Nitrite Reduction in Edible Bird's Nests Using *Pediococcus pentosaceus* and *Lactobacillus plantarum*

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Abstract. Edible bird's nests (EBNs), a highly valued ethnomedicinal product, are well-known for their nutritional and therapeutic benefits. Currently, Indonesia is the largest global producer and exporter of EBNs. However, due to high nitrite levels, China has banned the import of EBNs from Indonesia and Malaysia. Nitrite forms in EBNs naturally, driven by fluctuations in nitrogen levels within the swiftlet farmhouse. Since nitrite leads to the formation of methemoglobin, which hinders oxygen transport, it can be toxic and harmful. Therefore, reducing nitrite in EBNs is crucial. This study investigates the effectiveness of *P. pentosaceus* and *L. plantarum* in lowering nitrite levels in EBNs. The results indicate that applying *P. pentosaceus* and *L. plantarum* significantly ($p<0.05$) reduced nitrite levels of EBNs. Spraying EBNs with 1.2×10^8 CFU/g of *P. pentosaceus* and *L. plantarum* decreased nitrite by 84.65% (1.65±0.17 ppm) after 1 hour, 82% (1.94±0.02 ppm) after 3 hours, and 78% (2.27±0.2 ppm) after 6 hours. These findings demonstrate that nitrite levels in EBNs can be effectively reduced using *L. plantarum* and *P. pentosaceus*. This method is cost-effective, safe, easy to implement, leaves no residue, and yields excellent results, making it suitable for large-scale nitrite processing in EBNs factories.

Keywords: Edible bird nests, *P. pentaceous*, *L. plantarum*, Nitrite

1   Introduction

Edible bird's nests (EBNs), a highly valued ethnomedicinal product, are well-known for their nutritional and therapeutic benefits. Currently, Indonesia is the largest global producer and exporter of EBNs. EBNs contain protein, minerals, and omega-6 beneficial for physical health [1]. In Chinese society, EBNs is said to have health benefits such as anti-aging and boosting the immune system [2].

As the exporting country of EBNs, Indonesia is confronted with the challenge of providing high-quality products. One of the challenges is the high levels of nitrate found in the EBNs. The Indonesian National Standards Agency [3] stipulates that the maximum permitted level of nitrite in EBNs is 80 ppm. However, the regulatory requirement for EBNs exported to China is a lower maximum limit for nitrite. The Chinese government has set a maximum permitted nitrite level of 30 ppm.

The formation of nitrite in EBNs is a naturally occurring process driven by fluctuations in nitrogen levels within the Swiftlet farmhouses. The formation of nitrite in EBNs occurs naturally as a result of fluctuations in nitrogen levels in the environment. Nitrite can be synthesized from ammonia by bacteria through anaerobic fermentation. The nitrite is produced in the nests and absorbed by the swallow nest environment, mainly from the decomposition of organic waste in the soil [4]. Nitrogen, which is present in large amounts in the air, cannot be used directly by living organisms. The nitrogen in the form of ammonia must be converted to nitrite by nitrifying bacteria and then form nitrite to nitrate [5].

A variety of techniques have been developed for the degradation of nitrite, including oxidation, reduction, physical adsorption, bacterial degradation, water fertilization, and microbial degradation. The degradation of nitrite by microbes has been demonstrated to have a beneficial impact on the food industry with no adverse effects. The lactic acid bacteria present in probiotics are capable of degrading nitrite and nitrate, while other bacteria can also degrade nitrogen species, such as ammonia and nitrite.

Lactic acid bacteria (LAB) possess the capacity to degrade complex compounds into simpler compounds, ultimately converting them into lactic acid. The acidic product has the potential to inhibit the growth of pathogenic bacteria [8]. Some characteristics of LAB are classified as gram-positive, non-spore-forming, cocci or bacilli-forming, and generally catalase-negative. A number of studies have demonstrated that lactic acid bacteria are capable of degrading nitrite. Other studies have demonstrated that *L. plantarum*, *Leuconostoc mesenteroides*, *P. pentosaceus* and *L. casei* sub sp. *rhamnosus* are highly effective nitrite degraders. *P. pentosaceus* is capable of producing nitrite reductase (NiR), which is involved in the degradation of nitrite [11]. As stated by [12], *L. plantarum* exhibits an exceptional capacity to degrade nitrite levels. Beyond its capability to remove nitrite, the lactic acid bacteria promoters *L. plantarum*, *P. pentosaceus*, and *L. mesenteroides* have been demonstrated to effectively reduce nitrite formation by inhibiting the growth of nitrifying bacteria [13].

The investigation of methods to reduce the nitrite content of EBNs through the use of lactic acid bacteria represents a significant area of research, given the potential for such techniques to mitigate the toxic effects associated with nitrite. Nitrite has been identified as a toxic and dangerous substance capable of causing methemoglobinemia, a condition that results in a reduction in oxygen flow and respiratory difficulty. Lactic acid bacteria also demonstrate effective degradation of nitrite. In light of the description, as mentioned earlier, the objective of this study is to ascertain the efficacy of utilizing *P. pentosaceus* and *L. plantarum* in reducing nitrite levels in EBNs.

