# Studies on the color improvement of meat products using LAB that form ZnPP aerobically and the formation mechanism

（好気的にZnPPを形成する乳酸菌を用いた食肉製品の色調改善技術とそのメカニズムに関する研究）

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# Chapter 1

## General introduction

Meat and meat products purchasing decisions are affected by color more than any other determinant of quality; thereby, color is one of the main focusing viewpoints in the meat industry. The consumers adopt discoloration of the meat and meat products as a sign of freshness and wholesomeness. As a result, retail meat is discounted in price due to the surface discoloration, which corresponds to a vast amount of currency losses every year (Smith *et al*., 2000). The sarcoplasmic heme protein, myoglobin (Mb), is primarily responsible for meat color, although other heme proteins such as hemoglobin (Hb) and cytochrome C also keep a role in meat color to a lesser extent (Suman & Joseph 2013). The chemistry of Mb varies in the postmortem skeletal muscles and processed meats depending on the type of packaging materials used, and the processing technology applied. This tends to the variations in the color of meat and meat products from bright cherry-red (bloomed fresh meat) to dull brown (cooked meat). In packaged fresh meats (raw meat), Mb can be available in any of the four redox states (Mancini & Hunt 2005), namely deoxymyoglobin (DeoxyMb), oxymyoglobin (OxyMb), carboxymyoglobin (COMb), and metmyoglobin (MetMb). DeoxyMb, OxyMb, and COMb are in a ferrous state. The color of DeoxyMb is purplish-red. OxyMb and COMb are bright cherry-red in color, and the red color of these two redox forms is not identifiable by naked eyes (Cornforth & Hunt 2008). In contrast, oxidation of Mb forms MetMb, which remains in a ferric state and exhibits brown color and favors meat products to become discoloration. Cured meats and meat products without the addition of nitrites/nitrates will normally attain a dull brown color in raw products or a grey color in heating products, which

influence consumer acceptance negatively (Carpenter *et al*., 2001). The long-lasting, bright red color of cured meat products is due to the formation of nitrosylmyoglobin (NOMb). NOMb is formed by the reaction between nitric oxide (NO) derived from nitrites/nitrates and meat Mb in the presence of endogenous or added reductants (Honikel, 2008). Besides, nitrites/nitrates exhibit important bacteriostatic and bacteriocidal activity against several spoilage bacteria as well as foodborne pathogens found in meat products. The nitrites/nitrates salt (80-140 ppm) can control *Clostridium botulinum* germination and the growth of various foodborne pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, *Achromobacter*, *Aerobacter*, *Escherichia*, *Flavobacterium*, and *Micrococcus* spp. (Krause *et al*., 2011). Moreover, nitrites improve the antioxidant properties of the cured meat products (Bedale *et al*., 2016), and 20–50 ppm nitrite concentrations are required to retard rancidity (Rivera *et al*., 2019). Due to their multifunctional nature along with color promoting property, nitrites/nitrates has become a preferable curing agent in the meat processing industry over the past several decades.

Rather than beneficial effects, the adverse health effect caused by excessive nitrites/nitrates was first investigated in the 1950s and 1960s. Usage of excessive nitrites/nitrates burnt up a debate about the safety of ingested nitrites, which finally targeted cured meat and meat products. Over the years, great concerns have been expressed regarding certain harmful compounds that may be formed in cured meat and meat products during and after curing. The foremost concern is the involvement of nitrites/nitrates in cured meat products and the potential to form carcinogenic N-nitroso compounds (De Mey, 2014). Since the 1970s, the occurrence and mechanisms involved in the formation of N-nitroso compounds

have been studied. Generally, N-nitroso compounds are of two main groups, namely, N-nitrosamines and N-nitrosamides. They are formed by the reaction between secondary amines/amides derived from meat and meat products and nitrosating agents (e.g., $N_2O_3$) from nitrites under acidic conditions (Fig. 1) (Masuda *et al*., 2000). Secondary amines may be introduced into food products by different routes. The presence of biogenic amines, other protein, and lipid degradation products are considered to be an important source of amine precursors. The occurrence of some N-nitroso compounds is often related to the migration of

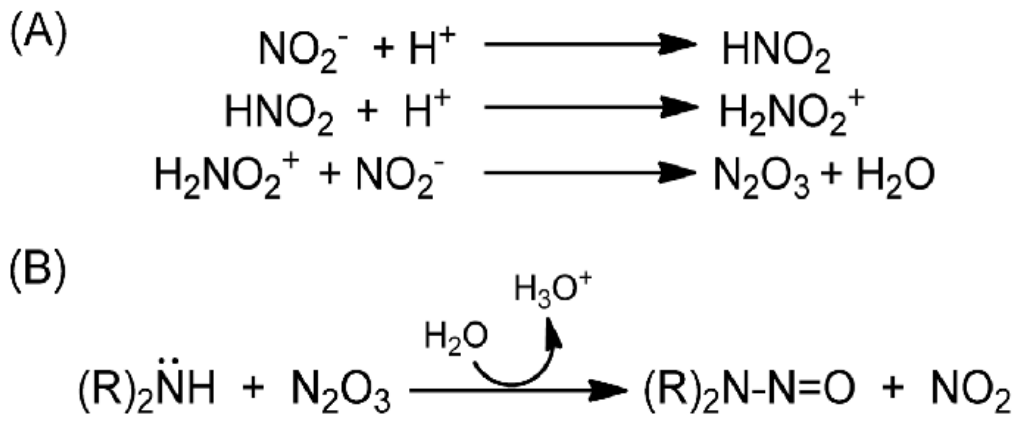



**Fig. 1. N-nitrosamine formation (inspired by Park *et al*., 2015). (A) Formation of a nitrous anhydride from nitrites and (B) nitrosation from a nitrous anhydride and an amine**

amine precursors from packaging materials (Domanska & Kowalski, 2003). Some N-nitroso compounds may originate from the use of pyrrolidine and piperidine containing spices like black pepper and paprika (Nakamura *et al*., 1981). In contrast, sodium nitrite (by curing) and gaseous nitrogen oxides (by smoking) are the main sources of nitrosyl donors in meat products (Hotchkiss & Parker, 1990). The higher N-nitroso compound contents are found in products as sausage, smoked meats, bacon, and luncheon meats (Stuff *et al*., 2009). N-

nitrosamides are directly alkylating agents that do not require metabolic activation and may be associated with a childhood brain tumor (Dietrich *et al*., 2005). Besides, N-nitrosamines are associated with colorectal cancer (Santarelli *et al*., 2008), stomach cancer (Larsson *et al*., 2006), pancreatic cancer (Larsson & Wolk, 2012). Recently, it was reported by the International Agency for Research on Cancer (IARC, 2018) that the risk of colorectal cancer increased due to taking processed meat (Bouvard *et al*., 2015) and classified processed meat as carcinogenic to humans (Group1) based on epidemiological studies. They estimated that the risk of colorectal cancer was increased by 18% for taking each 50 g of processed meat daily (Bouvard *et al*., 2015). The yield of N-nitrosamines is also variable depending on the time and temperature used during processing, the initial addition of nitrites/nitrates, the composition of the meat, pH, the addition of antioxidant components such as ascorbate, and the presence of microorganisms (Gibson *et al*., 1984; Honikel, 2008). However, due to the potential carcinogenic effect, the use of nitrites/nitrates could be limited in the meat products, although improvement of color without nitrites/nitrates is one of the challenges. Therefore, the concern regarding the adverse effects of N-nitrosamines on human health has led to seeking alternate ways of nitrites/nitrates for processed meat products.

Since the consumer urging for organic or natural meat products has increased due to the concerns of the health risk of synthetic nitrites/nitrates, the meat industry has currently concerned with the development of nitrites/nitrates alternatives. Over the past several decades, many studies have been conducted to identify a useful replacement material that possesses the properties of nitrites/nitrates. There is a significant interest in the development of alternatives from natural sources and other preservation techniques that are considered to

be comparatively healthier. Among the plant-based alternatives of nitrites/nitrates, vegetables such as celery, spinach, radish, and lettuce have been reported to contain more than 2500 mg nitrate/kg (Santamaria, 2006). Celery powder (3% nitrate) (Sindelar *et al*., 2007) is used by several meat processors, along with a bacterial starter culture (Sebranek & Bacus, 2007). Sometimes, off-flavors may develop if the addition of celery powder is at higher levels than 0.2-0.4% of the formulation weight (Sindelar *et al*., 2007). Recently, spray-dried Swiss chard powder (3.0-3.5% nitrate) was used as a natural source of nitrate, and it should also be used at a concentration of 0.15-0.3% (Sebranek *et al*., 2012). However, high concentrations may negatively affect the sensory attributes. Besides, green tea extract (1-2%) in ground beef meat (Mustafa, 2013), tea catechin with modified atmospheric packaging (200 mg/kg) in beef patties (Tang *et al*., 2006), rosemary and oregano extract (0.02% of each) in raw pork batters (Hernandez *et al*., 2009), and Anka rice (0.5%) with nitrite (25 ppm) in low-nitrite Chinese sausages were reported that reduce lipid oxidation and stabilize the color of meat and meat products. However, plant extract may impart a positive effect on sensory attributes. In addition, since nitrate derived from plants is finally converted to nitrite in meat products, the carcinogenic risk of nitrite intake is not reduced essentially. Therefore, searching for alternative sources other than plant extract is essential for the improvement of the color of meat products.

Apart from natural colorants, microbial conversion of Mb and MetMb to NOMb through the reaction with nitric oxide (NO) has generated considerable interest recently. Generally, NO generates in two ways in bacteria. (i) nitrate reductase pathway while nitrites/nitrates act as a substrate (Wang *et al*., 2007) and NO synthase pathway while L-arginine acts as substrate

(Ras *et al*., 2018). Several bacteria types have been tested, some of which demonstrated the ability to convert MetMb to NOMb, thereby providing a way to produce nitrite alternatives in meat products. There are many bacterial species, e.g., *Lactobacillus fermentum* in smoked fermented sausages (Møller *et al*., 2006) and in Chinese style sausage (Zhang *et al*., 2007); *Staphylococcus carnosus*, *Staphylococcus simulans*, *Staphylococcus saprophyticus* in sausages (Gotterup *et al*., 2008); *Chromobacterium violaceum*, *Kurthia* sp. in microbiological media (Arihara *et al*., 1993), and *Pediococcus acidilactici*, *Staphylococcus xylosus* in broth medium (Morita *et al*., 1998) and raw meat batters (Li *et al*., 2013) associated with the conversion of MetMb to NOMb were reported. Bacterial NO synthase has been detected in a *Nocardia* spp. (Chen & Rosazza, 1995) and *Lactobacillus fermentum* IFO 3956 (Morita *et al*., 1998). Generally, NO is derived from L-arginine by the bacterial NO synthase. However, without the addition of nitrites/nitrates, the production of NO by the *Staphylococcus* sp. remains unclear, and *Staphylococcus xylosus* does not contain NO synthase (Morita *et al*., 1998). Besides, there are several technologies such as high hydrostatic pressure (HHP), organic acids, plant extracts, a red pigment extracted from both plants or microorganisms, bacteriocins, and selected bacterial strains are also used and investigated in processed meats as nitrites/nitrates alternatives (Alahakoon *et al*., 2015; Munekata *et al*., 2020). However, no single substitute for nitrites/nitrates is available which can simultaneously provide all the functions of nitrites/nitrates, and especially the color improvement of the products always appeared as a major problem. Therefore, bacterial red pigment producing ability other than NO might be a single substitute of nitrites/nitrates to improve the color of meat and meat products.

On the other hand, zinc protoporphyrin IX (ZnPP) is a bright red colored pigment which is formed by the coordination of divalent zinc into protoporphyrin IX (PPIX) in nitrites/nitrates-free meat products, has been reported in different studies (De Maere *et al*., 2018; Møller *et al*., 2007; Wakamatsu *et al*., 2004a, 2009a). However, ZnPP was first introduced in Parma ham (*Prosciutto di Parma*), which is a traditional dry-cured ham of north Italian. Only legs of fattened pigs from heavier pigs (>150 kg in live weight) are used and processed for at least one year without the addition of nitrites/nitrates (Toldra, 2002) that has given the specialty to the ham. Though any coloring agents are not added, the color of the ham is extremely bright red. It has been reported that Parma ham has a red porphyrin derivative that is not NOMb or OxyMb (Møller *et al*., 2003; Morita *et al*., 1996; Parolari *et al*., 2003). Regarding this red color pigment, ZnPP was identified in Parma ham by Wakamatsu *et al*. (2004a). The lipophilic pigment extracted from Parma ham increased during the maturation of Parma ham (Adamsen *et al*., 2006b). Sixty to seventy percent of all porphyrins in Parma ham were ZnPP, and the amount of ZnPP in Parma ham was higher than that of heme (Bou *et al*., 2018; Wakamatsu *et al*., 2009a). The formation of ZnPP was also studied in other nitrite-free hams (Wakamatsu *et al*., 2009b), dry-cured Iberian hams (Adamsen *et al*., 2006a), and dry-cured fermented sausages (De Maere *et al*., 2016). The bright red color of ZnPP persists irrespective of light or heat exposure (Adamsen *et al*., 2004; Morita *et al*., 1996). Moreover, NO derived from nitrites/nitrates inhibited the formation of ZnPP in meat products (Wakamatsu *et al*., 2010). Thus, ZnPP might be a potential pigment to improve the color of meat products as bright red without adding nitrites/nitrates or any coloring agents.

Bacterial contribution regarding ZnPP formation in dry-cured meat products has been reported in several studies (Khozroughi *et al*., 2018; Morita *et al*., 1996; Sakata, 2000; Wakamatsu *et al*., 2020). Some bacteria, e.g., *Staphylococci epidermis*, *S. warneri*, and *S. lentus*, isolated from Parma ham, were reported to form red pigment though ZnPP was not identified at that time (Morita *et al*., 1996). Subsequently, it was proved that ZnPP formation depends not only on endogenous meat enzymes (Becker *et al*., 2012) but also on resident microorganisms since ZnPP formation was investigated in both sterile and non-sterile conditions (Wakamatsu *et al*., 2004a). Recently, a number of bacterial strains having ZnPP-forming ability in meat were reported (Asaduzzaman *et al*., 2020b; Kawazoe, 2013; Ohya, 2017; Wakamatsu *et al*., 2020), but these are not suitable for meat products either their lower ZnPP-forming ability or pathogenicity and overall considering the safety issue. It was also reported that microorganisms are involved in the formation of red pigments in cured meat products (Khozroughi *et al*., 2018). Therefore, the production of ZnPP by using high ZnPP-forming food-grade bacteria might be a potential alternative to nitrites/nitrates for the color improvement of meat products.

Microorganisms are widely used in different kinds of fermented meat products over a long time as a starter culture. The most widely used starter cultures in meat products are lactic acid bacteria (LAB), coagulase-negative staphylococci (*Staphylococcus carnosus* and *Staphylococcus xylosus*) (Stavropoulou *et al*., 2018), Micrococcaceae (*Kocuria* sp.) (Cocconcelli & Fontana, 2015), molds (*Penicillium nalgiovense*, *Penicillium gladioli*) (Berni, 2015; Laranjo *et al*., 2017) and yeasts (*Debaryomyces* sp., *Candida* sp.) (Laranjo *et al*., 2017). LAB are usually facultative anaerobes and mostly used in the fermented meat

products belonging to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* (Fraqueza *et al*., 2016). Other than lactic acid, LAB also produce acetic and propionic acids, ethanol, hydrogen peroxide, reuterin, antimicrobial peptides, and bacteriocins (Galvez *et al*., 2008; Reis *et al*., 2012), and these products are effectively involved in protecting or controlling microbiological hazards (Baka *et al*., 2011; Casquete *et al*., 2012; Pragalaki *et al*., 2013). The inhibitory action of LAB, namely *Lactobacillus plantarum* against *Clostridium perfringens* in fermented salami (Di Gioia *et al*., 2016), *Lactobacillus acidophilus* and *Lactobacillus sakei* against *Enterobacteria* in Romanian traditional dry sausage (Simion *et al*., 2014; Wang, 2013), a combined effect of *Pediococcus acidilactici* and *Staphyllococcus vitulinus* against *Enterobacteria* and coliforms in traditional Iberian dry sausage (Casquete *et al*., 2012), a combination of two strains of *Lactococcus* (*Lactococcus lactis* subsp. l*actis* and *Lactococcus casei* subsp. *casei*) against *Salmonella* sp., *Listeria* sp., and *Staphyllococcus aureus* in salami (Cenci-Goga *et al*., 2012) have been reported. However, LAB as a starter culture are very important to ensure the safety of fermented meat products by inhibiting or reducing the growth of spoilage or pathogenic microorganisms and by lowering the residual nitrites/nitrates. Therefore, LAB as a most protective starter culture impart multifarious functions and ensure the safety and quality of fermented meat products.

Hence, the objectives of the present study was to improve the color of meat products as an alternative to nitrites/nitrates. In chapter 2, high ZnPP-forming food-grade LAB was screened from various sources and applied in dry-cured sausage. Fortunately, aerobic ZnPP formation by some LAB was observed and the formation of ZnPP in the presence of oxygen

was responsible for the external bright red color of dry-cured sausage. Consequently, in chapter 3, it was investigated to elucidate the mechanism by which ZnPP is aerobically formed by the selected ZnPP-forming LAB during the processing of dry-cured sausage.

# Chapter 2

# Screening for high ZnPP-forming food-grade lactic acid bacteria to improve the color of dry-cured meat products

## 2.1. Introduction

Although ZnPP-forming bacteria have never been used in commercial meat products, ZnPP formation by the bacteria in salt-added minced meat and their effects on the color improvement of meat and meat products have been investigated. The addition of bacteria like *Carnobacterium divergens*, *Serratia liquefaciens* (Wakamatsu *et al*., 2020), *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Enterococcus faecium* (Asaduzzaman *et al*., 2020b) lead to the formation of high ZnPP in salt-added minced meat, thereby improving the color. Moreover, the content of ZnPP formed and the redness were significantly correlated. Interestingly, most ZnPP-forming bacteria reported were lactic acid bacteria (LAB). Generally, food-grade LAB are used as a bio-preservative against pathogens in meat products and are recognized as safe (da Costa *et al*., 2019). However, *Carnobacterium divergens* and *Serratia liquefaciens* are not food-grade bacteria. Moreover, accumulation of biogenic amines by *Leuconostoc mesenteroides* and *Enterococcus faecium* (Kučerová *et al*., 2010) makes their use inappropriate in meat products, although some strains of *Enterococcus faecium* are used as probiotics. However, ZnPP-forming LAB have not been practically applied for color improvement in the processing of meat products. Therefore, screening for high ZnPP-forming food-grade LAB is of utmost importance for their application in meat products for improving the color by forming plenty of ZnPP.

The development of bright red color by ZnPP in meat products was severely affected by the presence of oxygen. ZnPP is naturally present in meat and is effective in improving the color of meat products (Asaduzzaman, 2020b; Wakamatsu *et al*., 2020). However, the formation of ZnPP was inhibited in the presence of oxygen (Wakamatsu *et al*., 2004b). Moreover, the distribution of ZnPP in the periphery of the cross-section of Parma ham was partially indicated by the weak fluorescence of ZnPP due to penetration of oxygen (Wakamatsu *et al*., 2009b). An important meat-inherent enzyme, ferrochelatase (FECH), is a key contributor to ZnPP formation (Akter *et al*., 2019b; Khozroughi *et al*., 2018) strictly under anaerobic conditions (Chau *et al*., 2011). On the other hand, ZnPP formation by bacteria in salt-added minced meat has been observed in the absence of oxygen (Asaduzzaman *et al*., 2020b; Wakamatsu *et al*., 2020) and did not check under aerobic condition. Recently, ZnPP-forming bacteria were applied in dry-cured meat products and they can form ZnPP only inside the products, thereby improvement of internal color was the result (Asaduzzaman, 2020). The tan or brown color of uncured meat is due to the formation of a pigment called MetMb, which is formed due to the oxidation of iron in Mb and OxyMb upon continuous exposure to light and oxygen (Mancini & Hunt, 2005). Thus, the surface of the meat might not show a red coloring due to the inhibition of ZnPP formation by oxygen and the formation of MetMb. But, LAB are facultatively anaerobic organisms and grow well in both the presence and absence of oxygen (Moraes *et al*., 2013) by switching their metabolism. Hence, there is a possibility to form ZnPP by some LAB in the presence of oxygen and aerobic ZnPP formation by the LAB might improve the entire color of nitrite/nitrate-free meat products.

The ZnPP-forming LAB should have adequate salt tolerance ability because salt is an integral part of meat products. Moreover, ZnPP-forming LAB should be capable of growing at comparatively low temperature since dry-cured meat products are fermented under low temperature. Therefore, the present study was conducted to screen and identify the high ZnPP-forming food-grade LAB from various food products and environmental sources for the application in meat products to improve the color without using nitrites/nitrates by forming an abundance of ZnPP.

## 2.2. Materials and methods

### 2.2.1. Meat sample and commercial strains

#### 2.2.1.1. Meat sample

Porcine *Longissimus thoracis et lumborum* (LTL) muscle was used for the aseptic meat homogenate (aMH) model experiment, aseptic salt-added minced meat (aSMM) model experiment, and preparation of dry-cured sausages. LTL muscles were collected from three (for aMH and aSMM model experiments) or six (for each time preparation of dry-cured sausages) different primal cuts of common domestic crossbred pork loins, which were obtained from the local market in Hokkaido, Japan. For preparing the aseptic meat homogenate as a part of the meat homogenate model experiment and the aseptic minced meat for the salt-added minced meat model system, after removing the fat and major connective tissue from each pork loin primal cut, the muscle was cut into a small block (250-300 g), vacuum-packaged, and preserved at -30°C until use. For preparing sausage, the whole primal cut of pork loins without fat was used.

#### 2.2.1.2. Commercial strains

Several commercial strains of *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Lactococcus* spp. were used in this study. The AHU strains were transferred from the strain storage room of the Applied Mycology Laboratory, Department of Bio-functional Chemistry, Basic Research Division, Graduate School of Agriculture, Hokkaido University, and the

NBRC strains were purchased from the Biotechnology Center Product Evaluation Technology Infrastructure Organization, Tokyo, Japan.

### 2.2.2. Preparation of reagents

#### 2.2.2.1. Preparation of sterile physiological saline

Ultra-pure water and 9 g/L of NaCl (Kanto Chemical Co. Inc., Tokyo, Japan) was used for the preparation of physiological saline and autoclaved at 121°C for 15 min and stored at room temperature.

#### 2.2.2.2. Preparation of nutrient broth (NB), MRS broth (MRSB), and M17 broth (M17B)

For the preparation of NB medium, 5 g/L of tryptone (Difco Laboratories, New Jersey, USA), 2.5 g/L of yeast extract (Difco Laboratories), and 1 g/L of glucose (Kanto Chemical Co. Inc.) were dissolved in ultra-pure water and autoclaved at 121°C for 15 min. The normal pH of the medium was 7.0. The commercial MRSB (52.2 g/L) (Merck KGaA, Darmstadt, Germany) and M17B (Difco Laboratories) media were used, and the normal pH of the mediums were 5.7. All of the media were prepared just before use.

#### 2.2.2.3. Preparation of antibiotics and other solutions

A mixed solution of penicillin G potassium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and streptomycin sulfate (Wako Pure Chemical Industries, Ltd.) was prepared by dissolving them into 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). Similarly, gentamicin sulfate (Wako Pure Chemical Industries, Ltd.) was also dissolved in ultra-pure water so as to be 10 mg/mL and then filtered through a syringe filter. After filtration, the solutions were stored at -20°C until use. The solution of sodium chloride, sodium nitrite, sodium ascorbate, sodium sulphite, glucose, and polyphosphate were prepared and sterilized by passing them through a sterile syringe filter. The solutions were prepared just before use.

### 2.2.3. Isolation and identification of LAB

#### 2.2.3.1. Isolation of acid-producing bacteria (APB)

The bacteria were collected in two ways from various food products and environmental sources, e.g., fermented foods, meat and meat products, fish and fish products, fruits and vegetables and their products, dairy products, and miscellaneous (Table 1). One was that the bacteria from the samples were collected by swabbing the source with sterile cotton and dispersed in sterile physiological saline. Another was that the samples obtained from different foods and environmental sources (about 5 g) were homogenized in the stomacher (Exnizer 400, Organo Food Tech. Co. Tokyo, Japan) with sterile physiological saline. Then, to facilitate the growth of *Lactobacillus* spp. and *Leuconostoc* spp. among the LAB, an appropriate dilution of the prepared samples with sterile physiological saline was plated onto MRS agar medium (Oxoid, Hampshire, England) modified by supplementing with 3% salt with pH adjusted to 5.5 (mMRS); these are the conditions closest to those observed in fermented meat products. To isolate *Lactococcus* spp. (only from dairy products), M17 agar

**Table 1. Different food products and environmental sources used for high ZnPP-forming LAB isolation**

| Six broad groups | Sources |
|---|---|
| Fermented foods (13) | Fermented sausages (5), kimchi, miso, natto, soy sauce, fermented rice, cucumber pickle, cheese, fermented fin fry fish |
| Meat and meat products (3) | Parma ham, chicken skin, beef |
| Fish and fish products (2) | Dry shark fish, dry Indian carplet fish |
| Fruits, vegetables, and their products (20) | Cabbage, lemon, kiwi fruit, banana, orange, broccoli, tomato pulp, grape, orange marmalade, germinated bean, apple, germinated garlic, malta, lettuce, grapefruit, tomato ketchup, mango pickle, strawberry jam, pineapple, wine |
| Dairy products (5) | Milk tea, ghee, butter, chocolate butter, milk |
| Miscellaneous (12) | Stool, saliva, urine, teeth, nose, leg finger, coffee, processed noodles, tobacco, feces of crow, soil, bark of tree |
| Total: 55 sources | |

Number inside the parentheses indicates the number of individual source.

medium (Sigma-aldrich Co., St. Louis, Switzerland), modified as above (mM17), was used. After inoculating the appropriate dilution onto the respective modified agar medium, the plates were incubated at 30°C (mMRS) or 37°C (mM17) for 48 h under both aerobic and anaerobic conditions. An oxygen impermeable storage bag containing an oxygen absorbent (A-500HS, I.S.O. Inc., Yokohama, Japan) was used to regulate the anaerobic conditions. Colonies were randomly selected from mMRS and mM17 based on morphological characteristics and purified by streaking onto their respective agar plates. Bacterial colonies were then grown in the respective broth (MRSB or M17B). Evaluation of ZnPP-forming ability of all the isolates was performed as described in section 2.2.5 and the isolates of ZnPP-forming ability were considered as ZnPP-forming bacteria. The acid producing ability by the ZnPP-forming bacteria was investigated using a plate count agar with bromocresol purple (PCA-BCP) (Kanto Chemical Co.), and the ZnPP-forming bacteria having ability to change the color of the agar from purple to yellow were considered as ZnPP-forming acid-producing bacteria (APB).

#### 2.2.3.2. Identification of LAB

##### 2.2.3.2.1. Extraction of DNA from isolates

At first, 1 mL of freshly cultured broth of APB 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™ 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 the microtubes were heated in a dry thermo bath at 105°C for 10 min, allowed to cool at room temperature and centrifuged at 16,000 × g for 3 min at 4°C (CT15RE, Hitachi Koki). Then, 3 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 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 polymerase chain reaction (PCR). The concentration and quality of DNA samples were estimated using a UV-1800 spectrophotometer (Shimadzu Co., Kyoto, Japan) before starting PCR.

**2.2.3.2.2. PCR**

Amplification of the 16S rRNA gene sequence was carried out in a thermocycler (Life ECO thermal cycler, Hangzhou Bioer Technology, Co. Ltd., Binjiang, China) using a KOD-Plus-Neo (Toyobo Co. Ltd. Osaka, Japan) PCR kit, and the universal primers 10F (5´-AGTTTGATCCTGGCTCAG-3´) and 1500R (5´-CTACCTTGTTACGACTTAGTCC-3´), located in the conserved regions of the 16S rRNA gene (size of the full length approx. 1.5 kbp). The reaction conditions were 94°C, 120 sec → (94°C, 15 sec → 55°C, 30 sec → 68°C,

90 sec) × 45 cycles. Amplified products were subsequently purified and recovered using Fast Gene PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan).

#### 2.2.3.2.3. Gene sequencing

Next, 11.4 µL of purified DNA (5 µg/mL) and 9.6 µL of 1 µM 357F (5´-CTCCTACGGGAGGCAGCAG-3´) primer were mixed to produce samples for gene sequencing. The rRNA gene sequencing was performed by Eurofins Genomics K.K. (Tokyo, Japan). Finally, the sequence similarity analysis was carried out using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI), and the isolates whose sequences acquired an identification percentage of at least 97% compared to others in the databases were marked to the same species. After gene sequencing, ZnPP-forming APB were divided into ZnPP-forming food-grade and non-food-grade LAB.

### 2.2.3.3. Storage of bacteria

The isolated bacterium and commercial strains were preserved as a stock in an equal volume of NB and 40% glycerol (finally 20% glycerol) at -20°C until use. All stock cultures were propagated twice in NB as required for 18 h before starting each experiment.

### 2.2.4. Characterization of ZnPP-forming food-grade LAB

#### 2.2.4.1. Physiological and biochemical characterization of ZnPP-forming food-grade LAB

ZnPP-forming food-grade LAB were characterized based on morphological shape (Norris *et al*., 1981) and catalase testing (Endo *et al*., 2009).

#### 2.2.4.2. Phenotypic characterization of ZnPP-forming food-grade LAB

For phenotypic characterization, ZnPP-forming food-grade LAB were further grown under different culture and environment conditions, i.e., presence or absence of oxygen, and pH levels (4.5, 5.5, 6.5, and 8.5). The growth of ZnPP-forming food-grade LAB was also observed under a range of different temperatures (18 to 45°C). To check salt tolerance, ZnPP-forming food-grade LAB were again propagated at concentrations of 3 to 7% salt. MRS agar was used in every test except the pH tolerance test, where plate count agar was due to its improved pH adjustability.

### 2.2.5. Aseptic meat homogenate (aMH) and aseptic salt-added minced meat (aSMM) model systems of ZnPP formation

#### 2.2.5.1. Preparation of bacterial culture

The required bacteria were cultured in the NB medium and allowed to grow for 24-48 h anaerobically at 30°C. Before using the bacteria in the aMH and aSMM model systems, the number of bacteria was counted using a hemocytometer under the phase-contrast microscope

(Olympus Co., BX50, Tokyo, Japan), which was then adjusted to the appropriate number using sterilized physiological saline.

#### 2.2.5.2. Preparation of aMH model sample

To investigate the ZnPP-forming ability of bacterial isolates in meat, aMH model experiment was carried out as previously described (Wakamatsu *et al*., 2020) with minor modifications. Firstly, 30% pork homogenate was aseptically prepared from the core portion of LTL muscle using a sterilized cup and homogenizer (CELL MASTER CM-100, AZ ONE Co. Tokyo, Japan) at 10,000 rpm for 1.5 min. Then, 0.9 mL of 30% pork homogenate, 0.45 mL of 10% sterile salt solution, and 0.15 mL of NB medium containing specific bacterial isolate were transferred to sterilized test tubes (final concentrations; 20% pork homogenate, 3% salt, and $2.0\times10^6$ CFU/mL). In addition, to confirm sterility of the experiment, antibiotics were added in the antibiotic group with a final concentration of 70 µg/mL penicillin G potassium, 250 µg/mL streptomycin sulfate, and 50 µg/mL gentamicin sulfate. The samples (model mixtures) were then incubated at 25°C for 7 days in the dark under anaerobic and aerobic conditions. Anaerobic conditions was maintained using an oxygen impermeable storage bag containing an oxygen absorbent (A-500HS, I.S.O. Inc.).

#### 2.2.5.3. Preparation of the aSMM model sample

To investigate the ZnPP-forming and color improvement ability of ZnPP-forming food-grade LAB, an aSMM model experiment was carried out as previously described (Wakamatsu *et al*., 2020) with minor modifications. Briefly, 9 g of aSMM (porcine LTL muscle) including 3% salt (vs. meat) was packaged into a small bag (Standard bag Hiryu N-

1, Asahi Kasei Pax Corp., Tokyo, Japan) previously sterilized under UV light irradiation on a clean bench for overnight. aSMM was prepared using a pre-sterilized food processor (MK-K48P-W; Panasonic Co., Osaka, Japan). One mL of bacterial culture (finally $2.0\times10^6$ CFU/g) were added into the bag and then the bag was vacuum-packaged. In the case of the control and nitrite-added groups, autoclaved water and 300 ppm of sodium nitrite (final concentration) aseptically filtrated were added instead of the bacteria solution, respectively. Finally, the bags were incubated for 14 days at 18°C in the dark.

### 2.2.6. Manufacturing and analysis of dry-cured sausage

#### 2.2.6.1. Preparation of starter culture for sausage

According to ZnPP-forming ability of the isolated ZnPP-forming food-grade LAB, five selected ZnPP-forming LAB, *Enterococcus faecium* MP-1 (EF), *Lactobacillus curvatus* FS2(A)-1 (LC), *Lactobacillus plantarum* FS4-2 (LP), *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC), and *Leuconostoc lactis* SS(A)-1 (LL), were used as starter cultures in the preparation of dry-cured sausages. All LAB were cultivated anaerobically at 30°C. After 24 h of incubation, the concentration of bacteria in the cultured broth was quantified using the hemocytometer under a phase-contrast microscope (Olympus Co.) to calculate the required amount of bacterial cell suspension per the weight of the meat (6 log CFU/g of meat). The required bacterial cell suspension was then centrifuged (Tomy Digital Biology Co., CAX-371, Tokyo, Japan) at 10,000 ×*g* for 15 min at 4°C to remove the broth (supernatant). The pellets containing crude cells were then washed twice with a sterile physiological saline solution using the same centrifugation technique. Finally, the washed pellets were re-

suspended in 10 mL of sterile physiological saline solution, which was used as the starter culture. Freshly prepared bacterial culture were used.

#### 2.2.6.2. Preparation of sausage

Sausages were prepared twice using a mixture of porcine LTL muscle from six different primal cuts at each time. Sausage manufacturing was carried out in a pilot manufacturing scale plant at the Hokkaido University Agri-Food Center (Hokkaido, Japan). First, the LTL muscles were trimmed off the fat and major connective tissue and cut into small blocks. After that, the pieces of the block were chopped, transferring them into a meat grinder with a 3.2 mm plate hole size. Subsequently, salt (2.5%) and glucose (1%) were added to the chopped meat and firmly mixed. Then, the mixture was divided into seven groups. Among them, the non-inoculated and nitrite-added groups (300 ppm of final weight) were designated as the control and positive controls, respectively. The other five groups were inoculated with the five selected ZnPP-forming LAB (6 log CFU/g of meat). Sterile ultra-pure water was added to the non-inoculated group instead of the bacterial solution. Then, the mixture of each group was stuffed into a cellophane casing (Tohcello bista SP-S 450, Shikoku Tohcello Co. Ltd., Tokushima, Japan) with a stuffer machine and clipped with metal clips. Five sausages (approximately 150 g in weight and 12 cm in length) in each group were prepared and placed into an incubation chamber. A thermal hygrostat (QBX–132 HRST 1, Fukushima Galilei Co. Ltd. Osaka, Japan) was used as the incubation chamber. The sausages of each group were subjected to the following conditions: resting period at 1°C for 24 h, fermentation period at 18°C with 85% relative humidity (RH) for the next 7 days, drying period at 14°C with 75% RH for the next 7 days, and ripening period at 12°C with 80% RH for 14 days. Seven sausages

(one from each group) were chosen at random and analyzed on every 7th day of processing. The sausages were kept at -20°C during analysis. Each time from each sausage, three replications have been performed.

#### 2.2.6.3. Analysis of sausage

##### 2.2.6.3.1. The viable and lactic acid bacterial counts

Three grams of sample was obtained aseptically from the core portion of the sausages and transferred to a sterile plastic bag. Then, the sample was homogenized with 27 mL of sterile saline (0.9% salt) using a stomacher (Exnizer 400, Organo). Ten-fold serial dilutions were performed, and suitable dilutions (0.1 mL) were poured onto the growth media in duplicate. Two different growth media, standard plate count (SPC) agar (Eiken, Chemical Co. Ltd., Tochigai, Japan) for the viable count or MRS agar (Oxoid, Kanto Chemical Co. Inc.) for the total LAB count, were used. The dilutions were spread onto the agar plate using a sterilized glass rod. Subsequently, the SPC agar plate was incubated aerobically at 30°C for 24 h, and the MRS agar plate was incubated anaerobically at 30°C for 48 h. Finally, the number of bacteria was calculated and expressed as log CFU/g.

##### 2.2.6.3.2. Determination of water activity ($a_w$), pH, and titratable acidity

The $a_w$ of the sausages was determined using a LabMaster meter (Novasina AG, Lachen, Switzerland) at 25°C. The pH of the sausages was measured with a pH meter (Horiba, F-55 series, Horiba Ltd., Kyoto, Japan) by dipping the probe into a 20% meat homogenate. The titratable acidity of the sausages was estimated by titration of the sample with 0.1N NaOH using phenolphthalein (1% alcoholic phenolphthalein) as an indicator (AOAC, 1990).

### 2.2.7. ZnPP measurement and fluorescence analysis

The extraction of ZnPP by using cold acetone and subsequent analysis of fluorescence were carried out as previously mentioned methods (Wakamatsu *et al*., 2007) with minor modifications. As for aSMM, 20% homogenate of the sample was prepared, and 1.5 mL of the homogenate was taken for the measurement of ZnPP. In the case of dry-cured sausage, the chopped sausages was soaked into ultra-pure water (final volume 20%) for 1 h at room temperature to soften, and similarly, 20% homogenate of the sample was prepared. Next, 1.5 mL of meat homogenate was taken for the measurement of ZnPP. The ZnPP in the samples was extracted by adding three volumes of cold acetone (75% of the total volume). The samples were then vortexed and stored at 4°C for 30 min in the dark for better extraction of ZnPP. After extraction, the sample was filtered through a filter paper (No. 2, 90 mm, Toyo Roshi Kaisha Ltd., Tokyo, Japan). Finally, the fluorescence intensity of extracted ZnPP was measured using a spectrofluorophotometer (RF-5300PC; Shimadzu Co.) at Ex/Em: 420/590 nm. Fluorescence intensity was used as a metric for the quantity of ZnPP formed. In the case of dry-cured sausages, the fluorescence intensity was calculated and expressed as nmol/g DM (dry matter), whereas ZnPP (Aldrich Chem. Co., Milwaukee, WI) was used as the standard. Regarding fluorescent spectrum analysis, the fluorescence spectra of the extracts were measured using a spectrofluorophotometer (RF-5300PC; Shimadzu Co.) with a quartz microcell 2 mm thick (GL Sciences Inc., Tokyo, Japan). The excitation wavelength was fixed at 400 nm and the emission wavelength was scanned over the 420–750 nm range at 500 nm/minute. The entrance slit was set at 10 nm and the exit slit also at 10 nm.

### 2.2.8. Observation of visual image and ZnPP autofluorescence (ZAF) in aSMM and dry-cured sausage

To observe the appearance and ZAF of the aSMM after incubation and dry-cured sausages, a digital camera (Nikon D3300, Nikon Corporation, Tokyo, Japan) was used as previously described (Wakamatsu *et al*., 2020). Observation of ZAF was carried out using a digital camera equipped with a sheet-type band pass filter that transmits light around 600 nm (BPB-60, Fujifilm Co., Tokyo, Japan) and purple LED lighting devices equipped with a sheet-type band pass filter that transmits light around 420 nm (BPB-42, Fujifilm Co.).

### 2.2.9. Color analysis of aSMM and dry-cured sausage

The instrumental color analysis was carried out based on the 3-dimensional CIELAB color scale. The color of aSMM and dry-cured sausages was measured using a spectro-colorimeter (CM-700d, Konica Minolta, Inc., Tokyo, Japan) with the following specifications: target mask: 8 mm diameter; without stabilizer (SAV); color system: L* (lightness), a* (redness), b* (yellowness); illuminant: D65; viewing angle: 10° and regular reflection processing: SCI. Before measuring, the colorimeter was calibrated using a white standard plate. Measurements were performed in triplicate to obtain an average meat color value for each portion of aSMM. For measuring the outside color of the dry-cured sausage, the surface of the sausages was cut into 2 mm thick to get a uniform surface. For the measurement of the inside color of dry-cured sausage, the sausages were cut into 2.5 cm thick cross-sections with a uniform shape. Each cross-section was then exposed to air for about 1 h at room temperature to bloom (allowing the remaining heme pigments to oxygenate).

Finally, the color was measured carefully, applying gentle pressure to make sure no light entered or exited the aperture and avoiding any fat particles. The values were obtained from three different cross-sections of each group. In addition, the hue angle and chroma ($C^*$) were also calculated in both aSMM and dry-cured sausages.

### 2.2.10. Statistical analysis

Data were presented as mean ± standard error. Values were calculated using Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA) with Ekuseru-Toukei 2012 (Social Survey Research Information, Tokyo, Japan) as an add-in software. One-way analysis of variance (ANOVA) with Dunnett's comparison test was done to consider the high ZnPP-forming bacteria. Besides, ANOVA with Tukey's multiple comparison test was performed for the color parameters and ZnPP formation in aSMM and dry-cured sausages to evaluate the differences among individuals. $P < 0.05$ were considered to be statistically significant.

