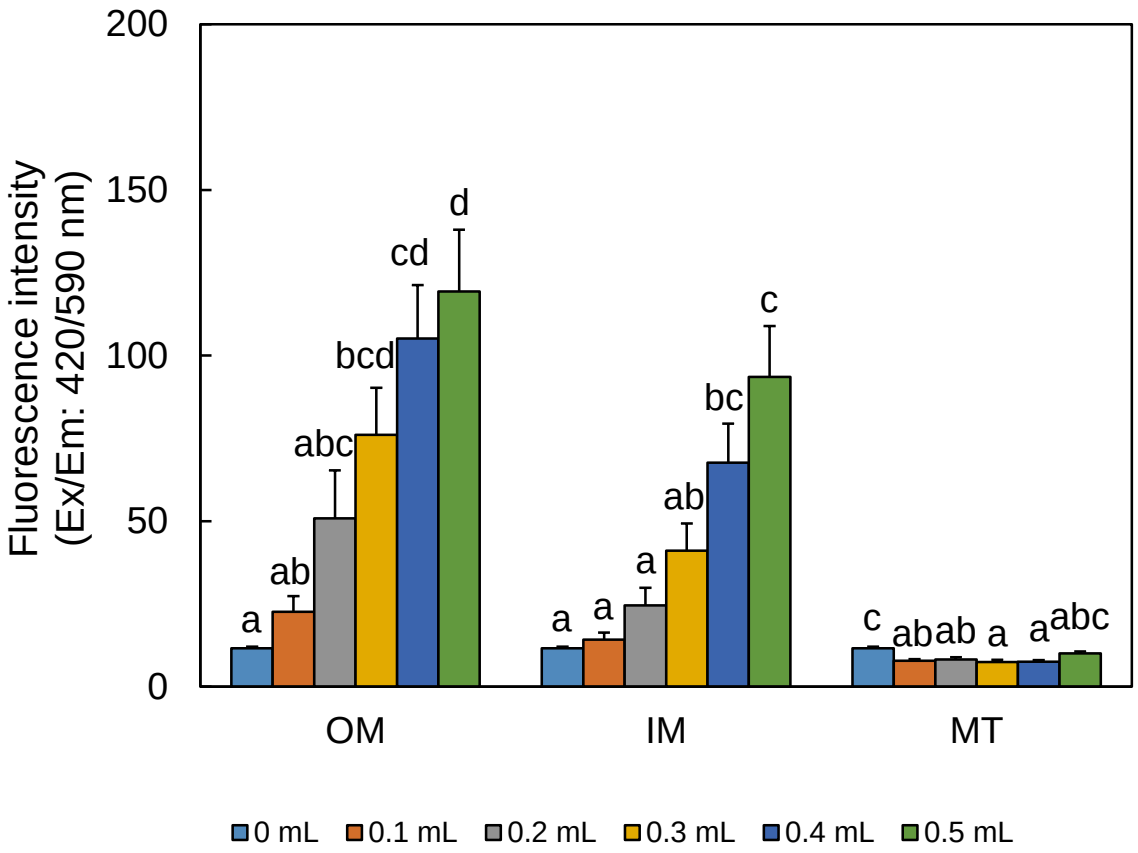


Fig. 22. Effect of different mitochondrial fractions on ZnPP formation in the model experiment inoculated with *Leuconostoc mesenteroides*

The sample was prepared as shown in Fig. 20 and after adding *L. mesenteroides* it was incubated under anaerobic condition at 25°C for 7 days. ZnPP was extracted with 75% acetone and the fluorescence intensity was measured (Ex/Em: 420/590 nm). OM: Outer membrane, IM: inner membrane and MT: mitoplast of mitochondria. Data expressed as means ± SE (n = 3). abcd: Values of different columns with different letter differ significantly within the group.

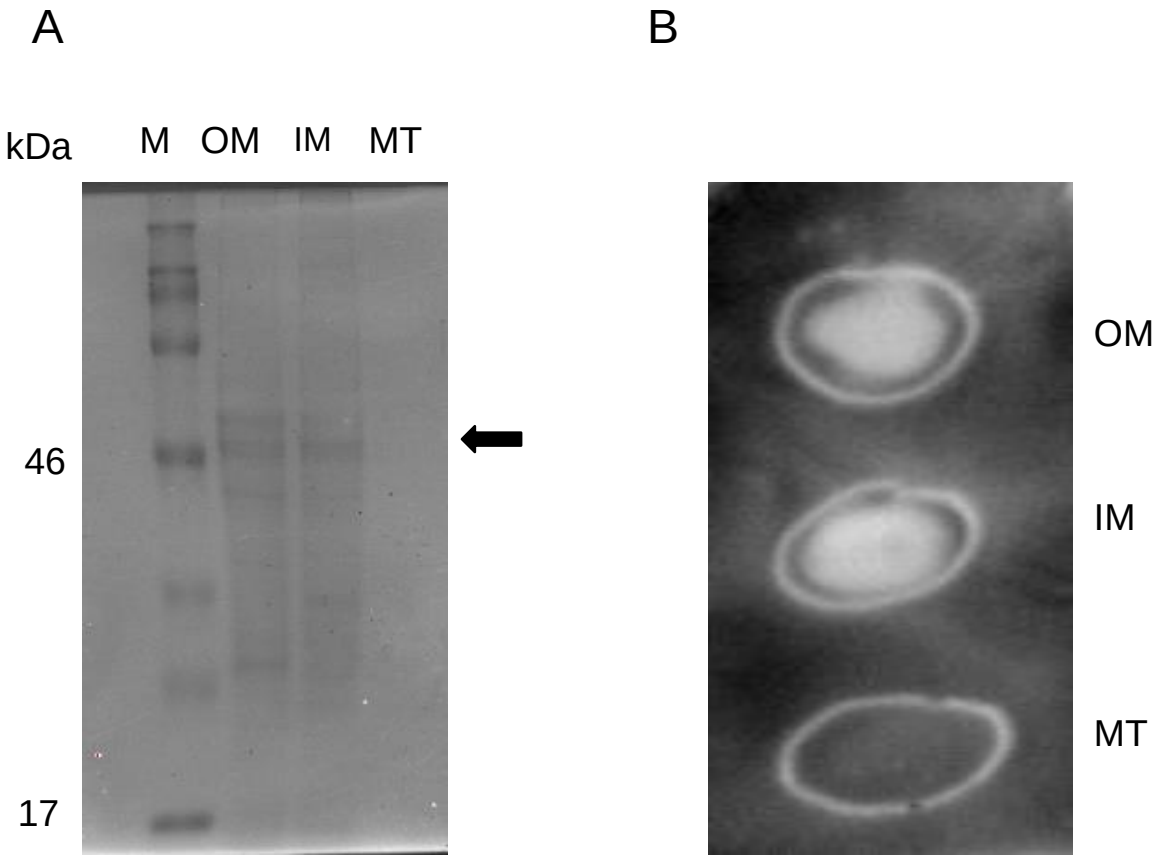


Fig. 23. SDS-PAGE and dot-blotting image of different mitochondrial fractions

(A) SDS-PAGE image of outer membrane (OM), inner membrane (IM) and mitoplast (MT) fraction of pork liver mitochondria was shown. The gel was stained with Coomassie brilliant blue. M: marker

(B) Confirmation of FECH by the dot-blotting using anti-FECH antibody.

Mitochondria is the key component for ZnPP formation in meat. The results of this research confirmed that mitochondrial FECH, which is localized in both IM and OM fraction of mitochondria, contributed to ZnPP formation in meat. FECH is the key enzyme that catalyzes the insertion reaction of Zn^{2+} into PPIX to form ZnPP (Chau *et al*., 2010; Ishikawa *et al*., 2007; Parolari *et al*., 2009). FECH also involved in the Fe^{2+} removal reaction from heme to form PPIX (Chau *et al*., 2011). FECH promoted ZnPP formation in a dose-dependent manner, suggesting that meat-inherent FECH is a rate-limiting enzyme in meat-inherent ZnPP formation. These results suggested that the supplementation of bacterial FECH to meat inherent FECH are responsible for higher ZnPP formation with the addition of high ZnPP-forming bacteria and the formation of ZnPP is dependent upon the concentration of FECH.

3.4. Proposed mechanism of the bacteria-assisted ZnPP formation

The mechanism of ZnPP formation by ZnPP-forming bacteria in meat and meat products was investigated in this study. Together with the obtained results and the previous reports, the mechanism of ZnPP formation by which the high ZnPP-forming bacteria is formed in meat and meat products is proposed in Fig. 24. In the formation of ZnPP, the precursor is derived from meat. The main meat components responsible for ZnPP formation is likely to be myoglobin and hemoglobin and heme is released after degradation of the components, whereas hemoglobin and myoglobin were not degraded by the high ZnPP-forming bacteria. Therefore, heme is derived from the meat mechanism; however, the contribution of the bacteria in heme synthesis is still unknown. The next step is the formation of PPIX from heme by the action of FECH. From his study, it was indicated that meat meat-inherent FECH is a rate-limiting enzyme of ZnPP formation. Thus, bacterial FECH and meat-inherent FECH cooperatively release PPIX from heme.

