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Suppression of histamine formation in processed tuna fish using probiotic (*Lactiplantibacillus plantarum* BY-45) approach

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ABSTRACT

Histamine producing foodborne pathogens pose a major microbiological risk in the overall seafood products. Specifically, negative health effects of histamine poisoning from seafood products after processing is a food safety and human health concern globally. Therefore, it is essential to advance sustainable and inexpensive post-harvest processing strategies to counter this serious food safety and health challenge and to improve overall food quality of common seafood product like tuna fish. Based on these food safety and health quality needs, the aim of this study was to investigate the effectiveness of the combination of *Lactiplantibacillus plantarum* BY-45 and salt concentration to control histamine formation *in vitro* and *in vivo* on sample filet of tuna fish during processing to reduce this microbiological associated health risk. Isolation and identification of histamine forming bacteria in tuna loin and optimal storage temperature for inhibiting histamine formation was determined with *L. plantarum* BY-45 treatment. In this study, *Escherichia coli* belonging to Enterobacteriaceae family was found to be the predominant histamine forming bacteria contaminating our tuna samples. This contamination was suspected to happen during handling after catch and prior to landing at Benoa harbor, Bali for further processing. However, the rate of histamine formation was significantly suppressed (<1 ppm) at temperature of ≤2°C, which was significantly lower than 79.73–88.33 ppm that was produced at 4°C. Additionally, in response to LAB and salt combination, histamine formation by *E. coli* was totally suppressed. The results of this study were consistent in the *in vivo* assay on loins of tuna samples. Overall, this study provides the foundation to reduce microbiological food safety risk from histamine poisoning by foodborne pathogens in tuna, and beneficial LAB based strategy can be targeted to achieve wider food safety and health quality benefits in processed seafood.

1. Introduction

Tuna fish is an important seafood commodity of Indonesia for exports targeting significant global consumption in countries, such as Japan, USA, Australia, New Zealand, and England [1]. Tuna fish is typically caught from the coral triangle zone of the Pacific Ocean (Indonesia, Philippine, Malaysia, East Timor, Papua New Guinea, Solomon Island, and Indian Ocean) and exported from Indonesia to above targeted countries where it is a delicacy [2]. According to a report published by *The International Seafood Sustainability Foundation* (ISSF) in 2009 and Lecomte et al. [3], species of tuna fish with the highest commercial value include yellowfin (*Thunnus albacares*), bigeye (*T. obesus*), bluefin (*T. thynnus*, *T. orientalis*, and *T. macoyii*), albacore (*T. alalunga*), and skipjack (*Katsuwonus pelamis*). In the last 2 decades, Indonesia has been one of the biggest tuna fish exporters [4].

Tuna fish contains high level of minerals, vitamins A and B, protein, and low level of fat [25]. Due to this high value of nutrient content, this product is easily contaminated by bacterial species, as it is used as an energy source by these contaminants [5,6]. Once contaminated, its shelf-life is reduced in post-harvest processed stages [7]. Contamination of this fish by *Vibrio spp.*, *Pseudomonas spp.*, and *Photobacterium spp.*, as well as by bacterial species belonging to Enterobacteriaceae family leads

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to histamine formation in this contaminated fish product [8]. Other bacterial species, such as *Morganella morganii*, *Klebsiella pneumoniae,* and *Hafnia alvei* are also able to produce high rate of histamine when incubated at temperatures of 20–30°C [9]. Histamine commonly exists due to contamination by histamine forming bacteria and its concentration increases during storage, even at low temperatures [10]. In general, histamine formation occurs during postmortem of the fish and this is due to the activity of histidine decarboxylase enzyme produced by either contaminating bacteria or indigenous microbiota of fish, including tuna fish [11]. This enzyme catalyzes decarboxylation of free histidine of fish and fishery products to form histamine. According to Visciano et al. [12,13] accumulation rate of such toxic compound is determined by the availability of free histidine (level of free histidine as substrate of the bacterial enzyme in the seafood products), the growth and activity of histidine decarboxylase producing bacteria, and environmental factors that favor the growth of histidine carboxylase producing bacteria in the seafood products. Histamine is a colorless and odorless compound, and therefore the presence of such compound does not change the color or smell of the sea products and often is not perceived by consumers [14–16]. Once the formation of histamine takes place in tuna fish, this amino acid derivative will be present permanently, even though it is heat treated at normal cooking temperature [12,17]. This toxic compound is known to induce inflammation-driven hyper allergic reaction but rarely causing death in some people who consume histamine-containing fish [18]. Histamine-mediated symptoms may occur in humans with histamine intolerance [19]. In such population, the ability of enzymes functioning in the degradation of histamine decreases or is even totally inhibited [20,21]. This leads to accumulation of histamine in the intestine which subsequently results in the increase of its absorption from the intestinal tract into the blood stream [20–22]. Accumulation of histamine in the blood leads to several health concerns, such as chronic headaches, dysmenorrhoea, flush, gastrointestinal discomfort, and intolerance of foods rich in histamine and alcohol [12,20].

Cases of histamine poisoning from fish processing stages are mostly due to poor handling of the fish during processing or storage [13]. Cases due to histamine poisoning with highest number of affected people are reported in the USA, Japan, and England [23]. In the USA alone, 223 outbreaks affecting 865 people with histamine poisoning were reported between 2000 and 2007 [24]. Due to these histamine poisoning cases, the maximum limits for histamine in fish and fishery products have been regulated by countries around the globe (by authorized organizations, such as FDA) [12].