## 2.3. Results

### 2.3.1. Screening for food-grade LAB with high ZnPP-forming ability and their characterization

#### 2.3.1.1. Isolation and identification of high ZnPP-forming food-grade LAB

A total of 55 samples from different food products and environmental sources categorized into six broad groups were used to screen for food-grade LAB with high ZnPP-forming ability (Table 1). The bacterial isolates were cultured anaerobically using two modified media (mMRS, mM17), which included 3% salt, and the pH was adjusted to 5.5 because of the application of isolated bacteria to meat products. A total of 450 isolates were separated from different food products and environmental sources. Next, the ZnPP-forming ability of these separated isolates using an aseptic meat homogenate (aMH) model experimental system was evaluated, in which *Serratia liquefaciens* (Wakamatsu *et al*., 2020) and antibiotic groups were used as positive and negative controls, respectively. Here, antibiotics were used to confirm that the operations were performed aseptically. After incubation of 7 days at 25°C under anaerobic conditions, some bacteria could form a significantly higher amount of ZnPP compared to the control group, meaning that the higher ZnPP was derived from ZnPP-forming bacteria as shown in Fig. 2 as an example of screening (ZnPP-forming ability by all the isolates was shown in appendix Fig. 1-2). From the 450 isolates, 69 isolates were recognized as ZnPP-forming bacteria based on ZnPP-forming ability (Table 2). Since no significant difference was found between the control and antibiotic groups in all experiments (data not shown), all operations were confirmed to be performed

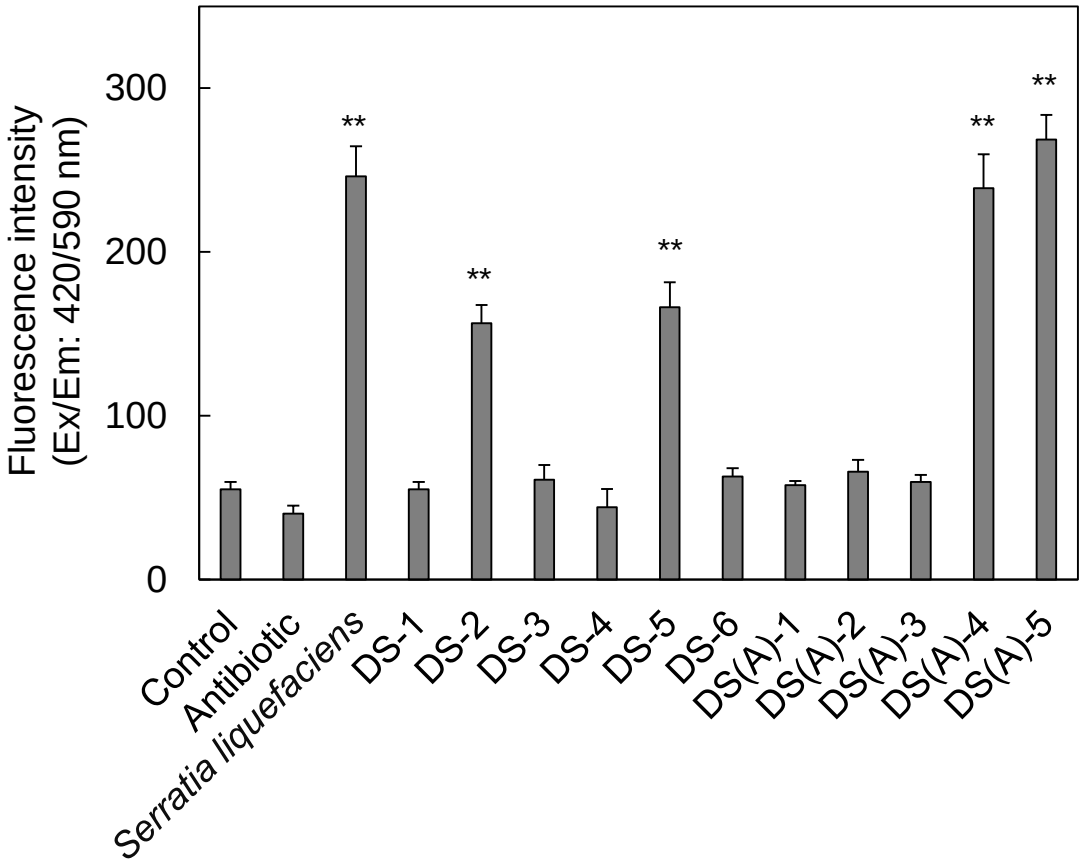



**Fig. 2. ZnPP-forming abilities of the bacteria from dry shark (DS) fish in aseptic meat homogenate (aMH) model (as an example)**

Aseptic 20% LTL muscle homogenate containing 3% salt was prepared and salt-added meat homogenate was inoculated with isolated bacteria ($2.0\times10^6$ CFU/mL). No bacterial isolate was added in the control and antibiotic groups. The *S. liquefaciens*-inoculated group was designated as positive control. After incubation at 25°C for 7 days under anaerobic condition, ZnPP of the mixture was extracted with 75% acetone method and the fluorescence intensity was measured. Data expressed as means ± SE (n = 3). **: Significant difference at $P < 0.01$ vs control.

**Table 2. Changes in the number of bacterial strains in different stages of ZnPP-forming LAB screening process**

| Sample groups | Number of isolates tested | ZnPP-forming | | |
|---|---|---|---|---|
| | | Bacteria | Acid-producing LAB (APB) | LAB |
| Fermented foods | 129 | 40 | 12 | 12 |
| Meat and meat products | 51 | 1 | 1 | 1 |
| Fish and fish products | 20 | 4 | 3 | 1 |
| Fruits, vegetables, and their products | 167 | 15 | 11 | 6 |
| Dairy products | 33 | 5 | 4 | 1 |
| Miscellaneous | 50 | 4 | 5 | 4 |
| Total | 450 | 69 | 36 | 25 |

aseptically. The high levels of ZnPP in the groups inoculated with the isolates such as MP-1, FS2(A)-1, FS2(A)-2, FS3(A)-1, FS4-2, CH-1, GB(A)-1, SS(A)-1, CB-1, OR-2 and FR(A)-1 was observed, compared to the positive control (appendix Fig. 1). Next, plate count agar with bromocresol purple (PCA-BCP) was used to confirm whether the screened ZnPP-forming bacteria were acid-producing bacteria (APB). From 69 ZnPP-forming bacteria, 36 bacteria were considered as ZnPP-forming APB (Table 2, 3).

Subsequently, the nucleotide sequences of the 16S rRNA gene of 36 ZnPP-forming APB were analyzed and determined to be LAB and food-grade species. Then, 25 bacteria were confirmed as ZnPP-forming LAB, and 23 isolates were found to be ZnPP-forming food-grade LAB (Table 2, 4). Among the identified LAB, a total of six genera; *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, S*treptococcus*, and *Weissella*, encompass 15 different species (avoiding duplication), whereas 13 were found to be food-grade, and 2 species of *Streptococcus*, *St. rubneri*, and *St. vestibularis*, were non-food-grade. Among the food-grade species, *Lactobacillus curvatus* FS2(A)-1, *Enterococcus faecium* MP-1, *Lactococcus lactis* subsp. *cremoris* GB(A)-1, *Lactobacillus plantarum* FS4-2, and *Leuconostoc lactis* SS(A)-1 were found to have the highest rates of ZnPP-formation within the species or strains of the same species (Appendix Fig. 1).

#### 2.3.1.2. Characterization of ZnPP-forming food-grade LAB

In this section, the physiological, biochemical, and phenotypic properties of the 23 ZnPP-forming food-grade LAB were investigated.

**Table 3. Confirmation of the ZnPP-forming APB by using plate count agar with bromocresol purple (PCA-BCP)**

| Source | ZnPP-forming bacterial isolates and **APB**[a] |
|---|---|
| Fermented sausage 1 | FS1-2, FS1-3, FS1(A)-3, FS1(A)-6 |
| Fermented sausage 2 | FS2-3, FS2-4, **FS2(A)-1**, **FS2(A)-2**, FS2(A)-4 |
| Fermented sausage 3 | FS3-2, FS3-3, FS3-5, **FS3(A)-1**, FS3(A)-3 |
| Fermented sausage 4 | FS4-1, **FS4-2**, FS4(A)-1, FS4(A)-5 |
| Cheese | **CH-1**, **CH-2**, CH-4, CH(A)-2 |
| Kimchi | KM-3, KM-5, KM-7, KM(A)-3, **KM(A)-5** |
| Miso | MS-2, **MS(A)-1** |
| Natto | NT-2, NT-4, NT(A)-2 |
| Soy sauce | SS-3, SS-4, **SS(A)-1** |
| Cucumber pickle | CP-2, **CP(A)-1**, CP(A)-2, **CP(A)-3** |
| Fermented rice | **FR(A)-1** |
| Chicken skin | **CS(A)-1** |
| Dry shark fish | **DS-2**, **DS-5**, **DS(A)-4**, DS(A)-5 |
| Cabbage | **CB-1**, CB(A)-4 |
| Lemon | LM(A)-3, **LM(A)-4** |
| Orange | **OR-1**, **OR-2**, **OR(A)-3** |
| Grape | **GP-2** |
| Orange marmalade | **OM(A)-1** |
| Germinated bean | **GB(A)-1** |
| Apple | **AP(A)-3** |
| Mango pickle | **MP-1** |
| Pineapple | **PA-6** |
| Malta | MT-1 |
| Lettuce | **LT(A)-2** |
| Ghee | **GH-1** |
| Butter | **BT-2**, **BT(A)-4**, **BT(A)-6** |
| Milk | ML(A)-4 |
| Stool | **ST(A)-4** |
| Saliva | **SL(A)-3** |
| Urine | **UR(A)-2** |
| Processed noodles | **PN-1** |
| | **APB**/Total: **36**/69 |

[a] Bold: Acid-producing bacteria (APB)

**Table 4. Identification of high ZnPP-forming LAB isolated from different food products and environmental sources**

| LAB isolates | Organism name (Closest strain) | Identity (%) | Gene bank accession No.[a] |
|---|---|---|---|
| MP-1 | *Enterococcus faecium* XL1747 | 97.83 | MH704178.1 |
| FS2(A)-1 | *Lactobacillus curvatus* JCM1096 | 99.39 | CP026116.1 |
| FS2(A)-2 | *Lactobacillus curvatus* CM-CNRG539 | 98.39 | MH935922.1 |
| FS4-2 | *Lactobacillus plantarum* SKB1234 | 99.18 | MK537376.1 |
| CH-1 | *Lactococcus lactis* RCB 787 | 98.23 | KT260999.1 |
| GB(A)-1 | *Lactococcus lactis* subsp. *cremoris* A76 | 99.29 | CP003132.1 |
| OM(A)-1 | *Lactococcus raffinolactis* MB8-2 | 99.49 | MG755389.1 |
| CS(A)-1 | *Leuconostoc citreum* D8 | 100.00 | MH229975.1 |
| CP(A)-1 | *Leuconostoc citreum* C21 | 99.11 | MH229970.1 |
| KM(A)-5 | *Leuconostoc holzapfelii* 51 | 99.59 | MH229961.1 |
| SS(A)-1 | *Leuconostoc lactis* SD501 | 99.39 | MH307960.1 |
| FS3(A)-1 | *Leuconostoc lactis* CAU10229 | 99.27 | MF098186.1 |
| LM(A)-4 | *Leuconostoc mesenteroides* RL1545 | 98.79 | MH704131.1 |
| ST(A)-4 | *Leuconostoc mesenteroides* OP9 | 99.37 | MF967225.1 |
| CB-1 | *Leuconostoc mesenteroides* K81 | 99.89 | MK311348.1 |
| OR-2 | *Leuconostoc mesenteroides* SRCM103453 | 99.78 | CP035271.1 |
| UR(A)-2 | *Leuconostoc pseudomesenteroides* SP89 | 98.21 | MG645308.1 |
| CP(A)-3 | *Leuconostoc pseudomesenteroides* CM-CNRG534 | 96.83 | MH935917.1 |
| GH-1 | *Leuconostoc pseudomesenteroides* A2-2 | 98.56 | MG694682.1 |
| FR(A)-1 | *Leuconostoc pseudomesenteroides* 2-58 | 98.52 | MK453316.1 |
| CH-2 | *Leuconostoc pseudomesenteroides* CAU7925 | 99.70 | MF108809.1 |
| SL(A)-3 | *Streptococcus rubneri* LMG27206 | 98.47 | JX861486.1 |
| PN-1 | *Streptococcus salivarius* XL1742 | 99.26 | MH704177.1 |
| DS-2 | *Streptococcus vestibularis* NCTC12167 | 99.57 | LR134275.1 |
| MS(A)-1 | *Weissella cibaria* HSP-WCi1 | 99.10 | MG669650.1 |
| Total: 25 | | | |

[a] GenBank accession numbers of the sequences obtained from the representative isolates.

#### 2.3.1.2.1. Physiological and biochemical characterization of ZnPP-forming food-grade LAB

Morphology of all 23 ZnPP-forming food-grade LAB was observed as cocci, rod, or cocco-bacilli shapes under microscopic observation (Table 5). All ZnPP-forming food-grade LAB showed catalase negative (Table 5), which was confirmed by a lack of or weak bubble production.

#### 2.3.1.2.2. Phenotypic characterization of ZnPP-forming food-grade LAB

Subsequently, the growth of these 23 ZnPP-forming food-grade LAB under different environmental conditions was tested to evaluate their growth capability for eventual application to different meat products (Table 6). All of the LAB were able to grow aerobically or anaerobically, but anaerobic conditions were preferred. In the case of pH, all the ZnPP-forming food-grade LAB grew optimally at pH 5.5 and 6.5 but also grew well at pH 8.5, except for FS2(A)-1, FS2(A)-2, and FS4-2 where limited growth were observed. These ZnPP-forming food-grade LAB grew best at pH 4.5, where the lowest growth was observed for all other LAB. In terms of salt concentration, six ZnPP-forming food-grade LAB; SS(A)-1, FS3(A)-1, LM(A)-4, ST(A)-4, CB-1, and OR-2 grew well even in 7% salt. FS2(A)-1, FS2(A)-2, and FS4-2 were able to propagate to their maximum potential in 5% salt, but all ZnPP-forming food-grade LAB preferred 3% salt conditions. Regarding temperature, maximum growth was observed at 30°C for all the ZnPP-forming food-grade LAB; no growth was observed at 45°C for all strains except MP-1. Optimal conditions were, therefore, aerobic or anaerobic conditions with 3% salt supplemented media, pH of 5.5, and

**Table 5. Physiological and biochemical characteristics of ZnPP-forming food-grade LAB**

| LAB isolates[a] | ZnPP-forming food-grade LAB | Medium (MRS/M17) | Cell shape | Catalase test |
|---|---|---|---|---|
| **MP-1** | *Enterococcus faecium* | MRS | Cocci | - |
| **FS2(A)-1** | *Lactobacillus curvatus* | MRS | Rod | - |
| FS2(A)-2 | *Lactobacillus curvatus* | MRS | Rod | - |
| **FS4-2** | *Lactobacillus plantarum* | MRS | Rod | - |
| **CH-1** | *Lactococcus lactis* | M17 | Cocci | - |
| **GB(A)-1** | *Lactococcus lactis* subsp. *cremoris* | MRS | Cocci | - |
| **OM(A)-1** | *Lactococcus raffinolactis* | MRS | Cocci | - |
| **CS(A)-1** | *Leuconostoc citreum* | MRS | Cocci | - |
| CP(A)-1 | *Leuconostoc citreum* | MRS | Cocci | - |
| **KM(A)-5** | *Leuconostoc holzapfelii* | MRS | Cocci | - |
| **SS(A)-1** | *Leuconostoc lactis* | MRS | Cocco-bacilli | - |
| FS3(A)-1 | *Leuconostoc lactis* | MRS | Cocco-bacilli | - |
| LM(A)-4 | *Leuconostoc mesenteroides* | MRS | Cocci | - |
| ST(A)-4 | *Leuconostoc mesenteroides* | MRS | Cocci | - |
| CB-1 | *Leuconostoc mesenteroides* | MRS | Cocci | - |
| **OR-2** | *Leuconostoc mesenteroides* | MRS | Cocci | - |
| UR(A)-2 | *Leuconostoc pseudomesenteroides* | MRS | Cocci | - |
| CP(A)-3 | *Leuconostoc pseudomesenteroides* | MRS | Cocci | - |
| GH-1 | *Leuconostoc pseudomesenteroides* | MRS | Cocci | - |
| **FR(A)-1** | *Leuconostoc pseudomesenteroides* | MRS | Cocci | - |
| CH-2 | *Leuconostoc pseudomesenteroides* | MRS | Cocci | - |
| **PN-1** | *Streptococcus salivarius* | MRS | Cocci | - |
| **MS(A)-1** | *Weissella cibaria* | MRS | Cocco-bacilli | - |
| Total: 23 | | | | |

[a] Bold: 13 LAB evaluated with aSMM model

**Table 6. Phenotypic properties of ZnPP-forming food-grade LAB**

| LAB Isolates | $O_2$ | | pH | | | | NaCl | | | Temperature | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Aerobic | Anaerobic | 4.5 | 5.5 | 6.5 | 8.5 | 3% | 5% | 7% | 18°C | 30°C | 45°C |
| MP-1 | +++ | ++ | + | +++ | +++ | +++ | +++ | ++ | + | - | +++ | +++ |
| FS2(A)-1 | + | +++ | ++ | +++ | +++ | + | +++ | +++ | + | + | +++ | - |
| FS2(A)-2 | + | +++ | ++ | +++ | +++ | + | +++ | +++ | + | + | +++ | - |
| FS4-2 | + | +++ | ++ | +++ | +++ | + | +++ | +++ | + | + | +++ | - |
| CH-1 | +++ | + | + | +++ | +++ | +++ | +++ | - | - | ++ | +++ | - |
| GB(A)-1 | +++ | +++ | + | +++ | +++ | ++ | +++ | + | + | + | +++ | - |
| OM(A)-1 | ++ | +++ | + | +++ | +++ | ++ | +++ | + | + | + | +++ | - |
| CS(A)-1 | + | +++ | + | +++ | +++ | +++ | +++ | ++ | + | ++ | +++ | - |
| CP(A)-1 | + | +++ | + | +++ | +++ | +++ | +++ | ++ | + | ++ | +++ | - |
| KM(A)-5 | ++ | +++ | + | +++ | +++ | ++ | +++ | + | + | +++ | +++ | - |
| SS(A)-1 | +++ | + | + | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - |
| FS3(A)-1 | +++ | + | + | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - |
| LM(A)-4 | + | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - |
| ST(A)-4 | + | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - |
| CB-1 | +++ | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - |
| OR-2 | +++ | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ | + | +++ | - |
| UR(A)-2 | + | +++ | + | +++ | +++ | ++ | +++ | + | + | ++ | +++ | - |
| CP(A)-3 | + | +++ | + | +++ | +++ | ++ | +++ | + | + | ++ | +++ | - |
| GH-1 | +++ | + | + | +++ | +++ | ++ | +++ | + | + | ++ | +++ | - |
| FR(A)-1 | + | +++ | + | +++ | +++ | ++ | +++ | + | + | ++ | +++ | - |
| CH-2 | +++ | + | + | +++ | +++ | ++ | +++ | + | + | ++ | +++ | - |
| PN-1 | +++ | ++ | + | +++ | +++ | ++ | +++ | + | - | - | +++ | - |
| MS(A)-1 | + | +++ | + | +++ | +++ | ++ | +++ | ++ | ++ | ++ | +++ | + |

-, no growth; +, minimum growth; ++, moderate growth; +++, maximum growth

temperature of 18°C with a long time or 30°C with a short time during ripening of fermented meat products. These conditions are commonly used in meat products; therefore, these ZnPP-forming food-grade LAB could be considered as promising starter cultures for use in meat products.

### 2.3.2. Evaluation of the screened ZnPP-forming food-grade LAB using aseptic salt-added minced meat (aSMM)

In order to apply the screened ZnPP-forming food-grade LAB identified in section 2.3.1.1 to dry-cured meat products, the ZnPP-forming ability of 13 different species (Bold in Table 5, avoiding duplication) of LAB was evaluated in aSMM which was closer to dry-cured meat products. In this experiment, nitrite and *Serratia liquefaciens* (Wakamatsu *et al*., 2020) were used along with the ZnPP-forming food-grade LAB as positive controls of color and ZnPP formation, respectively.

#### 2.3.2.1. Visual color and ZnPP autofluorescence (ZAF) of aSMM

To investigate the effects of the screened ZnPP-forming food-grade LAB on meat color, the samples of minced meat with 3% salt under sterile conditions were prepared and added to the screened ZnPP-forming food-grade LAB. Visual and ZAF images of aSMM were observed after 14-days of anaerobic incubation. The distinctive bright red color was observed in the nitrite-added group (Fig. 3). Among the ZnPP-forming food-grade LAB-inoculated groups, the *Lactobacillus curvatus*-inoculated group became more bright red in color compared to the non-inoculated group, with a color that was close to that of the nitrite-added group. The *Lactobacillus plantarum*-, *Lactococcus lactis* subsp. *cremoris*-, *Enterococcus*

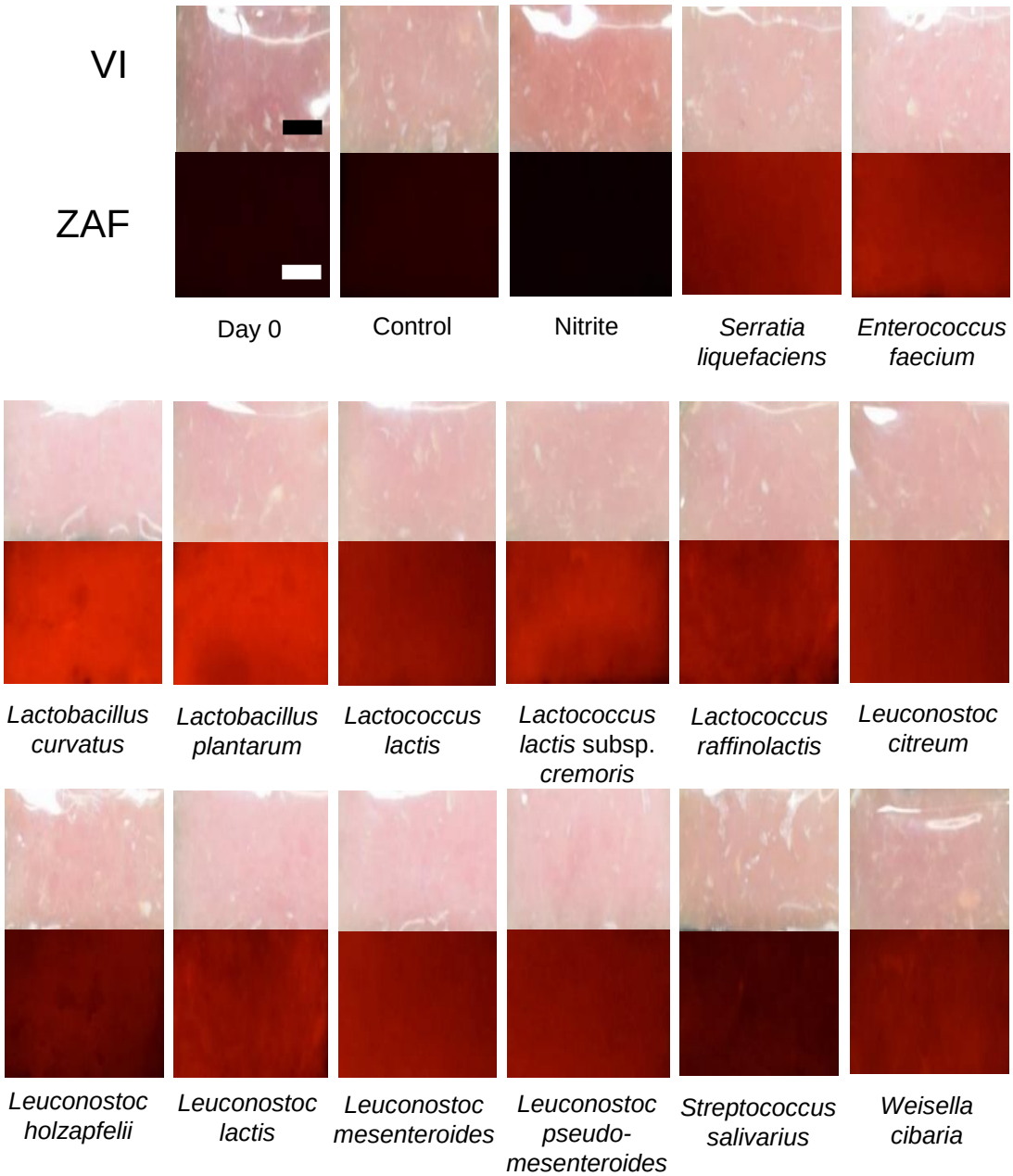



**Fig. 3. Effects of ZnPP-forming food-grade LAB on the visual color and ZnPP autofluorescence in aseptic salt-added minced meat (aSMM)**

aSMM prepared from the core portion of the porcine LTL muscle was inoculated with ZnPP-forming food-grade LAB and incubated anaerobically at 18°C for 14 days. The non-inoculated control and the nitrite-added groups were designated as the negative and positive controls, respectively, whereas ‘day 0’ indicates the state before incubation of the sample. The uppers are visible images (VI), and the bottoms are ZnPP autofluorescence (ZAF) images. Scale bars: 1 cm.

*faecium*-, *Leuconostoc lactis*-, *Leuconostoc mesenteroides*-, and *Leuconostoc pseudomesenteroides*-inoculated groups also exhibited improved color. In terms of ZAF, among the samples in which ZnPP-forming food-grade LAB had been added, the ZAF of the *Lactobacillus curvatus*-inoculated group was the strongest (Fig. 3). The ZAF was also enhanced in the *Lactobacillus plantarum*-, *Lactococcus lactis* subsp. *cremoris*-, *Enterococcus faecium*-, *Leuconostoc lactis*-, *Lactococcus lactis*-, *Lactococcus citrum*-, *Lactococcus raffinolactis*-, *Leuconostoc mesenteroides*-, *Leuconostoc pseudomesenteroides*-, *Weisella cibaria*-, *Serratia liquefaciens*-, *Leuconostoc holzapfelii*- and *Streptococcus salivarius*-inoculated groups as compared to the non-added group. On the other hand, almost no and very weak ZAF were observed in the nitrite-added and control groups, respectively.

#### 2.3.2.2. ZnPP formation and color profiles in aSMM

The fluorescence intensity of ZnPP and color of the aSMM were measured after 14 days of incubation (Fig. 4). The fluorescence intensity of ZnPP was higher in all the inoculated groups and significantly different compared to the control and nitrite-added groups (Fig. 4A). The color was measured using the CIE L* (lightness), a* (redness), and b* (yellowness) colorimetric system. The lightness (L* value) was significantly increased in all the high ZnPP-forming food-grade LAB compared to the control and nitrite-added groups (Fig. 4B). The redness (a* value) was greatly increased in the nitrite-added group (Fig. 4C). Samples inoculated with ZnPP-forming food-grade LAB also showed significantly higher a* values than the control group. The values of *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactococcus lactis* subsp. *cremoris* and *Leuconostoc lactis* were similar to the nitrite-added group. The b* values were significantly decreased in all the LAB-inoculated groups, and the

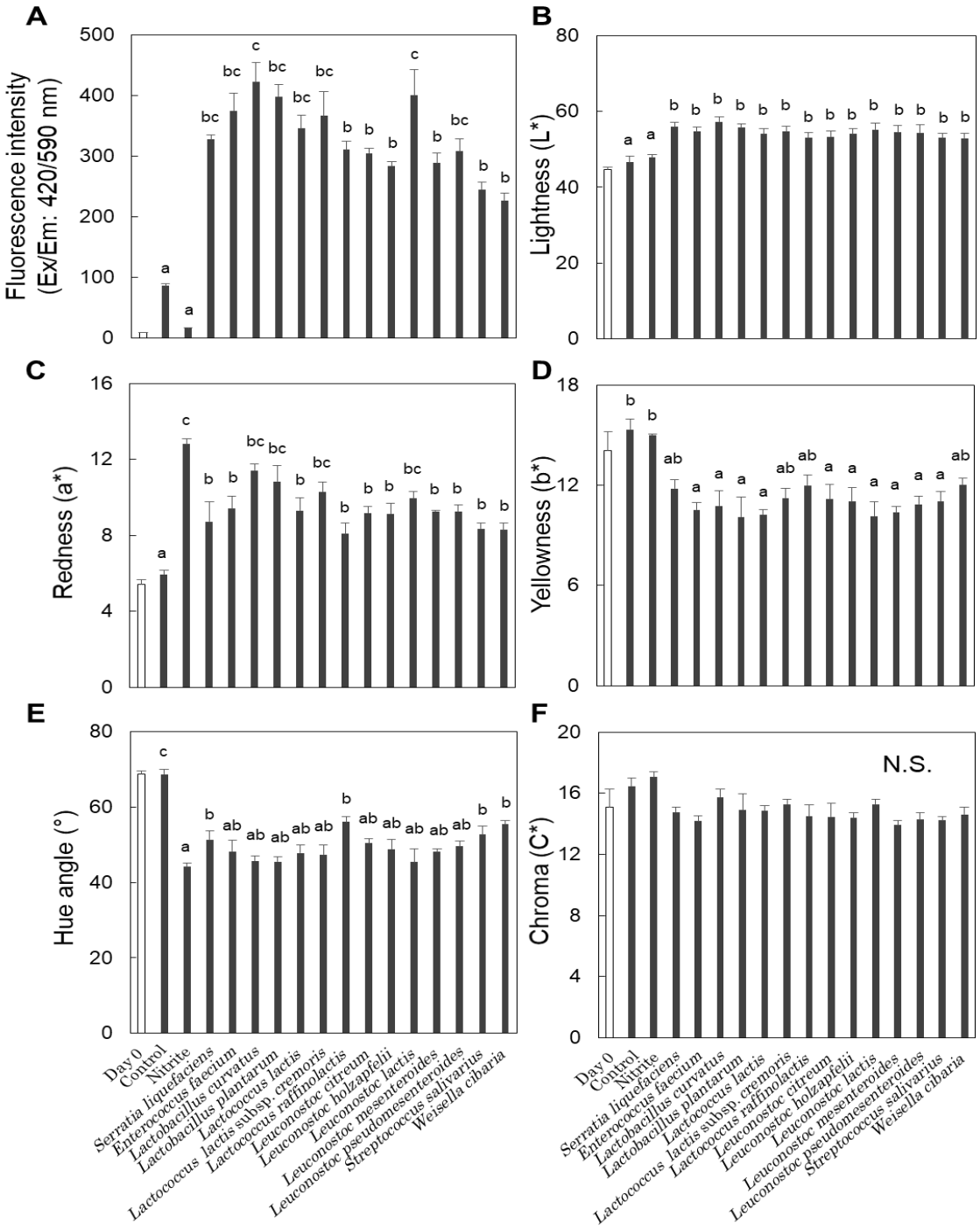



**Fig. 4. Effects of ZnPP-forming food-grade LAB on the formation of ZnPP and color profiles in aSMM**

Fluorescence intensity of ZnPP, CIE L*, a*, b*, hue angle, and chroma values of aSMM shown in Fig. 3 were measured. Data expressed as means ± SE (n = 3). abc: Significant differences among groups except the "day 0" group are indicated by different characters ($P < 0.05$). N.S.: Not significant among groups.

nitrite-added group showed only a non-significant difference compared to the control group (Fig. 4D). The hue angle was also lower in the nitrite-added group and LAB-inoculated groups compared to the control group (Fig. 4E), and the differences in C* values did not differ significantly among all the groups (Fig. 4F). These results illustrated that the visual appearance of the nitrite-added group and high ZnPP-forming food-grade LAB-inoculated groups were almost similar. Among the inoculated LAB, the colors of *Lactobacillus curvatus* FS2(A)-1, *Lactobacillus plantarum* FS4-2, *Lactococcus lactis* subsp. *cremoris* GB(A)-1, *Enterococcus faecium* MP-1, and *Leuconostoc lactis* SS(A)-1 were the closest to that of the nitrite-added group. Therefore, these five LAB were selected thereafter in the preparation of dry-cured sausages.

### 2.3.3. Preparation of dry-cured sausage using the selected ZnPP-forming food-grade LAB

#### 2.3.3.1. Changes in technological properties of dry-cured sausages

Initially, the technological properties of the sausages were inspected in terms of changes in weight and $a_w$, microbial profiles, pH, and titratable acidity as a percentage of lactic acid. The properties of the sausages were analyzed every 7 days for 28 days. The non-inoculated control and the nitrite-added groups were considered as negative and positive controls, respectively. Inoculated groups were referred to as *Enterococcus faecium* MP-1 (EF), *Lactobacillus curvatus* FS2(A)-1 (LC), *Lactobacillus plantarum* FS4-2 (LP), *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC), and *Leuconostoc lactis* SS(A)-1 (LL). The first and second weeks of processing were considered as periods of “fermentation” (0–7 days) and

"drying" (7–14 days), respectively, whereas the last 2 weeks were considered as a period of "ripening" (14–28 days) for the sausages.

#### 2.3.3.1.1. Changes in weight and $a_w$ of dry-cured sausages

The weight of each sausage was reduced noticeably during the fermentation and drying periods and then decreased gradually during the ripening stage (Fig. 5A). The weights of sausages substantially reduced by 45% to 48% over the first 14 days of processing and 56% to 57% by the end of ripening compared to the initial weight. The $a_w$ of the sausages decreased steadily throughout the process (Fig. 5B). Initially, the $a_w$ values of all groups were approximately 0.972–0.973. By the end of ripening, the $a_w$ was reduced to 0.822–0.827. However, regarding weight loss and $a_w$, there was no significant difference ($P > 0.05$) among the groups of sausages inoculated with the selected five LAB and non-inoculated ones.

#### 2.3.3.1.2. Changes in total viable and LAB counts

The microbiological profiles (total viable and LAB counts) were analyzed to determine the viability of the bacteria in the sausages (Fig. 6A & B). Significant differences ($P < 0.05$) were observed in the inoculated sausages compared to the control and nitrite-added groups in terms of the total viable count (Fig. 6A) and LAB count (Fig. 6B). The maximum value for the total viable count was noticed by day 7 of the fermentation period, and a slight decrease was observed at the end of ripening. As for the total viable count at the end of fermentation, the highest levels were observed in the LLC group (10.55 log CFU/g) followed by the LC group (10.52 log CFU/g) which showed significant difference compared to the control and nitrite-added groups ($P < 0.05$). In contrast, the control and nitrite-added groups

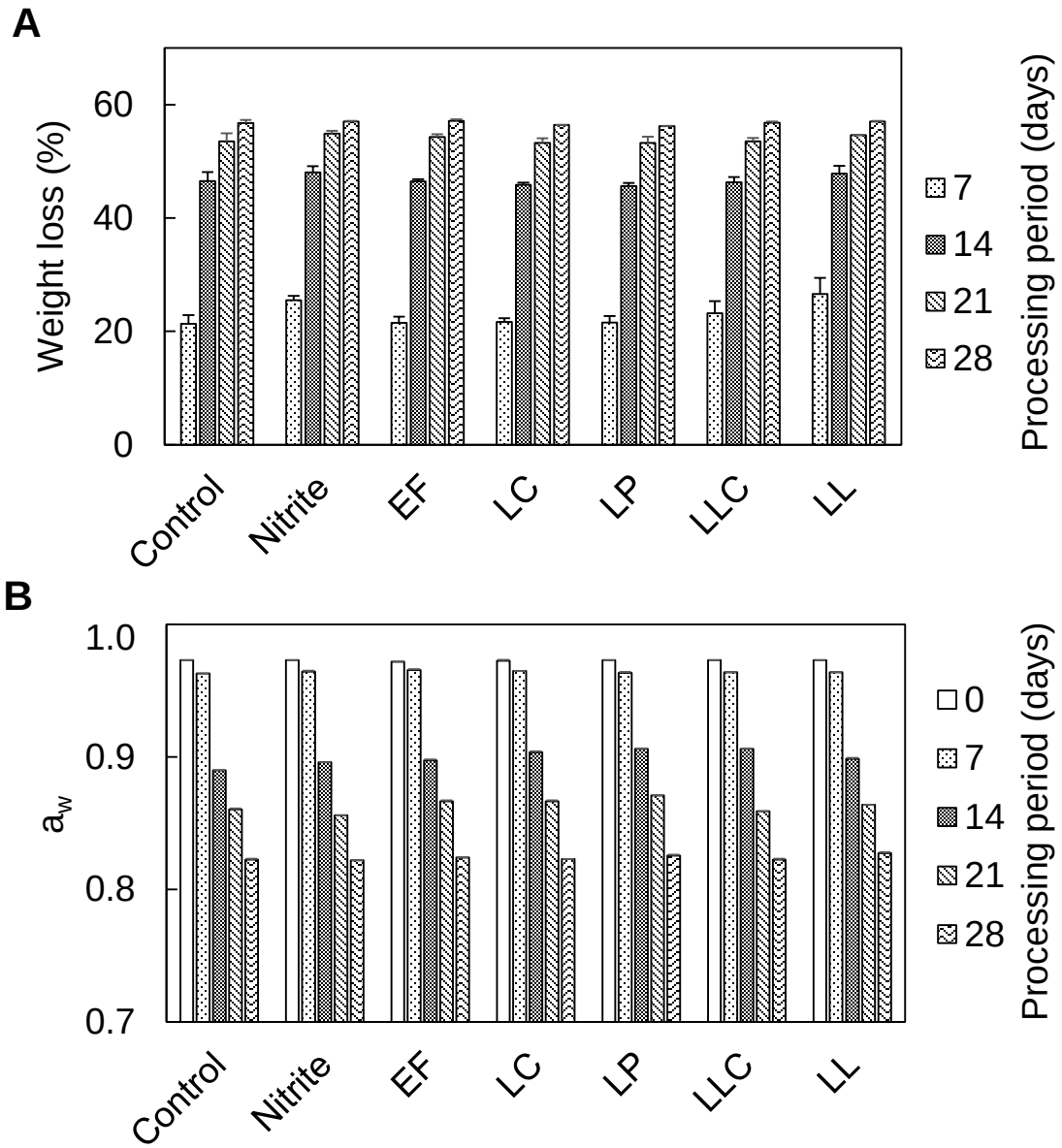



**Fig. 5. Changes in weight loss (A) and $a_w$ (B) of dry-cured sausages during processing**

Five selected high ZnPP-forming LAB (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) as starter cultures (6 log CFU/g) were used to prepare dry-cured sausages. The weight loss and $a_w$ of the sausages were analyzed up to 28 days. The non-inoculated control and the nitrite-added groups were designated as the negative and positive controls, respectively. Bars represent standard errors (n = 2).

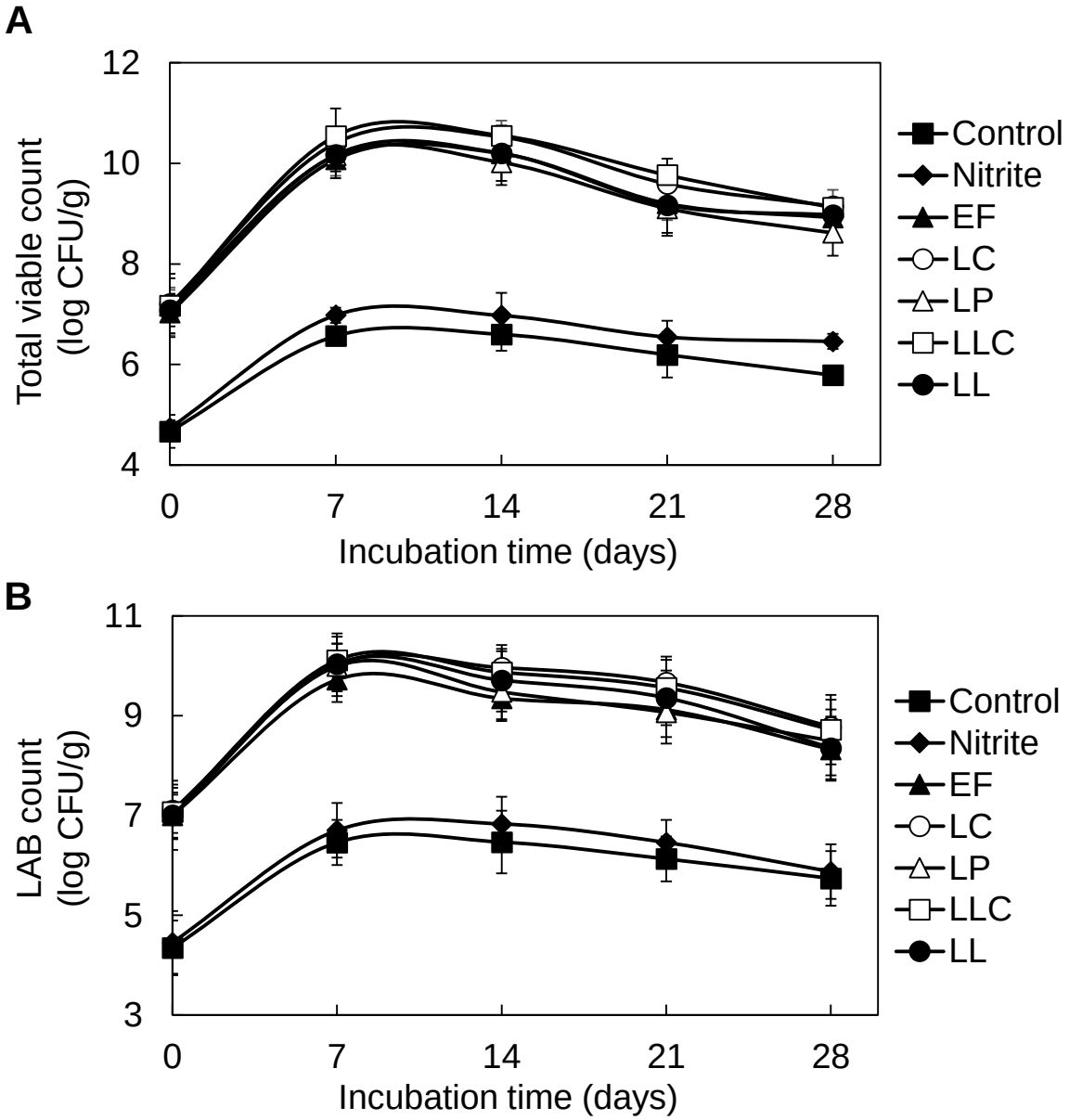



**Fig. 6. Changes in total viable (A) and lactic acid bacterial (LAB) counts (B) of dry-cured sausages during processing**

Total viable and total LAB counts of dry-cured sausages shown in Fig. 5 (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, and LL: *Leuconostoc lactis*) were performed using plate count agar and MRS agar media, respectively every seven days during processing. Bars represent standard errors (n = 2).

showed values of 6.60 and 6.97 log CFU/g, respectively. At the end of the ripening period, the highest count of 9.12 log CFU/g was observed in the LLC group, and the lowest count of 5.78 log CFU/g was observed in the control group. At the end of the fermentation period, the maximum value of the total LAB count (Fig. 6B) was observed in the LLC group (10.11 log CFU/g), whereas the minimum value was observed in the control group (6.46 log CFU/g). After fermentation, the LAB counts in all the groups slightly decreased. At the end of the ripening period, the counts were approximately 8.5 log CFU/g in all the LAB-inoculated groups and 5.8 log CFU/g in the control and nitrite-added groups.