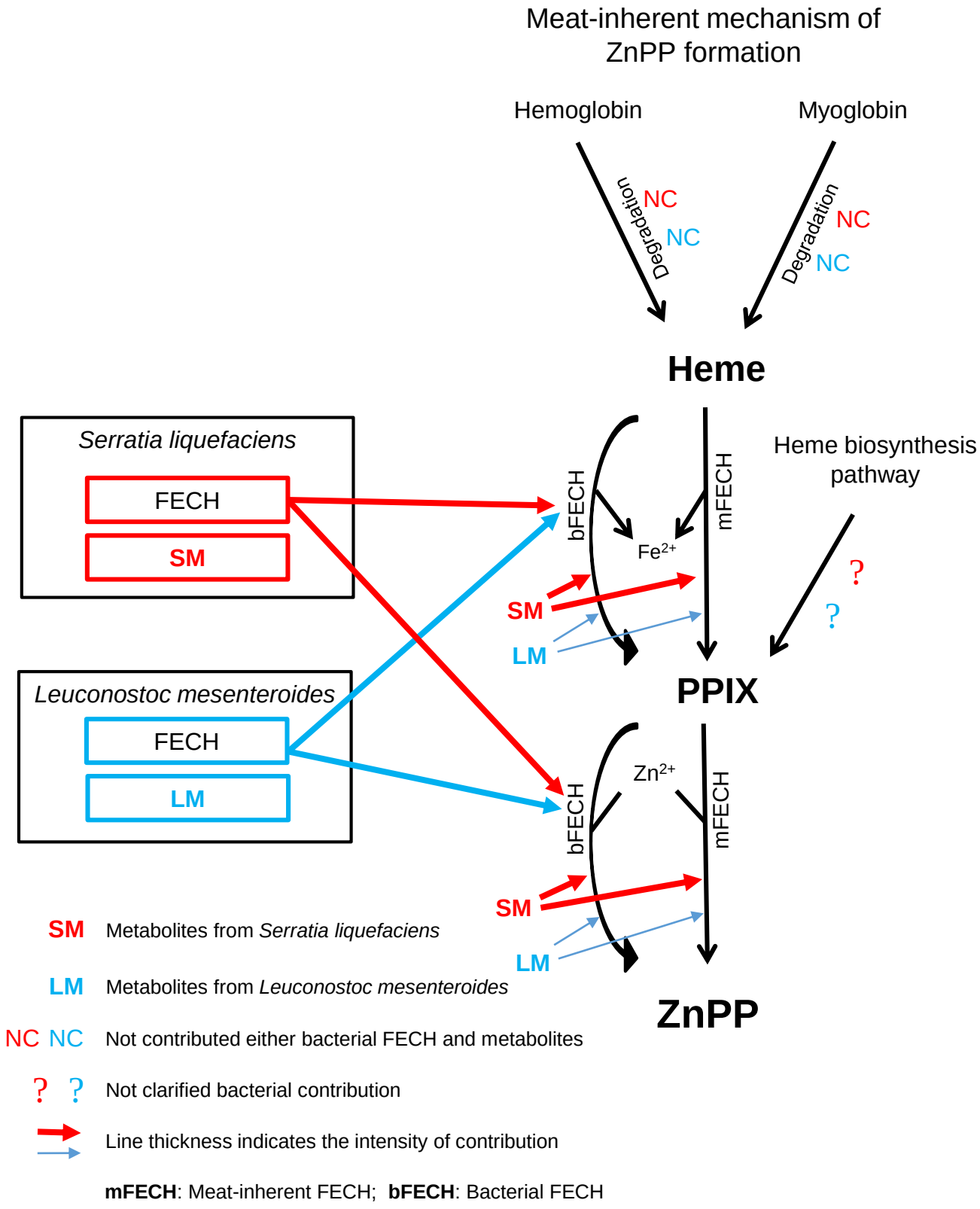


Fig. 24. Proposed mechanism of the bacteria-assisted ZnPP formation

The final step for ZnPP formation is the insertion of Zn^{2+} into PPIX and form ZnPP. Meat is an abundant source of Zn^{2+} (Hazell, 1982); therefore, it derives from meat. In the Zn^{2+} insertion process, both bacterial and meat-inherent FECH cooperatively form ZnPP. Therefore, in this mechanism, high ZnPP-forming bacteria acts as FECH supplier and promote the formation of ZnPP. In this research, the two high ZnPP-forming bacteria have different capabilities of ZnPP formation due to the activity of their metabolites. The metabolites of *S. liquefaciens* has shown higher ZnPP-forming ability than that of those metabolites of *Leuconostoc mesenteroides.* Therefore, ZnPP-forming ability of *S. liquefaciens* was suggested to be higher than that of *Leuconostoc mesenteroides* due to their secreted metabolites. Moreover, another meat-inherent component that was destroyed at 70°C could not be supplemented by both bacteria but it was essential for ZnPP formation in meat. This component is assumed to be a meat-inherent contributor other than FECH but it could not be clarified in this study. Thus, further study is needed to clarify the other contributors or other possible route for the elucidation of bacteria-assisted ZnPP formation mechanism.

Chapter 4

General discussion

In order to isolate and identify bacteria for a particular purpose, a suitable screening method is essential. In this study, the screening method used for the isolation of high-ZnPP-forming bacteria was the "meat homogenate model system". This method is suitable for the identification of high ZnPP-forming bacteria. However, meat is a complex medium and also easy to contaminate with bacteria or other microorganisms. It is challenging to maintain the sterility of meat components because meat products are not processed or preserved in a sterile condition. Therefore, an easy and suitable screening method will be helpful to find more suitable ZnPP-forming bacteria.

In this research, the formation of ZnPP was found to be a strain-specific. The formation of ZnPP in meat products by bacteria might depend on the metabolites secreted by the bacteria. Bacterial metabolites are the products of low molecular weight compound**,** necessary for the cell's or body metabolism processes, usually consist of aldehydes, ketones, esters, alcohols, organic acids, amines, and sulfur compounds etc. (Ercolini *et al.*, 2009; Samelis, 2006). It was also reported that incubation of FECH with myoglobin in the presence of ascorbic acid and cysteine resulted in the efficient conversion of myoglobin-heme to ZnPP (Chau *et al*., 2011). In this research, the metabolic activity on ZnPP formation was different for *Serratia liquefaciens* and *Leuconostoc mesenteroides*. The metabolites produced by the bacteria might be different from each other and their ZnPP-forming ability may also be different. Therefore, a detailed study of bacterial metabolites is helpful for not only the elucidation of bacterial-assisted ZnPP formation mechanism but also the development of non-bacterial ZnPP formation techniques.

The bacteria identified in this study improved only the internal color of sausages, which implies these bacteria can form ZnPP only under anaerobic conditions. The formation of ZnPP is generally dependent on several factors such as pH, temperature, environmental conditions, nitrite etc. (Akter, 2019). It was reported that ZnPP formation was higher under anaerobic than aerobic conditions (Wakamatsu *et al*., 2019). For this reason, the external color of the sausages was a dull brown. Therefore, the aerobic formation of ZnPP is important to improve the surface color of dry-cured meat products. Hence, it is necessary to search for other suitable bacteria that can form ZnPP both in aerobic and anaerobic environments.

In the present study, the prepared sausages were simple and spices or condiments were not used. However, different kinds of spices and condiments (cinnamon, ginger, onion, garlic, cloves, yellow mustard, sesame, black pepper, allspice, paprika etc.) are mostly used in commercial sausages as flavorings and coloring agents. (Lücke, 1997). Spices and condiments are also contributing to other substances, such as sugars, nitrates, and metallic ions into the cured meat products (Aguirrezábal *et al*., 1998). A variety of spices added to meat have been observed to accelerate lactic acid production by the lactic acid bacterial starter culture (Kivanç *et al*., 1991; Nes & Skjelkvåle, 1982). Accordingly, the use of ZnPP-forming bacteria in combination with spices and condiments might improve sausage color and food safety synergistically.