Until recently, control of formation rate of histamine at PT. Intimas Surya and other tuna exporters, particularly those located in Denpasar-Bali, Indonesia has relied on application of low temperature during storage of seafood products. The main drawback of this classical method is high electrical energy consumption to achieve this low temperature during storage. Additionally, it may also increase the risk of texture damage, loss of water holding capacity, and oxidation of the fishery products [25]. Alternative modern methods, such as high-pressure processing, pressure shift freezing, pulse light technology, pulsed electric field processing, modified atmospheric packaging, vacuum packaging, and irradiation have been proposed and reviewed [26]. In Indonesia, application of high salt concentration has also been traditionally practiced for decades to improve shelf life of the seafood products. However, the application of lactic acid bacteria (LAB) singly or in combination with salt concentrations is still limited in use during storage of these products, but it has significant potential to control histamine in processing and storage stages. In the present study, *E. coli* was found to be the predominant species, which potentially formed histamine in targeted tuna loin samples. Therefore, in the present study the efficacy of LAB applied singly or in combination with various salt concentrations was investigated during storage. The main objective of this research was to reduce the rate of *E. coli* related histamine formation in tuna fish so that its shelf-life can be prolonged at temperatures higher than minus 2°C (−2°C), potentially leading to improved safety, health benefits and reduced storage energy consumption.

2. Materials and method

2.1. Enumeration and isolation predominant suspected histamine forming bacteria

At the PT. Intimas Surya, a tuna exporter, in Denpasar-Bali, Indonesia, bacteria belonging to family of Enterobacteriaceae [27,28] have been the main concern in exported seafood products and are being targeted in routine quality control examination. Therefore, in this study, enumeration and isolation of histamine forming bacteria was focused on this bacterial family. Initially, 27 pieces of tuna loin samples (2 kg each) were tested for histamine levels and 5 samples with histamine levels of higher than 100 ppm (5 samples with highest histamine levels) were selected to obtain predominant isolate contributing to histamine formation. Enumeration was done by applying dilution and spread plate method as specified in Sintyadewi et al. [29] and Ramona et al. [30]. Samples of tuna loin in the amount of 10 g were diluted aseptically in 90 mL physiological saline solution to obtain dilution rate of 10^{-1}. These samples were further diluted with the same solution to obtain dilution rate up to 10^{-6}. Samples with dilution rates of between 10^{-4}–10^{-6} and sample volume of 100 μL were then spread on plates of *Violet Red Bile Dextrose Agar* (*VRBD Agar*) for enumeration of bacteria belonging to family of Enterobacteriaceae, incubated at 35 ± 2°C (inverted position) for 24 h, and plates with colonies of between 30 and 300 were counted and the results were averaged.

One type of bacterial colony appeared (visually observed) to be predominant and suspected to be histamine forming species and therefore was picked, purified (streak cultured for single colonies) on nutrient agar (NA) medium, and stored at 4°C until required in subsequent experiments.

2.2. Molecular identification of LAB and suspected histamine producing bacterium

The LAB isolate (BY-45) and the suspected histamine producing bacterium were identified by determination of their 16 s rDNA sequence (molecular technique). This was followed by aligning of their 16 s rDNA sequences with those of known isolates deposited in the GeneBank (http://www.ncbi.nlm.nih.gov). The stages of the procedures included isolation and purification, amplification, and sequencing of the isolate's 16 s rDNA.

2.2.1. Isolation of genomic DNA

Both LAB isolate and the suspected histamine producing bacterium were grown overnight in MRSB (DeMan Rogosa Sharpe Broth) medium and NA (nutrient agar) medium respectively, until cell density of approximately 10^8 cells/mL was reached. Subsequently, 1 mL each of these cell suspensions was transferred into an Eppendorf tube, centrifuged at 8000 *xg* for 3 min at 4°C, and the supernatants were decanted. This was followed by washing up of the pellets two times with sterile saline solution, centrifugation at 8000 *xg* at 4°C for 3 min, and decanting of the supernatants to obtain pellets of cells. This procedure was repeated several times until at least 50 mg pellet or bacterial cell mass was obtained. The cell mass obtained was next re-suspended in 200 μL of saline solution so that high density of cell suspension was obtained. The procedures specified in the *Quick*-DNA™ *Microprep Kit* (Zymo Research, USA) was applied in the DNA extraction. A volume of 200 μL of cell suspension previously prepared and 750 μL BashingBead™ Buffer was added into a ZR BashingBead™ Lysis Tube, shaken with a bead beater (TOMY micro Smash™ MS-100) at the speed of 4500 rpm for 6 min and centrifuged at 10,000 *xg* for 1 min at 4°C. The supernatant was transferred into a Zymo-Spin™ III-Filter in a collection tube and centrifuged at 8000 *xg* for 1 min at 4°C, and added with 1200 μL of Genomic Lysis Buffer. This mixture (800 μL) was next pipetted into a Zymo-Spin™ IICR

Column in a new collection tube, centrifuged at 10,000 *xg* (at 4°C) for 1 min, and its supernatant was decanted. This procedure was repeated twice, and followed by addition of 200 μL DNA Pre-Wash Buffer into a new collection tube of Zymo-Spin™ IICR Column, followed by centrifugation at 10,000 *xg* (at 4°C) for 1 min, and the supernatant was decanted. A volume of 500 μL g-DNA Wash Buffer was added into a Zymo-Spin™ IICR Column, further centrifuged at 10,000 *xg* (at 4°C) for 1 min, and 35 μL of DNA Elution Buffer was added into a Zymo-Spin™ IICR Column in a 1.5 mL capacity of Eppendorf tube. This mixture was again centrifuged at 10,000 *xg* (at 4°C) for 30 s in the DNA elution. A nano-photometer P-300 (Implen) was used in the quantification of the DNA obtained, at the wavelength of 260 nm. The DNA obtained from these procedures was then stored at −20°C before being used in the subsequent analysis.

2.2.2. *Amplification of 16 s rDNA using hot start master mix PCR kit, Qiagen*

Amplification of the 16 s rDNA was carried out using primers of 27-F (5′ AGA GTT TGA TCC TGG CTC AG3') dan 1492-R (5' TAC GGY TAC CTT GTT ACG ACTT 3′). A volume of 48 μL PCR master mix (5 μL dNTPs, 5 μL PCR buffer, 3.5 μL $MgCl_2$, 1 μL of 1 pmol primer 27-F, 1 μL of 1 pmol of 1492-R primer, 0.25 μL taq polymerase, and 34 μL deionized water) and 2 μL DNA sample were added into a PCR tube to obtain a total reaction volume of 50 μL. An Infinigen thermocycler machine (30 cycles) was used to amplify the targeted DNA sample. The DNA samples were first denatured at 94°C and followed by 30 cycles of PCR (polymerase chain reaction). The conditions of each cycle were: denaturing of DNA at 94°C for 30 s, 2 min of annealing at 55°C, and 2 min elongation at 72°C. The cycle was ended with a cycle of elongation for 5 min at 72°C. On the completion of PCR, the amplified 16 s rDNA was confirmed by running an electrophoresis on 1% *w/v* agarose gel in TAE buffer, with 1 μg/mL ethidium bromide in it. The electrophoresis was run for 45 min at 80 Volt and if the PCR was successful, bands with correct DNA size would appear on the gel, and this could be visualized under a UV trans-illuminator. Once these bands appeared on the gel, the PCR products were purified.