#### 2.3.3.1.3. Changes in pH and titratable acidity

The pH and acidity were used as parameters to evaluate the amount of lactic acid formed in the sausages. After 7 days of fermentation, the pH values significantly decreased from the initial value (~5.80) to 5.56, 5.00, 5.03, 5.24, and 5.46 in the EF, LC, LP, LLC, and LL groups, respectively ($P < 0.05$) (Fig. 7A). However, the pH values did not differ significantly between the control and nitrite-added groups. The lowest pH values were observed in the LP and LC groups (4.90), followed by the LLC group (5.14) after 14 days of drying. After 21 days, the pH value progressively increased until the end of ripening in each sausage. Lactic acid accumulated more rapidly during the fermentation and drying periods and then slightly decreased (Fig. 7B). On day 7, the acidity significantly increased from the initial value (~0.41%) to 0.74, 1.03, 0.95, 0.91, and 0.77% in the EF, LC, LP, LLC, and LL groups, respectively ($P < 0.05$). In contrast, there was no significant change in the control and nitrite-added groups. The maximum amount of lactic acid was measured in the LP (1.24%) and LC (1.19%) groups, followed by the LLC group (1.13%) after 14 days of drying.

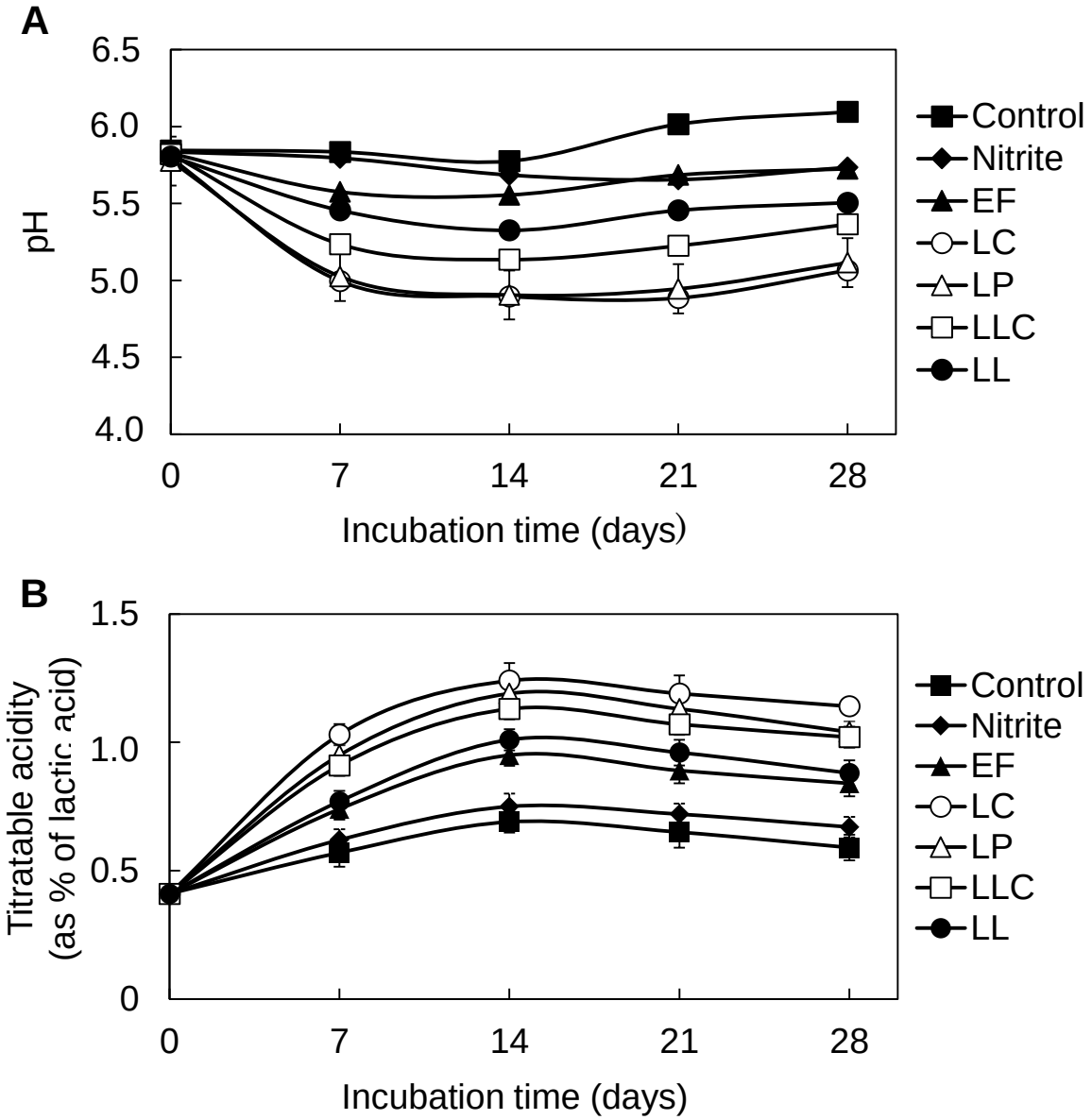



**Fig. 7. Changes in pH (A) and titratable acidity as % of lactic acid (B) of dry-cured sausages during processing**

Dry-cured sausages shown in Fig. 5 (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) were analyzed to measure pH and the content of lactic acid every 7 days during processing. Bars represent standard errors (n = 2).

#### 2.3.3.2. Color-improving ability of the selected ZnPP-forming food-grade LAB in dry-cured sausages

##### 2.3.3.2.1. The external color of dry-cured sausages

To investigate effects of the selected ZnPP-forming food-grade LAB on the external color, the visual images were observed throughout the process (Fig. 8). An increase in the bright red color was observed in the nitrite-added group after 14 days of drying, and this color stabilized until the end of ripening. Among the LAB-inoculated groups, the sausages of the LLC group showed a bright red color after 7 days of fermentation and retained the redness until the end of ripening compared to the non-inoculated group. The color of LLC group was similar to that of the nitrite-added group and more intensely red than those of the LL and EF groups. In contrast, the brown color on the surface of the sausages was observed in the LC and LP groups after 7 days of fermentation and gradually increased until the end of ripening compared to that in the non-inoculated and other inoculated groups.

##### 2.3.3.2.2. The internal color of dry-cured sausages and distribution of ZnPP

To investigate effects of the selected ZnPP-forming food-grade LAB on the internal color, the visual and ZAF images were observed in the cross-section of sausages throughout the process (Fig. 9). In visual images, the intensity of the bright red color gradually increased in the LLC and LC groups from 7 days, followed by the LP, LL, and EF groups, and the color was retained for 28 days. The color of all the inoculated groups was similar to that of the nitrite-added group. In ZAF images, the sausage in the LLC group also emitted the strongest red fluorescence, followed by those of the LC, LP, LL, and EF groups compared to those of

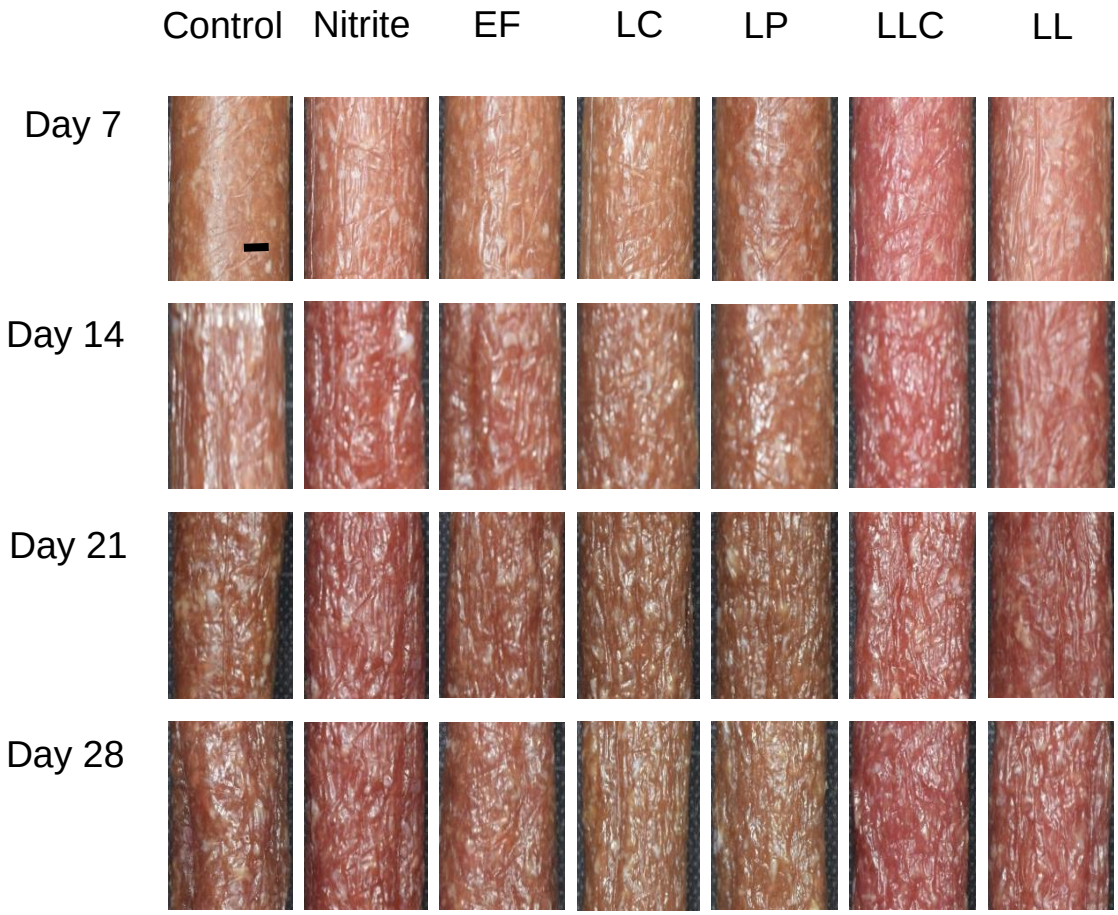



**Fig. 8. External visual images of the dry-cured sausages inoculated with the selected high ZnPP-forming LAB**

The external visual images of dry-cured sausages inoculated with the selected ZnPP-forming LAB shown in Fig. 5 (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) were observed up to the end of ripening every 7 days during processing. Scale bar: 1 cm.

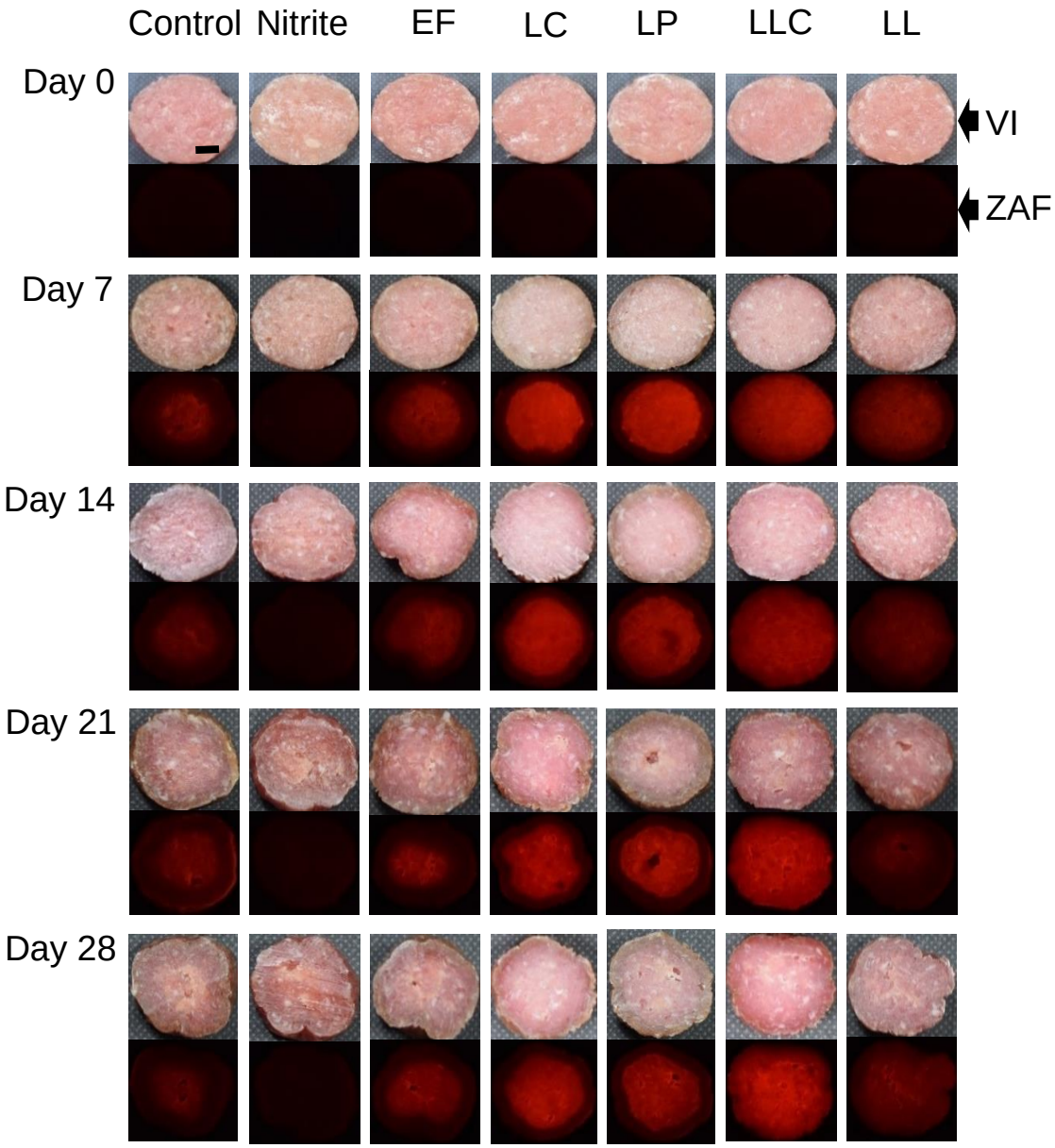



**Fig. 9. Internal visual and autofluorescence images of the dry-cured sausages inoculated with the selected high ZnPP-forming LAB**

The internal visual images (VI) and ZnPP autofluorescence (ZAF) images of dry-cured sausages inoculated with the selected ZnPP-forming LAB shown in Fig. 5 (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) were observed up to the end of ripening every 7 days during processing. Scale bar: 1 cm.

the non-inoculated group. Interestingly, a dark ring without ZAF was observed along the circumference of the cross-section of the sausages, especially in the LC and LP groups. In contrast, a dark ring without ZAF was not observed along the circumference of the sausages of LLC group. Besides, almost no or very weak ZAF was observed in the nitrite-added and control groups throughout the process.

#### 2.3.3.2.3. ZnPP content of dry-cured sausages

To verify the ZnPP-forming ability of LAB in dry-cured sausages, the amount of ZnPP was measured (Table 7). On day 7, the amount of ZnPP of the LAB-inoculated groups drastically increased and was significantly higher ($P < 0.01$) than those of the control and nitrite-added groups. The highest content of ZnPP was found in the LLC group, which showed non-significantly different compared to the LC group after 7 days of fermentation. Moreover, from day 7 onwards, the ZnPP content of the LAB-inoculated group steadily increased and was also higher than those of the control and nitrite-added groups ($P < 0.01$). At the end of ripening, the highest contents of ZnPP were observed in the LC and LLC groups. Although an increase in ZnPP with respect to time was measured to some extent in the control group, the amount of ZnPP of the nitrite-added group was negligible throughout the process.

#### 2.3.3.2.4. Color profiles of dry-cured sausages

The color profiles of both external and internal dry-cured sausages were analyzed using the CIE L* (lightness), a* (redness), and b* (yellowness) colorimetric system after 28 days of ripening (Fig. 10 and 11). In terms of external color, no significant difference in the L*

**Table 7. ZnPP content (nmol/g DM) in dry-cured fermented sausages during processing**

| | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| Control | 0.190±0.003 | 1.070±0.013[b] | 1.414±0.009[b] | 1.724±0.035[b] | 2.029±0.027[b] |
| Nitrite | 0.154±0.004 | 0.373±0.012[a] | 0.475±0.023[a] | 0.535±0.014[a] | 0.573±0.012[a] |
| EF | 0.190±0.004 | 2.041±0.300[c] | 2.923±0.049[c] | 3.579±0.048[c] | 3.799±0.083[c] |
| LC | 0.167±0.005 | 3.754±0.043[ef] | 4.661±0.051[ef] | 5.418±0.118[e] | 6.101±0.159[d] |
| LP | 0.166±0.006 | 3.577±0.029[e] | 4.373±0.124[e] | 4.941±0.114[e] | 5.659±0.078[d] |
| LLC | 0.160±0.001 | 3.891±0.018[f] | 4.869±0.089[f] | 5.395±0.132[e] | 6.076±0.120[d] |
| LL | 0.186±0.006 | 2.713±0.052[d] | 3.553±0.064[d] | 4.166±0.120[d] | 4.468±0.178[c] |

Data expressed as means ± SE (n = 2). Different letters in the same day differ significantly ($P < 0.01$).

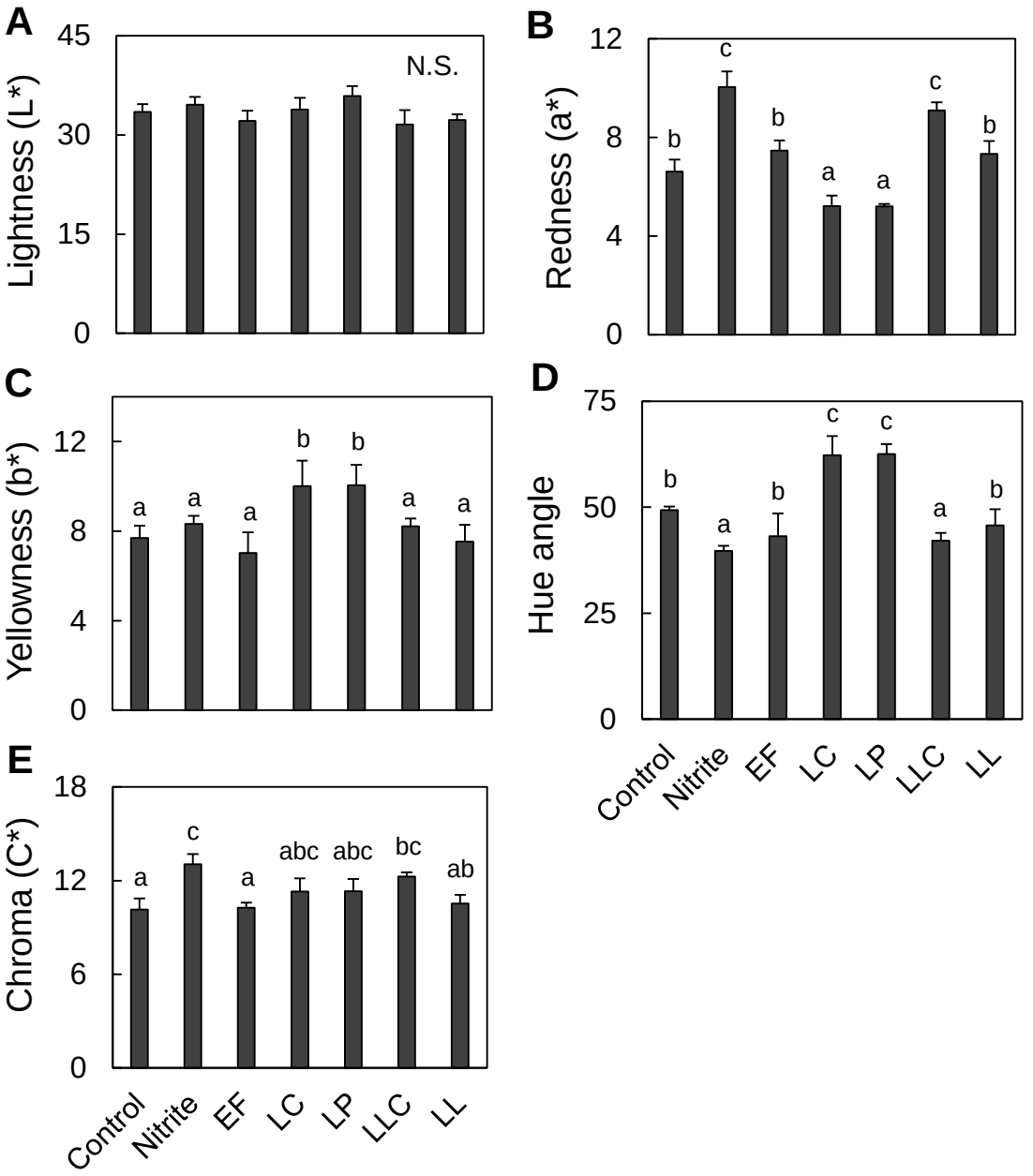



**Fig. 10. CIE L*a*b*, hue angle and C* as the external color profiles of dry-cured sausages after 28 days of processing**

CIE L* (A), a* (B), b* (C), hue angle (D), and chroma (E) values of the external part of the dry-cured sausages inoculated with the selected ZnPP-forming LAB shown in Fig. 5 (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) were measured at the end of ripening. Bars represent standard errors (n = 2). abc: Columns with different letters among the groups differ significantly ($P < 0.05$). N.S.: Not significant among groups.

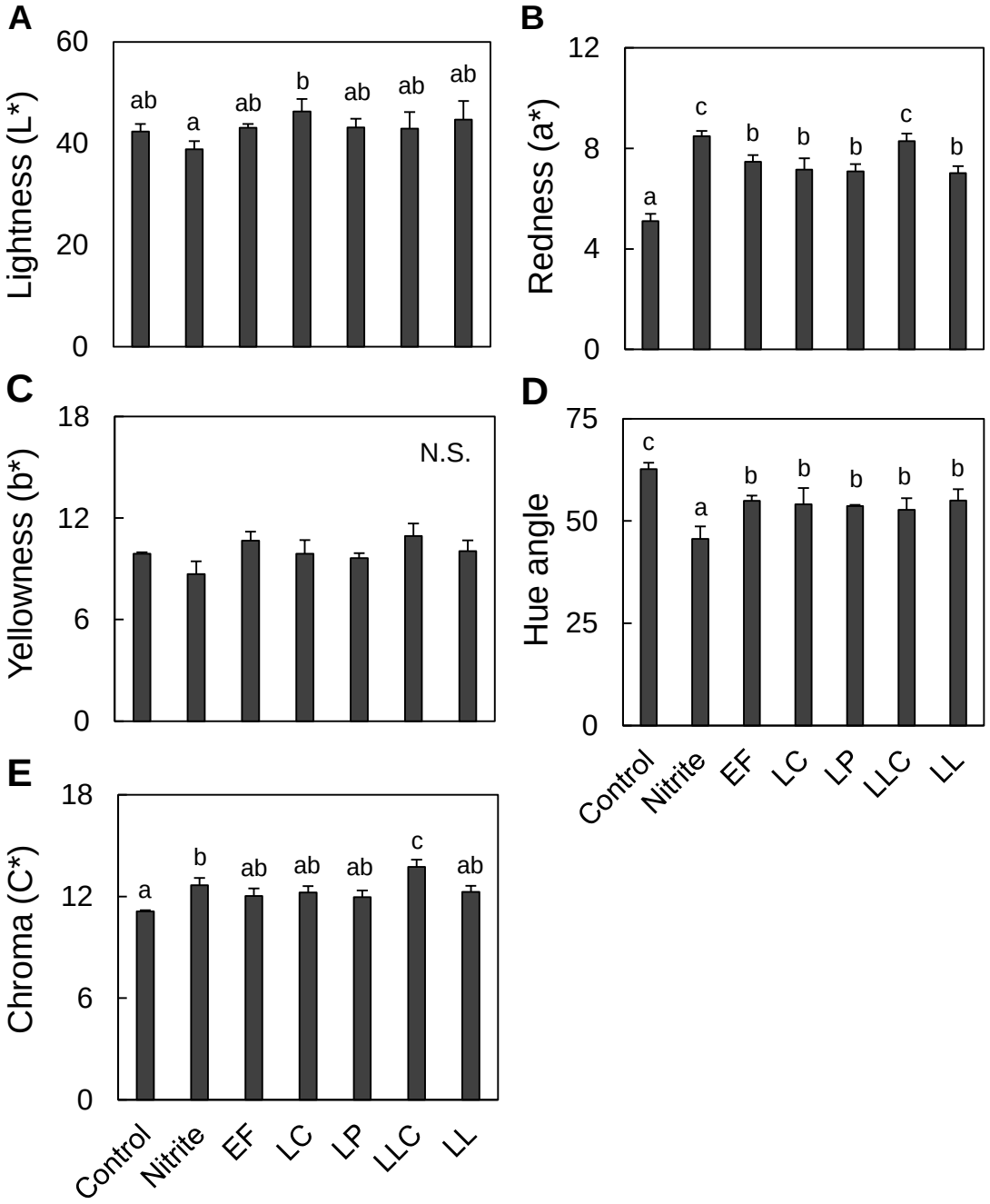



**Fig. 11. CIE L*a*b*, hue angle and C* as the internal color profiles of dry-cured sausages after 28 days of processing**

CIE L* (A), a* (B), b* (C), hue angle (D), and chroma (E) values of the internal part of the dry-cured sausages inoculated with the selected ZnPP-forming LAB shown in Fig. 5 (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) were measured at the end of ripening. Data expressed as means ± SE (n = 2). abc: Columns with different letters among the groups differ significantly ($P < 0.05$).

values was observed among the groups. However, the highest and lowest L* values among the groups were found in the LP and LLC groups, respectively (Fig. 10A). Moreover, among the LAB-inoculated groups, the LLC group showed a significantly higher a* value compared to the control group, and this result was close to that of the nitrite-added group. The LC and LP groups showed significantly lower a* values compared to the control group (Fig. 10B). Moreover, the b* values of the LC and LP groups were significantly higher than those of the other groups (Fig. 10C). Furthermore, the LLC group showed a significantly lower hue angle value compared to the control group, and this result was close to that of the nitrite-added group (Fig. 10D). In contrast, the LC and LP groups showed a significantly higher hue angle than the control group. However, the highest chroma (C*) value among the LAB-inoculated sausages was also found in the LLC group (Fig. 10E). The C* values of the LLC group were significantly higher than that of the control group (Fig. 10E), and this result was close to that of the nitrite-added group.

In terms of internal color (the core part of the sausages), the L* value of the nitrite-added group was significantly lower than that of the LC group (Fig. 11A). All the inoculated groups showed significantly higher a* values compared to the control group (Fig. 11B). The highest a* value among the inoculated groups was observed in the LLC group, similar to the nitrite-added group. No significant difference of the b* values was observed among the inoculated groups compared to the control and nitrite-added groups (Fig. 11C). Moreover, a significantly lower hue angle was observed in all the LAB-inoculated groups than that of the control group (Fig. 11D). However, the highest C* value (Fig. 11E) was observed in the LLC group, even higher than that of the nitrite-added group.

### 2.3.4. Effect of oxygen on LAB-associated ZnPP formation

From section 2.3.3.2.2, ZAF appeared in the entire sausages of the LLC group, whereas a dark ring without ZAF in the circumference of the sausages was observed in the LC and LP groups. In general, oxygen inhibits to form ZnPP (Wakamatsu *et al*., 2004a). Therefore, there is a possibility to form ZnPP by LLC in the presence of oxygen. In this section, in order to confirm whether the formation of LAB-associated ZnPP in the presence of oxygen is species or strain-specific, the formation of ZnPP with selected ZnPP-forming food-grade LAB which were used in dry-cured sausages, in the presence of oxygen was investigated using an aMH model experiment.

#### 2.3.4.1. Effect of oxygen on ZnPP formation by the selected ZnPP-forming food-grade LAB

To investigate the effect of oxygen on LAB-associated ZnPP formation, an aMH model experiment was performed, and the LAB-inoculated homogenate was incubated both aerobically and anaerobically. After 7 days of incubation, the high fluorescence intensity of ZnPP was observed in all the LAB-inoculated groups under anaerobic conditions (Fig. 12). However, the highest fluorescence intensity of ZnPP was observed in the LLC group and significantly different ($P < 0.01$) compared to the EF and LL groups under aerobic conditions. No remarkable fluorescence was observed in the LC and LP groups under aerobic conditions, although the highest fluorescence intensity of ZnPP was observed in the LC group under anaerobic conditions compared to that observed for the control and other LAB-inoculated groups (Fig. 12).

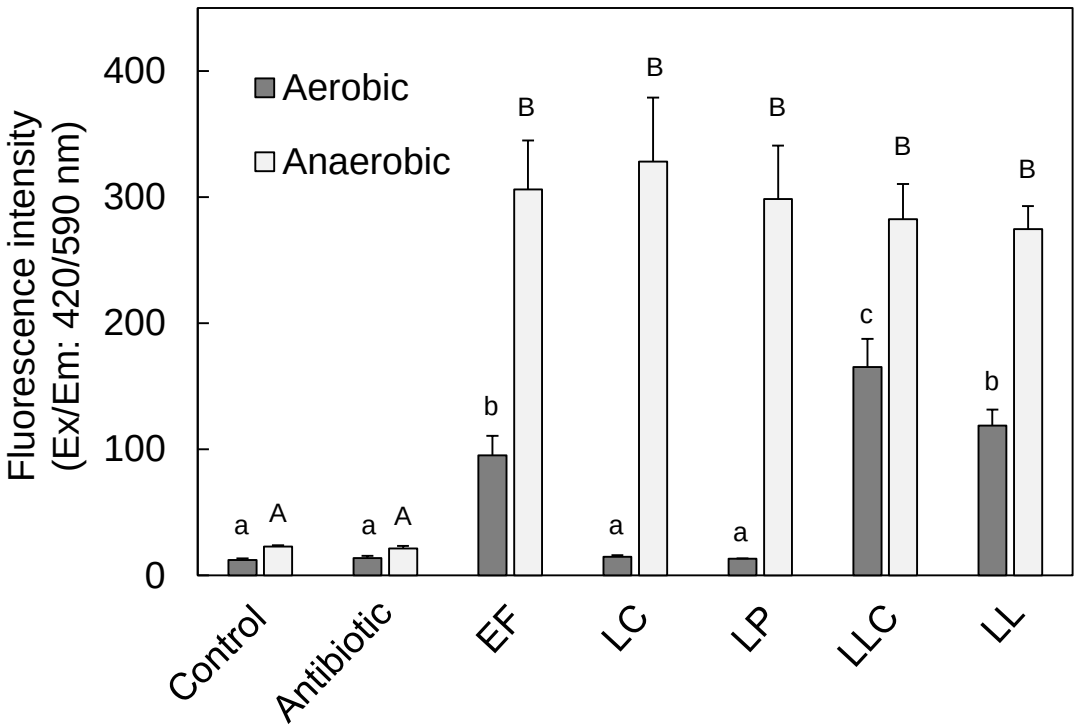



**Fig. 12. Effect of oxygen on ZnPP formation by the selected ZnPP-forming LAB in aMH model**

A mixture containing the final concentrations of 20% pork homogenate, 3% salt, and $2.0\times10^6$ CFU/mL of the selected ZnPP-forming LAB (EF: *Enterococcus faecium*, LC: *Lactobacillus curvatus*, LP: *Lactobacillus plantarum*, LLC: *Lactococcus lactis* subsp. *cremoris*, LL: *Leuconostoc lactis*) was incubated both aerobically and anaerobically at 25°C for 7 days in the dark. No bacterial isolate was added in the control and antibiotic groups. After incubation, ZnPP was extracted and measured. Data expressed as means ± SE (n = 3). abc and AB: Columns with different letters in the same incubation condition differ significantly ($P < 0.01$).

#### 2.3.4.2. Aerobic ZnPP formation by ZnPP-forming LAB within the species and strain level of the same genus

From section 2.3.4.1, it was observed that LAB-associated ZnPP formation under aerobic conditions seemed to be species-specific. Besides, from section 2.3.3.2.1, aerobic ZnPP formation was important for the entire color improvement of meat products. In this section, in order to confirm whether the ZnPP formation by the strains of the same LAB species or species of the same genus under aerobic condition exhibit the same result, the formation of ZnPP with high ZnPP-forming LAB and their strains in the presence or absence of oxygen was investigated using an aMH model experiment.

##### 2.3.4.2.1. Effects of *Lactobacillus* species and their strains on aerobic ZnPP formation

To investigate whether most of the strains of *Lactobacillus* species (e.g., *Lactobacillus curvatus* and *Lactobacillus plantarum*) are unable to form ZnPP aerobically or to find out the strain of high aerobic ZnPP-forming ability within the same species, an aMH model experiment was performed. The meat homogenate which was inoculated with several commercial strains were incubated both aerobically and anaerobically. After 7 days of incubation, the high fluorescence intensity of ZnPP was observed in all the strains of *Lactobacillus curvatus* and *Lactobacillus plantarum* under anaerobic conditions, and they showed significantly different from the control and antibiotic groups ($P < 0.01$), although no significant difference was noticed among the fluorescence intensity of ZnPP formed by the strains (Fig. 13). However, no increased fluorescence intensity of ZnPP was observed in both

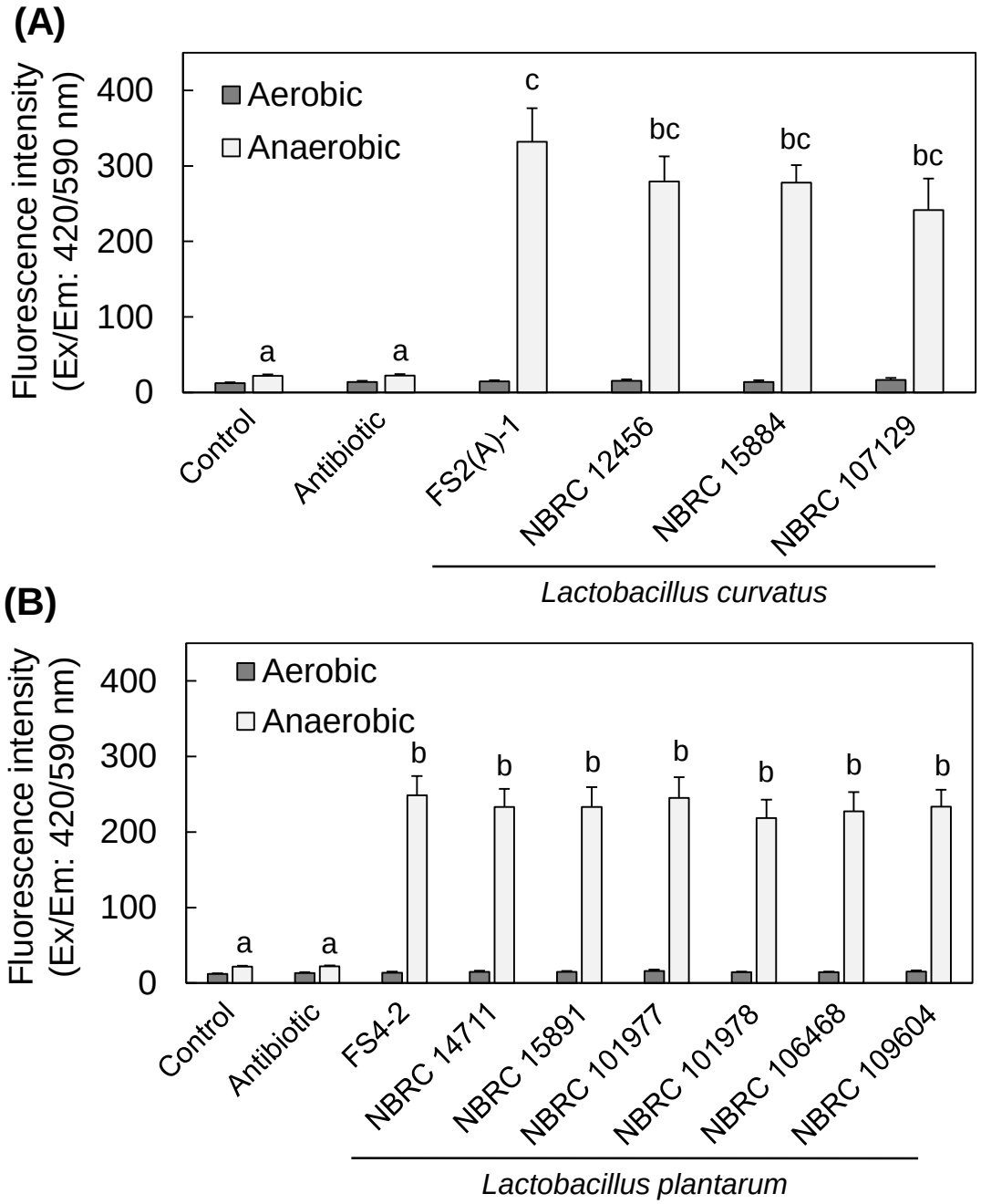



**Fig. 13. Effect of oxygen on ZnPP formation by different strains of *Lactobacillus curvatus* and *Lb. plantarum* in aMH model**

ZnPP formation ability of different species of *Lactobacillus curvatus* and *Lb. plantarum* was checked as shown in Fig. 12. Bars represent the standard error of the means (n = 3). abc: Columns with different letters in the same incubation condition differ significantly ($P < 0.01$).

the presence of *Lactobacillus curvatus* and *Lactobacillus plantarum* strains compared to the control under aerobic conditions.

#### 2.3.4.2.2. Effects of *Lactococcus* species, their subspecies and strains on aerobic ZnPP formation

To investigate whether *Lactococcus* species, their subspecies and strains form ZnPP aerobically or to find out the strain of high ZnPP-forming ability within the same species of *Lactococcus lactis* subsp. *cremoris*, aMH model experiment was performed, and the LAB-inoculated homogenate was incubated both aerobically and anaerobically. After 7 days of incubation, the high fluorescence intensity of ZnPP was observed in all the *Lactococcus* species, their subspecies, and strains-inoculated groups under anaerobic conditions, and they showed significantly different from the control and antibiotic groups ($P < 0.01$) (Fig. 14). In contrast, under aerobic conditions the highest fluorescence intensity of ZnPP was observed in the *Lactococcus lactis* subsp. *cremoris* GB(A)-1-inoculated group, followed by the *Lactococcus lactis* subsp. *lactis* NBRC 100933, *Lactococcus lactis* subsp. *lactis* NBRC 12007, *Lactococcus lactis* subsp. *hordinae* NBRC 100931 and *Lactococcus lactis* CH-1. The fluorescence intensity of ZnPP in all the *Lactococcus* species, their subspecies, and strains-inoculated groups were lower under aerobic conditions compared to the fluorescence intensities that were observed under anaerobic conditions by the same strains.

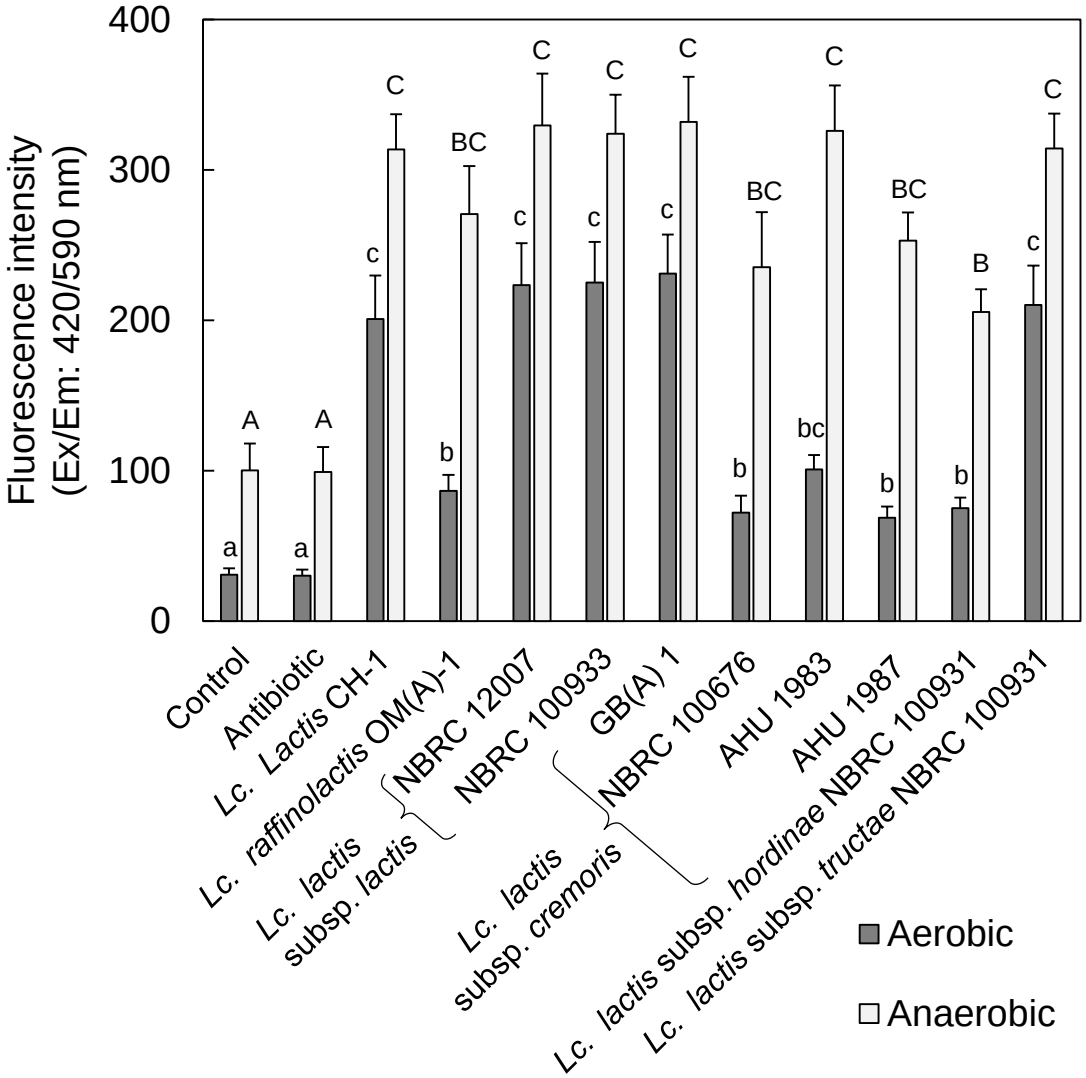



**Fig. 14. Effect of oxygen on ZnPP formation by the different *Lactococcus* species, subspecies, and their strains in aMH model**

ZnPP formation ability of species/subspecies/strains of *Lactococcus* was checked as shown in Fig. 12. Bars represent the standard error of the means (n = 3). abc and ABC: Columns with different letters in the same incubation condition differ significantly ($P < 0.01$).