Microorganisms have been used as starter cultures to manufacture dry-cured meat products exhibiting consistently high quality. In the production of dry-cured meat products, the main role of bacteria is the acidification of mixture realized through the production of lactic acid that occurs in order to inhibit the growth and production of toxins by undesirable microorganisms (Ammor & Mayo, 2007). Nowadays, the consumer pays

much attention to the relation between food and health. Prior to using bacteria in any meat products, it is necessary to confirm that if the strain of interest possesses protective characteristics against foodborne pathogens, especially *Clostridium* sp, which is commonly identified from raw or fermented meat products (Akhtar *et al*., 2009; Linton *et al*., 2014). Accordingly, high ZnPP-forming bacteria should have protective characteristics against foodborne pathogens besides color improving ability. The isolated bacteria in this study were less suitable in this regard. Thus, other suitable ZnPP-forming bacteria can be more prospective as a starter culture for improving the color and other quality characteristics of dry-cured meat products.

In conclusion, ZnPP is useful to improve the color of dry-cured meat products as an alternative use of nitrite. High ZnPP-forming bacteria could be the alternative of nitrite for the improvement of color in cured meat products. In this research, high ZnPP-forming bacteria were screened, which can improve the internal bright red color of meat products by forming ZnPP. In contrast, improvement of surface color by ZnPP-forming bacteria was suspicious and hence the further studies are is needed to emphasize the screening of potential bacteria having the ability to form ZnPP on the surface of the dry-cured meat products. This study confirmed that each particular bacteria has a different ZnPP formation mechanism, and their ZnPP-forming ability also different. Finally, it is expected that the problem of nitrite/nitrate about the food safety of the dry-cured meat products will be mitigated by using high ZnPP-forming bacteria as a starter culture.

Chapter 5

Abstract

Nitrite/nitrate has been added to improve the color of meat products, but it is pointed out that they could cause a carcinogenic effect. On the contrary, ZnPP is a main natural red pigment found in dry-cured meat products without nitrate/nitrite. Since some bacteria can stimulate to form ZnPP, they might be contributed to improve the color of meat products without nitrate/nitrite. It was hypothesized that some edible bacteria might be useful to improve the color of meat products by producing ZnPP. Moreover, it was also hypothesized that high ZnPP-forming bacteria promoted the endogenous ZnPP formation in meat. Thus, this study was undertaken to search for edible bacteria that can stimulate ZnPP production in meat products and also to clarify the formation mechanism of ZnPP by bacteria in meat products.

Bacterial isolates were searched from various sources and screened with modified standard plate count agar containing 3% salt and at pH 5.5 under anaerobic conditions. Total 126 isolates were assessed in the aseptic meat homogenate model system we established to check their ZnPP-forming ability. Only three edible bacteria *Lactococcus lactis, Leuconostoc mesenteroides*, and *Enterococcus faecium* were identified from the 44 high ZnPP-forming isolates with 16S rRNA partial gene sequencing. Then high ZnPP-forming edible bacteria were inoculated in aseptic salted minced meat, after incubation a brighter red color and a higher amount of ZnPP was observed in the inoculated groups than non-inoculated control. Furthermore, after heating, the color of inoculated minced meat was persisted to a degree. Finally, dry-cured sausages were prepared by using these bacteria to check their ZnPP-forming ability meat products. After ripening, a higher

amount of ZnPP was observed in the inoculated sausages than that of the control group and the color was almost similar to the nitrite group. Since these bacteria were proven to increase the microbiological safety by the suppression of pathogenic bacteria, it could be concluded that the screened bacteria are promising candidates as multi-functional starter cultures to improve the color of dry-cured sausage by forming ZnPP.

Regarding ZnPP formation mechanism by bacteria, initially, the ZnPP-forming ability of the bacteria was observed in commercial media in the presence of myoglobin, and meat components responsible for ZnPP formation. Next, the ZnPP-forming ability of the bacteria was checked in pre-heated meat and two components which deactivate at 50 and 70°C respectively are necessary for ZnPP formation, and the component that deactivates at 50°C was supplemented by ZnPP-forming bacteria. The ferrochelatase (FECH) activity of bacteria was confirmed by the FECH inhibitor in pre-heated meat and the bacterial metabolites also have the ability to form ZnPP. Moreover, ZnPP-forming bacteria did not degrade myoglobin and hemoglobin. When the meat homogenate was fractionated, only the FECH-containing fraction increased the ZnPP formation in a dose-dependent manner, and FECH was localized in the inner and outer membrane of mitochondria. It was clarified that not only FECH from bacteria but also their metabolites assist ZnPP formation in the meat-inherent mechanism.

The present study confirmed that *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *E. faecium* as high ZnPP-forming bacteria capable of forming ZnPP in dry-cured sausages and improves the color of sausage. High ZnPP-forming bacteria can facilitate the ZnPP formation in meat as FECH suppliers but cannot produce ZnPP by themselves.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Jun-ichi Wakamatsu for his invaluable advice, guidance, enthusiastic encouragement and useful critiques to complete this research work and writing of this dissertation. This dissertation would not have been possible without his massive contribution and supervision.

Besides my supervisor, I would also like to sincerely thank my co-supervisors Prof. Dr. Haruto Kumura, Prof. Dr. Masashi Takahashi and Assist. Prof. Dr. Toru Hayakawa, for their valuable suggestions and encouragement during my research work and finally, for the successful preparation of this dissertation.

I extend my sincere thanks to the staff of Field Science Centre for Northern Biosphere, Hokkaido University, for their friendly help during my research student period. I am thankful to the technical staff of the Agri-food Center (AFC), Mr. Shoji Hioki and Mr. Yu Toba for helping me for the preparation of sausages of my research.

I would like to thank Ms. Akiko Tanaka, Ms. Noriko Horibe and Ms. Yoko Hasegawa for their kind support in dealing with various official matters during the whole study period. I also wish to acknowledge the help provided by staff members in the Graduate School of Agriculture at Hokkaido University. My special thanks to Hokkaido University Agricultural Alumni Association for funding me in the participation of 65th ICoMST-2019 at Potsdam, Germany.

I am thankful to all the former and current students of the Laboratory of Applied Food Science, Graduate School of Agriculture, Hokkaido University, for their kindness, support, and friendship during my study in Japan.

I would like to thank my parents for their unconditional love and support throughout my life. My insightful and innumerable thanks go to my beloved wife, Dr. Mofassara Akter, for her mental support, patience and inspiration throughout my PhD study. I am also grateful to my daughter, Ibteda Ayman Pranti, who sacrifices a lot for the successful completion of my PhD study.

Finally, I would like to thank the Hokkaido University Frontier Fund Clark Scholarship, Kyoritsu International Foundation and Bangabandhu Science & Technology Fellowship Trust of Bangladesh for the scholarship, which supported my stay at Hokkaido University.

References

Acton, J. C., & Dick, R. L. (1976). A research note: composition of some commercial dry sausages. *Journal of Food Science*, 41, 971-972.

Adamsen, C. E., Møller, J. K. S., Hismani, R., & Skibsted, L. H. (2004). Thermal and photochemical degradation of myoglobin pigments in relation to color stability of sliced dry-cured Parma ham and sliced dry-cured ham produced with nitrite salt. *European Food Research and Technology*, 218(5), 403-409.

Adamsen, C. E., Moller, J. K. S., Laursen, K., Olsen, K., & Skibsted, L. H. (2006a). Zn-porphyrin formation in cured meat products. Effect of added salt and nitrite. *Meat Science*, 72(4), 672-679.

Adamsen, C. E., Møller, J. K. S., Parolari, G., Gabba, L., & Skibsted, L. H. (2006b). Changes in Zn-porphyrin and proteinous pigments in Italian dry-cured ham during processing and maturation. *Meat Science*, 74(2), 373-379.

Aguirrezábal, M., Mateo, J., Domínguez, C., & Zumalacárregui, J. M. (1998). Spanish paprika and garlic as sources of compounds of technological interest for the production of dry fermented sausages. *Science des Aliment*, 18(4), 409-414.

Ajioka, R. S., Phillips, J. D., & Kushner, J. P. (2006). Biosynthesis of heme in mammals. *Biochimica* and *Biophysica Acta – Molecular and Cell Biology of Lipids*, 1763(7), 723-736.

Akhtar, S., Paredes-Sabja, D., Torres, J. A., & Sarker, M. R. (2009). Strategy to inactivate *Clostridium perfringens* spores in meat products. *Food Microbiology*, 26(3), 272-277.

Akter, M. (2019). Study on the ZnPP formation mechanism in two different optimum pH at 4.75 and 5.5 in pork. Faculty of Agriculture, Hokkaido University, Doctoral Thesis.

Akter, M., Shiraishi, A., Kumura, H., Hayakawa, T., & Wakamatsu, J. (2019). Investigation of contributors to zinc protoporphyrin IX formation at optimum pH 5.5 in pork. *Animal Science Journal*, 90(6), 774-780.

Ammor, M. S., & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, 76(1), 138-146.

Arief, I. I., Wulandari, Z., Aditia, E. L., Baihaqi, M., Noraimah, & Hendrawan. (2014). Physicochemical and microbiological properties of fermented lamb sausages using probiotic *Lactobacillus plantarum* IIA-2C12 as starter culture. *Procedia Environmental Sciences*, 20, 352-356.