2.2.3. *Purification of PCR product and sequencing of purified 16 s rDNA*

The PCR products were purified using SUPRC™ PCR (Takara Bio-medicals, Otsu, Japan). The purified DNAs were then sequenced with Big Dye Primer Cycle Sequencing FS Ready Reaction Kit (Applied Biosystem) in an automated sequencing 3100 Genetic Analyzer (PE Applied Biosystems). This was carried out at the 1st base, Malaysia through the PT. Genetika Science Indonesia. The 16 s rDNA sequences of our isolates were then aligned with their counterparts in a clone library of known bacterial species deposited at the GenBank (http://www.ncbi.nlm.nih.gov), so that their molecular identities could be determined. Once the sequences of their 16 s rDNA were obtained, a phylogenetic tree was constructed using Custal W2 software which was combined with Njplot.

2.3. *Confirmation of histamine production by suspected species*

Histamine level produced by the suspected species in tuna loins was confirmed by applying *enzyme linked immunosorbent assay* (ELISA) method using *Veratox kits* AOAC-RI No 070.703 (Neogen's Veratox®) with histamine reading range of between 0 and 50 ppm. The suspected species was first grown in 5 mL TSB (Tripticase Soy Broth), incubated overnight at 35°C, harvested by centrifugation at 7000 rpm for 5 min [26], decanted, then the pelletes were washed twice with saline solution, resuspended in saline solution, and standardized with McFarland scale of 0.5 (approx 1.5×10^8 cells/mL). A volume of 10 μL of this bacterial suspension was then inoculated into Tripticase Soy Broth Histidine (TSBH) and its final volume was adjusted to 3 mL (to obtain cell density of 5×10^5 cells/mL), incubated at various temperatures (−2, 2, and 4°C, which are temperature ranges commonly applied to store tuna loins at the PT. Intimas) for 8 h, and assayed for histamine production at 4 and 8 h after inoculation. The data obtained in this experiment was used as a basis to determine appropriate incubation temperature for further studies (*in vitro* and *in vivo* experiments). TSBH is a trypticase soy broth (TSB) medium with 0.1% histidine in it, and this free amino acid is the main compound for histamine production following activity of histidine decarboxylase enzyme. This medium was used to confirm histamine formation by suspected bacteria isolated in our experiments. Prior to use in the experiments, the level of histamine in the sterile TSBH medium was confirmed to be zero (0 ppm). Additionally it was also confirmed that there was no interference in samples's histamine levels by salt addition (suplementary data), and this data obtained was representative and reproducible in an experimental setting.

Prior to measurement of histamine levels in TSBH or in loin samples inoculated with suspected bacteria, a standard curve that showed a relationship between absorbance at 650 nm and histamine levels was established. This was done by pipetting a volume of 100 μL conjugate into mixing wells for samples or standard histamine solutions. Standard histamine solution with concentrations of 0; 2.5; 10; 20; and 50 ppm in total volume of 100 μL were next added to standard mixing wells. All wells were then homogenized. A volume of 100 μL of each mixture were subsequently added into antibody-coated wells previously prepared. This was then incubated for 10 min at 30°C, decanted, rinsed 3 times with washing buffer solution, added with 100 μL anti-antibody linked enzyme, incubated for 10 min, decanted and then 100 μL substrate (Red Stop Solution) of the enzyme linked with anti-antibody was added before it was allowed to settle until color intensity appeared. Following this, the color development was measured with a spectrophotometer at the wave length of 650 nm. Triplicate measurement was done and the results were averaged.

The samples measured for histamine levels were prepared by weighing 10 g and added into 90 mL of deionized water to obtain dilution level of 10^{-1}, shaken for 20 s and allowed to settle for 5 min (this was conducted repeatedly three times), filtered, and measured for OD reading at 650 nm. In the case when the samples gave OD readings of higher than OD reading of the Veratox kit range (out of range), the samples were diluted in extract diluent buffer (EDB, as specified in the kit protocol) at appropriate dilution factor so that the OD readings of the samples fell in the detection range of histamine level of the kit, and the sample's histamine levels were redetermined. The actual histamine levels of the samples (samples with OD readings out of range) were calculated by multiplying the histamine levels of diluted samples with the dilution factor applied. Triplicate experiments were done for each sample and the results were averaged.

2.4. *The effect of LAB applied singly or in combination with salt on in vitro histamine formation by E. coli*

The main aim to conduct this assay was to investigate the effectiveness of salt at various concentration and LAB (isolate code of BY-45) applied singly or in combination to suppress histamine production by *E. coli* isolated in the absence of tuna loin. The LAB isolate (BY-45) is a collection belonging to the Udayana University Culture Collection Center (UnudCCC). This isolate had previously shown its probiotic potential (showed resistance to acidic conditions, resistance to high level of deoxicholic acid, and it did not convert cholic acid into deoxicholic acid) and has been targeted as a probiotic strain for further development. A factorial randomized design with two factors of treatments (triplicates with 3 levels of salt concentrations and 4 levels of LAB cell densities) was applied. Overall, there were 12 combinations of treatments with 3 replications per treatment. Therefore, a total of 36 unit systems were established in the assay (3x4x3), and this appeared to be representative statistically [31]. This triplicate *in vitro* assay was performed in TSB medium supplemented with 0.1% histidine (TSBH) with three level of salt concentrations (0, 2, and 4% *w/v*). In this assay, 2 mL of TSBH medium with these three levels of salt concentrations were first added