## 2.4. Discussion

Selected bacterial species as a starter culture have been used in the manufacturing of modified meat and meat products for a long time. The selection of starter culture for meat products is a very crucial factor for the final quality and safety of the meat products. It has been reported that the formation of ZnPP increased in the meat and salt-added minced meat due to the addition of some bacteria (Ohya, 2017; Wakamatsu *et al*., 2020; Asaduzzaman *et al*., 2020b). However, the bacteria previously proposed to facilitate color improvement in salt-added minced meat due to their ZnPP-forming ability are unsafe and consequently were inappropriate to be used in meat products. Besides, LAB are widely used in fermented meat products as a starter culture for the diversity in their functions. Therefore, in order to search for high ZnPP-forming food-grade LAB for improving the color of meat products, LAB from various food products and environmental sources were collected and evaluated in this chapter.

### 2.4.1. Screening for ZnPP-forming food-grade LAB

From 450 isolates, a total of six genera, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Weissella* encompassing 13 different species, were found to be ZnPP-forming food-grade LAB (Table 4). *Enterococcus* spp. 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). *Enterococcus faecium*, found in a Tunisian dried cured meat product “Dried Ossban,” can be used as a probiotic (Zommiti *et al*., 2018). *Lactobacillus* spp. act as competitive microflora in meat and meat products,

extending their shelf life by competing with pathogens such as *Listeria monocytogenes* and not allowing them to grow excessively (Agüero *et al*., 2020). *Lactococcus* spp. is the dominant acidifying mesophilic species used in the fermentation of various dairy products (Cavanagh *et al*., 2015). *Leuconostoc* spp. produces characteristic flavors and is used in the production of fermented milk beverages with a single or mixed culture (Farnworth, 2005). The most important thermophilic LAB is *Streptococcus salivarius* subsp. *thermophilus* are used in yogurt with the combination of *Lactobacillus bulgaricus* (Bashiti, 2010). *Weissella* plays an important role in fermentation processes such as the production of silage, as well as in food fermentations based on vegetables or meat as substrate (Björkroth *et al*., 2002). Therefore, these 13 high ZnPP-forming food-grade LAB could be the more appropriate candidates to apply to meat products manufacturing.

### 2.4.2. Anaerobic ZnPP formation and improvement of internal color of meat products

All high ZnPP-forming food-grade LAB found in this chapter facilitated to form ZnPP noticeably under anaerobic conditions (Fig. 9). Previously, it was reported that ZnPP-forming bacteria could form ZnPP in the absence of oxygen, but these were not suitable for the color improvement of meat products for their low ZnPP-forming ability (Asaduzzaman, 2020b). The ZnPP formation ability by the bacteria also varied depending on the species or strains (Asaduzzaman, 2020a; Wakamatsu *et al*., 2020). ZnPP-forming ability by the LAB also varied in their strain level (Fig. 13). The LAB that made the color brighter redder produced a large amount of ZnPP (Table 7). On the other hand, the higher a* of the LAB-inoculated groups than that of the control group was due to the formation of ZnPP (Morita *et al*., 1998).

In addition, very weak fluorescence intensity and ZnPP autofluorescence in the control group might be due to the meat-inherent ZnPP formation mechanisms (Khozroughi *et al*., 2018) or bacterial contamination. Moreover, the formation of a weak fluorescent ring of ZnPP on the outer surface of the sausage cross-section might be due to the absence of ZnPP. These results clearly demonstrated that some inoculated bacteria could form ZnPP only under anaerobic conditions, and it was consistent with the report of Wakamatsu *et al*. (2019) showing that ZnPP formation was checked under aerobic conditions. However, ZnPP-forming LAB having the ability to form ZnPP anaerobically exhibited limitations in the improvement of the external color of meat products.

### 2.4.3. Aerobic ZnPP formation and improvement of external color of meat products

Although aerobic conditions suppressed the bacteria-associated ZnPP formation in the previous study, some LAB found in this chapter could form ZnPP in the presence of oxygen (Fig. 12 & 14). The external surface of the sausages inoculated with *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC) and *Lactobacillus* spp. showed brighter and duller red coloration, respectively (Fig. 8). The higher external a* value of the sausage inoculated with LLC was consistent with the development of the bright red color (Fig. 10). Besides, the external bright red coloration of the sausages inoculated with LLC was due to the formation of ZnPP (Fig. 9). This external redness might be a result of LAB-specific contributors produced during their metabolism, and distinguishing LLC from the other LAB. The external brown color in the LAB-inoculated (LAB of aerobic ZnPP formation inability) sausages increased due to a lack of ZnPP content, indicating the inability of LAB to form ZnPP under aerobic conditions in

dry-cured sausages (Fig. 9). The formation of ZnPP in meat products that generally occurs due to meat-inherent functions was inhibited due to the presence of oxygen (Wakamatsu *et al*., 2004b). Chau *et al*. (2011) reported that meat-inherent FECH could not facilitate the formation of ZnPP in meat via an iron-removal reaction of heme in the presence of oxygen. In addition, the external brown color observed in the control group in this chapter is thought to have been produced by the oxidation of the major color pigment of fresh meat, namely, Mb (Suman & Joseph, 2013). However, some ZnPP-forming LAB can form ZnPP aerobically on the surface of the sausage. Therefore, ZnPP-forming LAB of aerobic ZnPP formation ability would be the most potential candidates to improve the entire color of nitrite-free meat products.

### 2.4.4. Species-specific properties of LAB that form ZnPP aerobically

Generally, bacteria of the same species showed almost dissimilarities in their physical or genetic characteristics. To uncover the evolutionary relationship among all 25 ZnPP-forming LAB found in this chapter, a phylogenetic tree was constructed and analyzed (Fig. 15) based on 16S rRNA total gene sequences of the closest strain.

The phylogenetic tree was mainly composed of 3 clusters. Cluster I, the *Leuconostoc* group, has several sub-clusters composed of *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc lactis*, *Leuconostoc. citreum*, *Leuconostoc holzapfelii*. Cluster II included only the *Weissella* group. Cluster III was mainly divided into three sub-clusters, leading by the *Lactobacilli*, *Lactococci*, and *Streptococci* groups. *Lactobacilli* group was consisted of two sub-clusters: sub-cluster I (*Lb. curvatus*) and sub-cluster II (*Lb.*

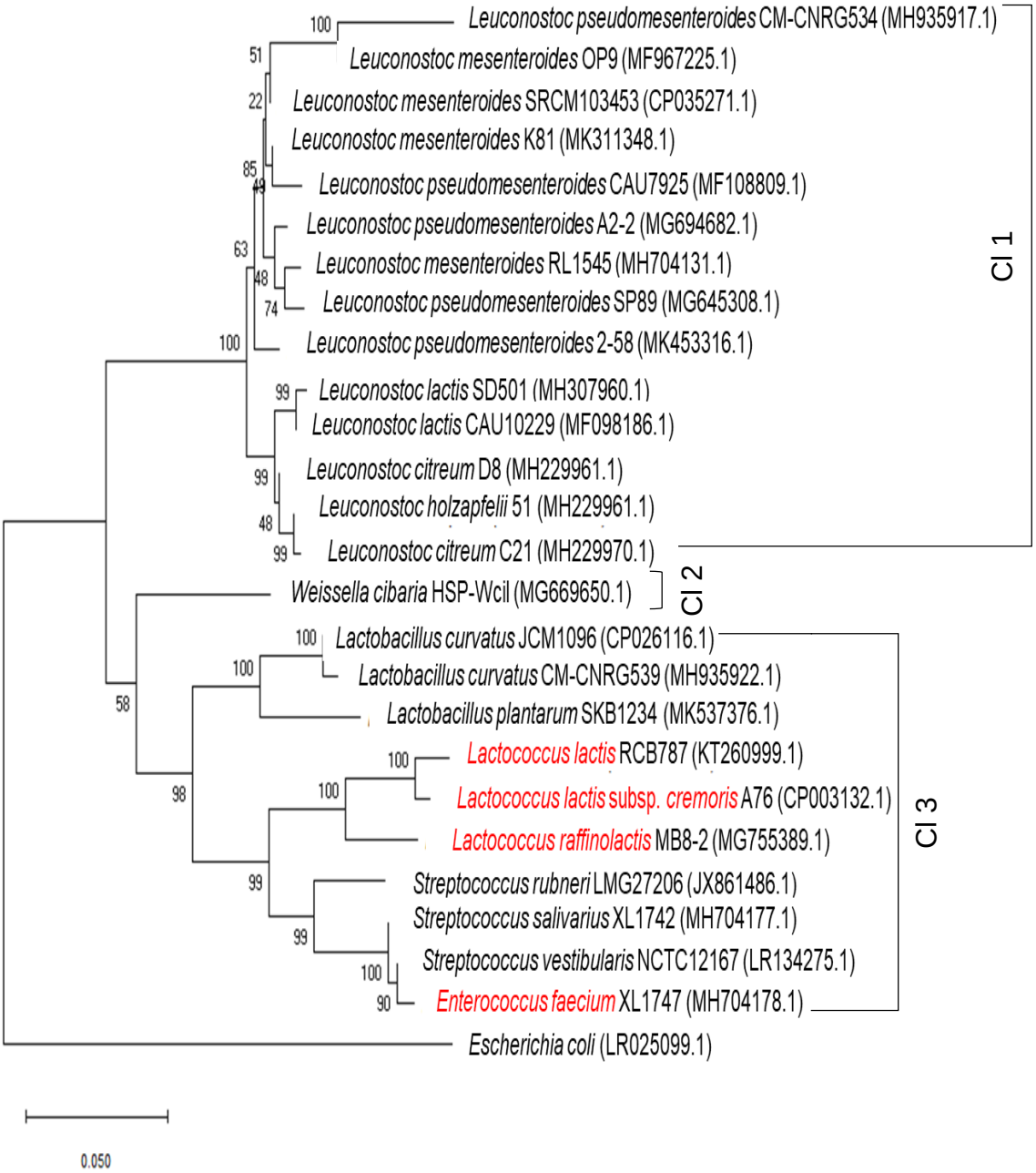



**Fig. 15. Phylogenetic tree of the screened ZnPP-forming LAB (the closest strain) based on 16S rRNA total gene sequence phylogenetics**

The scale bar indicates 0.050 nucleotide substitution per position. The tree was cut at same distance from the root, thereby the tree partitioned into three clusters. *Escherichia coli* was used a reference bacteria of other phylum. Red color indicates bacterial strains having ability to form ZnPP under aerobic and anaerobic conditions. Cl: Cluster

*plantarum*). The *Lactococcus* group had two sub-clusters: sub-cluster I (*Lactococcus lactis* and *Lactococcus lactis* subsp. *cremoris*) and sub-cluster II (*Lactococcus raffinolactis*). Bacteria in this group indicated aerobic ZnPP-forming ability (Red in Fig. 15). The *Streptococcus* group has one sub-cluster and includes *Enterococcus faecium* with aerobic ZnPP-forming ability. The bacterial strains with aerobic ZnPP-forming ability were most closely related, as shown by branch length, which has 100% bootstrap support. Cluster 1 showed the least confident relationship with clusters 2 and 3, which has 58% bootstrap support. Therefore, the strains of aerobic ZnPP-forming ability located in the same cluster and might be related in their characteristics.

### 2.4.5. Conclusion

In this chapter, for improving the color of dry-cured meat products, 13 high ZnPP-forming food-grade LAB were identified. It was shown that along with anaerobic ZnPP formation, aerobic ZnPP formation by the LAB is important to improve the entire color of nitrite-free meat products. The anaerobic as well as aerobic ZnPP-forming ability of three LAB, e.g., *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC), *Enterococcus faecium* MP-1, and *Leuconostoc lactis* SS(A)-1 were also confirmed. Among the LAB with aerobic ZnPP-forming ability, LLC showed the highest ZnPP-forming capability. These strains with aerobic ZnPP-forming ability were close genetically. As a result, the high ZnPP-forming LLC could be the most potential candidate as an alternative to nitrites/nitrates for improving the entire color of meat products.

# Chapter 3

# Mechanism involved in aerobic ZnPP-formation by ZnPP-forming LAB in dry-cured meat products

## 3.1. Introduction

ZnPP is not only formed endogenously in meat products during processing (Adamsen *et al*., 2006a), but also the formation was increased in the bacteria-inoculated dry-cured meat products (Asaduzzaman, 2020a). Under anaerobic conditions, ZnPP was formed without the addition of bacteria in meat and meat products (Wakamatsu *et al*., 2004b). Recently, the anaerobic formation of ZnPP by some bacteria has also been reported (Asaduzzaman *et al*., 2020b; Wakamatsu *et al*., 2020). In chapter 2, many LAB have been screened having the high ability to form ZnPP under anaerobic conditions and some LAB, especially *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC), showed high ZnPP-forming abilities under aerobic conditions. The ZnPP formation under aerobic conditions by the LAB was strain specific since the strain belonging to the same species did not show the similar capability in ZnPP formation. In addition, aerobic ZnPP formation by the LAB can improve the entire color of meat products and the external color was due to the aerobic ZnPP formation. It is possible that aerobic ZnPP formation by the LAB is different from meat-inherent or anaerobic bacterial ZnPP formation. Thus, it is important to elucidate the bacteria-associated ZnPP formation mechanism under aerobic conditions.

The mechanism by which aerobic ZnPP is formed by the ZnPP-forming bacteria is important with respect to its applicability for the entire reddening of meat products. Under

anaerobic conditions, bacteria promoted meat-inherent mechanism of ZnPP formation and bacterial ferrochelatase (FECH) and low molecular weight metabolites (LMMs) contribute to the anaerobic ZnPP formation (Asaduzzaman, 2020a). FECH activities have been detected in mammals, bacteria, and yeast (Dailey *et al*., 2000; Medlock *et al*., 2007). The formation of ZnPP greatly varies by the different factors like time, temperature, and environmental conditions in the presence of bacteria (Khozroughi *et al*., 2018; Ohya, 2017). Variation of ZnPP formation by the bacteria meaning that bacterial enzymes or metabolic end-products derived from ZnPP-forming bacteria might contribute to the ZnPP formation. Asaduzzaman (2020a) also reported that only the FECH-containing insoluble fraction of meat increased the ZnPP formation in a dose-dependent manner. The association of both a meat-inherent and a bacterial enzymatic reaction took place to form ZnPP during meat processing, and FECH from *Psudomonas fluorescens* partially participate in the $Fe^{2+}$-$Zn^{2+}$ substitution and contribute to ZnPP formation (Khozroughi *et al*., 2018). However, it was suggested that along with meat-inherent FECH (mFECH), bacterial FECH (bFECH) is involved in meat-inherent anaerobic ZnPP formation, but the ZnPP formation by the bacteria in the presence of oxygen is a new topic. However, aerobic ZnPP formation mechanism by the bacteria is not elucidated yet.

Bacteria produce and secrete different types of metabolites, which usually consist of aldehydes, ketones, esters, alcohols, organic acids, amines, and sulfur compounds, etc. (Ercolini *et al.*, 2009; Samelis, 2006). The production and number of metabolites vary in the bacterial species or strains. Extracellular metabolites are mainly produced as by-products of microbial metabolic activities, and their production is influenced by environmental

conditions such as temperature, oxygen, pH, nutrients, and others (Pinu & Villas-Boas, 2017). Low molecular weight metabolites (LMMs) serve as organic ligands in coordination complexes with various inorganic metals as central atoms and the function of LMMs is affected by oxygen (Hadacek & Bachmann, 2015). In addition, the activity of bacterial FECH was also inhibited in aerobic condition (Asaduzzaman, 2020a). Therefore, bacterial metabolites responsible for ZnPP formation cannot facilitate to form ZnPP in the presence of oxygen.

Some components and precursors are involved in the ZnPP formation pathway (e.g., Hb, Mb, free heme and zinc, and FECH) locate at different locations in meat products. An important meat-inherent enzyme, FECH, is a key contributor to ZnPP formation (Khozroughi *et al*., 2018), strictly under anaerobic conditions (Chau *et al*., 2011). Heme ($Fe^{2+}$), another important contributors for ZnPP formation principally derived from Mb and to a lesser extent from Hb is very unstable under aerobic conditions and converted to hemin which is the stable state of iron heme ($Fe^{3+}$) (Creek *et al*., 2009). Thus, the components and precursors derived from meat products responsible for ZnPP formation pathway cannot exert their functions to form ZnPP aerobically.

In the present study, it was hypothesized that aerobic ZnPP formation by LLC might be different from anaerobic ZnPP formation by other ZnPP-forming LAB. Therefore, the objectives of this chapter were to find out the contributors derived from either LLC or meat by which the ZnPP-forming LLC promoted to form ZnPP aerobically and how aerobic ZnPP formation differs from anaerobic one. The aerobic ZnPP formation in order to clarify the actual impact of ZnPP-forming LLC in improving the entire color of meat and meat products

will be focused in this chapter. LLC was used to elucidate the aerobic ZnPP formation mechanism in meat and meat products since LLC has high anaerobic and aerobic ZnPP-forming ability among the other screened LAB and within the strains of same species. On the other hand, *Lactobacillus curvatus* (LC) having ability to form ZnPP only under anaerobic conditions was used as a reference. Besides, the effects of temperature, pH and additives on aerobic ZnPP formation by the ZnPP-forming LLC were also investigated to apply to manufacturing meat products.

## 3.2. Materials and methods

### 3.2.1. Materials

#### 3.2.1.1. Sampling

Porcine loin and liver were obtained from common domestic crossbred pork, which were purchased from the local market at Hokkaido, Japan. Porcine *Longissimus thoracis et lumborum* (LTL) muscle were collected from loins and divided into a small block. For preparing the aseptic meat homogenate (aMH), a meat block (250-300 g) of the LTL muscle was dipped into 70% ethanol for one minute and taken the core portion after trimming out the surface of the meat block. Similarly, for the preparation of aseptic liver homogenate, the inner part of the liver was taken after wiping the surface of the liver with 70% ethanol. The block of the LTL muscle and aseptic samples of the LTL muscle and liver were vacuum-packed and stored at -20°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 (Tris: Nacalai Tesque Inc., Kyoto, Japan), adjusting the pH at 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.). For facilitating the solubility of digitonin, MMS buffer was heated at 60°C. After the preparation of MS and MMS buffers, they were kept in refrigerator (4°C) until use.

#### 3.2.1.3. Preparation of PPIX and heme solutions

A PPIX solution was prepared by dissolving 10 mg of PPIX disodium salt (Previously, 10 mg of PPIX disodium salt was dissolved with a few drops of 1 M HCl) (Aldrich Chem. Co., Milwaukee, WI, USA) in 50 ml of N, N-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd.). After dissolving 10 mg of hemin (Sigma–Aldrich Co., St Louis, MO, USA) with a few drops of 5% ammonia water, a heme solution was prepared by diluting with 50 ml of DMF. The solutions were stored at -20°C.

#### 3.2.1.4. Other reagents

The chemicals and reagents used in this study were acetone (HPLC grade), CBB R-250 (Wako Pure Chemical Industries, Ltd.); N-methyl mesoporphyrin IX (N-MMP) (Frontier scientific, Logan, USA); ethylenediaminetetraacetic acid (EDTA) (Kanto Chemical Co.); Hb, Mb, sodium dodecyl sulphate (SDS), acrylamide, N, N, N', N'- tetramethylethylenediamine (TEMED) and ammonium peroxodisulfate (APS) (Nacalai Tesque, Inc.).

### 3.2.2. Methods

#### 3.2.2.1. Culture of selected ZnPP-forming LAB and collection of culture fluid (CF)

Nutrient broth (NB) described in section 2.2.2.2 was used for the propagation of screened strains of *Lactobacillus curvatus* FS2(A)-1 (LC) and *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC). The inoculated NB broth was incubated anaerobically at 30℃ for 24-48 hours. Before using the bacterial culture in each experiment, the desired number of bacteria ($2.0\times10^6$ CFU/mL) from a cultured NB medium was calculated using a hemocytometer under a phase-contrast microscope. Bacterial CF was collected from the cultured NB medium. For the separation of CF from the cultured medium, the cultured medium was centrifuged for 7,700 × g for 15 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd., Tokyo, Japan) to separate the bacterial cell. After centrifugation, the supernatant was collected and passed through a sterile syringe filter (Minisart® syringe filter, 0.2 µm; Sartorius Stedim Biotech) to remove rest of the microbial cell and the filtrate was collected as CF. For the separation of low molecular weight metabolites (LMMs), CF solution was subjected to ultrafiltration spin tube with a 10 kDa molecular weight cut off membrane (VIVASPIN 20; 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd., Stonehouse, UK) and the filtrate was designated as LMMs-CF. Besides, the LMMs-CF was heated to 100°C for 10 min to get heated low molecular weight bacterial metabolites (hLMMs-CF). For the removal of volatile organic compounds from LMMs-CF, the non-volatile LMMs-CF were completely freeze dried (Freeze dryer, FD-1, Tokyo Rikakikai Co. Ltd. Tokyo, Japan) and then diluted up to the initial volume with sterile ultra-pure water.

#### 3.2.2.2. Aseptic fractionation of LTL muscle homogenate

The fraction of LTL muscle homogenate was performed as previously described method (Asaduzzaman, 2020a). Porcine aseptic LTL muscle homogenate (30%) (aMH) was prepared as mentioned in section 2.2.5.2. The aMH was centrifuged at 39,920 × g for 20 minutes at 4°C (himac CR20F, Hitachi Koki, Tokyo, Japan) to separate the soluble fraction (SF) from the insoluble one. After centrifugation, the filtration of the supernatant was performed through a filter paper (No. 2, 90 mm, Toyo Roshi Ltd.) which was previously sterilized by keeping in the heating chamber at 180°C for one hour, and the filtrated supernatant was considered as SF. The sterile ultra-pure water was added into the insoluble fraction (ISF), which was precipitated as pellet, up to the initial volume. Then, the pellet of the ISF was homogenized and centrifuged in the same manner as previously twice to completely washout the water-soluble components. The precipitated pellet was diluted with sterile ultra-pure water to a particular volume to prepare a specific concentration (20%) of the ISF. Moreover, the SF was passed out through an ultrafiltration spin tube (VIVASPIN 20, 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd.) which was previously sterilized by keeping it under UV light inside the clean bench for an overnight. The separation of the SF was performed by centrifuging at 8,000 × g for 90 minutes at 4°C (himac CR20F, Hitachi Koki). The filtrate and residue diluted with sterile ultra-pure water up to the initial volume were considered as “the <10 kDa SF” and “the >10 kDa SF”, respectively.

#### 3.2.2.3. Measurement of heme

For the extraction of heme from the insoluble fraction (ISF) of meat and the cultured broth, the addition of 75% acetone and 0.7% HCl of the final concentration was performed and then, the sample were kept at 4°C for 30 min in the dark for the proper extraction of heme. Finally, the absorbance of heme (Ex. 383 nm) was measured using spectrophotometer and the content of heme was calculated using a standard curve.

#### 3.2.2.4. Separation of mitochondria and the fractionation

##### 3.2.2.4.1. Separation of mitochondria

Mitochondria was aseptically separated from the pork liver as previously described method (Okabe, 1996) with minor modifications. MS buffer was used to prepare 10% liver homogenate. Firstly, 2 g liver and 18 mL of MS buffer were taken into a glass homogenizer. Homogenization was performed by fluctuating a pestle along the inner side of the glass ten times gently. During homogenization, the glass was kept in an ice container to ensure the intactness of the mitochondria. Then, the liver homogenate was centrifuged at 600 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd.). After centrifugation, the nucleus and other organelles were sedimented as pellet, and the supernatant containing mitochondria was collected. Afterward, the supernatant was again centrifuged at 8,000 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd.) to remove the soluble components, and the intact mitochondria was deposited as a pellet. Finally, the pellet was collected as mitochondria and diluted up to the initial volume with sterile ultra-pure water.

#### 3.2.2.4.2. Separation of the outer membrane (OM), inner membrane (IM), and matrix (MT)

The OM, IM, and MT of mitochondria were separated as previously described (Nishimura & Yano, 2014) with slight modifications. For the separation of OM, the separated mitochondria as a pellet from section 3.2.2.5 was taken and diluted to the initial volume with MMS buffer. For the solubilization of OM, the solution was mixed gently using a vortex mixer at maximum speed for 15 min. Then, the solution was centrifuged at 10,000 × g for 10 min at 4°C (CAX-371, Tomy Kogyo Co. Ltd.), and the supernatant was collected as OM. Thereafter, the pellet of the MT were collected and again diluted to initial volume with MMS buffer. Next, the solution was sonicated for 15 min in a sonication bath (Hitachi Koki) to disrupt the IM from the MT. Afterward, the sonicated solution was vortexed to separate the disrupted membrane from the MT into the solution at 4°C for 15 min. Finally, the solution was again ultra-centrifuged at 100,000 × g for 30 min at 4°C (himac CS120GXII, Hitachi Koki). The pellet and supernatant were collected as IM and MT, respectively, and diluted up to the initial volume with MMS buffer.

#### 3.2.2.5. Different model experiments for ZnPP formation

aMH and antibiotic solutions were prepared as described in sections 2.2.5.2 and 2.2.2.3, respectively. The ZnPP-forming selected LAB were inoculated in the specific group in the model experiment to investigate their role in ZnPP formation. Incubation of the samples of the model experiments was performed both aerobically and anaerobically at 25°C for 7 days for the experiment of investigating the external factors involved in ZnPP formation and 30°C

for 7 days for the experiment of investigating the internal mechanism of ZnPP formation. After incubation, the extraction of ZnPP from the sample and measurement of fluorescence intensity were performed as described in section 2.2.8.

#### 3.2.2.5.1. Investigating the effects of temperature, pH, and additives on LLC-associated aerobic ZnPP formation

To investigate the effect of different temperature on ZnPP formation by the ZnPP-forming LAB, an experiment with the addition of ZnPP-forming LAB in aMH was performed and incubated at 18, 25, 30, and 37°C. Similarly, the effect of pH at different levels was investigated, in which pH was adjusted to 5.80, 5.50, and 4,75. Moreover, the effects of different additives, e.g., salt (3%), sodium nitrite (300 ppm), sodium ascorbate (400 ppm), sodium sulphite (100 ppm), glucose (1%), and polyphosphate (0.3%) on LLC-associated aerobic ZnPP formation was also investigated by the similar way.

#### 3.2.2.5.2. Investigating the effects of enzymes, meat-inherent and bacterial FECH and CF on LLC-associated aerobic ZnPP formation

aMH with or without treated with blanching (immersed 20 sec in boiling water to inactivate meat-inherent enzymes) was performed to find out the role of meat inherent enzymes in LLC-associated aerobic ZnPP formation. To investigate the effect of FECH derived from either meat or LLC on aerobic ZnPP formation, a model experiment according to Table 8 was performed using FECH inhibitor, N-methyl mesoporphyrin (N-MMP). In order to investigate the effects of CF and LMMs-CF in LLC-associated aerobic ZnPP formation, these were applied in the ZnPP formation model experiment according to Table 9. In order to investigate

the effects of heated LMMs-CF (hLMMs-CF), the LMMs-CF was heated at 100°C for 10 min and cooled at room temperature, thereby hLMMs-CF was also applied in the model experiment according to Table 9.

**Table 8. Experimental model composition (mL): Effects of meat-inherent and bacterial FECH on LAB-associated ZnPP formation**

| Groups | aMH | N-MMP | sLAB | Antibiotics | UPW |
|---|---|---|---|---|---|
| aMH | 0.90 | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | 0.01 | 0.59 |
| + N-MMP | 0.90 | 0.10 | - | - | 0.50 |
| + N-MMP + antibiotics | 0.90 | 0.10 | - | 0.01 | 0.49 |
| + sLAB | 0.90 | - | 0.15 | - | 0.45 |
| + N-MMP + sLAB | 0.90 | 0.10 | 0.15 | - | 0.35 |

Total volume: 1.5 mL; aMH: Aseptic meat homogenate; N-MMP: N-methyl mesoporphyrin IX; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

**Table 9. Experimental model composition (mL): Effect of culture fluid/ low molecular weight metabolites (LMMs)/ heated LMMs on LAB-associated ZnPP formation**

| Groups | aMH | UF-NB/hNB | sLAB-CF/LMMs /hLMMs | Anti-biotics | UPW |
|---|---|---|---|---|---|
| UF-NB/hNB | 0.90 | 0.15 | - | - | 0.45 |
| UF-NB/hNB + Antibiotics | 0.90 | 0.15 | - | 0.01 | 0.44 |
| sLAB-CF/LMMs/hLMMs | 0.90 | - | 0.15 | - | 0.45 |
| sLAB-CF/LMMs/hLMMs + antibiotics | 0.90 | - | 0.15 | 0.01 | 0.44 |

Total volume: 1.5 mL; aMH: Aseptic meat homogenate; UF: Ultra-filtrated; NB: Nutrient broth; hNB: Heated nutrient broth; sLAB: Selected lactic acid bacteria; CF: Culture fluid; hLMMs: Heated low molecular weight metabolites; UPW: Ultra-pure water

#### 3.2.2.5.3. Investigating the effects of meat fractions and mitochondria and mitochondrial membranes on LLC-associated aerobic ZnPP formation

The separated fractions of <10 kDa and >10 kDa SF based on molecular weight, SF and ISF from the porcine LTL muscle were applied to the ZnPP formation model experiment according to Table 10 & 11. After homogenization of LTL muscle and separation of intact

mitochondria from pork liver, the effect of exogenous mitochondria on LAB-associated ZnPP formation were investigated in the model experiments according to Table 12. After separation of OM, IM, and MT fractions from liver mitochondria, the fractions were then added in the ZnPP formation model experiment as Table 13.

**Table 10. Experimental model composition (mL): Effects of meat fractions (soluble and insoluble fractions) on LAB-associated ZnPP formation**

| Groups | SF/ISF | ISF/SF | sLAB | Antibiotics | UPW |
|---|---|---|---|---|---|
| SF/ISF | 0.90 | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | 0.01 | 0.59 |
| + ISF/SF | 0.90 | 0.15 | - | - | 0.45 |
| + ISF/SF + Antibiotics | 0.90 | 0.15 | - | 0.01 | 0.44 |
| + sLAB | 0.90 | - | 0.15 | - | 0.45 |
| + ISF/SF + sLAB | 0.90 | 0.15 | 0.15 | - | 0.30 |

Total volume: 1.5 mL; SF: Soluble fraction; ISF: Insoluble fraction; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

**Table 11. Experimental model composition (mL): Effects of <10 kDa and >10 kDa fractions of soluble fraction on LAB-associated ZnPP formation**

| Groups | <10/>10 kDa | >10/<10 kDa | ISF | sLAB | Antibiotics | UPW |
|---|---|---|---|---|---|---|
| <10/>10 kDa | 0.90 | - | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | - | 0.01 | 0.59 |
| + ISF | 0.90 | - | 0.15 | - | - | 0.45 |
| + ISF + antibiotics | 0.90 | - | 0.15 | - | 0.01 | 0.44 |
| + sLAB | 0.90 | - | - | 0.15 | - | 0.45 |
| + ISF + sLAB | 0.90 | - | 0.15 | 0.15 | - | 0.30 |
| + >10/<10 kDa + ISF + sLAB | 0.90 | 0.15 | 0.15 | 0.15 | - | 0.15 |

Total volume: 1.5 mL; ISF: Insoluble fraction; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

**Table 12. Experimental model composition (mL): Effect of exogenous mitochondria on LAB-associated ZnPP formation**

| Groups | aMH | PLM | sLAB | Antibiotics | UPW |
|---|---|---|---|---|---|
| aMH | 0.90 | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | 0.01 | 0.59 |
| + PLM | 0.90 | 0.35 | - | - | 0.25 |
| + PLM + Antibiotics | 0.90 | 0.35 | - | 0.01 | 0.24 |
| + sLAB | 0.90 | - | 0.15 | - | 0.45 |
| + PLM + sLAB | 0.90 | 0.35 | 0.15 | - | 0.10 |

Total volume: 1.5 mL; aMH: Aseptic meat homogenate; PLM: Pork liver mitochondria; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

**Table 13. Experimental model composition (mL): Effects of mitochondria and its membranes on LAB-associated ZnPP formation**

| Groups | SF | MTC/OM/ IM/MT | sLAB | Antibiotics | UPW |
|---|---|---|---|---|---|
| SF | 0.90 | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | 0.01 | 0.59 |
| + MTC/OM/IM/MT | 0.90 | 0.15 | - | - | 0.45 |
| + MTC/OM/IM/MT + Antibiotics | 0.90 | 0.15 | - | 0.01 | 0.44 |
| + sLAB | 0.90 | - | 0.15 | - | 0.45 |
| + MTC/OM/IM/MT + sLAB | 0.90 | 0.15 | 0.15 | - | 0.30 |

Total volume: 1.5 mL; SF: Soluble fraction; MTC: Mitochondria; OM: Outer membrane; IM: Inner membrane; MT: Matrix; UPW: Ultra-pure water

#### 3.2.2.5.4. Investigating the effects of LLC and LLC-derived LLMs on LLC-associated aerobic ZnPP formation in the presence of exogenous Hb, Mb, heme, and PPIX

Different model experiments for the exogenous Hb and Mb (Table 14), hemin (Table 16), and PPIX (Table 16) were performed, which were mainly designed based on ISF and ZnPP-forming LAB or LMMs derived from these LAB to investigate their roles in LAB-associated ZnPP formation. Moreover, the formation of PPIX from the Hb and Mb as Table 15 and from heme as Table 17 by the effects of LMMs were performed.

**Table 14. Experimental model composition (mL): Formation of ZnPP from Hb/Mb in the presence of LAB and LAB derived low molecular weight metabolites (LMMs)**

| Groups | ISF | Hb/Mb | sLAB/LAB-LMMs | Antibiotics | UPW |
|---|---|---|---|---|---|
| ISF | 0.90 | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | 0.01 | 0.59 |
| + Hb/Mb | 0.90 | 0.10 | - | - | 0.50 |
| + Hb/Mb + Antibiotics | 0.90 | 0.10 | - | 0.01 | 0.49 |
| + sLAB/LAB-LMMs | 0.90 | - | 0.15 | - | 0.45 |
| + Hb/Mb + sLAB/LAB-LMMs | 0.90 | 0.10 | 0.15 | - | 0.35 |

Total volume: 1.5 mL; ISF: Insoluble fraction; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

**Table 15. Experimental model composition (mL): Formation of PPIX from Hb /Mb in the presence of low molecular weight metabolites (LMMs) derived from ZnPP-forming selected LAB**

| Groups | ISF | EDTA | Hb/Mb | LAB-LMMs | Antibiotics | UPW |
|---|---|---|---|---|---|---|
| ISF + EDTA | 0.9 | 0.015 | - | - | - | 0.59 |
| + Antibiotics | 0.9 | 0.015 | - | - | 0.01 | 0.58 |
| + Hb/Mb | 0.9 | 0.015 | 0.10 | - | - | 0.49 |
| + Hb/Mb + Antibiotics | 0.9 | 0.015 | 0.10 | - | 0.01 | 0.48 |
| + LAB-LMMs | 0.9 | 0.015 | - | 0.15 | - | 0.44 |
| + Hb/Mb + LAB-LMMs | 0.9 | 0.015 | 0.10 | 0.15 | - | 0.34 |

Total volume: 1.5 mL; ISF: Insoluble fraction; EDTA: Ethylenediaminetetraacetic acid; Hb: Hemoglobin; Mb: Myoglobin; LAB: Lactic acid bacteria; UPW: Ultra-pure water

**Table 16. Experimental model composition (mL): Formation of ZnPP from heme/protoporphyrin IX (PPIX) in the presence of LAB and LAB derived low molecular weight metabolites (LMMs)**

| Groups | ISF/ BL-ISF | Heme/PPIX | sLAB | Antibiotics | UPW |
|---|---|---|---|---|---|
| ISF/BL-ISF | 0.90 | - | - | - | 0.60 |
| + Antibiotics | 0.90 | - | - | 0.01 | 0.59 |
| + Heme | 0.90 | 0.04 | - | - | 0.56 |
| + Heme + Antibiotics | 0.90 | 0.04 | - | 0.01 | 0.55 |
| + sLAB | 0.90 | - | 0.15 | - | 0.45 |
| + Heme + sLAB | 0.90 | 0.04 | 0.15 | - | 0.41 |

Total volume: 1.5 mL; ISF: Insoluble fraction; BL: Blanched; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

**Table 17. Experimental model composition (mL): Formation of PPIX from heme in the presence of low molecular weight metabolites (LMMs) derived from ZnPP-forming selected LAB**

| Groups | ISF + AA + EDTA | UF-NB | Heme | sLAB-LMMs | Antibiotics | UPW |
|---|---|---|---|---|---|---|
| UF-NB | 0.90 + 0.40 + 0.015 | 0.15 | - | - | - | 0.04 |
| UF-NB + Antibiotics | Ditto | 0.15 | - | - | 0.01 | 0.02 |
| UF-NB + Heme | Ditto | 0.15 | 0.04 | - | - | - |
| UF-NB + Heme + Antibiotics | Ditto | 0.15 | 0.04 | - | 0.01 | - |
| sLAB-LMMs | Ditto | - | - | 0.15 | - | 0.04 |
| Heme + sLAB-LMMs | Ditto | - | 0.04 | 0.15 | - | - |

Total volume: 1.5 mL; ISF: Insoluble fraction; AA: Ascorbic acid; UF: Ultra-filtrated; NB: Nutrient broth; EDTA: Ethylenediaminetetraacetic acid; sLAB: Selected lactic acid bacteria; UPW: Ultra-pure water

### 3.2.2.6. SDS-PAGE

A conventional method was used to perform SDS-PAGE, 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 the sample. The mixture of the sample was prepared by heating for 5 minutes at 100°C and stored at -20°C until use. Both stacking and separating gel containing 0.1% SDS were composed of 4.5% and 15% acrylamide, respectively. After pouring the mixture into the electrophoresis plate (thickness 1.0 mm, ATTO, Tokyo, Japan), the polymerization of the gel was performed at room temperature. Then, a completely polymerized gel plate was put into the electrophoresis apparatus (AE-6530P, ATTO) with SDS-PAGE electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). After applying the previously prepared sample into the gel, the electrophoresis was performed by following conditions: 10 mA for 30 min, followed by 20 mA for 90 min. After completion of the electrophoresis, the gel was stained overnight with the conventional CBB method. Finally,

the gel was photographed with the ChemiDoc XRS Plus System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

#### 3.2.2.7. Western blotting

After completing electrophoresis (without staining), the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) using a semi-dry transfer system (AE-6677; ATTO Co.); the PVDF membrane was previously pre-treated with methanol for 5 min. The transferred membrane were 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 h at 4℃. Thereafter, horseraddish 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 h at 4℃. After applying chemiluminescence reagent (Chemi-Lumi One L, Nacalai Tesque Inc.) onto the PVDF membrane, the images were detected with ChemiDoc XRS Plus System (Bio-Rad Laboratories Inc.).

#### 3.2.2.8. Analysis of volatile compounds in CF

Volatile compounds in the LMMs-CF were determined by headspace solid phase micro-extraction (SPME) coupled with gas chromatography/mass spectrometry (GCMS-QP2010 Ultra; Shimadzu Co., Kyoto, Japan). The 3 mL of samples were taken into 20 mL vials, closed with a PTFE septa and magnetic cap. The volatile compounds of LMMs-CF were extracted using head space-SPME technique with auto-sampler (AOC-5000 plus; Shimadzu Co.). The vial containing sample was placed into heat block at 80°C for 5 min, then absorbed into

SPME fiber (2 mm of 50/30 μm DVB/CAR/PDMS fiber; Supelco, Bellefonte, PA) at 80°C for 20 min. Before analysis of the first sample, the fiber was baked at 250°C for 30 min. After absorption, the fiber was inserted into the gas chromatograph/mass spectrometry (GCMS-QP2010 Ultra; Shimadzu Co.). The analysis was conducted in split mode with the following conditions: injector temperature, 250°C; split ratio, 5:1; carrier gas, helium; linear velocity, 50 cm/sec. The volatile compounds were separated on a fused silica capillary column (DB-WAX 30 m × 0.25 mm internal diameter, Agilent Technologies Inc., Santa Clara, CA) with a temperature-rising condition (initial oven temperature at 40°C, held at 40°C for 1min, increased by 16°C/min to 120°C, increased by 8°C/min to 200°C, and held at 200°C for 19 min, total time of 35 min). The mass spectrometer was operated in the electron impact ionization mode at 70 eV; the mass range used was m/z 29 to 300. The mass spectrometer interface and ion source temperature were 210 and 200°C, respectively. Volatile compound was identified by comparison with the mass spectra of the National Institute of Standards and Technology (NIST) 11 Mass Spectral Library (NIST, Gaithersburg, MD). The peak area of the volatile compound was integrated from specific ions for each molecule to avoid overlapping between compounds.

#### 3.2.2.9. Statistical analysis

Data were 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

### 3.3.1. Effects of external factors on aerobic ZnPP formation by LLC

Temperature and pH are the important external factors that greatly affect the growth of bacteria. Sodium nitrite, sodium chloride, sodium ascorbate, glucose, and polyphosphate are commonly used in meat products for different purposes, although some of them have antibacterial effects beyond a certain limit. Therefore, in this section, to investigate the effects of different temperature, pH, and additives on aerobic ZnPP formation by *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC), aseptic meat homogenate (aMH) model systems were performed under both aerobic and anaerobic conditions.