Arihara, K., Kushida, H., Kondo, Y., Itoh, M., Luchansky, J. B., & Cassens, R. G. (1993). Conversion of metmyoglobin to bright red myoglobin derivatives by *Chromobacterium violaceum*, *Kurthia* sp., and *Lactobacillus fermentum* JCM1173. *Journal of Food Science*, 58(1), 38-42.

Arihara, K., Cassens, R. G., & Luchansky, J. B. (1994). Metmyoglobinreduktion durch Enterokokken. *Fleischwirtschaft*, 74, 1249-1250.

Atlas, R. M., & Snyder, J. W., (2014). Handbook of media for clinical and public health microbiology. CRC Press. Taylor & Francis Group, LLC.

Becker, E. M., Westermann, S., Hansson, M., & Skibsted, L. H. (2012). Parallel enzymatic and non-enzymatic formation of zinc protoporphyrin IX in pork. *Food Chemistry*, 130(4), 832-840.

Bedale, W., Sindelar, J. J., & Milkowski, A. L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, 120, 85-92.

Benedini, R., Raja, V., & Parolari, G. (2008). Zinc-protoporphyrin IX promoting activity in pork muscle. *LWT - Food Science and Technology*, 41(7), 1160-1166.

Berleur, M. P., & Cordier, S. (1995). The role of chemical, physical, or viral exposures and health factors in neurocarcinogenesis: implications for epidemiologic studies of brain tumors. *Cancer Causes Control*, 6(3), 240-256.

Bomdespacho, L. Q., Cavallini, D. C. U., Zavarizi, A. C. M., Pinto, R. A., & Rossi, E. A. (2014). Evaluation of the use of probiotic acid lactic bacteria in the development of chicken hamburger. *International Food Research Journal*, 21(3), 965-972.

Bou, R., Llauger, M., Arnau, J., & Fulladosa, E. (2018). Zinc-protoporphyrin content in commercial Parma hams is affected by proteolysis index and marbling. *Meat Science*, 139, 192-200.

Casaburi, A., Dimonaco, R., Cavella, S., Toldra, F., Ercolini, D., & Villani, F. (2008). Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits. *Food Microbiology*, 25(2), 335-347.

Casquete, R., Benito, M. J., Martín, A., Ruiz-Moyano, S., Aranda, E., & Córdoba, M. G. (2012). Microbiological quality of salchichón and chorizo, traditional Iberian dry-fermented sausages from two different industries, inoculated with autochthonous starter cultures. *Food Control*, 24(1-2), 191-198.

Cavanagh, D., Fitzgerald, G. F., & McAuliffe, O. (2015). From field to fermentation: The origins of *Lactococcus lactis* and its domestication to the dairy environment. *Food Microbiology*, 47, 45-61.

Chau, T. T., Ishigaki, M., Kataoka, T., & Taketani, S. (2010). Porcine ferrochelatase: the relationship between iron-removal reaction and the conversion of heme to Zn-protoporphyrin. *Bioscience, Biotechnology, and Biochemistry*, 74(7), 1415-1420.

Chau, T. T., Ishigaki, M., Kataoka, T., & Taketani, S. (2011). Ferrochelatase catalyzes the formation of Zn-protoporphyrin of dry-cured ham via the conversion reaction from heme in meat. *Journal of Agricultural and Food Chemistry*, 59(22), 12238-12245.

Contreras, H., Chim, N., Credali, A., & Goulding, C. W. (2014). Heme uptake in bacterial pathogens. *Current Opinion in Chemical Biology*, 19, 34-41.

Dailey, H. A., & Fleming, J. E. (1983). Bovine ferrochelatase. Kinetic analysis of inhibition by N-methylprotoporphyrin, manganese, and heme. *The Journal of Biological Chemistry*, 258(19), 11453-11459.

De Maere, H., Fraeye, I., De Mey, E., Dewulf, L., Michiels, C., Paelinck, H., & Chollet, S. (2016). Formation of naturally occurring pigments during the production of nitrite-free dry fermented sausages. *Meat Science*, 114, 1-7.

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M. C., Fraeye, I., Heyden, Y. B., & Paelinck, H. (2014). The occurrence of N-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. *Meat Science*, 96(2), 821-828.

Dietrich, M., Block, G., Pogoda, J. M., Buffler, P., Hecht, S., & Preston-Martin S. (2005). A review: dietary and endogenously formed N-nitroso compounds and risk of childhood brain tumors. *Cancer Causes Control*, 16(6), 619-635.

Doulgeraki, A. I., Paramithiotis, S., & Nychas, G. J. E. (2011). Characterization of the *Enterobacteriaceae* community that developed during storage of minced beef under aerobic or modified atmosphere packaging conditions. *International journal of Food Microbiology*, 145(1), 77-83.

Ercolini, D., Casaburi, A., Nasi, A., Ferricino, I., Di Monaco, R., Ferranti, P., Mauriello, G., & Villani, F. (2010). Different molecular types of *Pseudomonas fragi* have the same overall behaviour as meat spoilers. *International Journal of Food Microbiology*, 142(1-2), 120-131.

Ercolini, D., Russo, F., Nasi, A., Ferranti, P., & Villani, F. (2009). Mesophilic and psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. *Applied and Environmental Microbiology*, 75(7), 1990-2001.

Farnworth, E. R. (2005). Kefir–a complex probiotic. *Food Science and Technology Bulletin: Functional Foods*, 2(1), 1-17.

Faustman, C., Johnson, J. L., Cassens, R. G., & Doyle, M. P. (1990). Color reversion in beef. Influence of psychrotrophic bacteria. *Fleischwirtschaft*, 70(6), 676-679.

Fiddler, W., Pensabene, J. W., Piotrowski, E. G., Philips, J. G., Keating, J., & Mergens, W. J. (1978). Inhibition of formation of volatile nitrosamines in fried bacon by the use of cure-solubilized alpha-tocopherol. *Journal of Agriculture and Food Chemistry*, 26(3), 653-656.

Franken, A. C. W., Lokman, B. C., Ram, A. F. J., Punt, P. J., Van den Hondel, C. A. M. J. J., & De Weert, S. (2011). Heme biosynthesis and its regulation: Towards understanding and improvement of heme biosynthesis in filamentous fungi. *Applied Microbiology and Biotechnology*, 91(3), 447-460.

Franz, C. M. A. P., Huch, M., Abriouel, H., Holzapfel, W., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, 151(2), 125-140.

Giraffa, G. (2003). Functionality of enterococci in dairy products. *International Journal of Food Microbiology*, 88(2-3), 215-222.

Grossi, A. B., do Nascimento, E. S. P., Cardoso, D. R., & Skibsted, L. H. (2014). Proteolysis involvement in zinc-protoporphyrin IX formation during Parma ham maturation. *Food Research International*, 56, 252-259.

Hazell, T. (1982). Iron and zinc compounds in the muscle meats of beef, lamb, pork and chicken. *Journal of the Science of Food and Agriculture*, 33(10), 1049-1056.

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, 78(1-2), 68-76.

本間文佳（2016）至適pHの異なる2つの亜鉛プロトポルフィリンIX形成機構に関する研究、北海道大学大学院農学院共生基盤学専攻、修士論文

Inskip, P. D., Linet, M. S., & Heineman, E. F. (1995). Etiology of brain tumors in adults. *Epidemiologic Reviews*, 17(2), 382-414.

Ishikawa, H., Kawabuchi, T., Kawakami, Y., Sato, M., Numata, M., & Matsumoto, K. (2007). Formation of zinc protoporphyrin IX and protoporphyrin IX from oxymyoglobin in porcine heart mitochondria. *Food Science and Technology Research*, 13(1), 85-88.

ISO. (1973). Meat and meat products-Determination of moisture. ISO, 1442.

Jacobs, J. M., Sinclair, P. R., Sinclair, J. F., Gorman, N., Walton, H. S., Wood, S. G., & Nichols, C. (1998). Formation of zinc protoporphyrin in cultured hepatocytes: effects of ferrochelatase inhibition, iron chelation or lead. *Toxicology*, 125(2-3), 95-105.

Jafari, M., Mortazavian, A. M., Hosseini, H., Safaei, F., Khaneghah, A. M., & Sant'Ana, A. S. (2017). Probiotic Bacillus: Fate during sausage processing and storage and influence of different culturing conditions on recovery of their spores. *Food Research International*, 95, 46-51.

Kamarudheen, N., George, C., Priya, C. L., & Rao, B. K. V. (2014). Biopreservation of meat by probiotic bacteria isolated from dairy products. *Der Pharmacia Lettre*, 6(6), 266-271.