into sterile Falcon tubes, inoculated with 10 µL suspension of *E. coli*, and added with various volumes (0, 10, 20, and 40 µL) of specific LAB (*L. plantarum* BY-45) suspension (previously harvested from MRS broth medium in the same way as *E. coli* mentioned in Section 2.3 previously), and the final volume of each treatment was then adjusted to 3 mL by adding corresponding TSBH medium (TSBH with various salt concentrations in it). The optical density (OD) of *E.coli* and LAB suspensions at the wavelength of 600 nm was adjusted to McFarland standard of 0.5 that gave approximate cell density of 1.5×10^8 cells/mL [3]. By adjusting the final working volume to 3 mL, the LAB densities in the working solutions were 0; 5×10^5; 10×10^5; or 20×10^5 cells/mL, while the *E. coli* density was 5×10^5 cells/mL. Two sets experiments per treatment were prepared (1 set was assayed for histamine at 4 h after inoculation, the other set was assayed at 8 h after inoculation). All tubes were incubated for 8 h at 2°C with regular sample collection (4 h and 8 h after inoculation) for histamine assays as specified in the Section 2.3 previously. The LAB isolate used in this assay was isolate BY-45 (a collection of the Integrated Laboratory for Bioscience and Biotechnology, Udayana University). This LAB was isolated from feces of healthy infants in a previous study by Maha Uni [32]. The design of this *in vitro* assay is depicted in the supplementary data, or presented in Fig. 1.

2.5. The effect of LAB applied singly or in combination with salt to evaluate histamine formation by E. coli in loins of tuna fish (in vivo assay)

The experimental design mentioned in the previous section was applied for this *in vivo* assay. Pieces of tuna loins with a weight of 25 g each were immersed for 1 min in solutions containing combinations of LAB and salt concentrations and the same conditions were adjusted as in the *in vitro* assays above. The densities of LAB in various salt concentrations were adjusted either to zero (no LAB inoculation) or 20×10^5 cells/mL while the *E. coli* density in the same salt solutions (applied to immersed tuna loins) was adjusted at 5×10^5 cells/mL (5.7 log cfu/mL). Tuna loins immersed in sterile water only and those immersed in sterile water with *E. coli* only in it at cell density of 5.7 log cfu/mL, served as zero (untreated) control and control treatment, respectively. All treated tuna loins and controls were incubated for 8 h at 2°C and sampled at 4 h and 8 h after treatment (following 1 min immersion). Histamine level in each sample following treatments was evaluated according to the procedure as specified in Section 2.3 previously. Triplicate experiments were conducted, and the results were averaged. The diagram of the experimental design for this *in vivo* assay is shown in the supplementary data.