#### 3.3.1.1. Effects of temperature and pH

First, to investigate the effects of different temperatures on aerobic ZnPP formation by ZnPP-forming LLC, aMH model system was performed. The model solutions were incubated both aerobically and anaerobically at five different temperatures, i.e., 18, 25, 30, 37, and 45°C for 7 days, and ZnPP was measured. After 7 days of incubation under anaerobic conditions, the high fluorescence intensity of ZnPP was observed in both LAB-inoculated groups at 30 and 37°C (Fig. 16A). The drastic reduction of ZnPP formation was observed at 45°C in both LAB-inoculated groups and showed non-significantly different from the temperature at 18°C. However, under aerobic conditions, the high fluorescence intensity of ZnPP was observed only in the LLC-inoculated group at all incubated temperatures, and they showed significant difference ($P < 0.01$) compared to the other groups at the same incubation

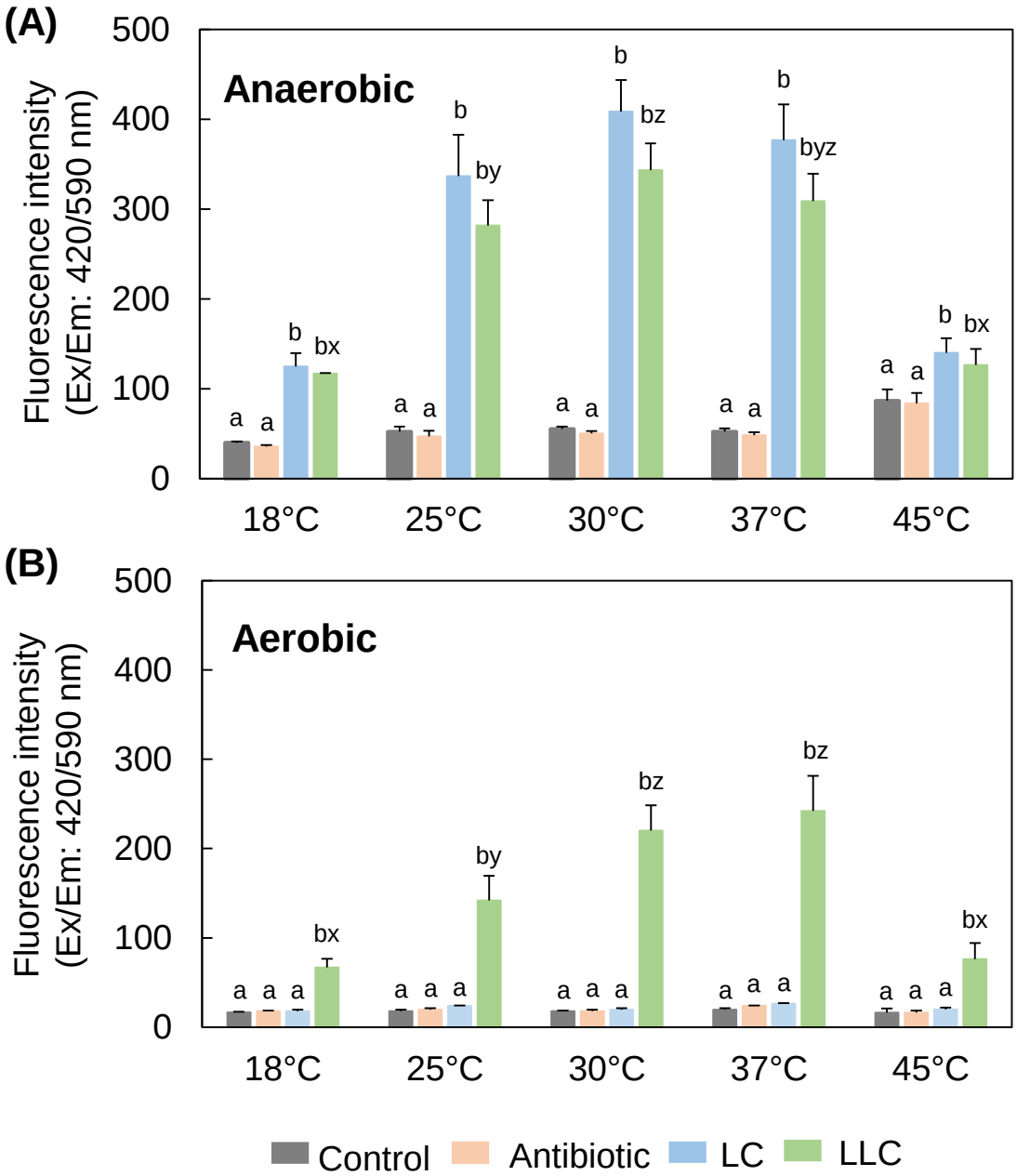



**Fig. 16. Effect of incubation temperature on LAB-associated ZnPP formation in aMH model experiment**

LAB-inoculated pork homogenate (20% meat, 3% salt) (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*, Control: non-inoculated, Antibiotic: antibiotics-added) was incubated both anaerobically (A) and aerobically (B) at five different temperatures (18, 25, 30, 37 and 45°C) for 7 days in the dark. After incubation, ZnPP of the mixture was extracted and measured. Data expressed as means ± SE (n = 3). Columns with different letters among the different groups in the same temperature (ab) and among the different temperatures in the same group (xyz) differ significantly ($P < 0.05$).

temperature (Fig. 16B). The maximum fluorescence intensity of ZnPP was observed at 37°C, followed by the 30 and 25°C. But, no significant difference for ZnPP formation in the LLC-inoculated group was observed between 30 and 37°C. Since ZnPP formation in the antibiotic group was almost the same as the control group, all operations were considered to be performed aseptically.

Next, to investigate the effect of pH on aerobic ZnPP formation by ZnPP-forming LLC, aMH model system was performed. In this model experiment, the pH was adjusted at three different levels, i.e., 5.8, 5.5, and 4.75. After the inoculation of ZnPP-forming LAB in the inoculated groups, the model solutions were incubated both aerobically and anaerobically at 25°C for 7 days, and ZnPP was measured. Under anaerobic conditions, the maximum ZnPP formation was measured at pH 5.5, followed by 5.8 and 4.75 in both LAB-inoculated groups, and the ZnPP formation was significantly ($P < 0.05$) increased in all the cases compared to the other groups (Fig. 17A). However, under aerobic conditions, the formation of ZnPP significantly increased only in the LLC-inoculated groups at all pH compared to the other groups ($P < 0.05$), and the maximum ZnPP formation was measured at pH 5.5, followed by the 5.8 and 4.75 (Fig. 17B). Moreover, the ZnPP formation significantly ($P < 0.05$) differed at three different pH levels in the LLC-inoculated group.

#### 3.3.1.2. Effect of additives

The effect of additives on aerobic ZnPP formation by the ZnPP-forming LLC was investigated using aMH model system. In this model experiment, various additives at different concentrations, i.e., salt (3%), sodium nitrite (300 ppm), sodium ascorbate (400

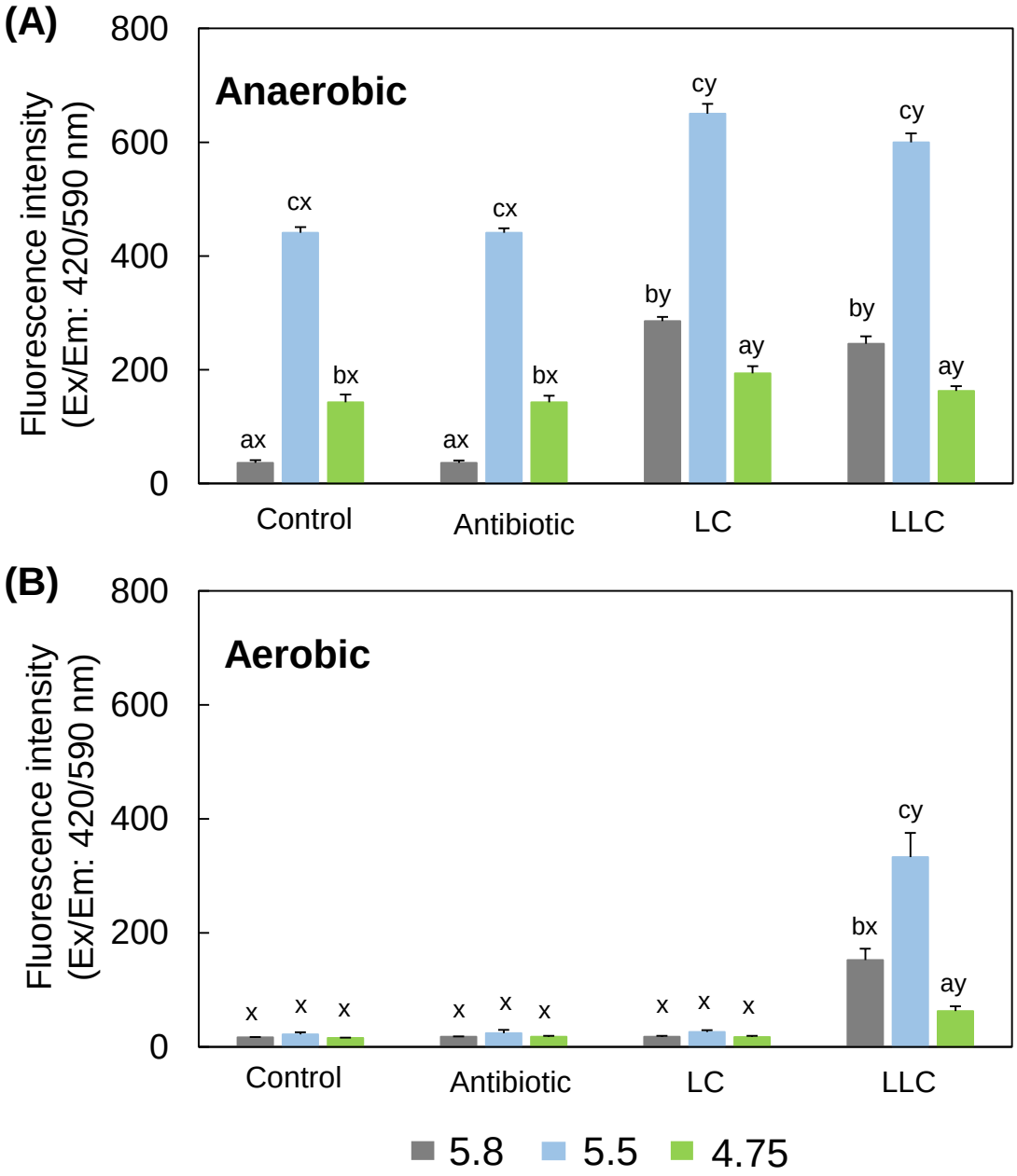



**Fig. 17. Effect of pH on LAB-associated ZnPP formation in aMH model experiment**

Pork homogenates were adjusted to pH 5.8 (■), 5.5 (■), and 4.75 (■). Pork homogenate mixture was prepared and incubated (25°C) both anaerobically (A) and aerobically (B) and the formed ZnPP was measured as described in Fig. 16. Data expressed as means ± SE (n = 3). Columns with different letters in the same group (abc) and among the different group at the same pH (xy) differ significantly ($P < 0.05$).

ppm), sodium sulfite (100 ppm), glucose (1%), and polyphosphate (0.3%) were used. The model solutions were incubated both aerobically and anaerobically at 25°C for 7 days, and ZnPP was measured. Under anaerobic conditions, the addition of salt, sodium ascorbate, and glucose showed minimum effects in ZnPP formation in both LAB-inoculated groups, whereas sodium nitrite completely inhibited ZnPP formation even in the presence of ZnPP-forming LAB (Fig. 18A). Besides, in the presence of sodium sulfite, a little formation of ZnPP was observed in both LAB-inoculated groups, and the LLC-inoculated group showed significantly higher ZnPP than that of the LC-inoculated group ($P < 0.05$). Moreover, with the addition of polyphosphate, no ZnPP was observed in the LC-inoculated group, although a little ZnPP was observed in the LLC-inoculated group and showed significant difference compared to the other groups. In contrast, under aerobic conditions, the formation of ZnPP significantly increased only in the LLC-inoculated groups compared to the other groups ($P < 0.05$) (Fig. 18B). However, sodium nitrite completely inhibited the formation of ZnPP in all the groups as was shown under anaerobic conditions. Additionally, the addition of sodium sulfite and polyphosphate also inhibited the formation of ZnPP, although a small amount of ZnPP was noticed in the LLC-inoculated groups. Moreover, the LLC-inoculated groups with salt, sodium ascorbate and glucose showed minimum effects in the reduction of ZnPP formation compared to the control group.

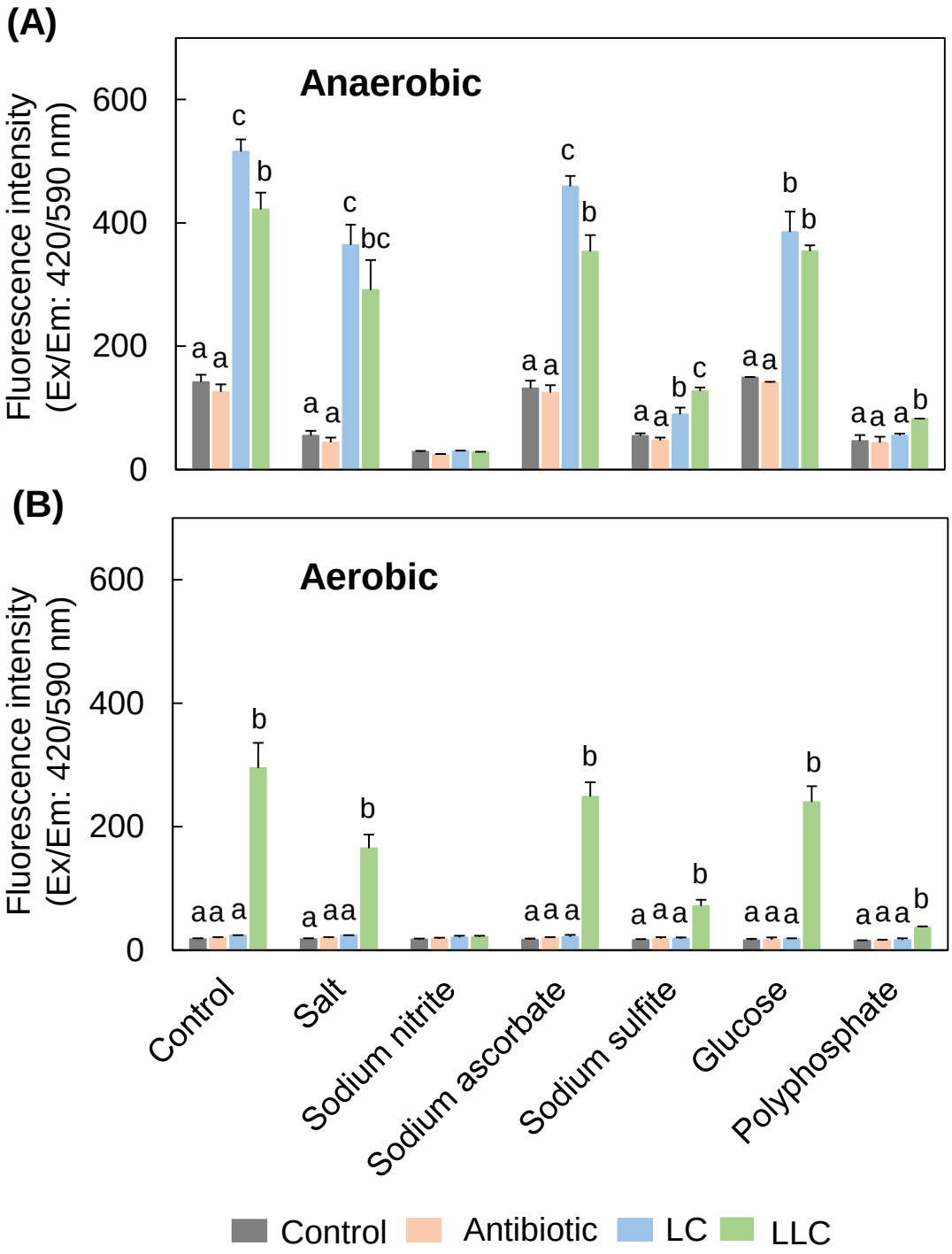



**Fig. 18. Effect of additives on LAB-associated ZnPP formation in aMH model experiment**

Additives-added pork homogenate mixture was prepared and incubated (25°C) both anaerobically (A) and aerobically (B) and the formed ZnPP was measured as described in Fig. 16. Data expressed as means ± SE (n = 3). abc: Columns with different letter in the same group of different additives differ significantly ($P$ < 0.05).

### 3.3.2. Effects of internal factors on aerobic ZnPP formation by LLC

#### 3.3.2.1. Effects of FECH and heating of meat homogenate

Most of the enzymes and proteins in meat are destroyed by applying a varying degree of heat. In the anaerobic mechanism of ZnPP formation, FECH is the key enzyme (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). However, not only meat-inherent FECH (mFECH) but also bacterial FECH (bFECH) contribute to the anaerobic formation of ZnPP (Asaduzzaman, 2020a). In order to clarify the LLC-associated aerobic ZnPP formation mechanism by separately considering the contribution of mFECH and bFECH derived from LLC, the effects of heated meat and FECH inhibitor (N-MMP) were investigated using the aMH model system with ZnPP-forming LLC.

##### 3.3.2.1.1. Effects of meat-inherent heat-sensitive compounds

First, in order to investigate the effects of meat-inherent heat-sensitive compounds in LLC-associated aerobic ZnPP formation, the LAB-inoculated cooked meat homogenate was incubated both aerobically and anaerobically at 30°C for 7 days, and the formation of ZnPP was measured. Under aerobic conditions, no ZnPP fluorescence peak at 590 nm was observed in the control and LAB-inoculated groups (Fig. 19A-C). A similar experiment was performed with the addition of exogenous Hb and Mb, but no fluorescence peak of ZnPP at 590 nm was observed in the inoculated groups as well as in the control group (Fig. 19D-I). Moreover, under anaerobic conditions, ZnPP was not formed in all groups (data not shown).

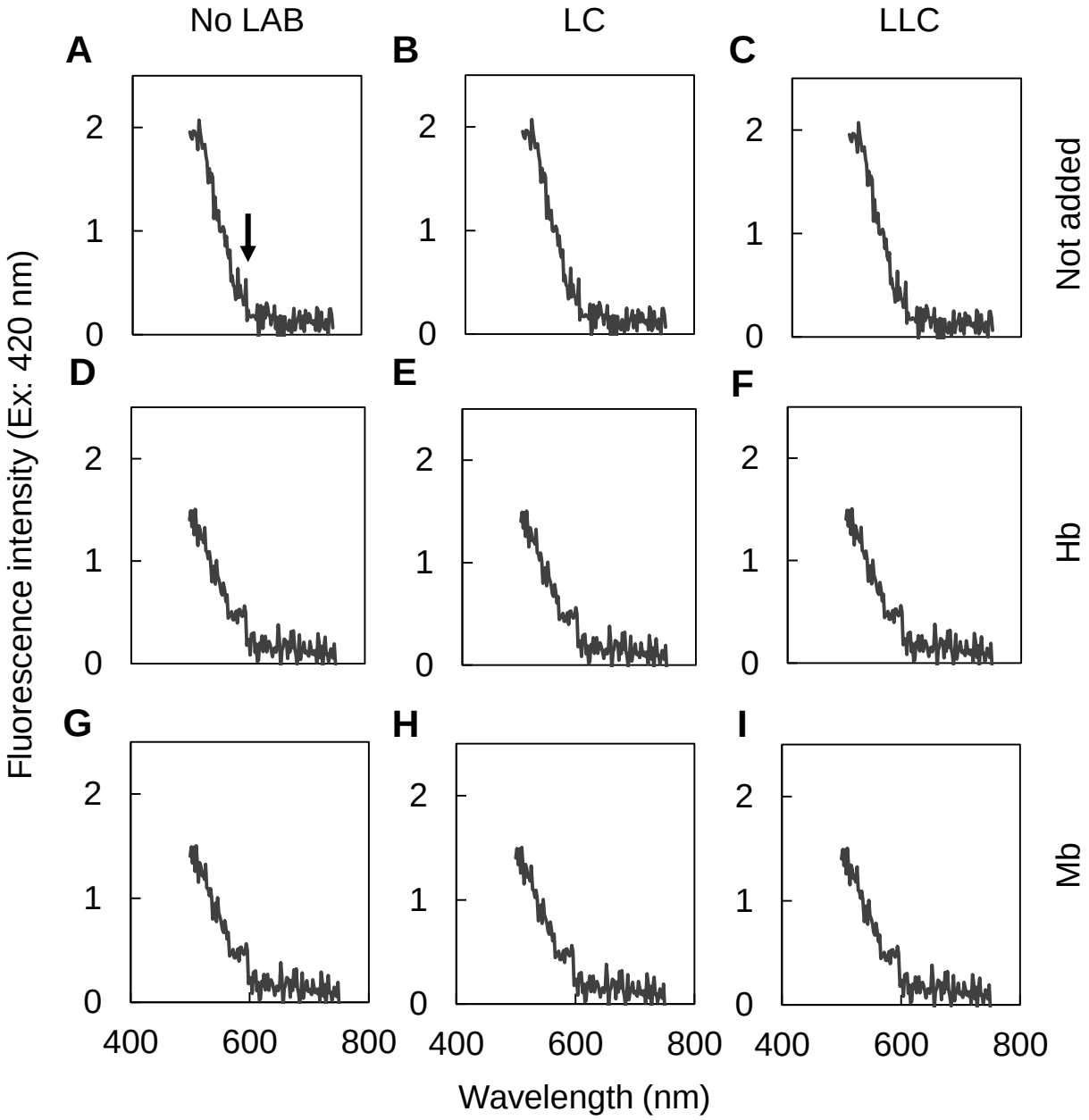



**Fig. 19. Aerobic ZnPP formation by ZnPP-forming LAB in cooked meat homogenate**

Hemoglobin (Hb) and myoglobin (Mb) were added onto LAB-inoculated cooked meat homogenate (CMH) (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*). The CMH was then incubated aerobically at 30°C for 7 days. ZnPP was extracted from the incubated CMH and the fluorescence spectrum of ZnPP was measured. Allow indicates the position of ZnPP fluorescence peak (590 nm). A,D,G: No addition of LAB; B,E,H: LC-inoculated; C,F,I: LLC-inoculated; D,E,F: Hb-added; G,H,I: Mb-added

#### 3.3.2.1.2. Effect of meat-inherent enzyme

Next, in order to investigate the effects of meat-inherent enzymes in LLC-associated aerobic ZnPP formation, the experiments with blanched meat homogenate were performed. LAB-inoculated raw and blanched meat homogenates were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured. Under anaerobic conditions, the formation of ZnPP drastically reduced in the blanched meat homogenate groups without the addition of LAB, but the ZnPP formation was significantly higher in both LAB-inoculated groups ($P < 0.01$) than those in the non-inoculated groups (Fig. 20A). Under aerobic conditions, the formation of ZnPP was only observed in raw meat homogenate of LLC-inoculated groups compared to those of the other groups (Fig. 20B). The ZnPP formation was suppressed in the LLC-inoculated groups with blanched meat homogenate, but significantly higher compared to the other groups ($P < 0.01$).

#### 3.3.2.1.3. Effects of mFECH and bFECHs

Next, in order to investigate the effects of bFECH derived from LLC on LLC-associated aerobic ZnPP formation, FECH inhibitor-added aMH model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured (Table 8). Under anaerobic conditions, the formation of ZnPP was drastically suppressed in the non-LAB-inoculated group in the presence of FECH inhibitor (Fig. 21). The ZnPP formation was also suppressed in both LAB-inoculated groups with the addition of FECH inhibitor but showed significantly higher value than those of the non-inoculated groups ($P < 0.05$). However, under aerobic conditions in the absence of FECH inhibitor, the formation of ZnPP only in the LLC-

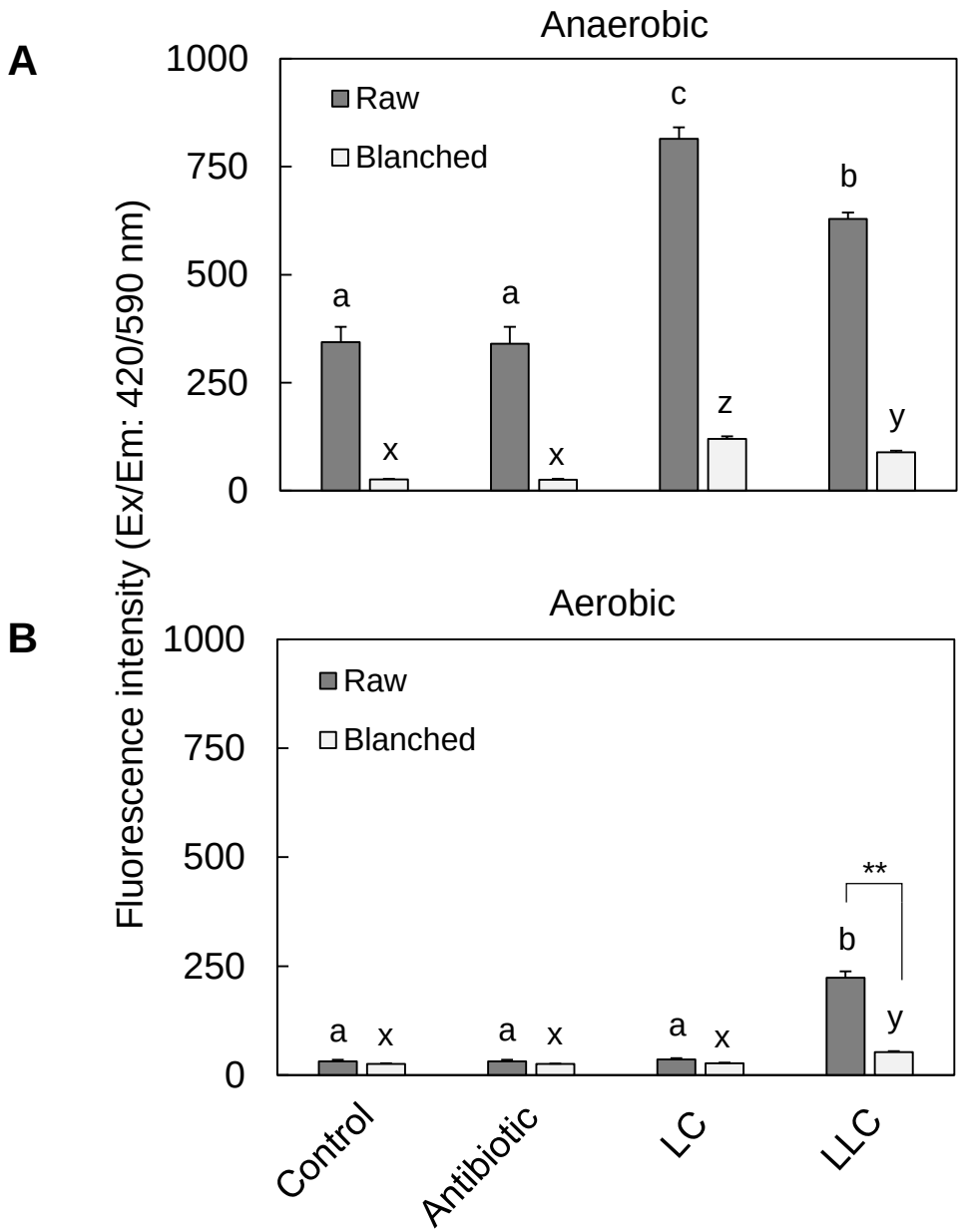



**Fig. 20. Effect of meat-inherent enzymes on ZnPP formation by the ZnPP-forming LAB in the model experiment**

LAB-inoculated pork homogenate (20% meat) (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*, Control: non-inoculated, Antibiotic: antibiotics-added) was incubated both anaerobically (A) and aerobically (B) at 30°C for 7 days in the dark. After incubation, ZnPP of the mixture was extracted and measured. Data expressed as means ± SE (n = 3). ab and xyz: Columns with different letters in the different groups differ significantly ($P < 0.01$). ** ($P < 0.01$) significantly different between groups.

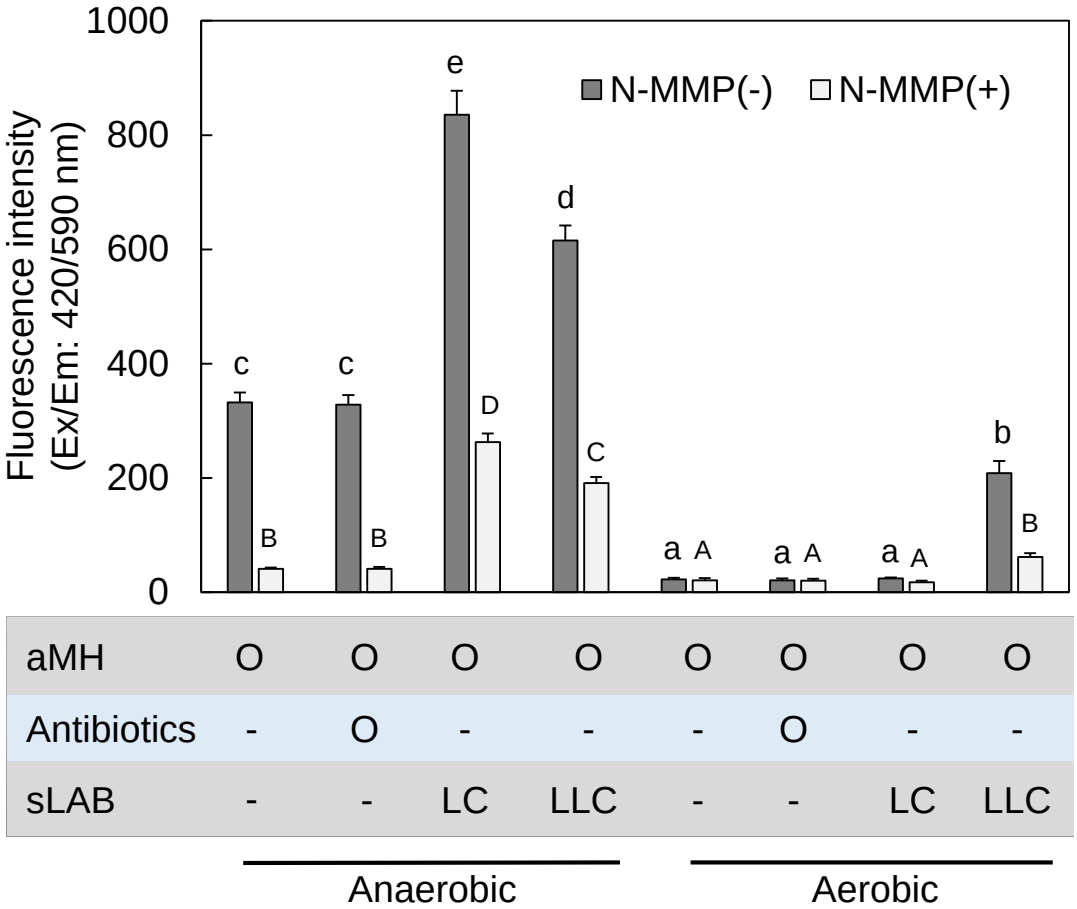



**Fig. 21. Effect of FECH inhibitor on ZnPP formation by the ZnPP-forming LAB in the model experiment**

N-methyl mesoporphyrin IX (N-MMP) as FECH inhibitor was added to LAB-inoculated (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*) aseptic meat homogenates (aMH). Then, aMH mixture was incubated and the formed ZnPP was measured as described in Fig. 25. Data expressed as means ± SE (n = 3). abcde and ABCD: Columns with different letters differ significantly ($P < 0.05$). sLAB: Selected LAB

inoculated groups was significantly increased compared to the other groups ($P < 0.05$) (Fig. 21). The ZnPP formation was also suppressed in the presence of FECH inhibitor, but the amount of formed ZnPP was higher than those of the other groups ($P < 0.05$).

#### 3.3.2.2. Effects of different fractions of meat homogenate on LLC-associated aerobic ZnPP formation

In section 3.3.2.4.1, it was mentioned that ZnPP-forming LLC itself could not form ZnPP aerobically, and components of fresh meat are mandatory in bacteria-associated ZnPP formation. Therefore, it is essential to clarify the particular components from fresh meat responsible for plenty of ZnPP formation in the presence of oxygen with the association of ZnPP-forming LLC. Moreover, to elucidate which fraction comprises the rate-limiting components and where there is a synergistic effect between each fraction and ZnPP-forming LLC, pork homogenate was fractionated, and different model experiments were designed using each fraction of pork homogenate and ZnPP-forming LAB.

##### 3.3.2.2.1. Effects of meat fractions

First, in order to clarify the effects of meat fractions on LLC-associated aerobic ZnPP formation, meat homogenate was fractionated into soluble and insoluble fractions. In this section, the insoluble fraction (ISF) with or without ZnPP-forming LLC along with a constant concentration of the soluble fraction (SF) and SF with or without ZnPP-forming LLC along with a constant concentration of the ISF were performed (Table 10) to clarify the effects of ISF and SF in LLC-associated aerobic ZnPP formation, respectively. The mixture of the model solutions was incubated both aerobically and anaerobically. Under anaerobic

conditions, all groups containing ISF significantly increased ZnPP formation compared to the group without ISF, and the addition of LAB drastically increased the ZnPP formation ($P < 0.01$) (Fig. 22A). On the other hand, under aerobic conditions, ZnPP was not formed in all groups in the absence of ISF. However, the formation of ZnPP was observed only in the LLC-inoculated group with ISF (Fig. 22A).

In the experiment, regarding effect of the addition of SF into ISF under anaerobic conditions, the formation of ZnPP in the non-inoculated groups with SF significantly increased compared to the non-added SF groups ($P < 0.01$) (Fig. 22B). Moreover, the addition of SF in both LAB-inoculated groups also significantly differed the formation of ZnPP compared to the LAB-inoculated groups without SF ($P < 0.01$). Under aerobic conditions, the formation of ZnPP was noticed only in the LLC-inoculated group without SF, but the addition of SF increased the formation (Fig. 22B).

**3.3.2.2.2. Effect of meat soluble fraction**

In a previous experiment, the role of SF in the presence of ZnPP-forming LLC and ISF is not clear. It is essential to clarify the role of SF on LLC-associated aerobic ZnPP formation, and hence the SF was fractionated into the <10 kDa and >10 kDa fractions.

First, to clarify the effect of >10 kDa SF on LLC-associated aerobic ZnPP formation, a model experiment was performed using a constant concentration of <10 kDa SF (Table 13). The mixture of model solutions was incubated aerobically and anaerobically at 30 °C for 7 days, and ZnPP was measured. Under anaerobic conditions, the addition of ISF led to slight increase in the non-inoculated groups. ZnPP also significantly increased in both LAB-

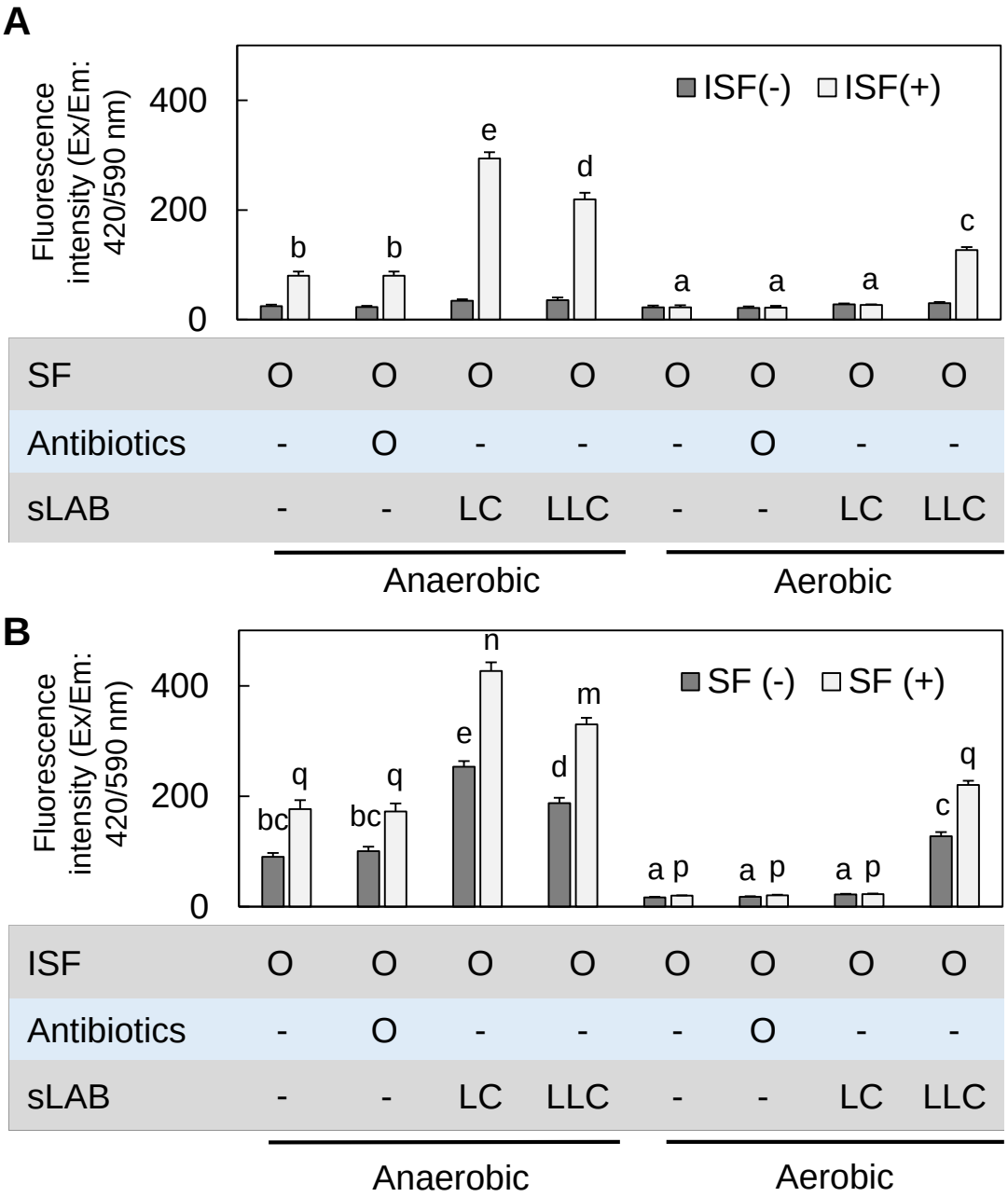



**Fig. 22. Effect of meat fractions on ZnPP formation by the ZnPP-forming LAB in the model experiment**

Soluble fractions (SF) (A) or insoluble fractions (ISF) (B) of pork, which was inoculated with LAB (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*), was incubated both aerobically and anaerobically at 30°C for 7 days in the dark. ZnPP of the mixture was extracted and measured. Data expressed as means ± SE (n = 3). abcde and pqmn: Columns with different letters in the different groups differ significantly ($P < 0.01$). sLAB: Selected LAB

inoculated groups due to the addition of ISF but the addition of >10 kDa SF increase markably ($P < 0.05$) (Fig. 23A). However, under aerobic conditions, the addition of the ISF and >10 kDa SF significantly increased the ZnPP formation only in the LLC-inoculated group compared to the other groups ($P < 0.05$) (Fig. 23A).

Next, the additional effect of <10 kDa SF on ZnPP formation, was investigated using a constant concentration of the >10 kDa SF (Table 11). In all the groups of both aerobic and anaerobic conditions, the addition of the ISF facilitated remarkable ZnPP formation but the addition of the <10 kDa SF did not affect the ZnPP formation (Fig. 23B).

**3.3.2.2.3. Effects of ISF concentrations on ZnPP formation and heme content in the ISF**

In the previous section, ISF was suggested to affect aerobic ZnPP formation by LLC highly. To clarify whether the ZnPP formation by the ISF is dose-dependent manner, an experiment was performed using different concentrations of ISF and incubated anaerobically at 30°C for 7 days. As the amount of ISF increased, the fluorescence intensity of ZnPP increased linearly (Fig. 24). According to this result, the rate-limiting component for ZnPP formation was suggested to be present in the ISF of meat.

The >10kDa SF was essential to form ZnPP formation in the previous study (Akter, 2019a) but ZnPP was formed in the absence the of >10kDa SF in this study. Therefore, to investigate whether ISF contains heme or not (mainly heme is water-insoluble), the content of heme was measured just after separation and after several washing of ISF. It was found that the content of heme was significantly reduced after the first washing of ISF from the separation of ISF and steadily reduced to third washing ($P < 0.05$) (Fig. 25). Besides, the

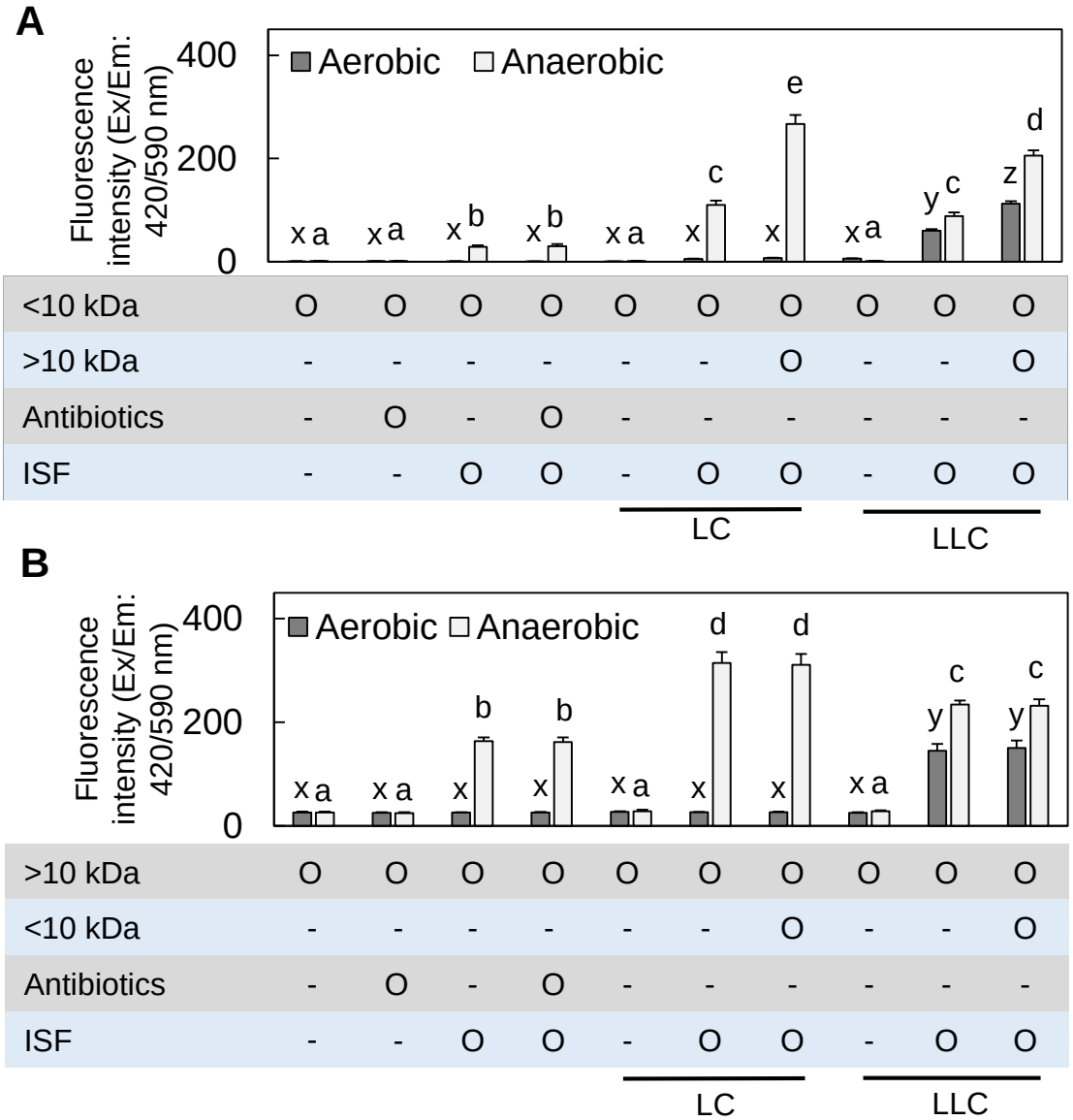



**Fig. 23. Effect of soluble fractions (SF) on ZnPP formation by the ZnPP-forming LAB in the model experiment**

LAB-inoculated (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*) <10 kDa (A) or >10 kDa (B) SF of pork (where <10 and >10 kDa SF as common components) was incubated both aerobically and anaerobically at 30°C for 7 days in the dark. ZnPP of the mixture was extracted and measured. Data expressed as means ± SE (n = 3). abcde and xyz: Columns with different letters in the different groups differ significantly ($P < 0.05$). ISF: Insoluble fraction

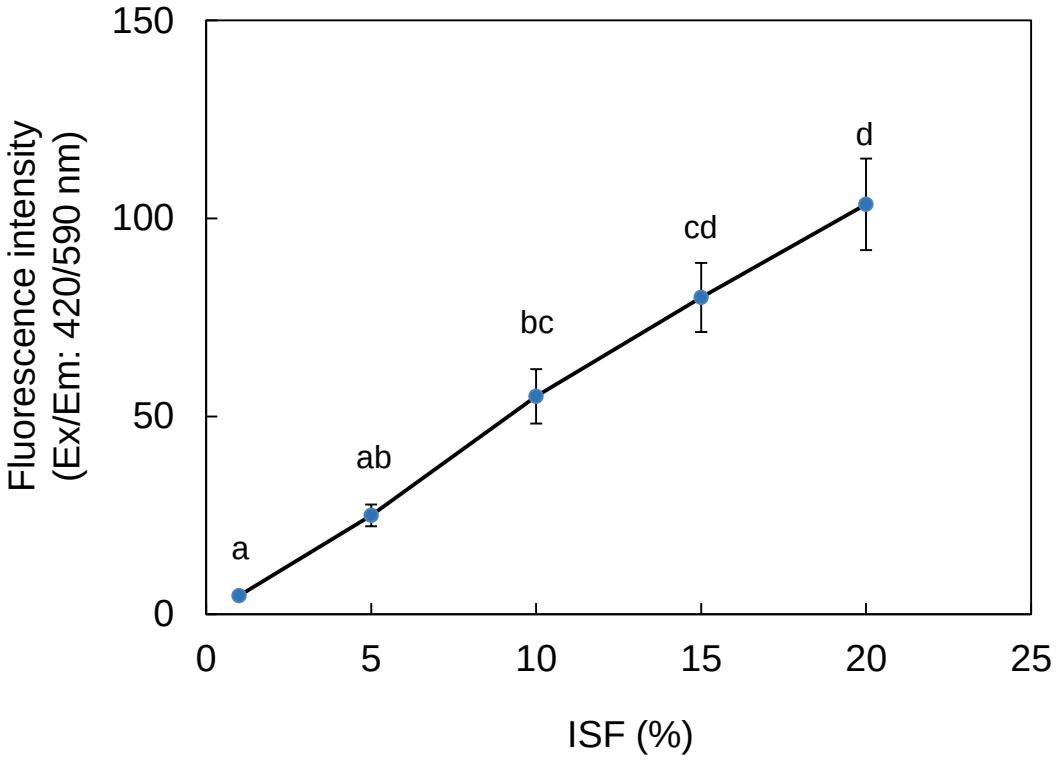



**Fig. 24. Effects of different concentrations of insoluble fraction (ISF) on ZnPP formation in the model experiment**

Pork ISF of different concentrations were incubated anaerobically at 30°C for 7 days in the dark. ZnPP of the incubated samples was extracted and the fluorescence intensity was measured. Data expressed as means ± SE (n = 3). abcd: Columns with different letters differ significantly ($P < 0.05$).