川添穂高 (2013) 酸の種類、pHおよび微生物が食肉製品中の亜鉛プロトポルフィリンIX形成に及ぼす影響. 北海道大学大学院農学院共生基盤学専攻, 修士論文

Khozroughi, A. G., Jander, E., Schirrmann, M., Rawel, H., Kroh, L. W., & Schlüter, O. (2017). The role of myoglobin degradation in the formation of zinc protoporphyrin IX in the longissimus lumborum of pork. *LWT-Food Science and Technology*, 85, 22-27.

Khozroughi, A. G., Krohb, L. W., Schlütera, O., & Rawel, H. (2018). Assessment of the bacterial impact on the post-mortem formation of zinc protoporphyrin IX in pork meat. *Food Chemistry*, 256, 25-30.

King, J. N., & Whyte, R. (2006). Does it look cooked? A review of factors that influence cooked meat color. *Journal of Food Science*, 71(4), 31-40.

Kivanç, M., Akgül, A., & Doğan, A. (1991). Inhibitory and stimulatory effects of cumin, oregano and their essential oils on growth and acid production of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. *International Journal of Food Microbiology*, 13(1), 81-86.

Labbé, R. F., Vreman, H. J., & Stevenson, D. K. (1999). Zinc protoporphyrin: A metabolite with a mission. *Clinical Chemistry*, 45(12), 2060-2072.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature*, 227(5259), 680-685.

Laursen, B. G., Bay, L., Cleenwerck, I., Vancanneyt, M., Swings, J., Dalgaard, P., & Leisner, J. J. (2005). *Carnobacterium divergens* and *Carnobacterium maltaromaticum* as spoilers or protective cultures in meat and seafood: Phenotypic and genotypic characterization. *Systematic and Applied Microbiology*, 28(2), 151-164.

Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology*, 15(2), 67-78.

Lijinsky, W. (1987). Structure-activity relations in carcinogenesis by N-nitroso compounds. *Cancer Metastasis Reviews*, 6(3), 301-356.

Linton, M., Connolly, M., Houston, L., & Patterson, M. F. (2014). The control of *Clostridium botulinum* during extended storage of pressure-treated, cooked chicken. *Food Control* 37, 104-108.

Lücke, F. K. (1997). Fermented sausages. In B. J. B. Wood (Ed.), *Microbiology of fermented foods* (pp. 441-483). London: Blackie Academic & Professional.

Mahlen, S. D. (2011). *Serratia* infections: From military experiments to current practice. *Clinical Microbiology Reviews*, 24(4), 755-791.

Mancini, R. A., & Hunt, M. C. (2005). Current research in meat color. *Meat Science*, 71(1), 100-121.

Marianthi, S., Alex, G., Anastasios, N., Maria, K., & Yiannis, K. (2014). Evaluation of *Lactobacillus casei* ATCC 393 protective effect against spoilage of probiotic dry-fermented sausages. *Food Control*, 42, 315-320.

Moretti, M. B., Garcia, S. C., & Batlle, A. (2000). Porphyrin biosynthesis intermediates are not regulating δ-aminolevulinic acid transport in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications*, 272(3), 946-950.

Morita, H., Sakata, R., Sonoki, S., & Nagata, Y. (1994). Metmyoglobin conversion to red myoglobin derivatives and citrate utilization by bacteria obtained from meat products and pickles for curing. *Animal Science and Technology*, 65(11), 1026-1033.

Morita, H., Niu, J., Sakata, R., & Nagata, Y. (1996). Red pigment of Parma ham and bacterial influence on its formation. *Journal of Food Science*, 61(5), 1021-1023.

Morita, H., Sakata, R., & Nagata, Y. (1998). Nitric oxide complex of iron(II) myoglobin converted from metmyoglobin by *Staphylococcus xylosus*. *Journal of Food Science*, 63(2), 352-355.

Nes, I. F., & Skjelkvåle, R. (1982). Effect of natural spices and oleoresins on *Lactobacillus plantarum* in the fermentation of dry sausages. *Journal of Food Science*, 47(5), 1618-1625.

Nishimura, M., & Yano, M. (2014). Separation of the inner and outer mitochondrial membrane in HeLa cells. *Bio-protocol*, 4(22), 1-4.

Ohgaki, H. (2009). Epidemiology of brain tumors. In: Verma, M., editor. Methods of molecular biology, *cancer epidemiology* (pp. 323-42), 472, Totowa, NJ: Humana Press.

大屋桃 (2017) 亜鉛プロトポルフィリンIX形成能の高い微生物の探索と応用に関する研究. 北海道大学大学院農学院共生基盤学専攻, 修士論文

岡部潤 (1996) 動物細胞成分の分離法. 新・生物化学実験のてびき 1 生物試料の調製法. (下西康嗣, 永井克也, 長谷俊治, 本田武司編) 55-69. 化学同人. 京都

Paganelli, M. O., Grossi, A. B., Dores-Silva, P. R., Borges, J. C., Cardoso, D. R., & Skibsted, L. H., (2016). Limited proteolysis of myoglobin opens channel in ferrochelatase-globin complex for iron to zinc transmetallation. *Food Chemistry*, 210, 491-499.

Parolari, G., Benedini, R., & Raja, V. (2008). Zinc-protoporphyrin IX promoting activity in pork muscle. *LWT-Food Science and Technology*, 41(7), 1160-1166.

Parolari, G., Benedini, R., & Toscani, T. (2009). Color formation in nitrite-free dried hams as related to Zn-protoporphyrin IX and Zn-chelatase activity. *Journal of Food Science*, 74(6), C413-C418.

Pearson, A. M., & Gillett, T. A. (1996). Processed Meats, 3rd edn. Chapman & Hall, New York, NY.

Preston-Martin, S., Munir, R., & Chakrabarti, I. (2006). Nervous system. In: Schottenfeld, D., Fraumeni, Joseph F., Jr, editors. *Cancer Epidemiology and Prevention* (pp. 1173-95), 3, New York: Oxford University Press.

Renerre, M. (1990). Factors involved in the discoloration of beef meat. *International Journal of Food Science and Technology*, 25(6), 613-630.

Ruiz-Moyano, S., Martin, A., Benito, M. J., Hernandez, A., Casquete, R., & De Guia Cordoba, M. (2011). Application of *Lactobacillus fermentum* HL57 and *Pediococcus acidilactici* SP979 as potential probiotics in the manufacture of traditional Iberian dry-fermented sausages. *Food Microbiology*, 28(5), 839-847.

Sakaino, M., Ishigaki, M., Ohgari, Y., Kitajima, S., Masaki, R., Yamamoto, A., & Taketani, S. (2009). Dual mitochondrial localization and different roles of the reversible reaction of mammalian ferrochelatase. *FEBS Journal*, 276(19), 5559-5570.

Sakata, R. (2000). Studies on physicochemical characteristics of red pigments in meat products. *Journal of Animal Science*, 71(1), 1-16.

Samelis J. (2006). Managing microbial spoilage in the meat industry. *In* Blackburn C. D. W. (ed.). *Food spoilage microorganisms* (pp. 213–286). Cambridge, United Kingdom: Woodhead Publishing Limited.

下田啓介（2014）非加熱乾塩漬食肉製品における亜鉛プロトポルフィリンIXの形成機構解明に向けた形態学的アプローチ、北海道大学大学院農学院、共生基盤学専攻、修士論文

Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide - Biology and Chemistry*, 24(4), 176-183.

Sparo, M. D., Confalonieri, A., Urbizu, L., Ceci, M., & Bruni, S. F. (2013). Bio-preservation of ground beef meat by *Enterococcus faecalis* CECT7121. *Brazilian Journal of Microbiology*, 44(1), 43-49.

Taketani, S. (1993). Molecular and genetic characterization of ferrochelatase. *The Tohoku Journal of Experimental Medicine*, 171(1), 1-20.

Taketani, S., & Tokunaga, R. (1981). Rat liver ferrochelatase. Purification, properties, and stimulation by fatty acids. *The Journal of Biological Chemistry*, 256(24), 12748-12753.

Taketani, S., Ishigaki, M., Mizutani, A., Uebayashi, M., Numata, M., Ohgari, Y., & Kitajima, S. (2007). Heme synthase (Ferrochelatase) catalyzes the removal of iron from heme and demetalation of metalloporphyrins. *Biochemistry*, 46(51), 15054-15061.

Tephly, T. R., Gibbs, A. H., & Dematteis, F. (1979). Studies on the mechanism of experimental porphyria produced by 3,5-diethoxycarbonyl-1,4- dihydrocollidine – Role of a porphyrin-like inhibitor of protoheme ferro-lyase. *Biochemical Journal*, 180(1), 241-244.

Toldra´, F. (1998). Proteolysis and lipolysis in flavor development of dry-cured meat products. *Meat Science*, 49(1), 101-110.

Troller, J. A., & Christian, J. H. B. (1978). Water activity and food, Academic Press, New York.