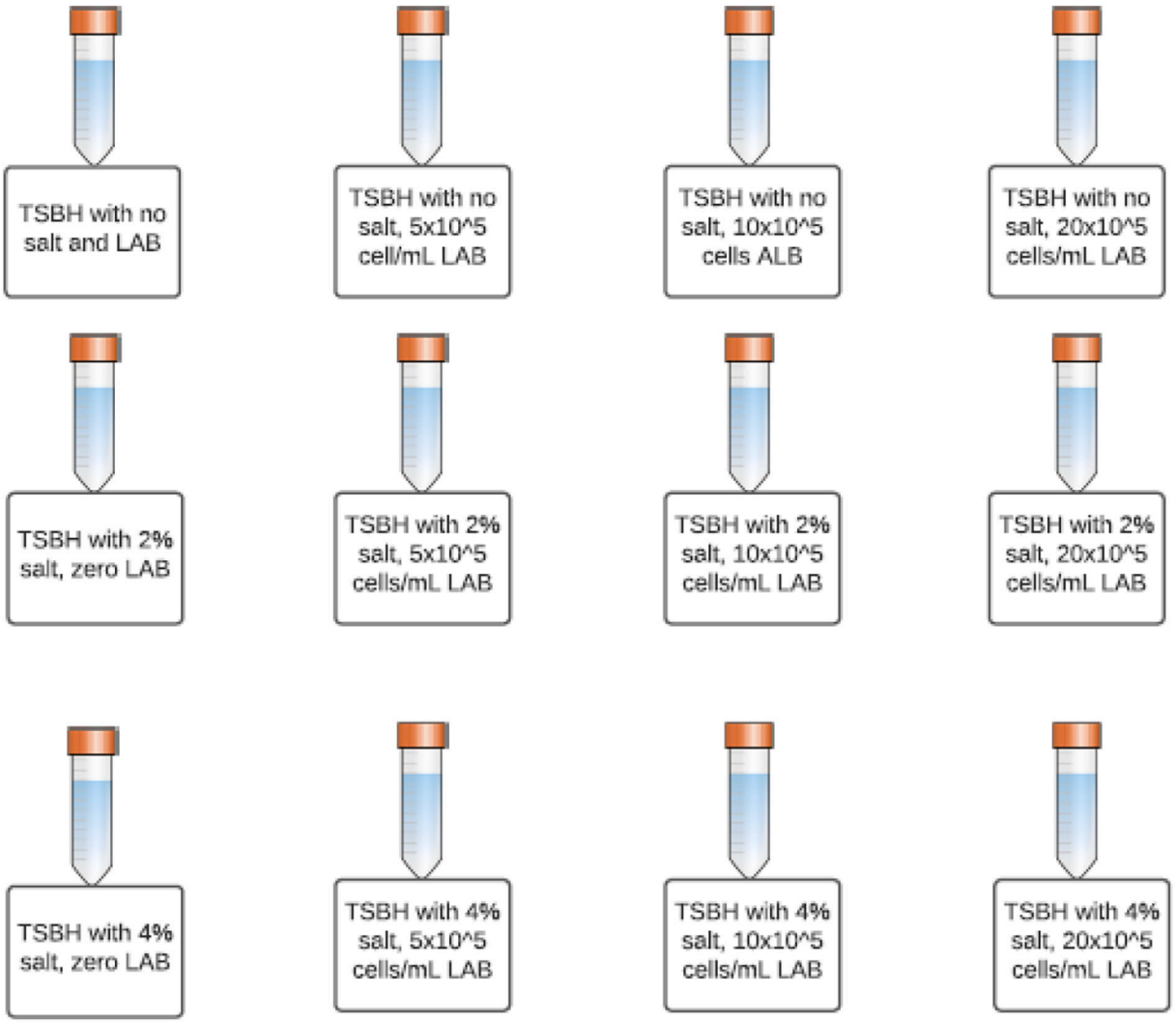

Fig. 1. Experimental design for *in vitro* assay of histamine formation by *E. coli* in TSBH medium. Triplicates were used in this *in vitro* experiment. Each tube was inoculated with suspension of 10 µL *E. coli* of cell density of 1.5×10^8 cells/mL. A total volume of 3 mL was set to establish *E. coli* density of 5×10^5 cells/mL in each triplicate working treatment.

2.6. Data analysis

Data obtained from this research was analysed with *Analysis of Variance* (ANOVA) with help of Minitab for windows version 16. When significant differences at $p < 0.05$ was indicated, the data was further analysed with multiple range of Duncan test.

3. Results and discussion

The histamine level of the 27 tuna loin samples fell in the range of 80.43 ± 0.21 and 152.37 ± 18.39 ppm, and these are presented in Table 1. Five samples with histamine level of >100 ppm (5 samples with highest levels of histamine) were subjected to total bacterial count, and the results are shown in Table 2. The histamine levels of these 5 samples were in the range of 103.43 ± 5.42 ppm to 152.37 ± 18.39 ppm. High levels of histamine observed in our samples (although bacterial count was relatively low) was potentially due to histamine production by other bacterial contaminants which could not grow in the specific medium for family of Enterobacteriaceae used in the total plate count. Although colonies of such contaminants did not appear in the enumeration, their previously released enzymes might still be active and catalyze free histidine to histamine [19]. Such enzyme driven changes potentially resulted in an increase of histamine levels in samples, although there was lower enumeration of Enterobacteriaceae. As our research focused on the histamine forming bacteria belonging to family of Enterobacteriaceae (routinely examined in the quality control department of the PT. Intimas Surya), *Violet Red Bile Dextrose Agar* (*VRBD Agar*) medium was applied to determine the total counts of bacteria belonging to family of Enterobacteriaceae, and the results are shown in Table 2. Therefore, the presence of histamine forming bacterial contaminants in addition to those belonging to family of Enterobacteriaceae would result in high level of histamine detected in the samples plated onto such medium, although the cfu/mL was lower than expected. According to Chung et al. [17] and Visciano et al. [12,13] once produced in the seafood products, histamine will remain stable although the contaminated foods are processed by cooking, freezing, or canning, as this compound is heat stable.

Histamine is an undesired compound in seafoods, as intoxications of such compound in human body will produce the same allergenic symptoms with the endogenously released histamine [33], although the mechanisms of allergenic reactions could be different. Such symptoms will occur in people who consume seafoods with histamine levels beyond their physiological ranges [13,33,34]. According to Hungerford [33] therefore, it is important to develop clinical methods with capability to distinguish whether the allergenic reactions are caused by dietary histamine or endogenously released histamine by human body.

Bacteria especially those belonging to the family of Enterobacteriaceae have been reported to increase histamine level in seafoods and their derivatives [35,36]. As contamination by mesophilic histamine producing bacteria commonly happens while the seafood products are in fresh conditions, the activity and growth of such contaminants need to be controlled before stable low temperature is reached during storage. *Escherichia coli* (being a member of mesophilic bacteria) is easily found in fresh seafood products [37], because contamination by such bacterial species may occur during handling (after catch but before storage at low temperature).

Histamine levels found in this investigation (Table 2) were much higher than that of allowable levels specified by Japan quality control standard (<50 ppm) [38], but still in the range (100–200 ppm) allowed by European countries [12,13]. Although the presence of histamine at certain levels is still acceptable, efforts to reduce its level in processed seafood products need to be continuously investigated and optimized to improve seafood safety and avoid rejection of such seafood products by importers. The histamine level of samples is not necessarily related to bacterial total count (Table 2). It implicitly suggested that levels of histamine depend on types of bacteria contaminating the samples and their metabolic process in producing histamine and not on the total cfu/mL [39]. Therefore, histamine producing bacteria likely contributed to the histamine content of seafood products, instead of the non-producers [40,41]. Highest rate of histidine decarboxylase enzyme production by its producers occurred at temperature of between 20 and 37°C [42]. This mesophilic stage might contribute to the histamine production by *E. coli* in the tuna loin before stable low temperatures (4, 2, and −2°C) were reached in our experiments (Tables 2–4). In addition, histidine decarboxylase enzyme which potentially accumulated during this mesophilic stage remain active (with reduced activity rate) at temperature of > −8°C or might totally become inactive at temperature of <−8°C [43]. This suggested that histamine accumulation in seafood products investigated in our study can be due to active growth of histamine producing bacteria, accumulation of histidine decarboxylase enzyme previously produced by its producers, or combinations of these. This result corroborated with findings of previous study by Garcia-Tapia et al. [15].

Table 1
Histamine levels of the 27 tuna loin samples.

Sample codes	Histamine levels (ppm)	Sample codes	Histamine levels (ppm)	Sample codes	Histamine levels (ppm)
1	102.13 ± 6.07	10	84.43 ± 2.30	19	96.07 ± 4.69
2	80.43 ± 0.21	11	104.17 ± 4.61	20	93.13 ± 1.83
3	82.67 ± 3.73	12	92.77 ± 3.29	21	121.17 ± 8.43
4	81.50 ± 0.75	13	82.3 ± 1.80	22	96.03 ± 3.46
5	96.95 ± 0.43	14	90.83 ± 5.24	23	97.3 ± 1.55
6	96.87 ± 8.44	15	92.57 ± 2.25	24	103.43 ± 5.42
7	83.57 ± 2.44	16	152.37 ± 18.39	25	99.13 ± 7.08
8	102.27 ± 4.90	17	99.27 ± 4.30	26	92.03 ± 4.04
9	105.7 ± 11.28	18	97.93 ± 0.40	27	101.43 ± 9.35

Values in Table 1 ± standard deviation are averages of triplicates. Sample codes 9, 11, 16, 21, & 24 were analysed in further studies (subjected to Enterobacteriaceae enumeration).