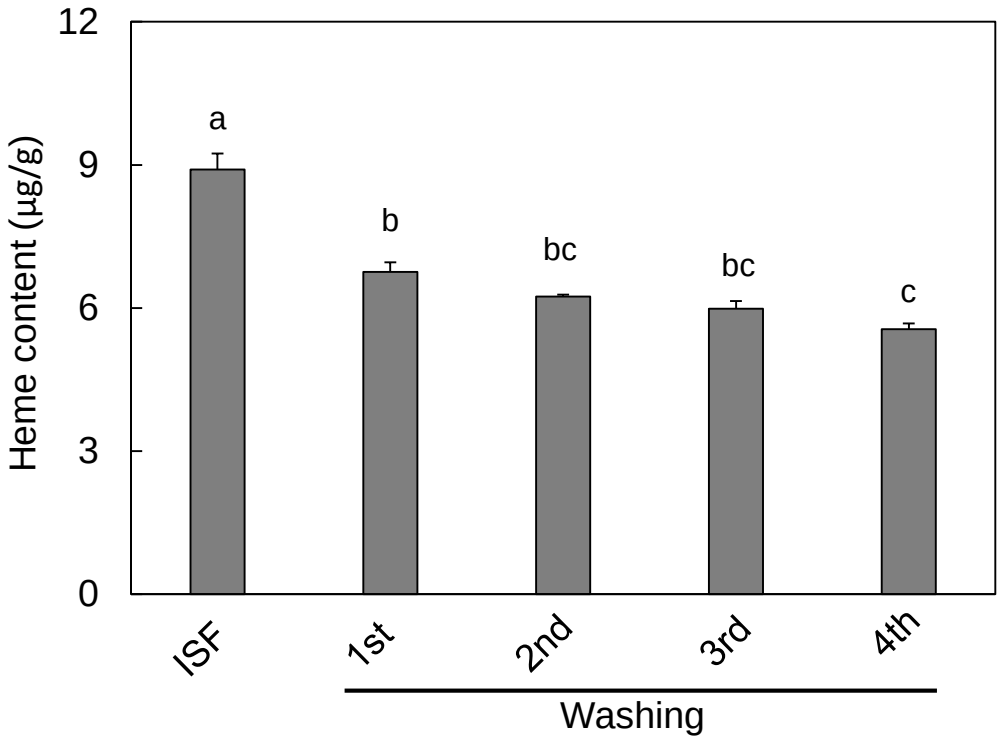



**Fig. 25. Effect of washing on retaining the amount of heme in insoluble fraction (ISF)**

Pork ISF was prepared and washed several times. Heme content of the washed ISF was extracted and the absorbance of the heme was measured. Data expressed as means ± SE (n = 3). abc: Columns with different letters differ significantly ($P < 0.05$).

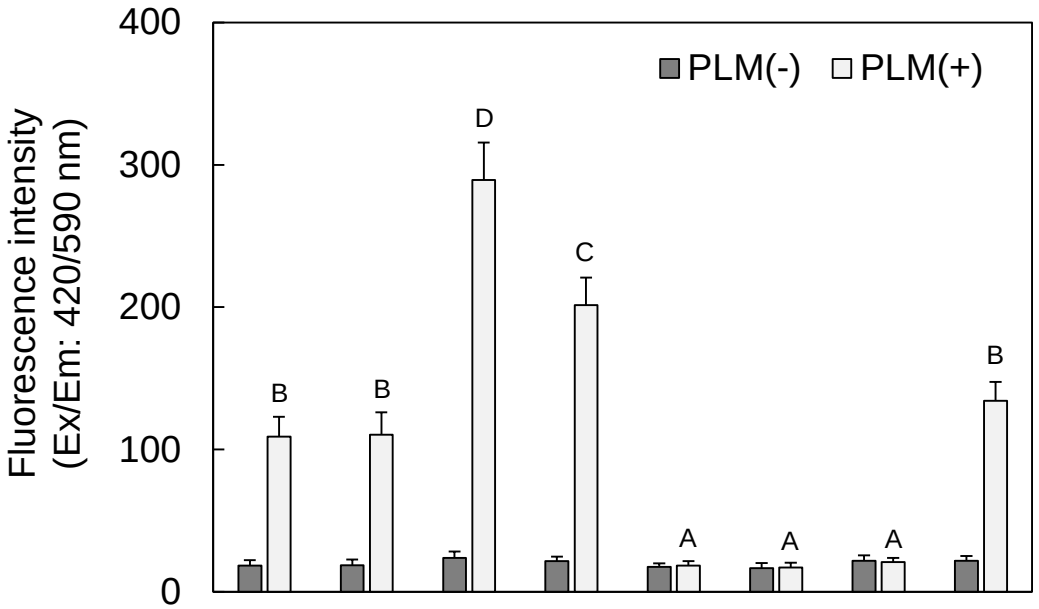
heme content was non-significantly reduced among the second, third, and fourth washing of ISF but the heme could not be removed completely.

#### 3.3.2.3. Effect of mitochondria on LLC-associated aerobic ZnPP formation

From section 3.3.2.2.3, it was observed that the higher concentration of ISF significantly increases the amount of ZnPP. It is known that ISF contains mitochondria and the key enzyme FECH for the formation of ZnPP locates in the membranes of mitochondria (Taketani *et al*., 2007; Asaduzzaman, 2020a). Hence, in this section, in order to clarify whether FECH contributes to the aerobic formation of ZnPP with the ZnPP-forming LLC, the effects of mitochondria and its membranes on the formation of ZnPP in association with ZnPP-forming LLC were investigated using the model experiment system.

##### 3.3.2.3.1. Effect of exogenous mitochondria

To investigate the effect of exogenous mitochondria on LLC-associated aerobic ZnPP formation, exogenous mitochondria-added SF solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured (Table 12). Overall, no ZnPP was observed in exogenous mitochondria free-SF under both aerobic and anaerobic conditions. Under anaerobic conditions, the addition of exogenous mitochondria derived from pork liver increased the formation of ZnPP in all groups (Fig. 26). The formation of ZnPP was significantly increased in both LAB-inoculated groups treated with exogenous mitochondria compared to the non-inoculated groups as well ($P < 0.05$). However, under aerobic conditions, the addition of mitochondria increased ZnPP only in the LLC-inoculated group compared to the other groups (Fig. 26).




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| SF | O | O | O | O | O | O | O | O |
| Antibiotics | - | O | - | - | - | O | - | - |
| sLAB | - | - | LC | LLC | - | - | LC | LLC |
| | Anaerobic | | | | Aerobic | | | |

**Fig. 26. Effect of exogenous mitochondria on ZnPP formation by the ZnPP-forming LAB in the model experiment**

LAB-inoculated soluble fraction (SF) of pork (20%) (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*, PLM: Pork liver mitochondria) was incubated both aerobically and anaerobically at 30°C for 7 days in the dark. ZnPP was extracted and measured. Data expressed as means ± SE (n = 3). ABCD: Columns with different letters in the different groups differ significantly ($P < 0.05$). sLAB: Selected LAB

#### 3.3.2.3.2. Effects of mitochondrial membranes

FECH is bound to mitochondrial membrane in mammals (Sakaino *et al*., 2009). In order to investigate the effect of mitochondrial membranes on LLC-associated aerobic ZnPP formation, outer membrane (OM)-, inner membrane (IM)-, and matrix (MT)-added model solutions were incubated, both aerobically and anaerobically (Table 13). After 7 days of incubation under anaerobic conditions, ZnPP increased in the non-inoculated groups containing OM and IM significantly compared with the non-added group of mitochondrial fraction and MT-added groups ($P < 0.05$) (Fig. 27), moreover the addition of OM and IM in both LAB-inoculated groups significantly increased the ZnPP formation compared to the non-inoculated groups ($P < 0.05$). On the contrary, under aerobic conditions, the ZnPP formation was observed only in the LLC-inoculated groups with OM and IM (Fig. 27). However, no ZnPP formation was observed in all the LAB-inoculated and non-inoculated groups containing MT in both aerobic and anaerobic conditions (Fig. 27).

Next, SDS-PAGE and western blotting were performed to investigate the existence of FECH among the mitochondria and mitochondrial fractions. The FECH of mitochondria and mitochondrial fractions were separated by SDS-PAGE, and the presence of FECH was identified by western blotting (Fig. 28). In each lane of the mitochondria (MTC), OM, and IM fractions, a FECH band of nearly 45 kDa was observed, and no band of FECH in MT fraction lane was observed.

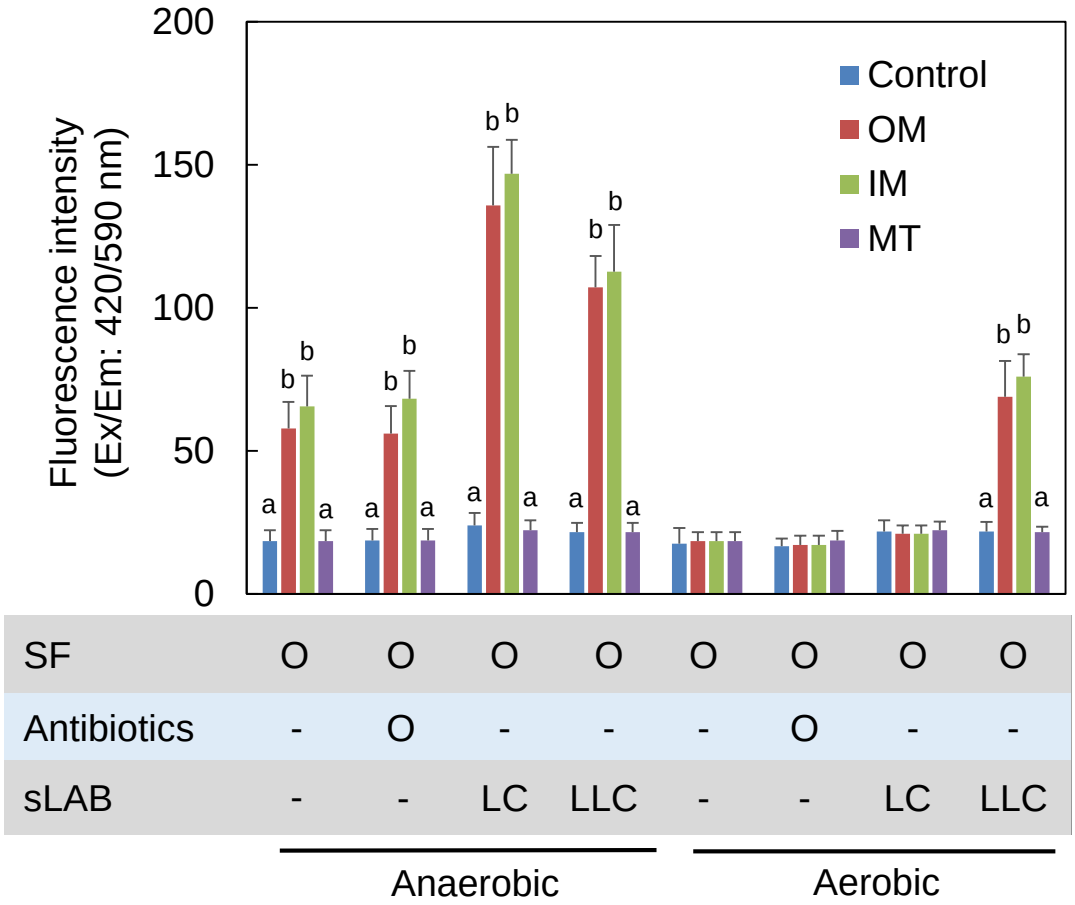



**Fig. 27. Effects of mitochondrial fractions (MF) on ZnPP formation by the ZnPP- forming LAB in the model experiment**

LAB-inoculated soluble fraction (SF) of pork (20%) (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*, Control: non-added MF, OM: Outer membrane-added, IM: Inner membrane-added, MT: Matrix-added) was incubated both aerobically and anaerobically at 30°C for 7 days in the dark. ZnPP was extracted and measured. Data expressed as means ± SE (n = 3). ). ab : Columns with different letters in the same groups differ significantly ($P < 0.05$).

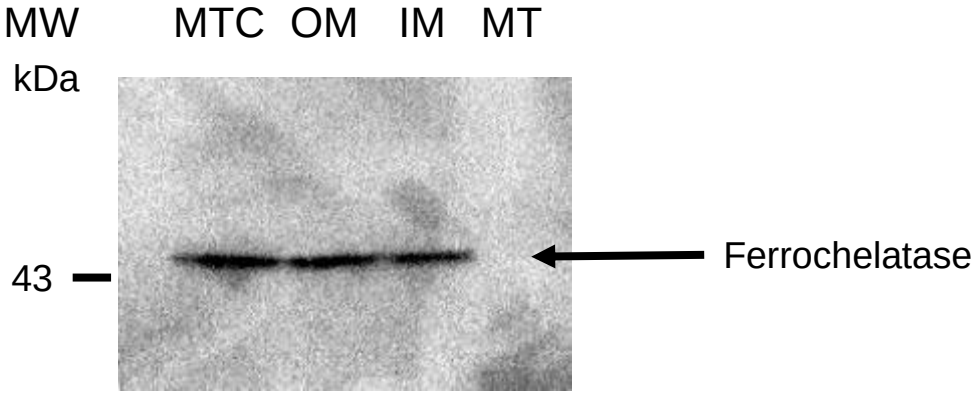



**Fig. 28. Western blotting image of mitochondria and mitochondrial fractions extracted from pork liver**

FECH in mitochondria (MTC), the outer membrane (OM), inner membrane (IM) and matrix (MT) was confirmed by the western blotting using anti-FECH antibody.

#### 3.3.2.4. Direct contribution of LLC on aerobic ZnPP formation

Next, the aerobic ZnPP formation capability of LLC themselves was checked in the absence of meat. In this section, two commercial media were used, e.g., nutrient broth (NB) and MRS broth (MRSB). NB is a universal medium, whereas MRSB is a selective medium for some kind of LAB. Moreover, the aerobic synthesis of heme by LLC was also investigated using the commercial medium.

##### 3.3.2.4.1. Aerobic ZnPP formation by LLC in culture media

The high ZnPP-forming LC and LLC were inoculated in the NB and MRSB medium and incubated aerobically at 30°C for 7 days. After extraction with 75% acetone, the fluorescence spectrum was measured. No ZnPP fluorescence peak at 590 nm was observed in the two media of the control and both LAB-inoculated groups (Fig. 29A-C & 30A-C). Although exogenous Hb or Mb as heme sources was added to the media, no fluorescence peak of ZnPP at 590 nm was observed in both LAB-inoculated groups as well as in the control group under aerobic conditions (Fig. 29D-I & 30D-I). Similarly, under anaerobic conditions, ZnPP was not formed in all groups (data not shown).

##### 3.3.2.4.2. Heme synthesis by LLC in culture media

The heme synthesis ability of the ZnPP-forming LLC under aerobic conditions was investigated. LAB inoculated-NB medium was incubated aerobically at 30°C for 7 days, and heme was measured. No absorbance peak of heme at 383 nm was observed in all groups under aerobic conditions (Fig. 31). Similarly, under anaerobic conditions, heme was not newly formed in all groups (data not shown).

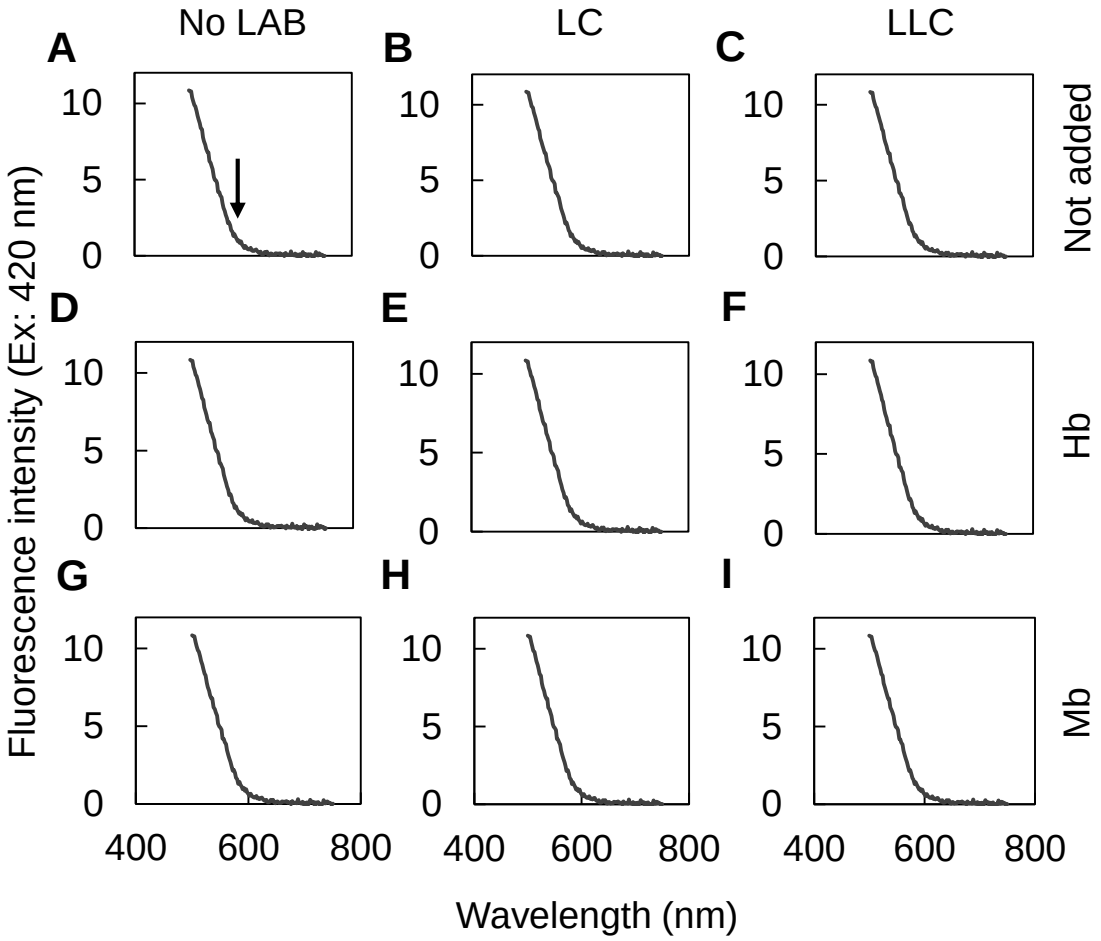



**Fig. 29. Aerobic ZnPP formation by ZnPP-forming LAB in nutrient broth (NB) medium**

NB medium was inoculated with LAB (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*), and then hemoglobin (Hb) and myoglobin (Mb) were added. The medium was incubated aerobically at 30°C for 7 days and then ZnPP was extracted and the fluorescence spectrum of the medium was measured. Allow indicates the position of ZnPP fluorescence peak (590 nm). A,D,G: No addition of LAB; B,E,H: LC-inoculated; C,F,I: LLC-inoculated; D,E,F: Hb-added; G,H,I: Mb-added

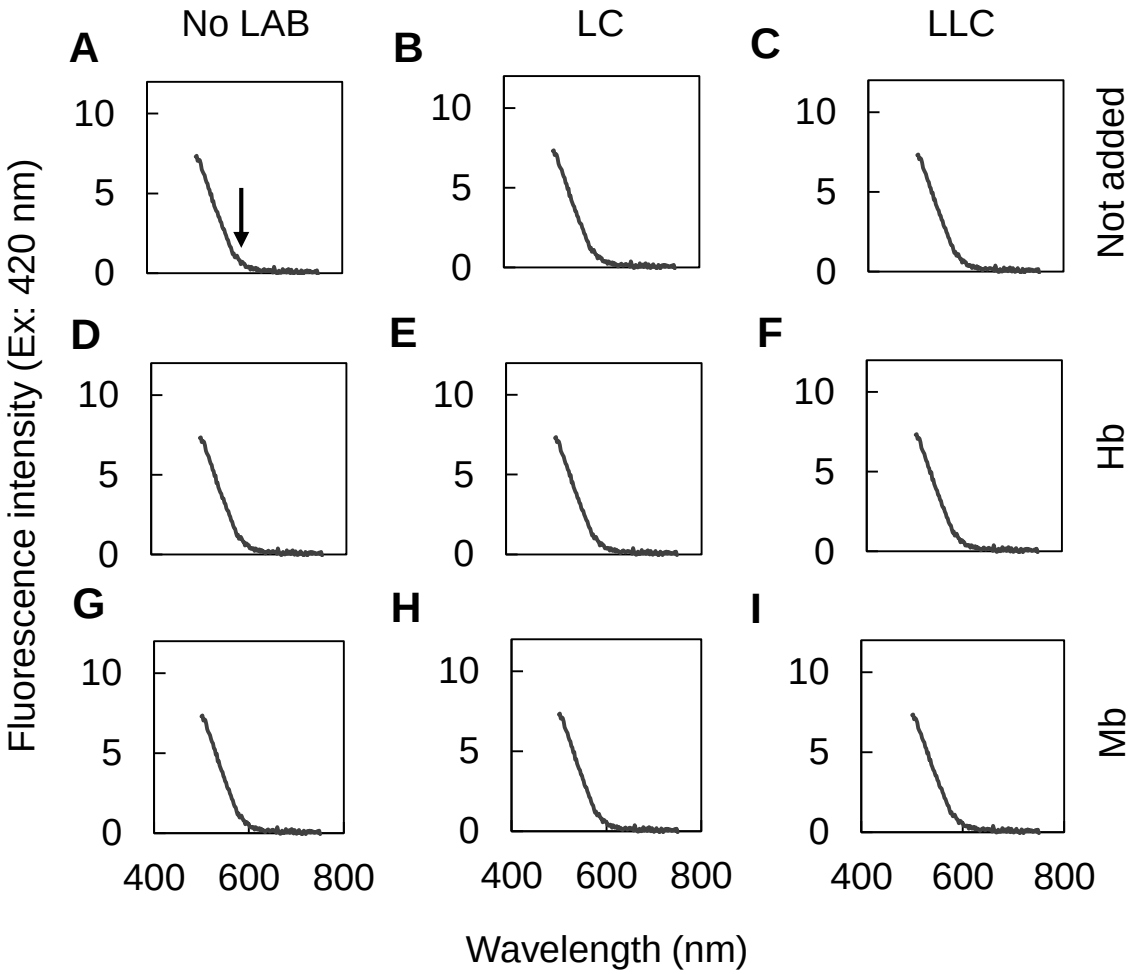



**Fig. 30. Aerobic ZnPP formation by ZnPP-forming LAB in MRS broth (MRSB) medium**

After the inoculation of LAB and the addition of Hb and Mb into MRSB medium, the incubation of the samples and measurement of formed ZnPP were performed as described in Fig. 29. Allow indicates the position of ZnPP fluorescence peak (590 nm). A,D,G: No addition of LAB; B,E,H: LC-inoculated; C,F,I: LLC-inoculated; D,E,F: Hb-added; G,H,I: Mb-added

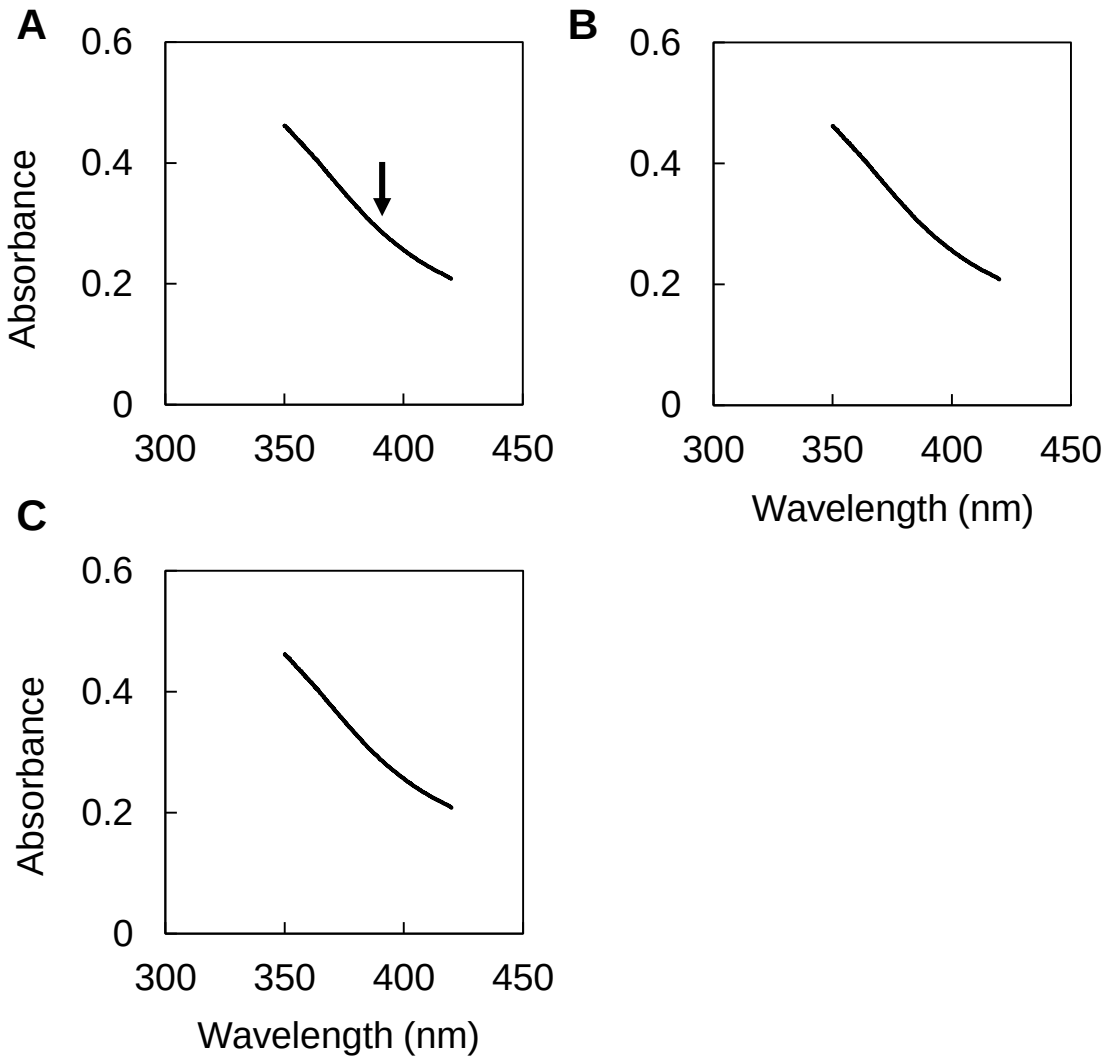



**Fig. 31. Heme formation by ZnPP-forming LAB in nutrient broth (NB) medium under aerobic conditions**

LAB-inoculated (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*) NB medium was incubated aerobically at 30°C for 7 days. Heme was extracted and the absorbance spectrum was measured. Allow indicates the position of ZnPP fluorescence peak (590 nm). A: Control, B: LC-inoculated; C: LLC-inoculated

#### 3.3.2.4.3. Degradation of heme proteins by LLC in culture medium

It was reported that the degradation of Mb during meat salting and ham maturation is necessary for the formation of ZnPP (Grossi *et al*., 2014). The degradability of Hb and Mb by the ZnPP-forming LLC in culture medium under aerobic conditions was investigated. LAB-inoculated NB media with the addition of Hb/Mb were incubated aerobically as well as anaerobically for 7 days at 30°C. The culture media were collected every day and then SDS-PAGE was performed. The band thickness of Hb in the LC- and LLC-inoculated groups was almost constant throughout the 7-days of both anaerobic (Fig. 32A) and aerobic (Fig. 32B) incubations compared to the non-LAB-inoculated group (lane Hb), consequently no degradation of Hb was observed. Similarly, the band thickness of Mb in the LAB-inoculated groups showed almost constant throughout the incubations compared to the non-LAB-inoculated group (lane Mb) under both anaerobic (Fig. 33A) and aerobic (Fig. 33B) conditions.

### 3.3.2.5. Effects of low molecular weight metabolites (LMMs) derived from LLC on aerobic ZnPP formation

Asaduzzaman (2020a) reported that low molecular weight metabolites (LMMs) in culture fluid (CF) of high-ZnPP-forming bacteria play an important role in the bacteria-associated anaerobic ZnPP formation. It was assumed that LMMs contribute to the aerobic ZnPP formation. In this section, the effects of LMMs on aerobic ZnPP formation were investigated using the aMH model system.

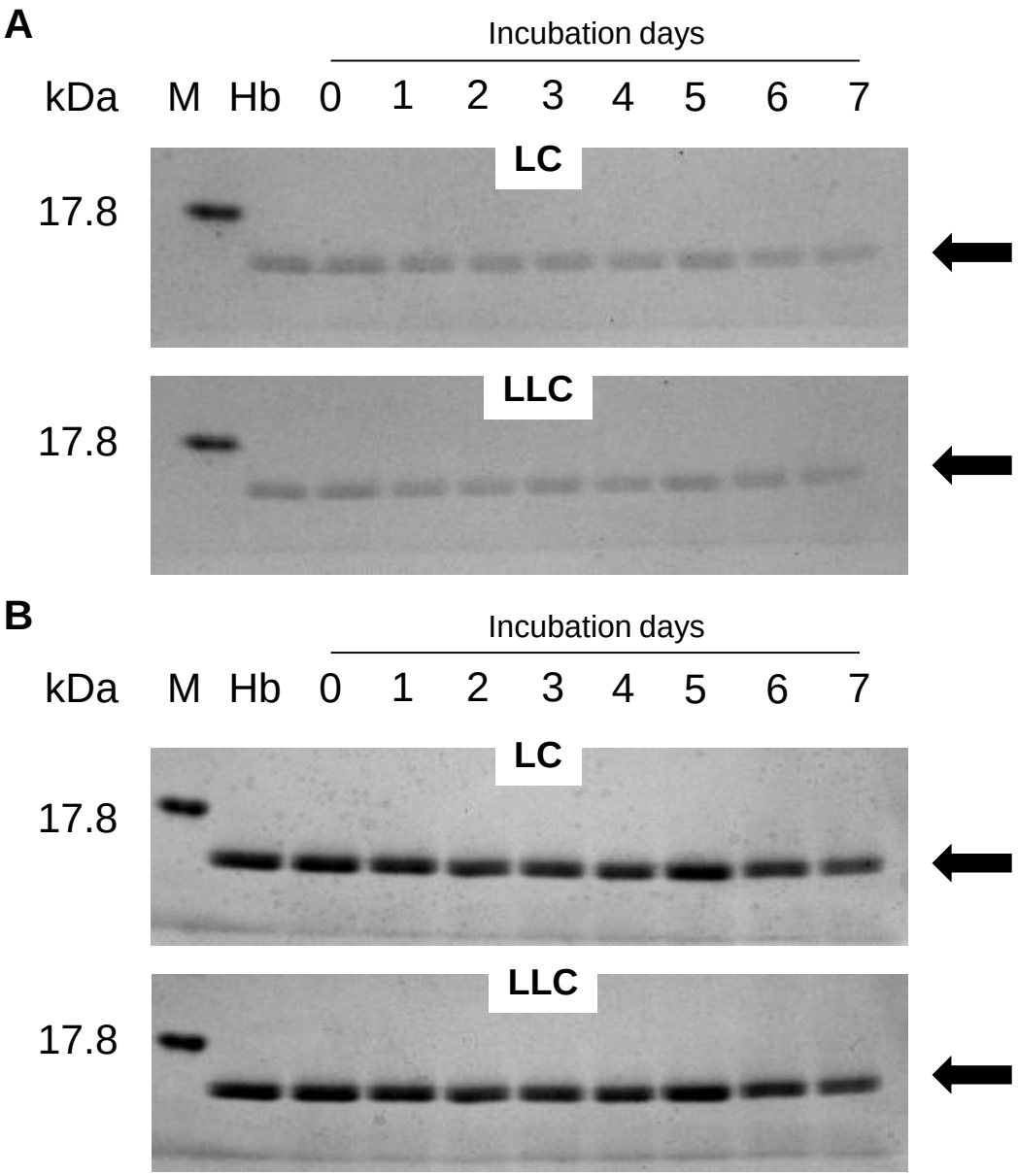



**Fig. 32. Degradation of hemoglobin (Hb) in NB medium inoculated with ZnPP-forming LAB**

LAB-inoculated (LC: *Lactobacillus curvatus* and LLC: *Lactococcus lactis* subsp. *cremoris*) and Hb-added NB medium was incubated both anaerobically (A) and aerobically (B) at 30°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 Hb. Lane M: marker and lane Hb: non-inoculated Hb-added broth.

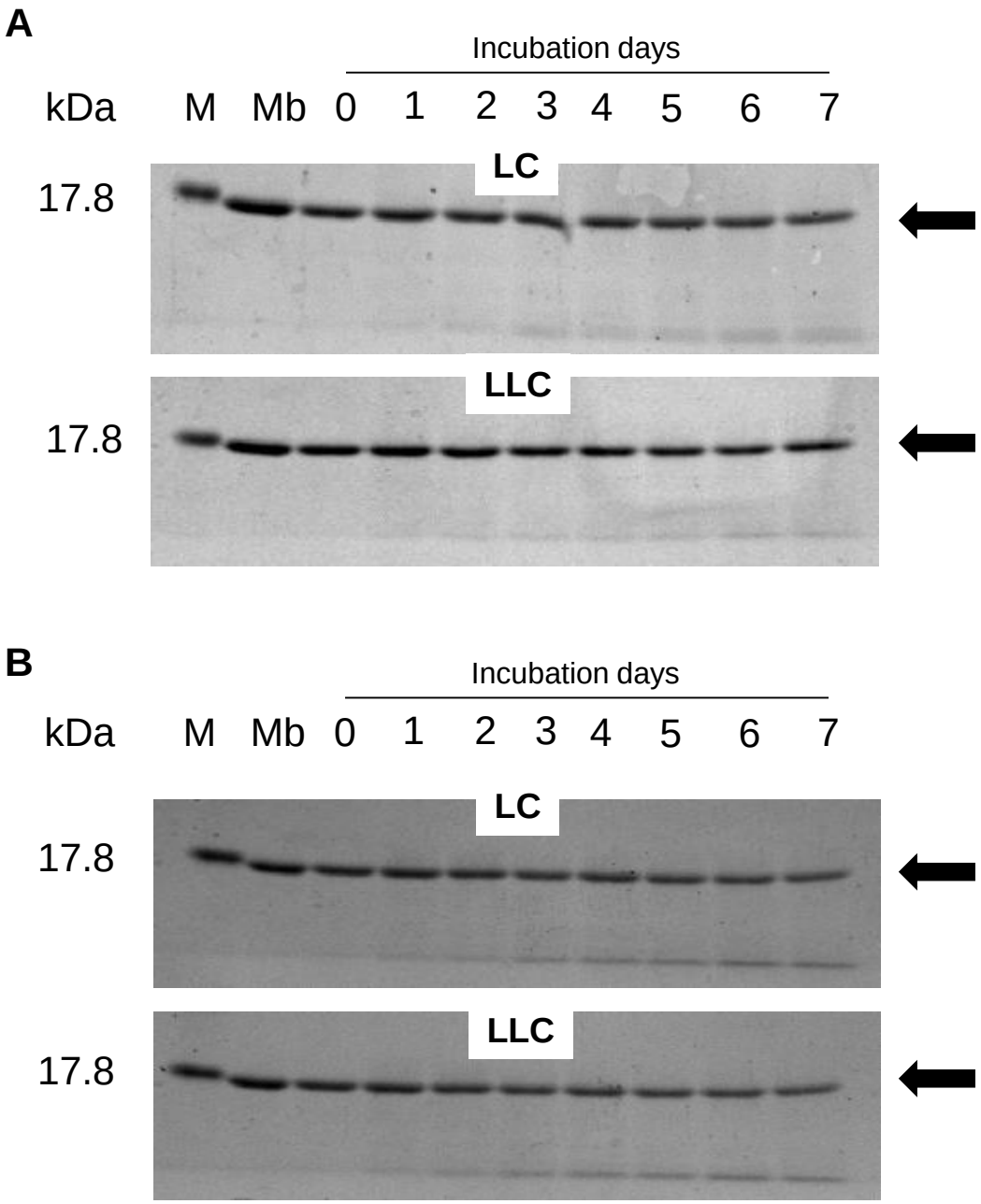



**Fig. 33. Degradation of myoglobin (Mb) in NB medium inoculated with ZnPP-forming LAB**

Mb was added to NB medium and inoculated with LC and LLC and incubated both anaerobically (A) and aerobically (B) at 30°C for 1 to 7 days. The staining of SDS-PAGE gel was performed as described in Fig. 32. Arrows indicate the band of Mb. Lane M: marker; lane Mb: non-inoculated Mb-added broth.

#### 3.3.2.5.1. Effects of culture fluid (CF) of LLC

First, to investigate the effects of metabolites of LLC on aerobic ZnPP formation, CF-added aMH model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured (Table 9). Under anaerobic conditions, the addition of CF including metabolites derived from both LAB significantly increased ZnPP formation compared to the group with the addition of only NB ($P < 0.01$) (Fig. 34). However, under aerobic conditions, the formation of ZnPP was significantly increased only in the groups containing CF of LLC compared to the other groups ($P < 0.01$) (Fig. 34). Under both conditions, a similar results were also observed in the antibiotics-added groups.

#### 3.3.2.5.2. Effects of LMMs of LLC and the heating

Next, to investigate the effects of LMMs of LLC and the heating (heat sensitive or heat resistant) on aerobic ZnPP formation, LMMs- and heated LMMs (hLMMs)-added aMH model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured (Table 9). Under anaerobic conditions, the addition of LMMs derived from both LAB and hLMMS significantly increased ZnPP formation compared to the group with the addition of NB ($P < 0.01$) (Fig. 35A & B). However, under aerobic conditions, the formation of ZnPP was significantly increased only in the groups containing LMMs (Fig. 35A) and hLMMs (Fig. 35B) of ZnPP-forming LLC compared to the other groups ($P < 0.01$). Under both conditions, a similar results were also observed in the antibiotics-added groups.