Wakamatsu, J., Akter, M., Honma, F., Hayakawa, T., Kumura, H., & Nishimura, T. (2019). Optimal pH of zinc protoporphyrin IX formation in porcine muscles: Effects of muscle fiber type and myoglobin content. *LWT - Food Science and Technology*, *101*, 599-606.

Wakamatsu, J., Hayashi, N., Nishimura, T., & Hattori, A. (2010). Nitric oxide inhibits the formation of zinc protoporphyrin IX and protoporphyrin IX. *Meat Science*, 84(1), 125-128.

Wakamatsu, J., Ito, T., Nishimura, T., & Hattori, A. (2007a). Direct demonstration of the presence of zinc in the acetone-extractable red pigment from Parma ham. *Meat Science*, 76(2), 385-387.

Wakamatsu, J., Kawazoe, H., Ohya, M., Hayakawa, T., & Kumura, H. (2020). Improving the color of meat products without adding nitrite/nitrate using high zinc protoporphyrin IX-forming microorganisms. *Meat Science*, 161, 107989.

Wakamatsu, J., Nishimura, T., & Hattori, A. (2004a). A Zn-porphyrin complex contributes to bright red color in Parma ham. *Meat Science*, 67(1), 95-100.

Wakamatsu, J., Odagiri, H., Nishimura, T., & Hattori, A. (2009b). Quantitative determination of Zn protoporphyrin IX, heme and protoporphyrin IX in Parma ham by HPLC. *Meat Science*, 82(1), 139-142.

Wakamatsu, J., Okui, J., Ikeda, Y., Nishimura, T., & Hattori, A. (2004b). Establishment of a model experiment system to elucidate the mechanism by which Zn-protoporphyrin IX is formed in nitrite-free dry-cured ham. *Meat Science*, 68(2), 313-317.

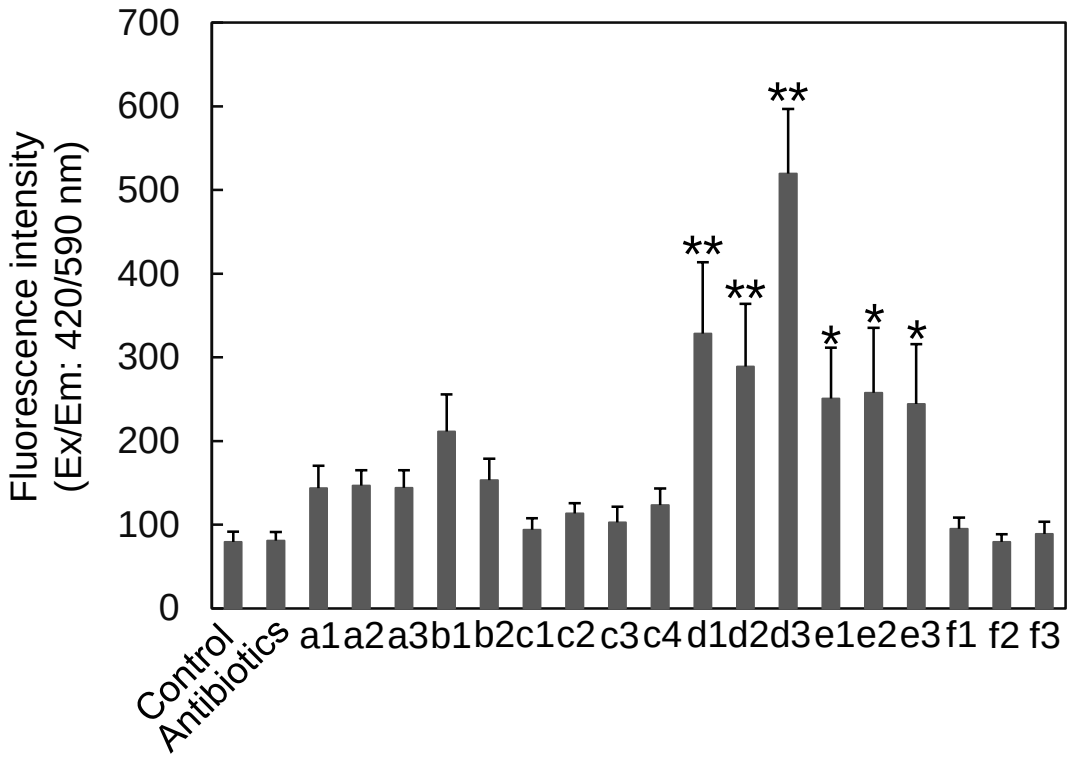
Wakamatsu, J., Okui, J., Hayashi, N., Nishimura, T., & Hattori, A. (2007b). Zn protoporphyrin IX is formed not from heme but from protoporphyrin IX. *Meat Science*, 77(4), 580–586.

Wakamatsu, J., Uemura, J., Odagiri, H., Okui, J., Hayashi, N., Hioki, S., Nishimura T., & Hattori, A. (2009a). Formation of zinc protoporphyrin IX in Parma-like ham without nitrate or nitrite. *Animal Science Journal*, 80(2), 198-205.

Wrensch, M., Minn, Y., Chew, T., Bondy, M., & Berger, M.S. (2002). Epidemiology of primary brain tumors: current concepts and review of the literature. *Neuro Oncology*, 4(4), 278-299.

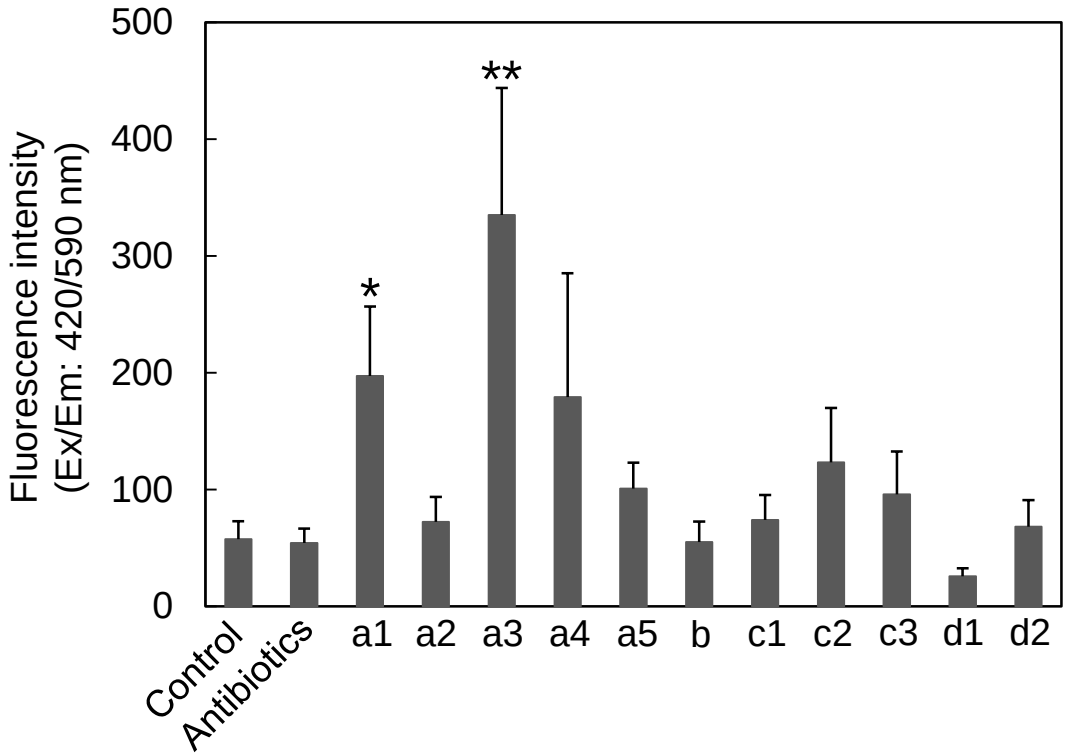
Young, O. A., Zhang, S. X., Farouk. M. M., & Podmore, C. (2005). Effects of pH adjustment with phosphates on attributes and functionalities of normal and high pH beef. *Meat Science*, 70(1), 133-139.

Zommiti, M., Cambronel, M., Maillot, O., Barreau, M., Sebei, K., Feuilloley, M., Ferchichi, M., & Connil, N. (2018). Evaluation of probiotic properties and safety of *Enterococcus faecium* isolated from artisanal Tunisian Meat “Dried Ossban”. *Frontiers in Microbiology*, 9, 1-12.