Table 2
Histamine levels and bacterial total counts in the 5 tuna loin samples.

Sample	Histamine level (ppm.)*	Total count of bacteria belong to family of Enterobacteriaceae (logcfu/g sample)*
9	105.7 ± 11.28	2.31 ± 0.12
11	104.17 ± 4.61	1.90 ± 0.09
16	152.37 ± 18.39	2.20 ± 0.14
21	121.17 ± 8.43	2.48 ± 0.11
24	103.43 ± 5.42	2.65 ± 0.03

* Values in Table 2 ± standard deviations are averages of triplicate measurements.

Table 3
Histamine formation by *E. coli* in TSBH medium following 8 h incubation at various temperatures.

Temperature (°C)	Histamine concentration (ppm)*	
	4 h incubation	8 h incubation
−2	0.10 ± 0.10^{c}	0.40 ± 0.10^{c}
2	0.40 ± 0.10^{c}	0.63 ± 0.12^{c}
4	79.73 ± 3.15^{b}	88.33 ± 2.76^{a}

Values in Table 3± standard deviation are averages of triplicate measurements. Values followed by the same letter in the same column are not statistically different ($p > 0.05$) based on Duncan analysis following ANOVA. TSBH medium was frozen at −2°C, and thawed prior to analysis. Prior to use in this experiment, the level of histamine in this medium was confirmed to be zero (0 ppm).

Table 4
Histamine formation (ppm histamine) by *E. coli* in TSBH medium treated singly with LAB or in combination with various concentrations of salt (NaCl) and incubated at 2°C for 8 h.

LAB suspension at density of (10^5 cells/mL)	Salt concentrations (% *w/v*)*		
	0.0	2.0	4.0
0.0	1.73 ± 0.25 a	0.87 ± 0.25 b	0.33 ± 0.35 bc
5	0.30 ± 0.10 bc	0.20 ± 0.10 c	0.10 ± 0.10 c
10	0.20 ± 0.26 c	0.20 ± 0.00 c	0.03 ± 0.06 c
20	0.10 ± 0.10 c	0.10 ± 0.10 c	0.00 ± 0.00 c

* Values in Table 4± standard deviations are averages of triplicate measurement. Values followed the same letter(s) are not statistically different ($p > 0.05$) based on Duncan analysis following ANOVA.

The predominant colonies contaminating our loin samples were confirmed to be *E. coli*, following alignment of their 16 s rDNA sequence with those deposited at the GeneBank (Fig. 2A). This species showed its ability to convert histidine into histamine *in vitro* in the TSBH medium when incubated at various temperatures (Table 3). The histamine level indicated in Table 3 must be due to activities of *E. coli.* The rate of histamine formation was the highest at temperature of 4°C following incubation for 4 and 8 h with histamine levels of 79.73 ± 3.15 and 88.33 ± 2.76 ppm, respectively. A decrease in temperature of incubation appeared to significantly suppress histamine formation by this isolate (Table 3). It can be seen in Table 3 that histamine produced by *E. coli* at −2°C was lower than that produced at 2°C, but these histamine levels in these two temperatures are not statistically different ($p > 0.05$). Therefore, incubation temperature of 2°C was then chosen in the main experiment (*in vitro* and *in vivo* experiments), as it provides greater advantages in term of reducing electrical energy expenditure.

Although *E. coli* was found not to be a significant contributor to accumulate histamine level in seafood products in a previous study by Behling and Taylor [44], our results are in line with those reported by investigators, such as Bjornsdottir et al. [35] who found that this species has capability to convert free amino acid histidine into histamine. This indicates that *E. coli* isolated in our study also has capability to produce histamine decarboxylase involved in the conversion of free histidine into histamine (Table 3). The reason why *E. coli* produced different levels of histamine following incubation at 2°C for 8 h (as shown in Tables 3 and 4) is still unclear.

Results presented in the Table 3 revealed that histamine formation by *E. coli* is significantly affected by incubation temperature. An increase in temperature by 4°C (from −2°C to 2°C) was found to increase histamine levels in tuna loins by about 300% and 50% in samples incubated for 4 and 8 h, respectively from their baseline levels at −2°C. However, these values were not statistically significant ($p > 0.05$) when compared to other samples at 2°C. Significant increase in histamine levels occurred when the incubation temperature was increased to 4°C, where its levels approximately were 800 and 220 folds higher than those incubated at −2°C for 4 h and 8 h incubation, respectively (Table 3). At low temperature (lower than water freezing point) enzyme activity tends to be very low or even decrease to zero [13,45]. According to Ghaly et al. [46] and van der Sman [27], water activity of frozen materials is equal to zero and this slows or stops catalytic activity of enzymes. Data in Table 2 indicated that potential activity of *E. coli* enzyme started to increase significantly when the samples were incubated at 4°C or higher. This aligned to that reported by Rossano et al. [47]. Also, at 4°C or more, the levels of histamine in samples appeared to be affected by length of incubation period.