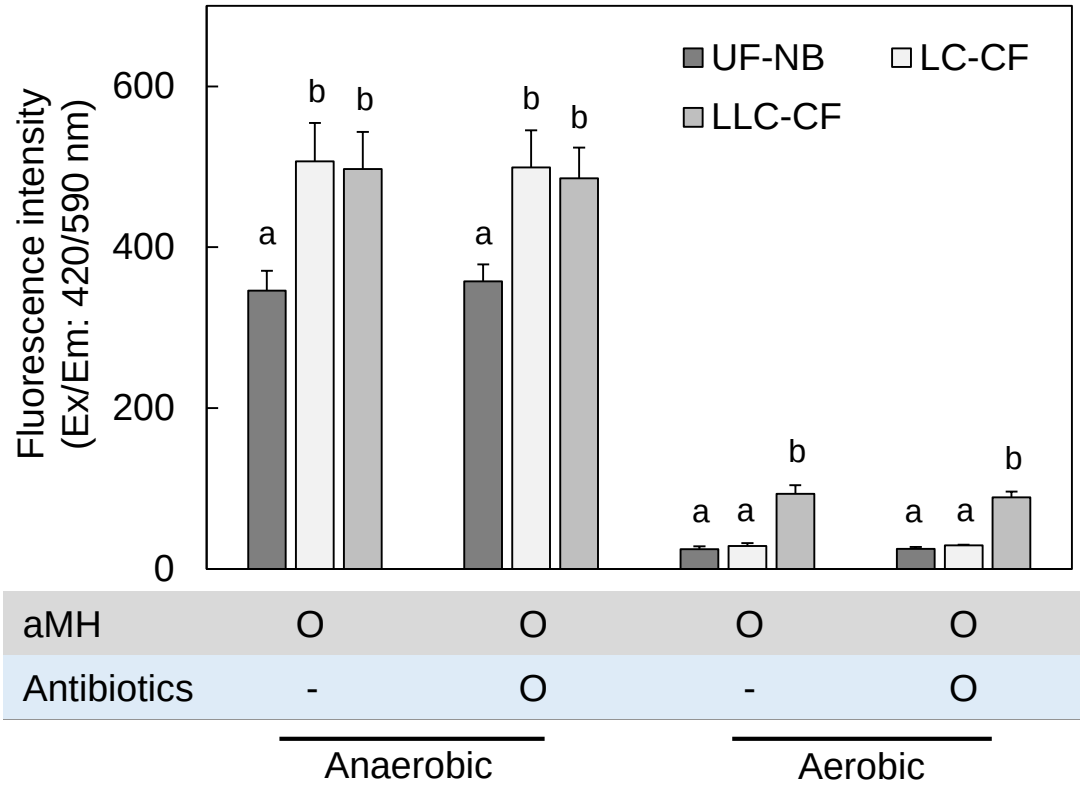



**Fig. 34. Effect of culture fluid (CF) of ZnPP-forming LAB on ZnPP formation in the model experiment**

The CF of LAB (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*) was added to aseptic meat (aMH) and the mixtures were incubated both anaerobically and aerobically at 30°C for 7 days in the dark. After incubation, ZnPP of the mixture was extracted and measured. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.01$). UF: Ultra filtrated; NB: Nutrient broth

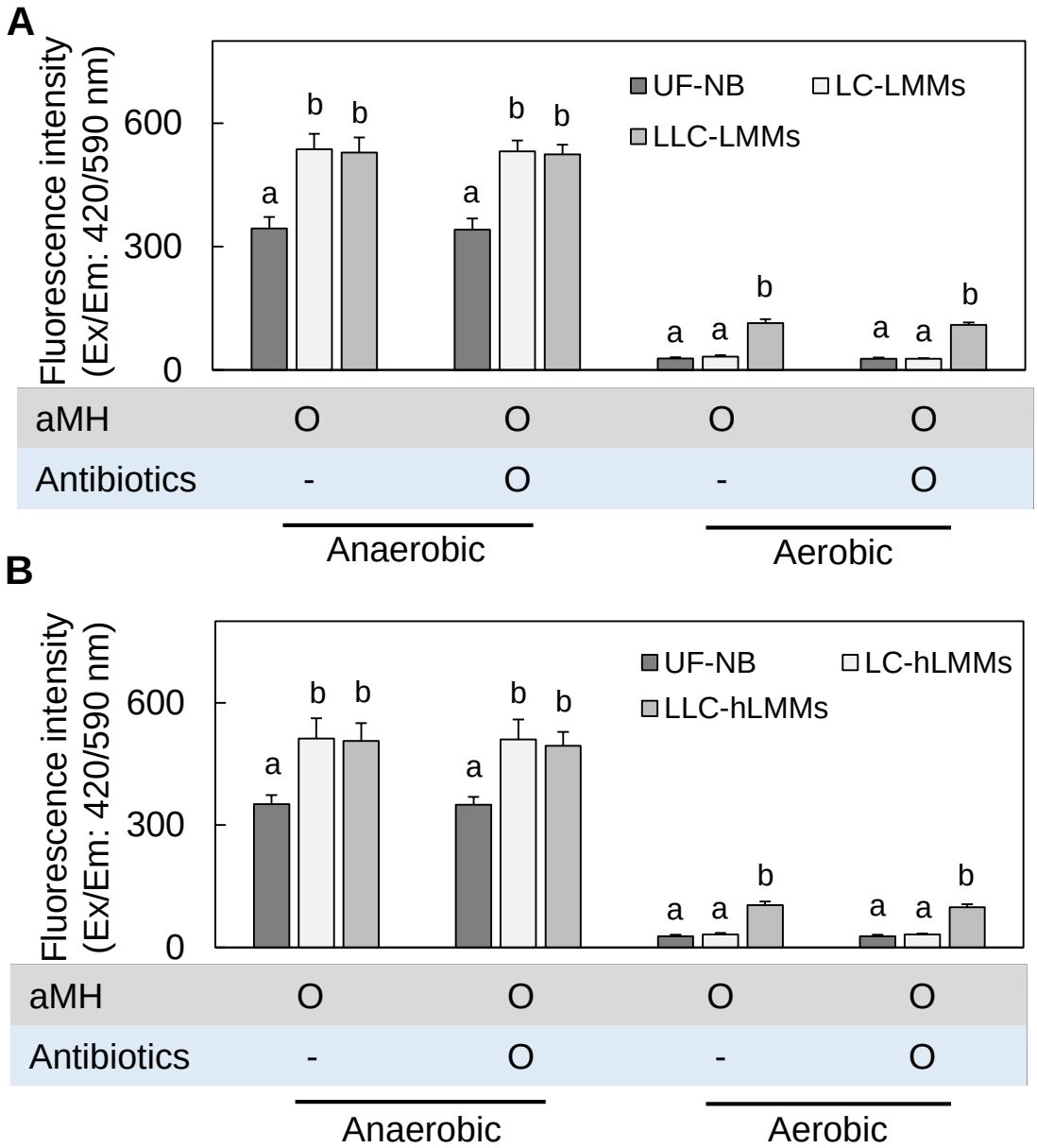



**Fig. 35. Effects of low molecular weight metabolites (LMMs) of ZnPP-forming LAB on ZnPP formation in the model experiment**

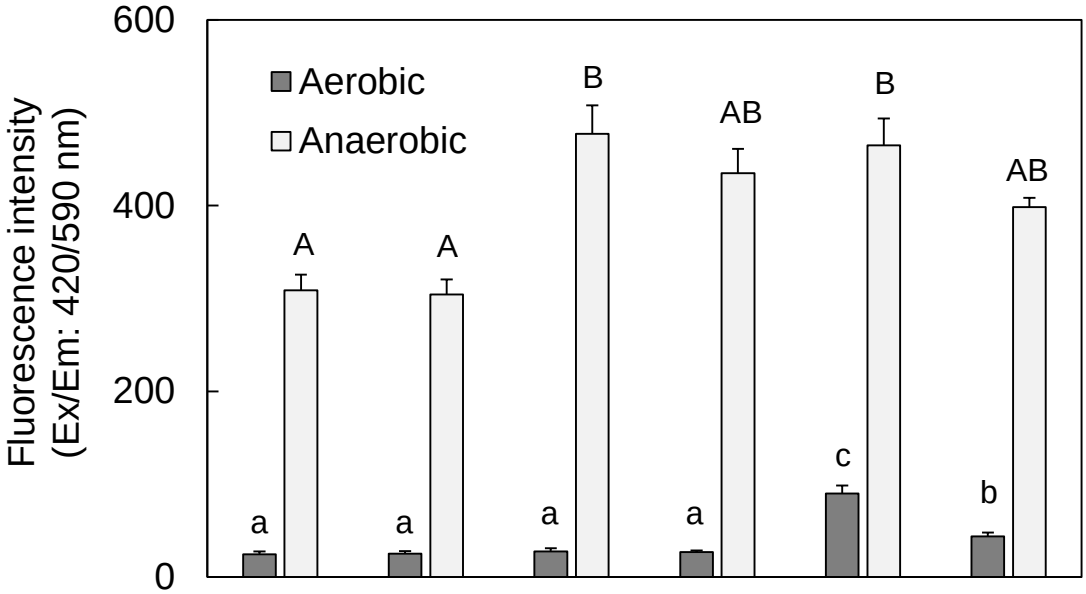
The low molecular weight metabolites (LMMs) (A) and heated LMMS (hLMMs) (B) were added to aseptic meat homogenates (aMH). The mixture was incubated and the formed ZnPP was measured as described in Fig. 34. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.01$).

#### 3.3.2.5.3. Effect of freeze-drying of LLC-LMMs

Next, to investigate the effects of freeze-dried LMMs (FD-LMMs) (having reduced volatile compounds) of LLC on aerobic ZnPP formation, FD-LMMs-added pork homogenate solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured. After 7 days of incubation under anaerobic conditions, the ZnPP formation non-significantly decreased in FD-LMMs groups derived from both LAB and the behavior was almost the same between the LC and LLC groups (Fig. 36). However, under aerobic conditions, the ZnPP formation significantly decreased in FD-LMMs group derived from only LLC compared to the LLC group of non-FD-LMMs, but significantly higher compared to the other groups ($P < 0.05$) (Fig. 36).

#### 3.3.2.5.4. Screening for the volatile LMMs (vLMMs) of LLC responsible for aerobic ZnPP formation

In the previous section, the decrease of aerobic ZnPP formation by freeze drying of LMMs in the LLC group might be due to the removal of volatile compounds. In order to investigate which vLMMs derived from LLC contribute to the aerobic ZnPP formation, the vLMMs in LC-LMMs, LLC-LMMs, and freeze-dried LLC-LMMs were analyzed with gas chromatography-mass spectrophotometry. A total of 54 vLMMs were identified and the screening was performed using the relative change of peak area of each compound. First, to investigate the difference between metabolites secreted by LC and LLC, the ratio of each metabolite in non-freeze-dried LLC-CF (N(LLC)) against that in non-freeze-dried LC-CF (N(LC)) was examined (Table 18, N(LLC) vs. N(LC)). Sixteen compounds which showed




| | | | | | | |
|---|---|---|---|---|---|---|
| aMH | O | O | O | O | O | O |
| UF-NB | O | O | - | - | - | - |
| Antibiotics | - | O | - | - | - | - |
| LC-LMMs | - | - | O | - | - | - |
| LC-LMMs (FD) | - | - | - | O | - | - |
| LLC-LMMs | - | - | - | - | O | - |
| LLC-LMMs (FD) | - | - | - | - | - | O |

**Fig. 36. Effects of freeze drying of low molecular weight metabolites of ZnPP-forming LAB on ZnPP formation in the model experiment**

LMMs and LMMs treated with freeze drying and redissolution (FD) were applied in aseptic meat homogenate (aMH) model system and the model solutions were incubated both aerobically and anaerobically at 30°C for 7 days in the dark. ZnPP of the mixture was extracted and measured. Data expressed as means ± SE (n = 3). ab and AB: Columns with different letters in the different groups differ significantly ($P < 0.05$); UF: Ultra filtrated; NB: Nutrient broth; LC*: Lactobacillus. curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*

**Table 18. LLC/LC-derived LMMs detected with GC-MS analysis from cultured broth and the comparison of their relative peak areas between species or between freeze drying treatment and non-treatment of LLC-CF**

| LMMs | Increase ratio of N(LLC) from N(LC) (%)[a] | Decrease ratio of FD(LLC) from N(LLC) (%)[b] |
|---|---|---|
| Formic acid, ethenyl ester | -37.3 ± 5.5 | 18.7 ± 4.3 |
| Acetone | 20.0 ± 4.5 | 55.6 ± 3.5 |
| Ethyl Acetate | 54.4 ± 7.5 | 50.0 ± 2.3 |
| Butanal, 3-methyl | -4.9 ± 12.6 | -1.5 ± 3.7 |
| Ethanol | 50.0 ± 4.5 | 70.8 ± 2.4 |
| 2,3-Butanedione | -82.1 ± 3.5 | -15.6 ± 4.6 |
| 2-Butenal | 2.2 ± 6.4 | -115.6 ± 5.4 |
| Benzene, 1,3-dimethyl | 25.9 ± 10.5 | 69.5 ± 3.5 |
| 1-Butanol | -76.1 ± 4.6 | 34.7 ± 2.6 |
| Heptanal | 44.5 ± 11.7 | 82.6 ± 2.0 |
| 1-Butanol, 3-methyl | -29.5 ± 5.4 | 56.5 ± 1.6 |
| Octanal | 111.2 ± 8.6 | 57.8 ± 4.8 |
| Acetoin | -93.6 ± 8.4 | 59.5 ± 3.2 |
| Pyrazine, 2,5-dimethyl | -9.6 ± 9.4 | 27.7 ± 4.2 |
| Dimethyl trisulfide | -34.9 ± 4.6 | 47.1 ± 2.3 |
| Nonanal | 167.4 ± 3.6 | 64.8 ± 3.9 |
| Pyrazine, 2-ethyl-5-methyl | -7.2 ± 5.6 | 19.7 ± 6.5 |
| Pyrazine, trimethyl | -9.3 ± 3.5 | 15.0 ± 4.3 |
| 1-Octen-3-ol | 121.2 ± 2.8 | 74.7 ± 5.3 |
| Acetic acid | -17.7 ± 4.6 | 41.6 ± 2.4 |
| Methional | 14.9 ± 4.6 | 8.7 ± 4.1 |
| 1-Hexanol, 2-ethyl | 42.6 ± 8.5 | 20.7 ± 3.0 |
| Benzaldehyde | 0.1 ± 2.4 | 28.4 ± 3.3 |
| Propanoic acid | 149.6 ± 7.5 | -347.0 ± 2.6 |
| 1-Octanol | 33.8 ± 4.3 | 32.9 ± 4.9 |
| Propanoic acid, 2-methyl | -10.2 ± 3.8 | 30.1 ± 5.6 |
| Ethanol, 2-(2-methoxyethoxy) | -72.9 ± 2.4 | -157.4 ± 8.5 |
| Butanoic acid | -8.9 ± 1.6 | 26.7 ± 4.3 |
| Benzeneacetaldehyde | 40.2 ± 5.8 | 15.2 ± 2.3 |
| Butanoic acid, 3-methyl | -10.3 ± 3.1 | 25.8 ± 2.4 |
| 2-Hydroxyethyl methacrylate | 4.5 ± 4.9 | 27.8 ± 1.9 |
| Benzaldehyde, 2,4-dimethyl | -19.5 ± 6.5 | -418.8 ± 3.6 |
| Benzaldehyde, 4-propyl | -15.6 ± 3.3 | 64.6 ± 2.5 |
| Hexanoic acid | 6.3 ± 3.9 | 15.9 ± 2.4 |
| Benzaldehyde, 2,4,6-trimethyl | -3.5 ± 5.4 | 52.9 ± 8.6 |
| Benzyl alcohol | 18.0 ± 8.6 | 9.4 ± 3.5 |
| Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl) propyl ester | -10.1 ± 4.3 | -72.6 ± 4.3 |
| Phenylethyl Alcohol | -45.5 ± 2.4 | 15.0 ± 3.8 |
| Benzeneacetaldehyde, .alpha.-ethylidene | 34.7 ± 2.5 | -1328.7 ± 2.4 |
| Hexanoic acid, 2-ethyl | -62.5 ± 1.5 | -133.4 ± 3.2 |
| 1-Dodecanol | -51.5 ± 5.8 | -491.4 ± 1.3 |
| 2-Hydroxy-iso-butyrophenone | -10.8 ± 4.7 | 10.9 ± 2.0 |
| Hexadecanal | -17.2 ± 8.6 | -221.5 ± 5.4 |
| Benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester | -8.1 ± 3.2 | -9.7 ± 7.5 |
| Nonanoic acid | -8.5 ± 5.3 | -135.4 ± 7.4 |
| 1-Tetradecanol | -34.9 ± 4.6 | -433.4 ± 2.6 |
| Caprolactam | -43.6 ± 3.4 | -18.5 ± 6.5 |
| Hexadecanoic acid, methyl ester | -32.1 ± 2.0 | -136.6 ± 7.4 |
| Hexamethylene diacrylate | 52.1 ± 3.4 | 76.9 ± 8.4 |
| n-Decanoic acid | 60.5 ± 3.2 | -27.5 ± 6.4 |
| Phenol, 2,4-bis (1,1-dimethylethyl) | 3.8 ± 6.4 | -58.3 ± 2.9 |
| Dodecanoic acid | 73.7 ± 3.9 | -18.0 ± 4.8 |
| n-Nonadecanol-1 | -37.7 ± 4.3 | -8.3 ± 5.3 |
| Methanone, (1-hydroxycyclohexyl) phenyl | -17.2 ± 2.7 | -2.4 ± 2.3 |

Values were means ± SE (n = 6)

LC: *Lactobacillus curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*; LMMs: Low molecular weight metabolites; CF: Culture fluid; FD: Freeze-dried; N: Not freeze-dried

[a] Red-colored values indicate the compounds of over 20% increase

[b] Blue-colored values indicate the compounds of over 50% decrease

more than 20% increase were selected as abundant compounds contained in CF of LLC. Next, to clarify the compounds removed by freeze drying, the ratio of each metabolite in freeze dried LLC-CF (FD(LLC)) against that in N(LLC) of each metabolite was examined (Table 18, FD(LLC) vs. N(LLC)). Thirteen compounds which showed below than -50% reduction with freeze drying were selected. Finally, nine compounds, i.e., acetone, ethyl acetate, ethanol, benzene 1,3-dimethyl, heptanal, octanal, nonanal, 1-octen-3-ol, hexamethylene diacrylate, were considered as conformable contributors to both conditions.

#### 3.3.2.6. Effect of ZnPP-forming LLC on aerobic ZnPP formation in each ZnPP formation step

From the previous experiments, it was observed that the LLC assisted to form plenty of ZnPP aerobically in the aMH model system. Therefore, to investigate how ZnPP-forming LLC contributes to the pathways of meat-inherent ZnPP formation aerobically, exogenous Hb, Mb, heme, PPIX, and zinc were used in a model system that was designed keeping ISF as a common component, with the association of ZnPP-forming LLC.

##### 3.3.2.6.1. Effect of LLC on aerobic ZnPP and PPIX formation from Hb and Mb

###### 3.3.2.6.1.1. Effects of LLC-derived contributors

In order to investigate the effect of LLC on aerobic ZnPP formation from heme proteins (Hb and Mb), LAB-inoculated and exogenous heme proteins-added model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured (Table 14). Under anaerobic conditions, the formation of ZnPP significantly increased in both

LAB-inoculated groups compared to the other groups ($P < 0.05$) (Fig. 37A & 37B). Besides, heme proteins' addition increased the ZnPP formation in both LAB-inoculated and non-inoculated groups and showed significant differences between them ($P < 0.05$). However, under aerobic conditions, the formation of ZnPP significantly increased only in the LLC-inoculated groups compared to the other groups ($P < 0.05$) (Fig. 37A & 37B). Besides, the addition of exogenous heme proteins (e.g., Hb & Mb) drastically increased the ZnPP formation compared to the same group of non-added heme proteins.

#### 3.3.2.6.1.2. Effects of LLC-derived LMMs on aerobic ZnPP formation

In order to investigate the effect of LLC-LMMs on aerobic ZnPP formation from heme proteins (Hb and Mb), LAB-derived LMMs- and exogenous heme proteins-added model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured (Table 14). The model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured. A similar result was observed in the cases of both heme proteins. Under anaerobic conditions, the formation of ZnPP significantly increased in both LAB-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 38A & 38B). Besides, the addition of heme proteins increased the ZnPP formation in both LAB-LMMs-added and non-added groups and the ZnPP formation in both LAB-LMMs-added groups was significantly higher than those in the non-inoculated groups ($P < 0.05$). However, under aerobic conditions, the formation of ZnPP significantly increased only in the LLC-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 38A & 38B). The ZnPP formation was also drastically increased with exogenous heme proteins (e.g., Hb & Mb) compared to the same group of non-added heme proteins.

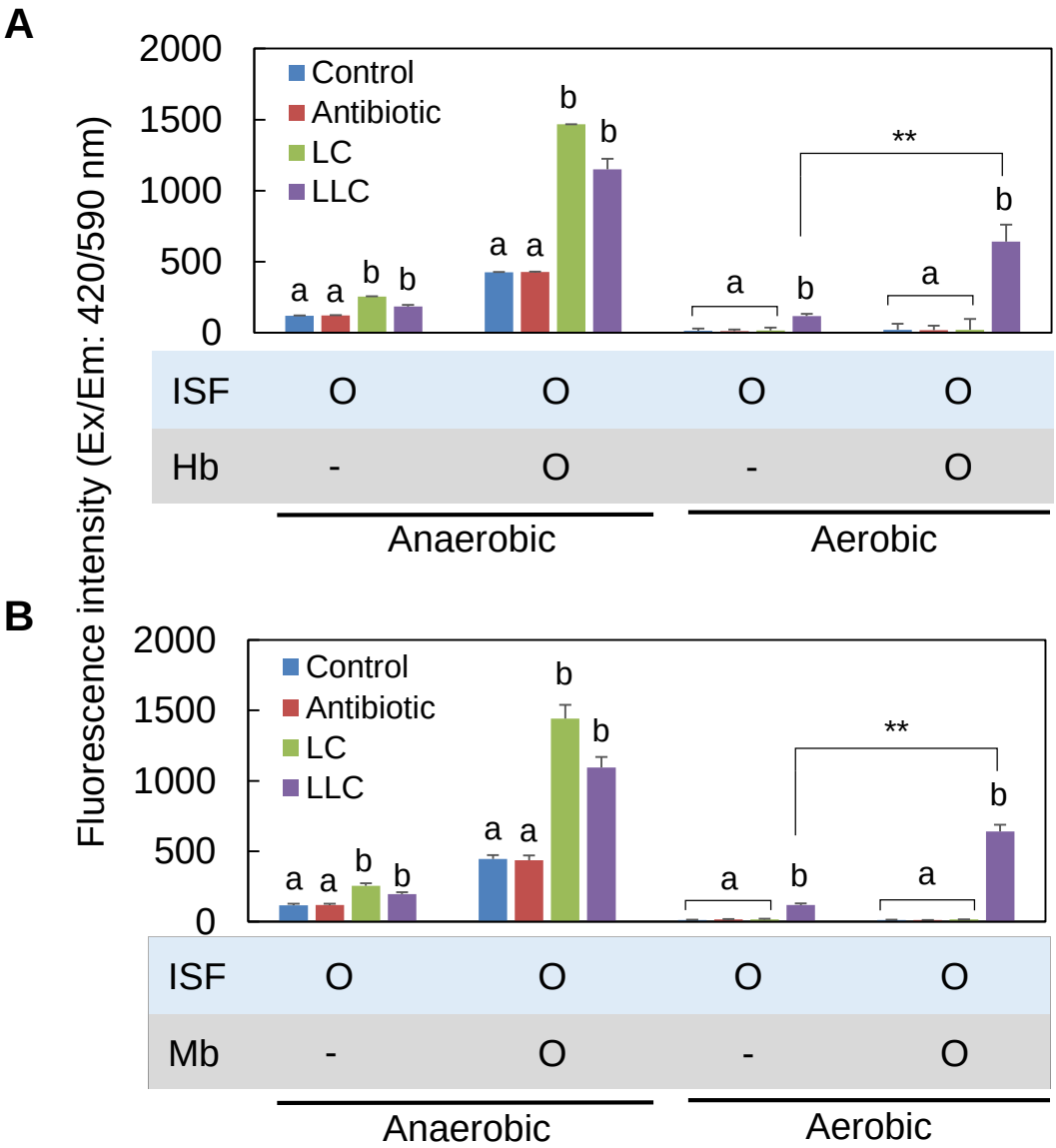



**Fig. 37. Effect of ZnPP-forming LAB on ZnPP formation from hemoglobin (Hb) and myoglobin (Mb) in the model experiment**

LAB-inoculated insoluble fraction (ISF) of pork (20%) (LC: *Lactobacillus curvatus*, LLC: *Lactococcus lactis* subsp. *cremoris*, Control: non-inoculated, Antibiotic: antibiotics-added) and Hb- (A)/Mb-added (B) mixture was incubated both aerobically and anaerobically at 30°C for 7 days in the dark. ZnPP was extracted and measured. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.05$). ** ($P < 0.01$) significantly different between groups.

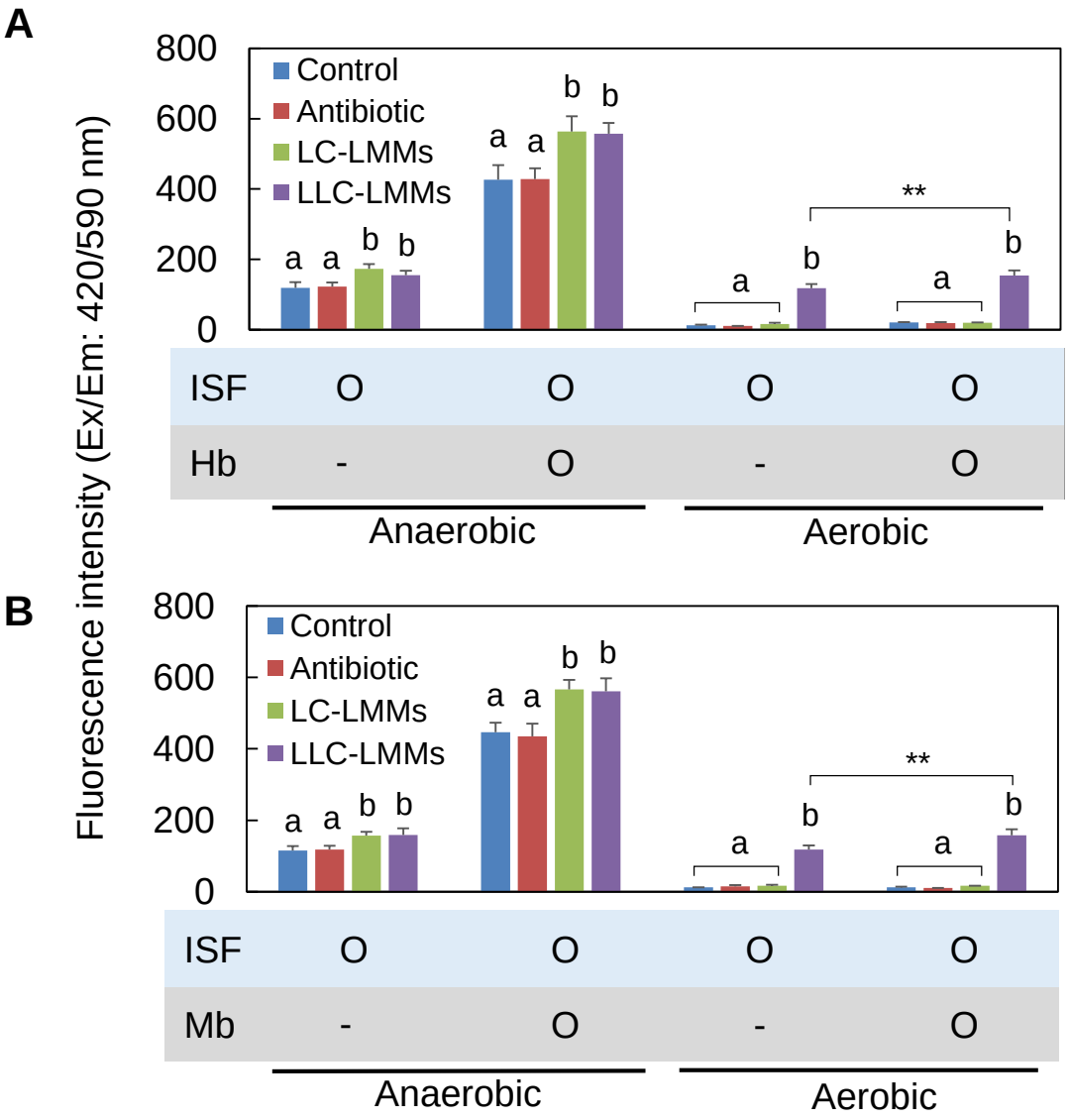



**Fig. 38. Effects of low molecular weight metabolites (LMMs) of ZnPP-forming LAB on ZnPP formation from Hb and Mb in the model experiment**

LAB (LC*: Lactobacillus curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*)-LMMs and Hb- (A)/Mb-added (B) ISF mixture was prepared and incubated, and the formed ZnPP was measured as described in Fig. 37. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.05$). ** ($P < 0.01$) significantly different between groups.

#### 3.3.2.6.1.3. Effects of LLC-derived LMMs on aerobic PPIX formation

Next, to investigate the effect of LLC-LMMs on aerobic PPIX formation from heme proteins (Hb and Mb), LAB-derived LMMs- and exogenous heme proteins-added model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and PPIX was measured (Table 15). Under anaerobic conditions, the LAB-LMMs-added groups showed a significant difference for PPIX formation compared to the other groups ($P < 0.05$) (Fig. 39A & 39B). Besides, the addition of heme proteins increased the PPIX formation in both of the LAB-LMMs-added and non-added groups and the ZnPP formation in both LAB-LMMs-added groups was significantly higher than those in the non-inoculated groups ($P < 0.05$). However, under aerobic conditions, the formation of PPIX significantly increased only in the LLC-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 39A & 39B). The PPIX formation was also drastically increased with exogenous heme proteins (e.g., Hb & Mb) compared to the same group of non-added heme proteins.

#### 3.3.2.6.2. Effect of LLC on aerobic ZnPP and PPIX formation from heme

#### 3.3.2.6.2.1. Effect of LLC on aerobic ZnPP formation

In order to investigate the effect of LLC on aerobic ZnPP formation from heme, the model solutions containing exogenous heme were incubated, both aerobically and anaerobically (Table 16). After 7 days of incubation under anaerobic conditions, the ZnPP was formed in both LAB-inoculated groups and exhibited a significant difference compared to the other groups ($P < 0.05$) (Fig. 40A). Besides, the ZnPP formation was drastically promoted with exogenous heme in the LAB-inoculated groups compared to the non-

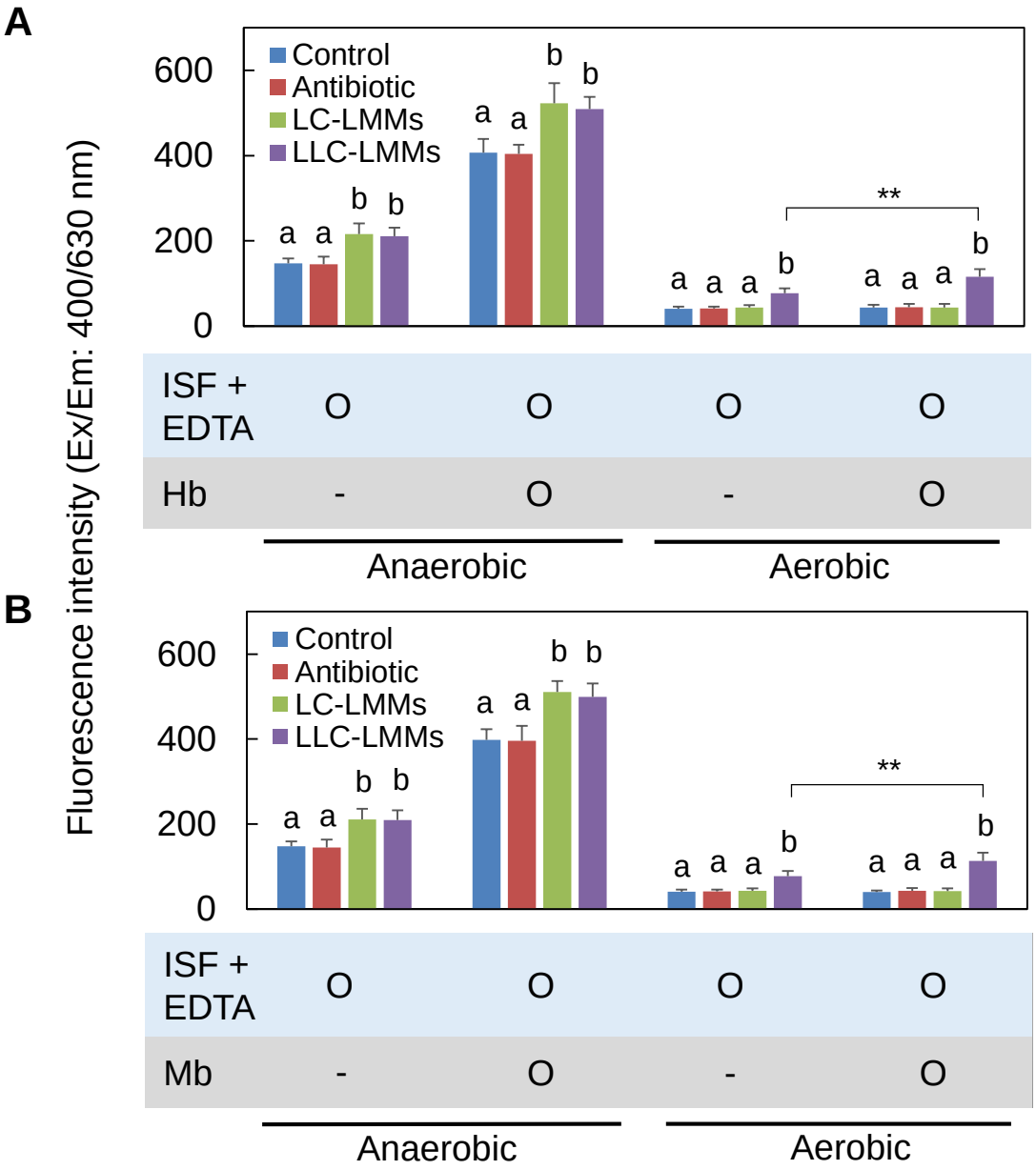



**Fig. 39. Effects of low molecular weight metabolites (LMMs) of ZnPP-forming LAB on PPIX formation from Hb and Mb in the model experiment**

LAB (LC*: Lactobacillus curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*)-LMMs and Hb- (A)/Mb-added (B) ISF mixture was prepared with EDTA. The mixture was then incubated at 30°C for 7 days in the dark and the formed PPIX was measured. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.05$). ** ($P < 0.01$) significantly different between groups.

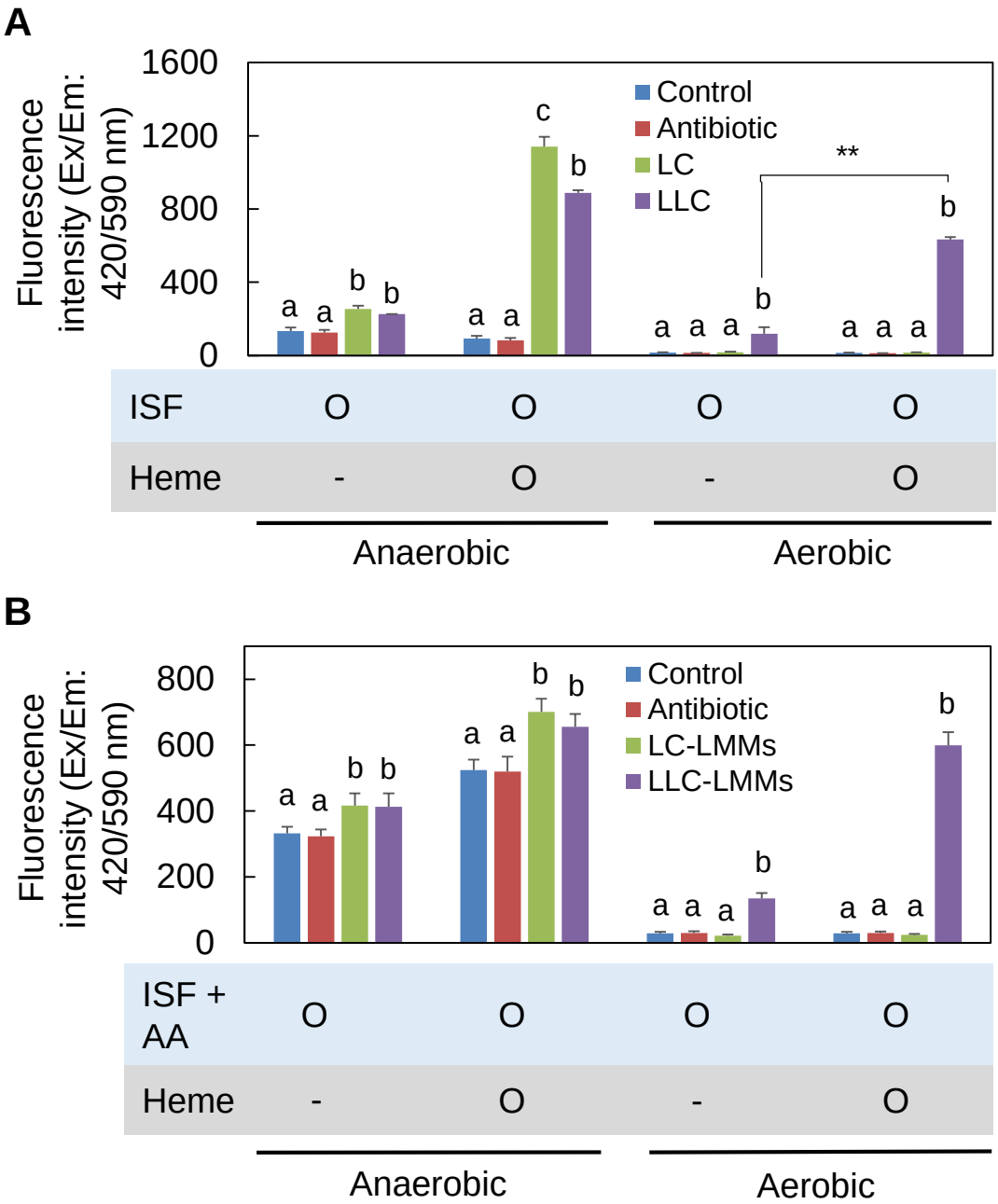



**Fig. 40. Effect of ZnPP-forming LAB and LAB-derived low molecular weight metabolites (LMMs) on ZnPP formation from heme in the model experiment**

LAB-inoculated (LC*: Lactobacillus curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*) (A)/LAB-derived LMMs with ascorbic acid (AA)- (B) and heme-added ISF mixture was prepared. Then, the mixture was incubated and the formed ZnPP was measured as described in Fig. 37. Data expressed as means ± SE (n = 3). abc: Columns with different letters in the same groups differ significantly ($P < 0.05$). ** ($P < 0.01$) significantly different between groups.

inoculated heme-added groups. However, under aerobic conditions, the formation of ZnPP significantly increased only in the LLC-inoculated groups compared to the other groups, and the addition of exogenous heme drastically increased the ZnPP formation compared to the same group of non-added heme ($P < 0.05$) (Fig. 40A).

#### 3.3.2.6.2.2. Effects of LLC-derived LMMs and ascorbic acid on aerobic ZnPP formation

In order to investigate the effects of LLC-LMMs on aerobic ZnPP formation from heme, the model experiment with the addition of heme and ascorbic acid (AA) was performed. AA was used as a reductant to convert heme (III) to heme (II) (Table 16). The model solutions were incubated both aerobically and anaerobically at 30°C for 7 days, and ZnPP was measured. Under anaerobic conditions, the formation of ZnPP significantly increased in both LAB-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 40B). Besides, the addition of heme (II) increased the ZnPP formation in both of the LAB-LMMs-added and non-added groups and showed a significant difference between them ($P < 0.05$). Under the aerobic condition, the formation of ZnPP significantly increased only in the LLC-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 40B). Besides, the addition of exogenous heme (II) (e.g., Hb & Mb) drastically increased the ZnPP formation compared to the same group of non-added heme (II).

#### 3.3.2.6.2.3. Effects of LLC-derived LMMs on aerobic PPIX formation

In order to investigate the effect of LAB-LMMs on aerobic PPIX formation from heme, a model experiment with the addition of heme and EDTA (as a metal chelator) was performed (Table 17). The mixture of model solutions was incubated both aerobically and

anaerobically. After 7 days of incubation under anaerobic conditions, the formation of PPIX significantly increased in both LAB-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 41). Besides, the addition of heme increased the PPIX formation in both of the LAB-LMMs-added and non-added groups and showed a significant difference between them ($P < 0.05$). However, under aerobic conditions, the formation of PPIX significantly increased only in the LLC-LMMs-added groups compared to the other groups ($P < 0.05$) (Fig. 41), although the addition of exogenous heme drastically increased the PPIX formation compared to the same group of non-added heme.

#### 3.3.2.6.3. Effect of LLC and the LMMs on aerobic ZnPP formation from PPIX

First, in order to investigate whether ZnPP-forming LAB contribute to the insertion of $Zn^{2+}$ into PPIX in aerobic conditions, a meat model experimental system with the addition of exogenous PPIX was performed (Table 16). The model solutions were incubated, both aerobically and anaerobically. Under anaerobic conditions, the formation of ZnPP significantly increased in both LAB-inoculated groups compared to the other groups ($P < 0.05$) (Fig. 42A). Besides, the addition of PPIX increased the ZnPP formation in both LAB-inoculated and non-inoculated groups and the increase in the both LAB-inoculated groups were notable ($P < 0.05$). However, under aerobic conditions, the formation of ZnPP significantly increased only in the LLC-inoculated groups compared to the other groups ($P < 0.05$) (Fig. 42A), and the ZnPP formation was also increased with exogenous PPIX compared to the same group of non-added PPIX. On the other hand, since ZnPP was aerobically formed in the non-LAB and LC-inoculated groups with the addition of PPIX, the insertion of zinc into PPIX was suggested not to be inhibited with oxygen.