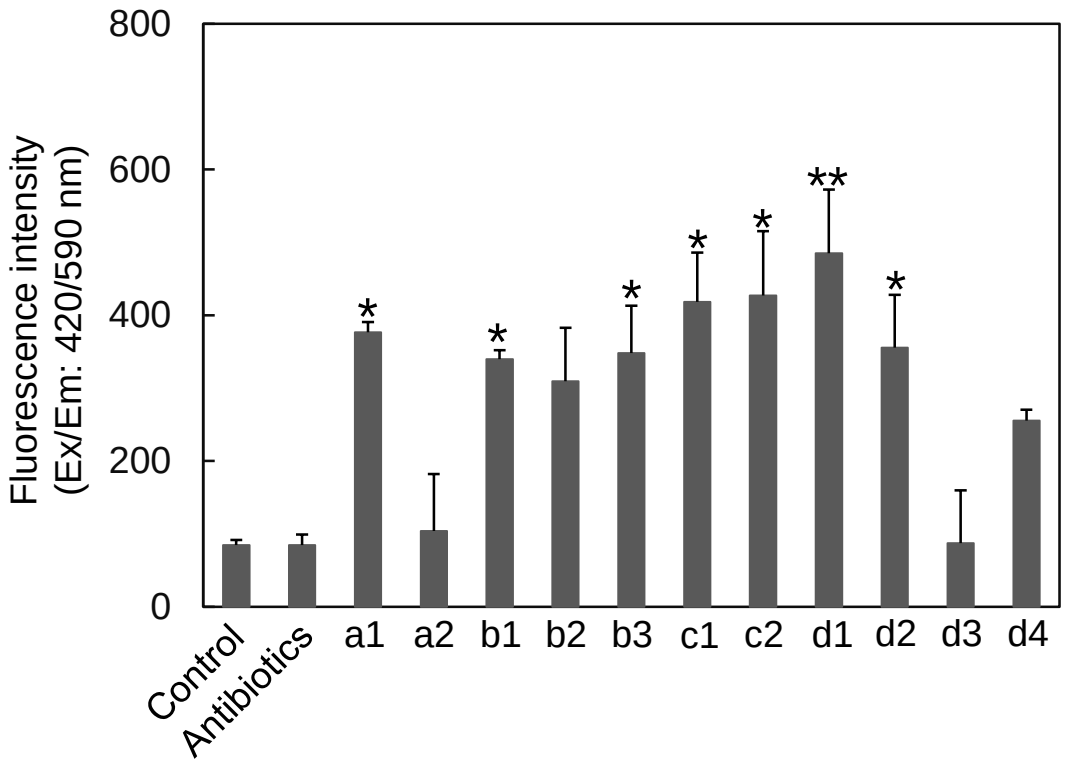
Appendix Fig. 1. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a3: Salami, b1-b2: Kimuchi, c1-c4: Cheddar cheese, d1-d3: Plant leaves e1-e3: Tap water and f1-f3: Human skin) Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control and *: Significant difference at $P < 0.05$ vs control.



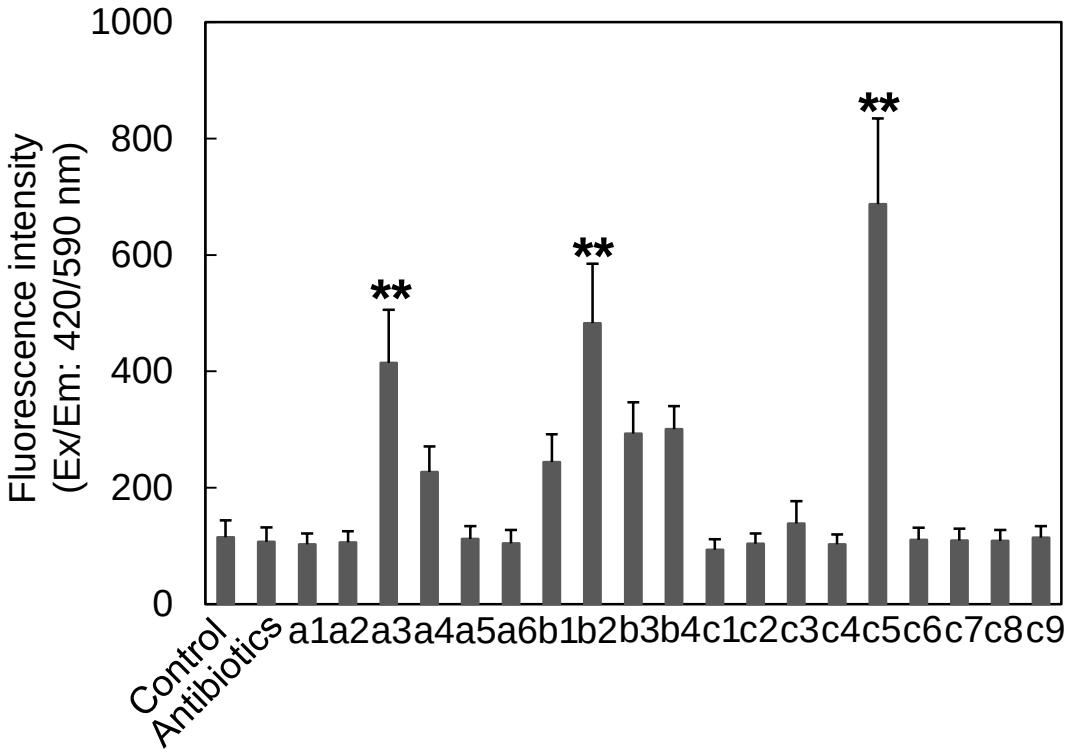
Appendix Fig. 2. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a5: Raw milk, b: Pasteurized milk, c1-c3: Gouda cheese and d1-d2: Yoghurt). Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control and *: Significant difference at $P < 0.05$ vs control.



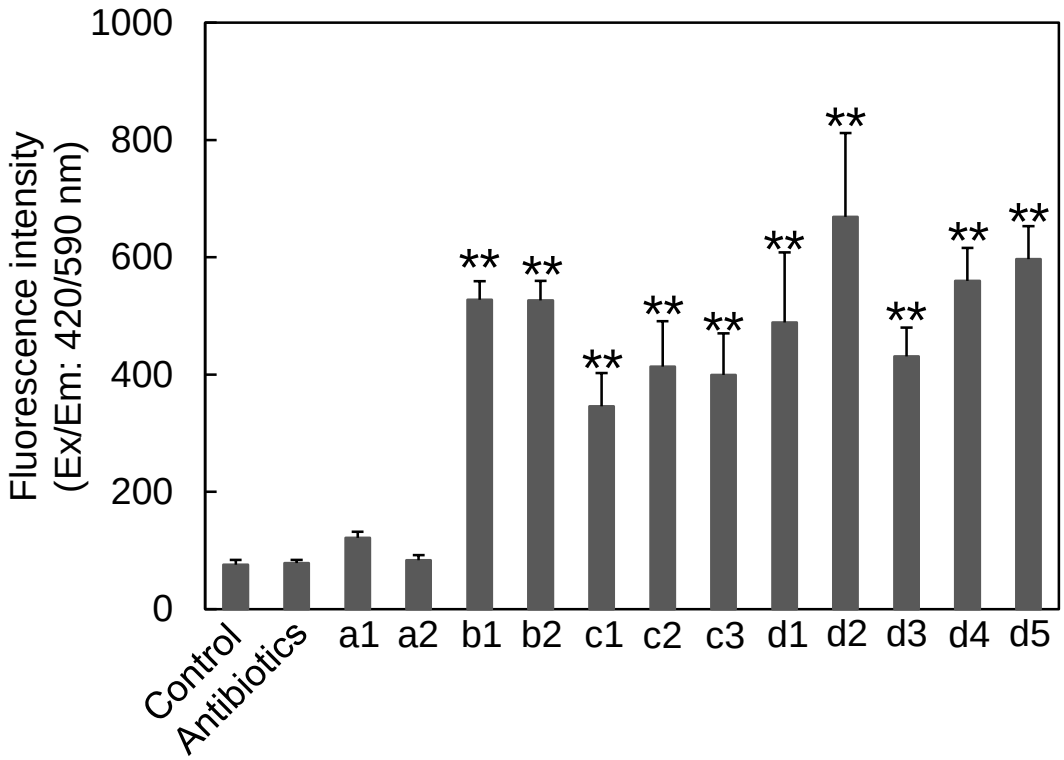
Appendix Fig. 3. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a2: Poultry wings, b1-b3: Poultry cases, c1-c2: Poultry feces and d1-d4: Pig feces). Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control and *: Significant difference at $P < 0.05$ vs control.