The LAB (BY-45) used in our study was identified as *Lactiplantibacillus plantarum,* as its 16 s rDNA sequence aligns with those deposited at the GeneBank. The position of this isolate in the phylogenetic tree is shown in Fig. 2B. Suppression of histamine formation by *E. coli* following exposure to our LAB isolate (*L. plantarum* BY-45), salt, or combination of L. *plantarum* BY-45 and salt at various concentrations was studied *in vitro* in TSBH medium at 2°C. The results are presented in Table 4. In this *in vitro* test, inoculation of L. *plantarum* BY-45 to TSBH medium with *E. coli* in it significantly suppressed histamine formation. Our result is in line with those reported by Lee et al. [48] and Kung et al. [49] who found that L. *plantarum* decreased the histamine formation during *miso* fermentation. They concluded that the biogenic amine reduction was due to the activity of their isolate to degrade such compounds. The efficacy of L. *plantarum* isolate to inhibit formation of biogenic amine in salted mackerel (*Scomberomorus niphonius*) and Chinese rice wine fermentation were also recently reported by Zhang et al. [50] and Xia et al. [51], respectively. The mechanisms by which our LAB isolate (*L. plantarum* BY-45) suppressed *E. coli* to produce histamine in tuna

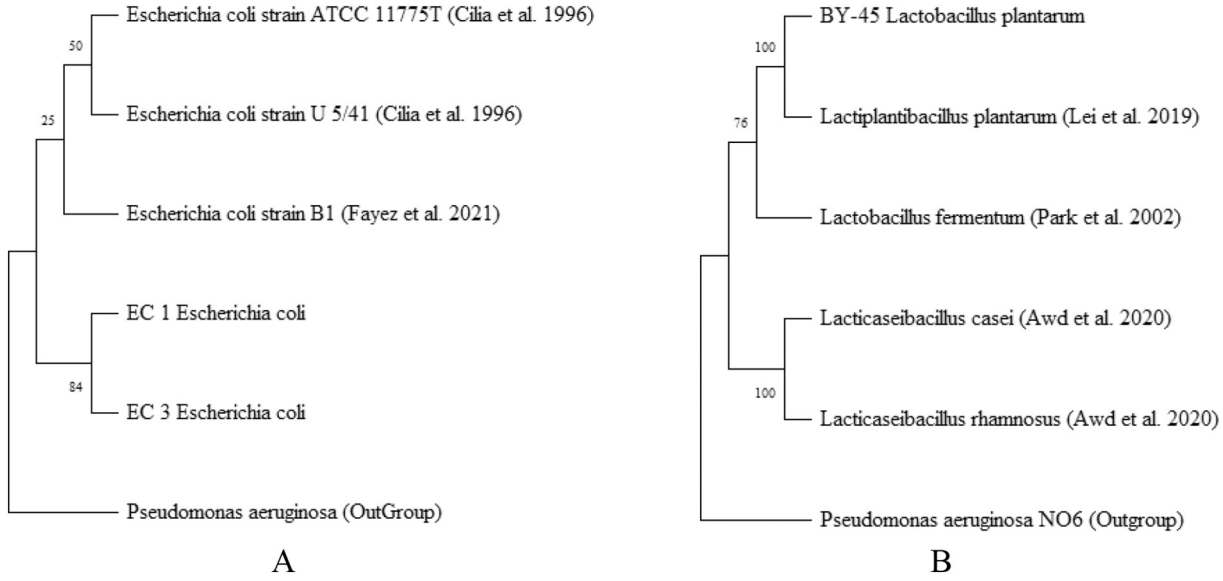

Fig. 2. The predominant colonies (suspected to be histamine producing bacterium) isolated in our study and LAB isolate (*L. plantarum* BY-45) used in this study to suppress histamine formation both *in vitro* and *in vivo*. They were identified as *E. coli* (isolates EC1 and EC3 obtained in our study) (A) and *Lactiplantibacillus plantarum* (B), following alignment of their 16 s rDNA sequences with their counterparts deposited in the GeneBank.

loins are not completely clear yet (but there is an indication of histamine degradation by our isolate). This was initially indicated by reduction of histamine level in the TSB with added histamine in it, following inoculation with viable cells of L. *plantarum* BY-45, but not with heat killed cells of this isolate (Supplementary data). Similar results were also reported by Kung et al. [52], Jiang et al. [53], and Li et al. [54]. Therefore, there is a need to further study our isolate in order to comprehensively elucidate the mechanisms of this phenomenon. Some reports have also suggested that the inhibition of undesired microorganisms by LAB could be due to one or more of the following mechanisms: (i) production of acidic compounds [55], (ii) competition [56], or (iii) production of toxic compounds, such as bacteriocin or antibiotics [57]. Our results appeared to be in line with that suggested by Khalid [55] as our isolate tended to decrease the pH of medium following incubation for at least 24 h. This indicated that acid formation by our LAB isolate had contribution to the inhibition of histamine formation by *E. coli*.

Suppression of histamine formation in *E. coli* appeared to be more significant when L. *plantarum* BY-45 was combined with various concentrations of salt (Table 4). The levels of suppression were proportional with the concentration of salt and cell density of the LAB in the suspension, either applied singly or in combination. Histamine formation at 2°C incubation was even eliminated when the density of the LAB in the suspension of 4% *w/v* salt (NaCl) was 20×10^5cfu/mL. *E. coli* plays minor role in histamine production at 2°C as only 1.73 ± 0.25 ppm histamine was produced by this isolate in cultures without inoculation of LAB and addition of salt, and therefore low temperature limited contribution to increased histamine level in the samples. These results may suggest that high histamine level in tuna loin samples might be produced by bacteria belonging to non-Enterobacteriaceae families but did not show growth response in the selective medium used in our study.

When compared to zero control (treatment with no salt and no L. *plantarum* BY-45), salt at concentrations of 2 and 4% were found to significantly ($p < 0.05$) decrease the rate of histamine formation in the *in vitro* experiments in TSBH medium inoculated with *E. coli*, although the results indicated that these two salt concentrations were not statistically significant ($p > 0.05$) with other treatments (cultures of TSBH medium with salt and *E. coli* in it and inoculated with L. *plantarum* BY-45 suspension at various levels) (Table 4). This means that the presence of L. *plantarum* BY-45 slightly suppressed histamine formation either in the absence or in the presence of salt, but non-significant statistically at $p < 0.05$ (Table 4). Inhibitory effect of salt on *E. coli* was also reported by Paramasivam et al. [58], who found that salt concentration of 10% apparently suppressed the growth of histamine producing bacteria in fish samples which in turn decreased the rate of histamine formation. Similar phenomenon was also reviewed by Ghaly et al. [46] where application of salt at concentration of 1 to 2% *w*/w on meat can stabilize its microbial composition and hence prolonged its storage time. The presence of salt in a solution will increase its osmotic pressure, and this would have inhibitory effect on the growth of microorganisms [59]. In extremely high salt concentration (hypertonic), this condition may kill all microbes, and therefore high salt concentration is often used to preserve seafood products [46,60].