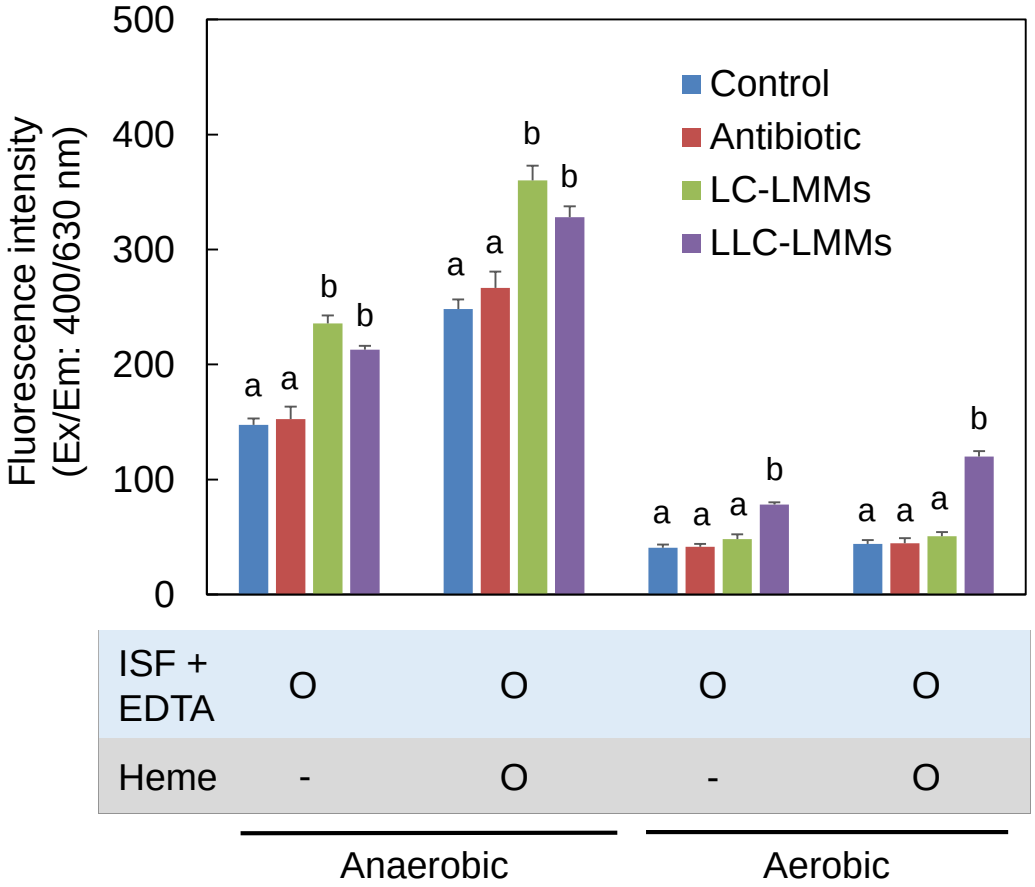



**Fig. 41. Effects of low molecular weight metabolites (LMMs) of ZnPP-forming LAB on PPIX formation from heme in the model experiment**

LAB (LC*: Lactobacillus curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*)-LMMs and heme-added ISF mixture was prepared and incubated, and the formed PPIX was measured as described in Fig. 39. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.05$).

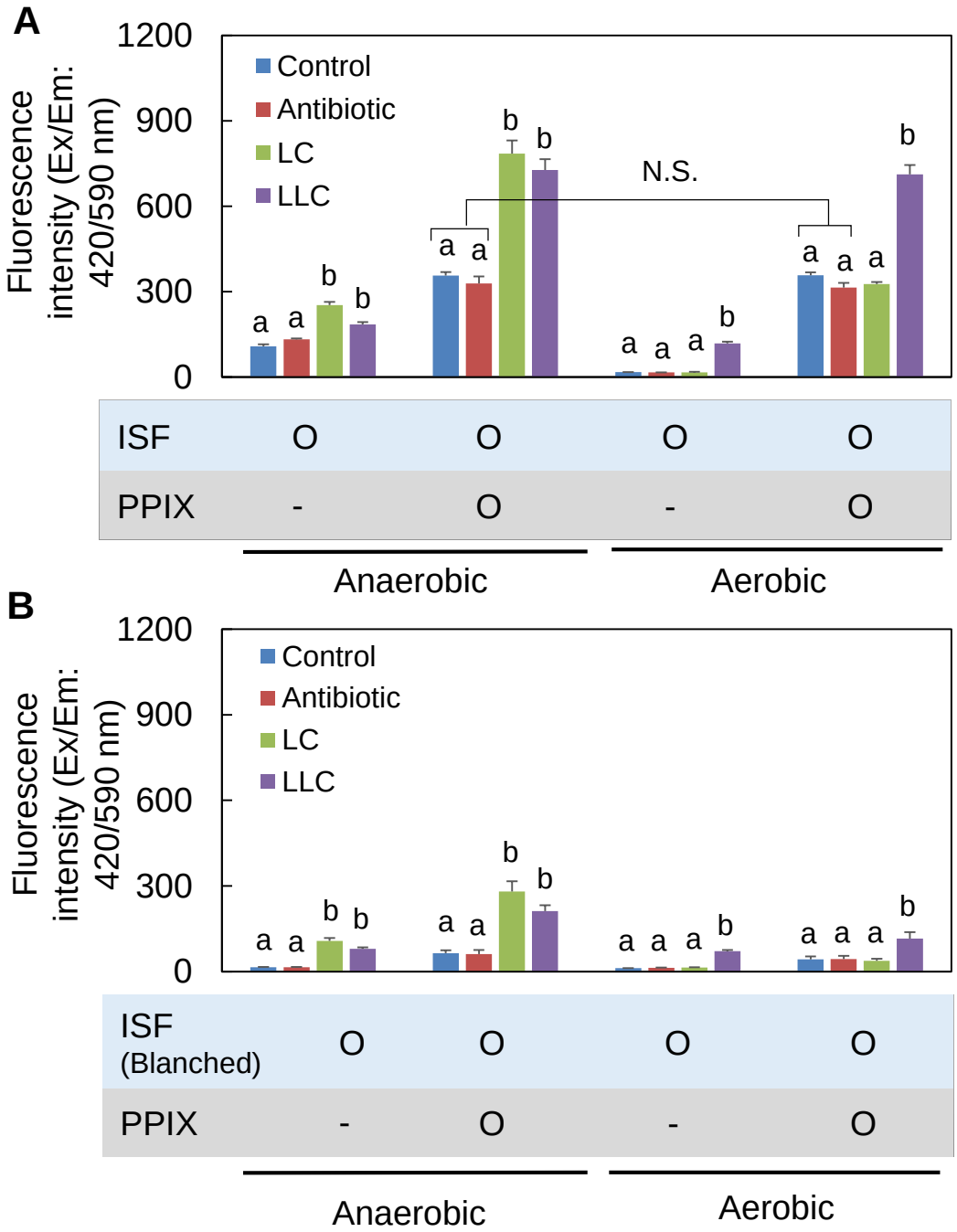



**Fig. 42. Effect of ZnPP-forming LAB on ZnPP formation from PPIX in the model experiment**

LAB-inoculated and PPIX-added ISF (A)/blanched ISF (B) mixture was prepared and incubated, and the formed ZnPP was measured as described in Fig. 37. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.05$). N.S.: Not significant between groups.

Next, to clarify whether ZnPP is formed non-enzymatically by inserting zinc into PPIX when enough PPIX in the medium, a model experiment using blanched meat homogenate as previous was performed (Table 16). The model solutions were incubated, both aerobically and anaerobically. Under anaerobic conditions, the formation of ZnPP was suppressed (Fig. 42B) compared to the experiment using raw meat homogenate (Fig. 42A) but significantly increased in both LAB-inoculated groups compared to the other groups ($P < 0.05$). Besides, the addition of PPIX increased the ZnPP formation in all groups. However, under aerobic conditions, the formation of ZnPP was suppressed except in the LLC-inoculated groups and exhibited a significant difference among the other groups ($P < 0.05$).

In order to investigate the effect of LLC-LMMs on aerobic ZnPP formation from PPIX, the model solutions with exogenous PPIX were incubated, both aerobically and anaerobically (Table 16). After 7 days of incubation under anaerobic conditions, the promotion of ZnPP was observed in the LAB-LMMs-added groups and showed significantly different compared to the other groups ($P < 0.05$) (Fig. 43). Besides, the addition of PPIX increased the ZnPP formation in both of the LAB-LMMs-added and non-added groups and the increase in the both LAB-inoculated groups were notable ($P < 0.05$). However, under aerobic conditions, the formation of ZnPP was observed only in the LLC-LMMs-added groups without the addition of PPIX ($P < 0.05$) (Fig. 43). Besides, the ZnPP formation with exogenous PPIX was drastically promoted in all groups like the above result (Fig. 42A). Moreover, ZnPP formation with endogenous PPIX in the LLC-LMMs-added groups was significantly higher than those in the other groups. A meat-inherent enzyme was suggested to be involved in the

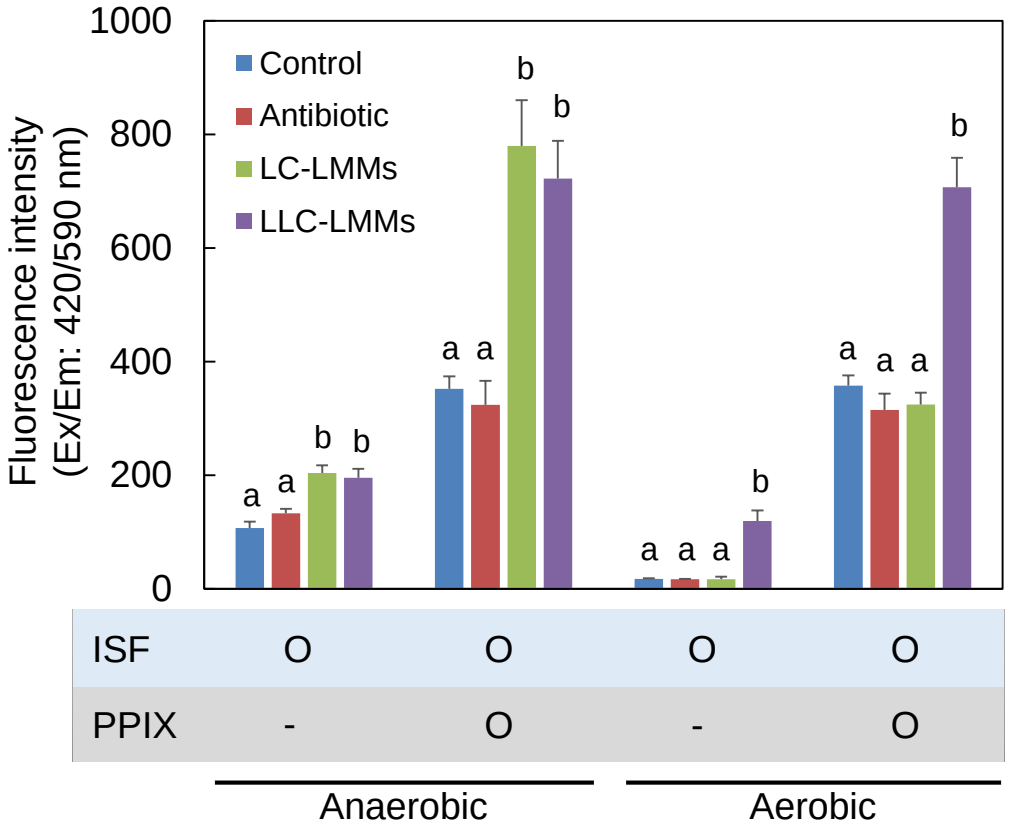



**Fig. 43. Effects of low molecular weight metabolites (LMMs) of ZnPP-forming LAB on ZnPP formation from PPIX in the model experiment**

LAB- (LC*: Lactobacillus curvatus*; LLC: *Lactococcus lactis* subsp. *cremoris*) LMMs and PPIX-added ISF mixture were prepared and incubated, and the formed ZnPP was measured as described in Fig. 37. Data expressed as means ± SE (n = 3). ab: Columns with different letters in the same groups differ significantly ($P < 0.05$).

insertion of zinc into PPIX in spite of the existence of oxygen but LLC might be also slightly involved.

## 3.4. Discussion

In this chapter, the promotion mechanism of aerobic ZnPP formation by ZnPP-forming *Lactococcus lactis* subsp. *cremoris* GB(A)-1 (LLC) in meat products was investigated and *Lactobacillus curvatus* FS2(A)-1 (LC) was used as a reference.

### 3.4.1. Effect of oxygen on meat-inherent ZnPP formation mechanism without bacteria

Generally, oxygen inhibits the formation of ZnPP (Wakamatsu *et al*., 2004b) but in some cases ZnPP was formed aerobically with the addition of LLC and/or the metabolites (Fig. 42 & 43, Control & antibiotic). Although ZnPP was not formed with the incubation of only ISF and heme (Fig. 40, Control & antibiotic), with the incubation of only ISF and PPIX ZnPP was remarkably formed (Fig. 42A & 43, Control and antibiotic). However, the formation of PPIX from heme proteins and heme was inhibited by oxygen (Fig. 39 & 41). The main meat components responsible for ZnPP formation is likely to be heme proteins (e.g., Hb and Mb) and heme is released after degradation of the components mainly by proteolysis. After that, protoporphyrin IX (PPIX) is formed from heme by the action of meat-inherent FECH (mFECH) and finally, $Zn^{2+}$ inserts into PPIX, and form ZnPP (Becker *et al*., 2012; Chau *et al*., 2011; Labbé *et al*., 1999; Taketani *et al*., 2007). Hence, it was suggested that the ISF, i.e. meat insoluble fraction, inserts zinc into PPIX but the reaction was not inhibited by oxygen. However, ZnPP was not formed with the incubation of blanched ISF and PPIX, and the amounts of ZnPP formed under aerobic and anaerobic conditions were almost equal (Fig. 42B, Control and antibiotic). These results suggested that the heat-sensitive compounds in ISF, e.g. enzymes, catalyzed the insertion of zinc into PPIX in spite of the existence of oxygen

and the formation of ZnPP depends on the availability of PPIX. Therefore, since oxygen affects the step from heme proteins (Hb and Mb) up to PPIX in the meat-inherent ZnPP mechanism, to elucidate the promotion mechanism of aerobic ZnPP by LLC the insertion step of zinc into PPIX is not so important and can be ignored.

### 3.4.2. Difference between LLC and LC in ZnPP promotion mechanism in meat under anaerobic conditions

The formation of ZnPP was noticeably promoted under anaerobic conditions by both LLC and LC, whereas LC showed slightly higher fluorescence intensity than that of the LLC (Fig. 20, 21). This result implied that LC has slight stronger promotion effect in ZnPP formation than LLC. Both LLC and LC have promotion effect in the formation of PPIX from heme proteins (e.g., Hb and Mb) and heme under anaerobic conditions (Fig. 39 & 41). Instead of direct LAB, CF was used to observe the effects of both LAB on PPIX formation from heme proteins and heme, since a metal chelator EDTA was used to accumulate PPIX in the medium. It was reported that EDTA inhibits the formation of ZnPP and helps in the accumulation of PPIX in the model solutions (Adamsen *et al*., 2006a). On the other hand, LAB needs some metal ions in trace amounts for their growth. No difference in PPIX formation was observed using CF under anaerobic conditions, although the slight variation by the LAB was observed in ZnPP formation and this variation in ZnPP formation might be due to the contribution of LAB itself. However, under anaerobic conditions, both LC and LLC promoted ZnPP formation and they did not show remarkable variation in their ZnPP formation.

### 3.4.3. Difference of FECH between LLC and other FECHs

The activity of FECH derived from meat and bacteria varied under aerobic and anaerobic conditions. Under aerobic conditions, the meat-inherent FECH and FECH derived from LC did not promote ZnPP formation, whereas LLC-FECH promoted ZnPP formation (Fig. 21). On the other hand, under anaerobic conditions the formation of ZnPP increased in meat but the addition of LC and LLC promoted ZnPP formation (Fig. 20, 21). Besides, the formation of ZnPP drastically increased with the addition of heme proteins and heme by the LC and LLC under anaerobic conditions (Fig. 37, 40). Generally, since FECH catalyzes the insertion of divalent metal into PPIX (Chau *et al*., 2010), FECH is mainly assumed to be involved in the conversion from PPIX to ZnPP in meat products (Akter *et al*., 2019a; Chau *et al*., 2010). Under anaerobic conditions, the meat-inherent FECH is a rate-limiting enzyme of ZnPP formation, and bacterial FECH, and meat-inherent FECH might cooperatively release PPIX from heme (Asaduzzaman, 2020a). The addition of mitochondrial membrane fraction including FECH increased the formation of ZnPP (Fig. 27). However, FECH derived from LLC did not affect by oxygen and exhibit their functions in the presence of oxygen. On the other hand, meat-inherent FECH has a strong activity in iron-zinc substitution process under anaerobic conditions (Chau *et al*., 2011; Khozroughi *et al*., 2018) and strictly restricted under aerobic conditions. Similarly, FECH derived from LC did not perform their functions under aerobic conditions to form ZnPP. These results suggested that the activities of mFECH and LC-FECH were strongly suppressed by oxygen, whereas LLC-FECH was oxygen resistant. As a result, LLC could form ZnPP aerobically by forming PPIX from heme.

### 3.4.4. Impact of LMMs derived from LLC on aerobic ZnPP formation

Aerobic ZnPP formation was increased due to the addition of culture fluid (CF) of LLC in the meat model (Fig. 34). This ZnPP formation was due to the presence of metabolites in the CF secreted by the LLC during incubation (Fig. 35, 36). LMMs derived from LLC also promoted ZnPP (Fig. 38, 40A) and PPIX (Fig. 39, 40B) formation from heme proteins and heme. Bacterial metabolites usually consist of aldehydes, ketones, esters, alcohols, organic acids, amines, and sulfur compounds, etc. (Ercolini *et al*., 2009; Samelis, 2006). Besides, most metabolites in the culture fluid were low molecular weight compounds since most of the primary metabolites derived from bacteria are low molecular weight compounds (Hadacek and Bachmann, 2015). It was reported that exogenous acid, e.g. gluconic acid, significantly promoted the ZnPP formation in the model solution (Kawazoe, 2013). Besides, the metabolic activity of bacteria is species or strain-specific (Doulgeraki *et al*., 2011; Ercolini, 2010). Sixteen compounds out of 54 volatile compounds clarified in this study, were found as abundant compounds included in CF of LLC (more than 20% increase) compared to those in CF of LC (Table 18).The low molecular weight metabolites (LMMs) derived from LLC (LLC-LMMs) had promotion effect in the formation of PPIX from heme proteins (e.g., Hb and Mb) and heme, but no PPIX formation was observed by the addition of LMMs derived from LC (LC-LMMs) under aerobic conditions from the same sources (Fig. 39 & 41). Therefore, some specific compounds found in LLC-LMMs might protect the oxygen sensitive mFECH or inhibit the effect of oxygen on inactivation of mFECH. Moreover, LMMs from LLC might be stable under aerobic conditions or the concentration of particular metabolite in LLC-LMMs might be responsible for aerobic ZnPP formation. Subsequently,

volatile low molecular weight metabolites (vLMMs) were found as a facilitator for aerobic ZnPP formation (Fig. 29). Thirteen compounds which showed below than -50% reduction with freeze drying were found in CF of LLC (Table 18). The vLMMs in the CF of ZnPP-forming LLC were suggested to have a promotion effect on aerobic ZnPP formation. It is difficult for vLMMs to catalyze directly due to the low molecular weight. Therefore, LLC-vLMMs might act as an accelerator to promote the activity of mFECH or other meat-inherent enzymes in the formation of ZnPP under aerobic conditions.

### 3.4.5. Proposed mechanism of the LLC-associated aerobic ZnPP formation

In this study, the promotion mechanism of aerobic ZnPP formation by ZnPP-forming LLC in meat and meat products was investigated. A scheme of aerobic ZnPP formation by the ZnPP-forming LLC was illustrated (Fig. 44) in which the obtained results from this study and the previous reports were arranged. In LLC associated aerobic ZnPP formation, LLC did not produce ZnPP and heme directly (Fig. 19, 29, 30, 31), and did not degrade heme proteins. As described in section 3.4.1, no effect of oxygen for the insertion of zinc into PPIX was observed. This result suggested that abundance of ZnPP formation depends on the availability of PPIX. As described in section 3.4.3, the activity of mFECH and LC-FECH was inhibited by the oxygen but FECH derived from LLC facilitated ZnPP formation under aerobic conditions due to their oxygen resistant ability by forming PPIX from heme. On the other hand, Hb and Mb are thought to be the main components responsible for ZnPP formation, and heme is released after degradation of the components or dissociated from the components. LLC-LMMs also promoted PPIX formation from heme proteins as well as

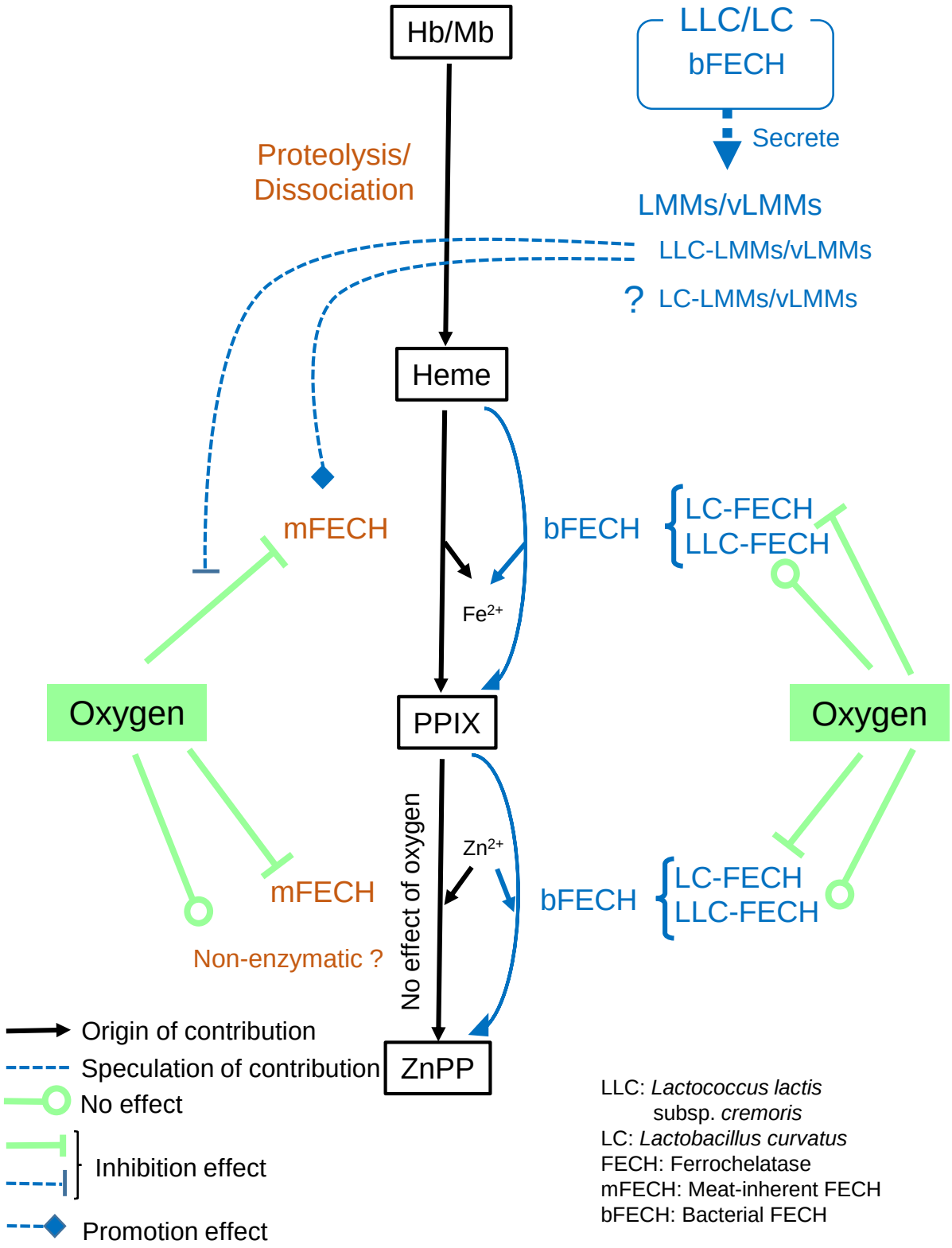



**Fig. 44. Proposed mechanism of the LLC-associated aerobic ZnPP formation**

heme. However, the effect of LLC-LMMs on the dissociation of heme from heme proteins were not examined. Therefore, as described in 3.4.4, LLC-LMMs might act as accelerator to promote the activity of mFECH or protect the activity from oxygen. The contributors in LLC-LMMs were clarified to be volatile and some candidates were screened.

Based on the above discussion, the mechanism by which ZnPP is formed aerobically by LLC in meat was proposed. Since mFECH and most bFECH, which are the most important contributors of ZnPP formation, were oxygen-sensitive, ZnPP could be only formed under anaerobic conditions. However, since FECH derived from LLC was oxygen-resistant, the LLC rescued mFECH activity lost in the presence of oxygen. Moreover, some species-specific volatile metabolites secreted from LLC were also likely to rescue the activity of ZnPP formation under aerobic conditions. In the meat-inherent mechanism, the pathway from heme proteins up to PPIX was clarified to be affected notably by oxygen.

### 3.4.6. LLC-associated aerobic ZnPP formation influenced by external factors

For the improvement of meat products by forming ZnPP, the processing-associated factors might affect in ZnPP formation. The temperature, pH, oxygen, and additives are the main external factors that can affect the promotion of aerobic ZnPP formation in meat products. ZnPP-forming patterns were remarkably different among the temperatures and pH levels investigated in this study. At temperature 37°C and pH 5.5, it was shown that the maximum formation of ZnPP in the presence of ZnPP-forming LLC, and the magnitude of ZnPP formation in anaerobic conditions was higher than that of the aerobic conditions (Fig. 16 & 17). Along with FECH activity, vegetative propagation of the ZnPP-forming LAB

might play an important role for the abundance of ZnPP formation in the inoculated groups. In this study, it was observed that bacterial growth of LAB was correlated to the amount of ZnPP formation in anaerobic conditions (data not shown). The optimum growth temperature for the genus *Lactobacilli* lies between 30-40°C, whereas 30-37°C for *Lactococcus* and they can grow at temperatures as low as 10°C but not at 45°C (Ahmed *et al*., 2006). Therefore, bacterial growth is important, although the growth is hampered due to the addition of additives in the meat products, thereby affected ZnPP formation. The ability of salt to decrease water activity is thought to be due to the ability of sodium and chloride ions to associate with water molecules (Fennema, 1996) and ultimately affects LAB growth. Besides, NO produced from nitrite inhibited the formation of ZnPP in cured meat products since nitrite is used as a curing agent in meat products (Wakamatsu *et al*., 2010; Adamsen *et al*., 2006a) and it has anti-bactericidal effects. Besides, ZnPP are formed by two independent mechanisms in porcine skeletal muscles whose optimal pH values are 5.5 and 4.75 (Wakamatsu *et al*., 2019). Moreover, the optimum pH in porcine hearts for ZnPP formation at 37°C is 5.0-6.0 (Wakamatsu *et al*., 2015). An increase in the number of LAB in the medium lower the pH of the medium due to the production of lactic acid. In addition, oxygen markedly inhibits the formation of ZnPP in pork homogenate (Wakamatsu *et al*., 2004a, 2007). Moreover, since some metabolites secreted from LLC promoted to form ZnPP without bacteria, the color of non-fermented meat products might be improved with ZnPP formation even under the LAB free-system. Therefore, the external factors could be counted for the improvement of entire color of meat products by forming ZnPP aerobically.

# Chapter 4

## General discussion

In this study, LAB having aerobic ZnPP-forming ability has been obtained in the same cluster of a phylogenetic tree, which indicates a close genetic relationship among the *Lactococcus* strains (Fig. 15). They might harbor the gene encoding specific compounds which are responsible for the ZnPP formation in the presence of oxygen. LAB are a phylogenetically diverse but functionally related group of bacteria comprising the families *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae* (Börner *et al*., 2019) and are clustered due to similarities in their metabolic pathways (Zhang *et al*., 2002). The outside of the sausages was closely related to oxygen and salt was used in the manufacturing of sausage. It was reported that oxygen stress affects the metabolic function of *Lactococcus lactis*, mostly its lactic acid production. Other metabolites such as $CO_2$, ethanol, acetate, acetoin, and diacetyl which tend to be increased when bacteria are exposed to oxygen reagents (Miyoshi *et al*., 2003). Therefore, the strains of *Lactococcus* can tolerate oxygen and salt stress to form ZnPP. Besides, the higher concentration of salt in a medium causes osmotic stress in bacteria and affects cell functions such as gene expression, protein synthesis, and water retention, which is necessary for cell nutrition and stability (Rallu *et al*., 1996). Moreover, ZnPP-formation might depend on gene expression, which greatly relies on the composition of the growth medium, as procaryotic regulatory proteins are often controlled by nutrient availability (England *et al*., 2010). Consequently, irregular cluster differences among high ZnPP-forming

LAB were observed in the phylogenetic tree, indicating that the formation of ZnPP by identified LAB was independent of phylogeny.

Color improvement by using ZnPP-forming LAB would be suitable for fermented meat products, although non-fermented meat products occupies the majority of meat market. In this study, ZnPP formation in meat by the LAB is species-specific and might depend on the metabolites secreted by the ZnPP-forming LAB. Metabolites resulted from metabolism are the intermediate products of low molecular weight compounds produced by microbes, and also necessary for the development of their own growth usually consisting of aldehydes, ketones, esters, alcohols, organic acids, amines, and sulfur compounds, etc. (Ercolini *et al.*, 2009; Samelis, 2006). It was also reported that the efficient conversion of Mb-heme to ZnPP happened, while the FECH was incubated with Mb in the presence of ascorbic acid and cysteine (Chau *et al*., 2011). The metabolites produced by the ZnPP-forming bacteria might be different, since only few bacteria can form ZnPP both aerobically and anaerobically among the ZnPP-forming bacteria. It might be possible to identify the metabolites responsible for aerobic ZnPP formation by comparing the whole gene sequence of aerobic ZnPP-forming LAB with anaerobic ZnPP-forming one. A detailed study regarding bacterial metabolites will be helpful not only for the elucidation of the LAB associated ZnPP formation mechanism but also for the improvement of non-fermented meat products.

Commercial meat products composed of salt, spices and other flavoring agents in response to the consumer demands. In this study, the dry-cured sausage was prepared experimentally without the addition of any fat and spices or condiments. Generally, about 20% of fat is used in sausage products for binding or textural properties, processing yields,

sensory characteristics, and storage stability (Badpa and Saghir, 2014). Different kinds of spices and condiments are widely used in making commercial sausages such as cinnamon, ginger, onion, garlic, cloves, sesame, black pepper, allspice, paprika, coriander, nutmeg, cloves, garlic, vinegar, mace, pepper, chili peppers, or pistachio nuts, etc. as flavorings and coloring agents. (Lücke, 1997). Manganese was considered as a factor in spices (clove, cardamom, ginger, celery seed, cinnamon, and turmeric) claimed for the enhancement of acid production by meat starter bacteria (Zaika and Kissinger, 2006). Spices and condiments also contribute to other substances, such as sugars, nitrates, and metallic ions into the cured meat products (Aguirrezábal *et al*., 1998). However, maintenance of the lean-to fat ratio and the addition of spices or condiments are important to keep the quality attributes in the sausages. Therefore, it is essential to investigate the ZnPP-forming ability by the ZnPP-forming LAB in combination with the common curing and seasoning agents to establish a suitable method for the color improvement of meat products by forming ZnPP.

In conclusion, ZnPP formation by the LAB can be considered as an alternative of nitrite since the bright red color of dry-cured meat products has been improved through ZnPP. High ZnPP-forming LAB under aerobic conditions could be the potential candidates as ZnPP former in the dry-cured meat products. In this study, high ZnPP-forming LAB were screened and *Lactococcus lactis* subsp. *cremoris* GB(A)-1 could improve both the external and internal bright red color of meat products by forming ZnPP both aerobically and anaerobically. For the improvement of external bright red color, aerobic ZnPP formation is important, and food-grade bacteria with aerobic ZnPP forming ability has been limitedly found. This study confirmed that the LAB-associated ZnPP formation mechanism under aerobic conditions

differed from that under anaerobic conditions since aerobic ZnPP formation by the LAB was species-specific, and their ZnPP-forming ability was also different. Therefore, further studies are needed to investigate the components responsible for aerobic ZnPP formation. Finally, it is assumed that both the risk of human health arising due to the addition of nitrite/nitrate in the dry-cured meat products and the safety of the meat products will be relieved by using high ZnPP-forming LAB having ability to form ZnPP both aerobically and anaerobically as a starter culture.

# Chapter 5

## Abstract

Zinc protoporphyrin IX (ZnPP) is a red pigment formed in nitrites/nitrates free meat products and the promotion of ZnPP formation in meat by some bacteria has been reported. It was hypothesized that some food-grade LAB might be useful to improve the entire color of meat products by forming ZnPP aerobically and mechanism of the aerobic ZnPP formation by the bacteria might be different from that of anaerobic ZnPP formation. Therefore, this study was carried out to screen high ZnPP-forming food-grade LAB to improve the color of meat products and also to clarify the aerobic formation mechanism of ZnPP by bacteria in meat products.

Screening of LAB was performed using modified MRS agar and M17 mediums supplemented with 3% salt with pH adjusted to 5.5. A total of 25 ZnPP-forming LAB were confirmed by 16S rRNA gene sequencing, whereas 13 species were found to be ZnPP-forming food-grade LAB avoiding duplication. All 13 LAB could form ZnPP in salt-added minced meat. Next, to investigate the effects of LAB on the color improvement of meat products, nitrite-free dry-cured sausages using five ZnPP-forming food-grade LAB were prepared. The bright red color on both inside and outside was observed in the *Lactococcus lactis* subsp. *cremoris* (LLC)-inoculated sausages, although the brown color in the outside was noticed for the *Lactobacillus* spp. Besides, the redness of the LLC-inoculated sausages was close to the nitrite-added group. Moreover, the external bright red color improved by the LLC was due to the aerobic formation of ZnPP. Therefore, the screened LAB which could

form ZnPP in both presence and absence of oxygen can be used to improve the appearance of fermented meat products.

Regarding aerobic ZnPP formation mechanism by LLC, initially, the ZnPP-forming ability of the LLC was observed in commercial media in the presence of Hb and Mb, but meat components essential for aerobic ZnPP formation. When the meat homogenate was fractionated, only the FECH-containing insoluble fraction increased the ZnPP formation. No effect of oxygen for the insertion of zinc into PPIX was observed. The activity of mFECH and LC-FECH was inhibited by the oxygen but FECH derived from LLC facilitated ZnPP formation under aerobic conditions. LLC-LMMs also promoted PPIX formation from heme proteins as well as heme. Additionally, temperature at 37°C and pH 5.5 showed maximum formation of ZnPP, whereas the formation of ZnPP completely affected by the addition of sodium nitrite and polyphosphate.

The present study confirmed that LLC could be an alternative of nitrites/nitrates for the improvement of entire color of meat products. It was suggested that LLC themselves could not form ZnPP aerobically and aerobic ZnPP formation by the LLC was different from anaerobic ZnPP formation by other LAB, whereas vLMMs facilitated aerobic ZnPP formation.

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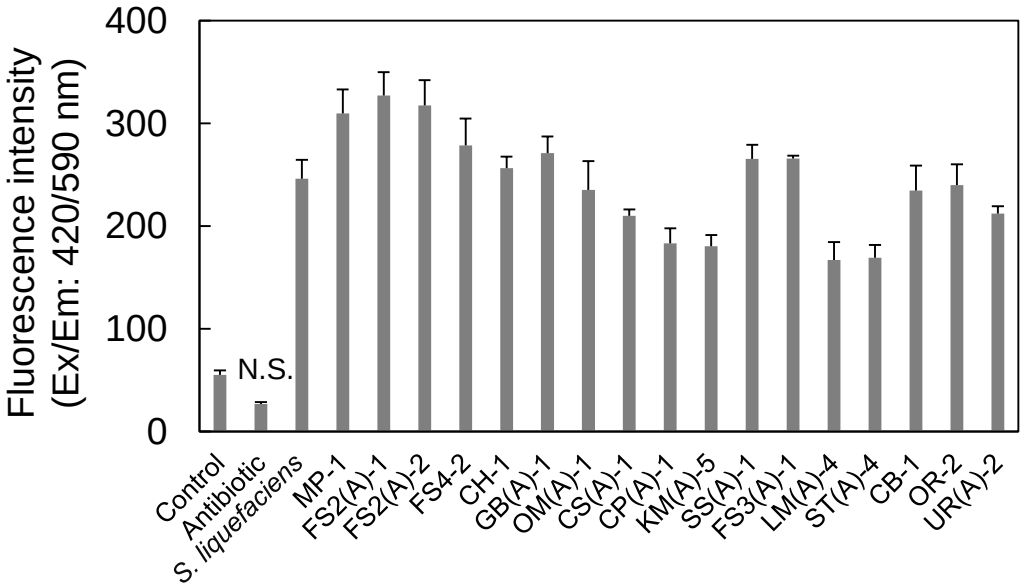
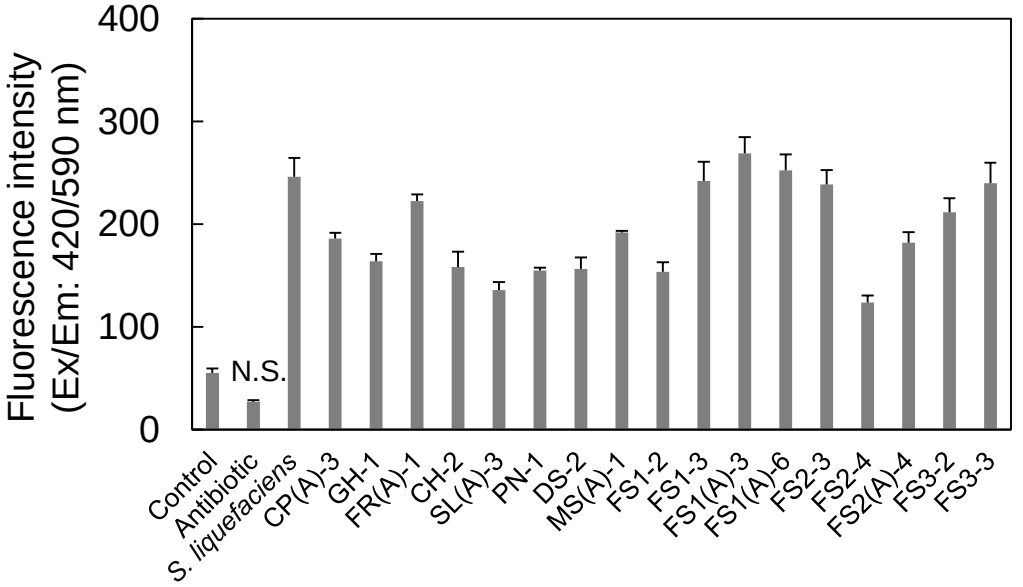
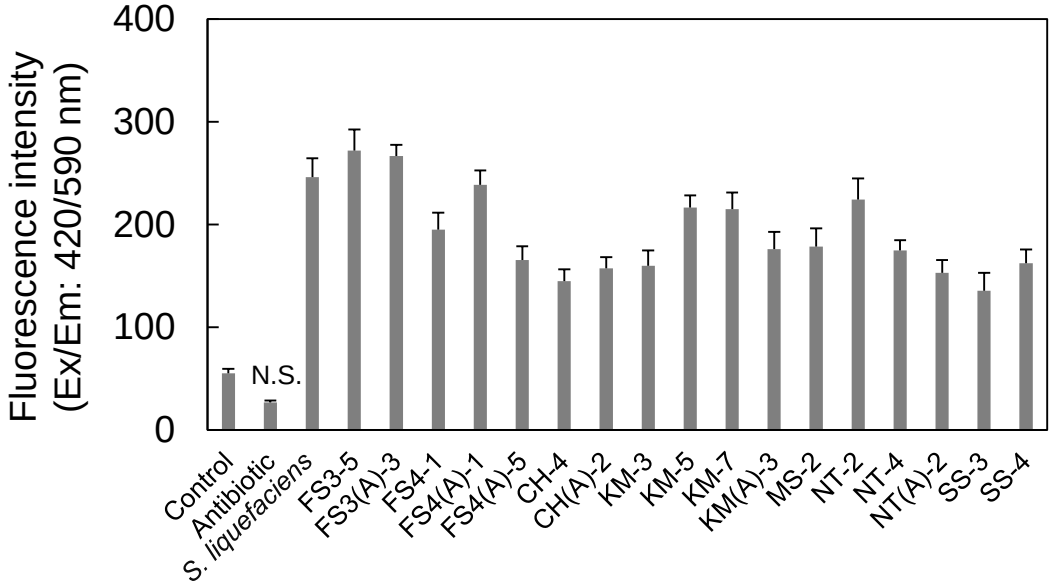
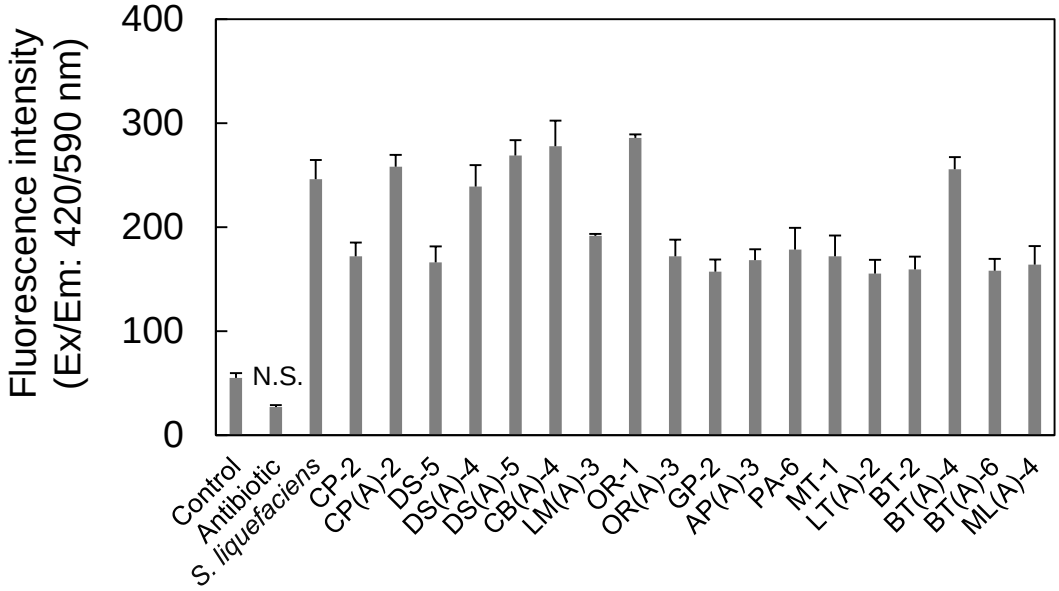



**Appendix Fig. 1. ZnPP-forming abilities of the bacteria isolated from various sources**

The preparation of pork homogenate, inoculation of bacterial isolates (source enlisted in Table 3), incubation of the pork homogenate mixture, and the formed ZnPP was measured as described in Fig. 2 (Chapter 2). Data expressed as means ± SE (n = 3). N.S.: Not significant vs. control.




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

The preparation of pork homogenate, inoculation of bacterial isolates (source enlisted in Table 3), incubation of the pork homogenate mixture, and the formed ZnPP was measured as described in Fig. 2 (Chapter 2). Data expressed as means ± SE (n = 3). N.S.: Not significant vs. control.