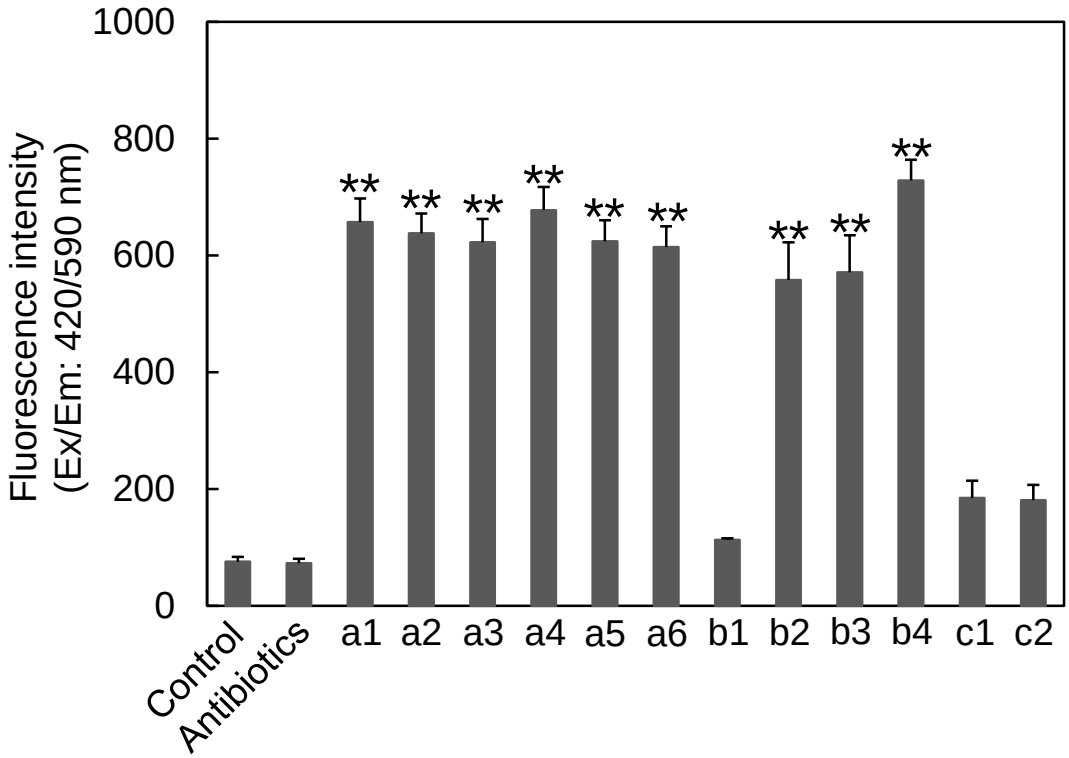
Appendix Fig. 4. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a6: Fermented Mackerel, b1-b4: Dry shrimp and c1-c9: Fermented Saury). Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control.



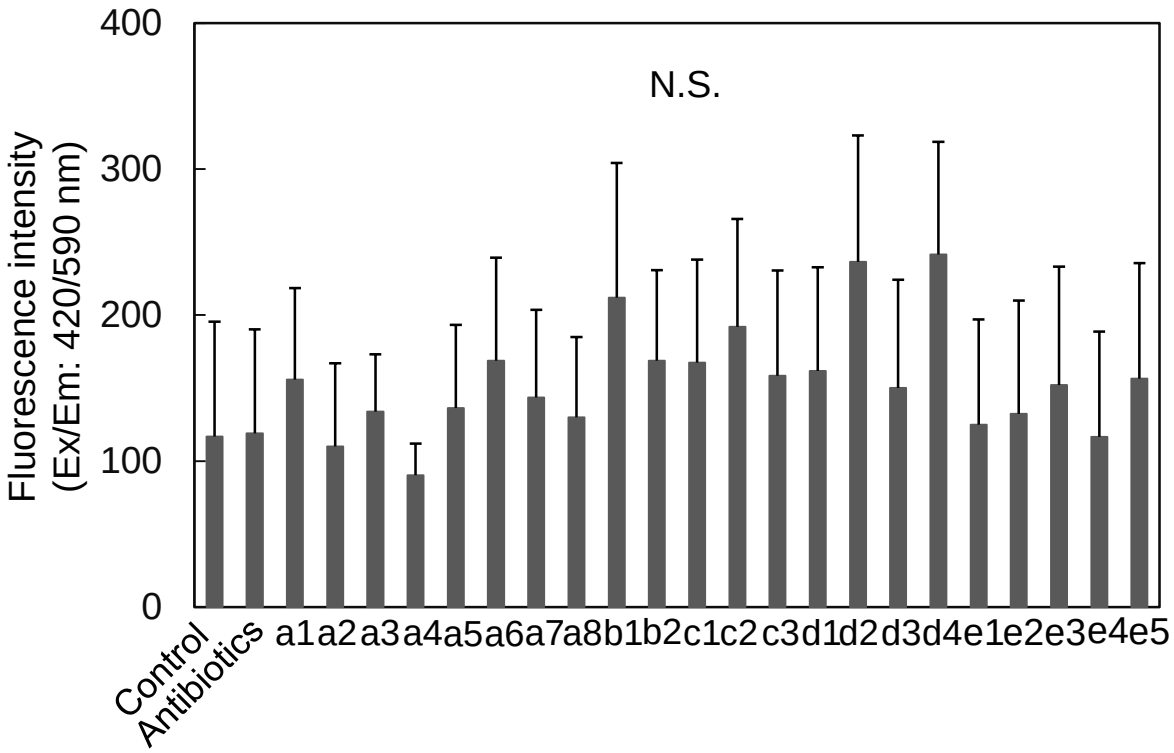
Appendix Fig. 5. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a2: Orange, b1-b2: Fermented meat, c1-c3: Biofermin probiotics and d1-d5: Grape). Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control.



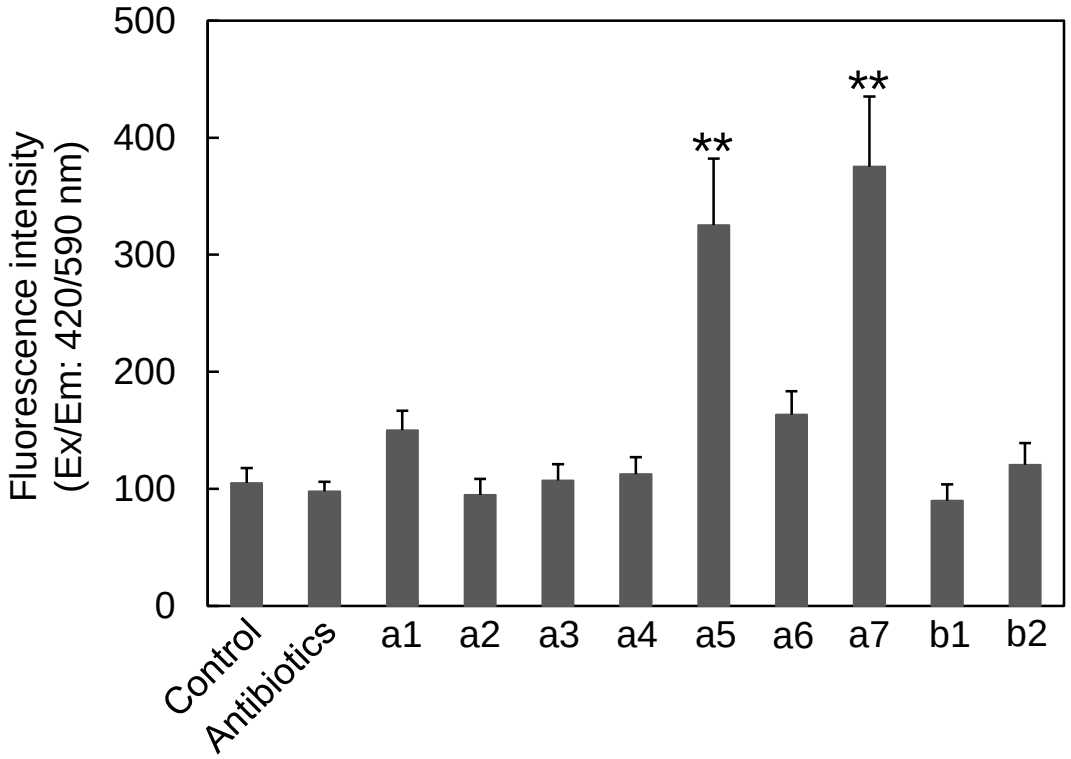
Appendix Fig. 6. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a6: Parma ham, b1-b4: Fermented milk and c1-c2: Natto). Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control.



Appendix Fig. 7. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a8: Sausage1, b1-b2: Sausage2, c1-c3: Sausage3, d1-d4: Sausage4 and e1-e5: sausage5). Data expressed as means ± SE (n = 3). N.S.: Non-significant.



Appendix Fig. 8. ZnPP-forming abilities of the bacteria isolated from various environmental sources

Aseptic 20% meat homogenate containing 3% NaCl was prepared and inoculated with bacteria. After incubation at 25°C for 7 days under anaerobic condition, ZnPP was extracted with 75% acetone and the fluorescence intensity of ZnPP was measured (Ex/Em: 420/590 nm). (a1-a7: Cow dung and b1-b2: Miso). Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control.