Suppression of histamine formation by L. *plantarum* BY-45 and salt concentration was found to be consistent in the *in vivo* experiment, where 4% salt in combination with 20×10^5 cell/mL of L. *plantarum* BY-45 suspension (with *E. coli* added at density of 5×10^5cfu/mL) (A1B1) reduced histamine level by 94%, relative to control treatment (tuna loins dipped in *E. coli* suspension only at density of 5×10^5cfu/mL) (A1B0) (Table 5). This result was found to be relatively improved when compared to those obtained from loins treated with salt (A0B0) or LAB (A0B1) only, but not significant statistically at $p < 0.05$ (Table 5).

All samples treated with salt (2 or 4% salt concentration) showed lower histamine level when compared with those of control (samples without salt addition). *L. plantarum* BY-45 and *E. coli* at cell densities of 20×10^5 cfu/mL and 5×10^5 cfu/mL, respectively in various concentrations of salt solutions (A1B1) was found to significantly suppress histamine formation in tuna loins previously dipped in it, when compared to those inoculated with *E. coli* only (A1B0) at all levels of salt (Table 5). This indicated antagonistic activity of L. *plantarum* BY-45 against *E. coli*.

Table 5
Histamine formation (ppm histamine) on 25 g of tuna loins dipped in various salt concentrations with LAB (*L. plantarum* BY-45) density of 20×10^5 CFU/mL and *E. coli* density of 5×10^5 CFU/mL, and incubated at 2°C for 8 h.

Treatments	Salt concentrations (% w/v)*		
	0	2	4
Tuna loins dipped in solution without LAB and *E. coli* (A0B0)	1.13 ± 0.23 b	1.03 ± 0.06 b	0.90 ± 0.10 b
Tuna loins dipped in solution with *E. coli* only at density of 5×10^5 cells/mL (A1B0)	13.27 ± 1.50 a	13.30 ± 0.85 a	13.10 ± 0.72 a
Tuna loins dipped in solution with LAB only at density of 20×10^5 cells/mL (A0B1)	1.07 ± 0.06 b	0.93 ± 0.12 b	0.87 ± 0.12 b
Tuna loins dipped in solution with LAB at density of 20×10^5 cells/mL and *E. coli* at density of 5×10^5 cells/mL(A1B1)	0.93 ± 0.23 b	0.90 ± 0.10 b	0.80 ± 0.10 b

* Values in Table 5±standard deviation are averages of triplicate measurement. Values followed the same letter(s) are not statistically different ($p > 0.05$) based on Duncan analysis following ANOVA.

The levels of histamine on tuna loins inoculated with L. *plantarum* BY-45 only (A0B1) was not statistically significant ($p > 0.05$) with that observed in zero control (A0B0) in all salt concentrations applied (Table 4), indicating that L. *plantarum* BY-45 used in this study did not contribute to the histamine formation. If only *E. coli* was present in tuna loin, 2% *w/v* salt solution with L. *plantarum* BY-45 density of 20 x10^5cfu/mL and incubation temperature of 2°C could be recommended to reduce the rate of histamine formation on tuna loins in post-harvest storage conditions.

Exporters in Indonesia generally adjust their storage temperature for seafood products at minus 2 degree (−2°C). In our findings, application of L. *plantarum* BY-45 in combination with salt during storage decreased the rate of histamine formation, although the storage temperature was higher at 2°C (4°C higher than that normally applied by seafood exporters). In term of energy expenditure, an increase in storage temperature by 4°C will result in significant energy savings during storage and this will lead to reduction of seafood production costs. To obtain ideal storage temperature for seafood products, comprehensive studies on histamine forming bacteria other than *E. coli* contaminating seafood products (particularly major histamine producers) need to be further investigated, because many of them have ability to produce histidine decarboxylase at temperature of lower than −2°C [36]. It must be stressed in this study that if only *E. coli* (as indicated in a study by Bjornsdottir et al. [35] and a minor histamine producer in an older study by Behling and Taylor [44]) is the main contaminant in seafood products, increasing the storage temperature up to 2°C can then be recommended following application of L. *plantarum* BY-45 suspension singly or in combination with salt solution to such products.

Although our L. *plantarum* BY-45 showed significant contribution to increasing storage temperature in this experiment, its long term use in fishery industries need to consider criteria specified by Ghaly et al. [46] and Ghanbari et al. [56], as follows: (1) This LAB strains must be genetically stable; (2) Bacteriocins produced by this LAB must have wide spectrum for control of bacterial pathogens; (3) This LAB should be able to survive well and be active to produce inhibitory metabolites at low temperature; (4) Application of this LAB as food preservative need to consider the regulation of countries where raw or processed seafood products are exported.

4. Conclusion

This study clearly demonstrated that L. *plantarum* BY-45 showed

significant contribution to suppress the rate of histamine formation by *E. coli* isolated from tuna loin samples during post-harvest storage. When applied in combination with salt, suppression of histamine formation in processed tuna loin was significantly improved. Combination of L. *plantarum* BY-45 suspension at density of 20×10^5 cfu/mL in 4% *w/v* NaCl totally eliminated histamine formation in an *in vitro* assay even though incubated at 2°C (4°C higher than storage temperature applied by most Indonesian seafood exporters) and indicating potential energy use efficiency. This provides a foundation to integrate beneficial lactic acid bacteria for managing food safety and quality while enhancing overall health benefits from reduced histamine production.

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Author contributions

YR designed the experiment, supervised the work, and wrote the manuscript; AFO carried out the experiments, analysed data, did administration; IMAGW supervised the work, analysed data, and drafted the MS; NMT carried out the experiment and analysed data; DS edited the MS and analysed data statistically; KS supervised the work and edited plus fine tuned the MS prior to submision.

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KS: Kalidas Shetty

Ethical statement

NA.
This study did not use any human and animal subjects.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nfs.2023.05.001.

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