2   Materials and Methods

2.1   Samples of EBNs

A total of 20 EBN samples, with a weight range of 4 to 6 grams, were obtained from Kateng Village in the Central Lombok Regency. The samples were initially subjected to a cleaning process with the objective of removing any flecks, feathers, and fine dirt adhering to the EBNs. The cleaning process was conducted through two methods: spraying and brushing. Subsequently, the samples were subjected to drying via a blower. This was done to ensure that the samples remained free from contamination by microorganisms.

2.2   Isolation of *P. pentosaceus* and *L. plantarum*

Isolation was conducted by preparing liquid MRS media and allowing the growth of bacterial isolates of *Pediococcus pentosaceus* and *Lactobacillus plantarum*. The isolates were obtained from glycerol stocks stored in the Microbiology Laboratory of the Faculty of Animal Science and cultured on liquid MRS media using a 37°C incubator for 24–48 hours in an anaerobic environment.

2.3   Incubation of EBNs Samples and Nitrite Measurement

Following the isolation of the bacteria *P. pentosaceus* and *L. plantarum*, the subsequent step is to mix the bacteria with 9 mL of a NaCl solution. The mixture was homogenized using a Vortex Mix II. Samples were sprayed on every 1 gram sample that had been placed on aluminum foil. Each sample consisted of spraying 1 mL and 2 mL. Subsequently, each sample was marked and incubated for three different times (1 hour, 3 hours, and 6 hours) at an incubator temperature of 37 °Celcius. Nitrite levels were then measured by the spectrophotometric method.

2.4   Experimental Design

Microbial cell counts were conducted indirectly via the Total Plate Count (TPC) method. The objective was to determine the concentration of bacteria present prior to inoculation with an edible bird nest's. Subsequently, 1 ml of microbial suspension was diluted in physiological saline solution to a volume of 9 ml, forming dilution 10-1, and this process was repeated until dilution 10-8 was reached. A volume of 1 ml from each of the three highest dilutions was transferred to a sterile petri dish, followed by the addition of 15 ml of PCA medium. The Petri dishes were agitated in order to ensure that the suspension and the medium were thoroughly mixed (the Pour Plate Method was employed). Following the solidification of the medium, the samples were incubating in the incubator 'Gallenkamp' within 370C for 24 hours. Following the incubation period, the number of colonies was enumerated using a colony counter. Subsequently, the results of the colony count were converted into colony-forming units (CFU) per

gram. After that the nitrite concentration measure with the spectophotometer in the Laboratory.

A completely Randomized Design with 2 x 3 pattern was employed to determine the effect of *P. pentosaceus* and *L. plantarum* in reducing nitrite in EBNs. The use of factorial RAL has two factors, namely, the first factor (F1) is the concentration of bacteria *P. pentosaceus* and *L. plantarum* with two levels and the second factor (F2) is the length of incubation using three levels. Each level was replicated three times. 1.2 x 108 CFU/g concentration (1 ml and 2 ml) and three incubation times (1 hour, 3 hours, and 6 hours).

2.5   2.5 Data Analysis

Data from the study were analyzed using Analysis of Variance (ANOVA). If there are any differences in mean, Duncan's multiple range test (DMRT) will be applied. The data analysis was done using IBM SPSS version 21 software. The statistical model used was the analysis of variance (ANOVA):

$$yijk = \mu + \alpha i + \beta j + \alpha\beta(ij) + \varepsilon ij \tag{1}$$

Where yijk represents the observed value of each individual, μ denotes the overall mean, αi represents the effect of the concentration of bacteria factor, βj represents the effect of the length of incubation factor, $\alpha\beta(ij)$ signifies the interaction between these two factors, and εij denotes the residual error (variation among replicates for each treatment).

2.6   Reduction of Nitrite Levels and Data Analysis

Reduction of nitrite content using UV Spectrophotometer method. Nitrite (NO_2) levels were obtained by substituting the sample absorbance into the formula :

$$Nitrite\ (NO_2) = (ppm\ from\ curva\ regression\ x\ fp)/(\ sample\ weight)\ x\ 100\% \tag{2}$$

3   Results and Discussion

The EBN samples were procured during the rainy season. During this period, EBNs exhibit robust growth, resulting in increased salivary production. Additionally, swallow nest samples are observed to be larger, thicker, and markedly swollen, with a reduced quantity of feces [16]. In comparison to the rainy season, the dry season is characterized by a higher prevalence of feces and feathers within EBNs. This is believed to be closely associated with the rate of nest formation in the rainy season in comparison to the dry season, which is influenced by the availability of food resources during the rainy season. It is hypothesized that the longer the nest is formed, the greater the likelihood of swallow feathers falling out and adhering to the nest. This may increase the quantity of dirt observed [17].

The number of colonies was calculated indirectly using the Total Plate Count (TPC) method. The isolate suspension was diluted with physiological saline solution (NaCl

0.9%) in increments of 9 ml, up to a dilution of 10^{-8}. A total of 1 ml was extracted from each of the three highest dilutions and transferred to a sterile Petri dish. The recommended number of live lactic acid bacteria cells in the digestive tract to achieve a beneficial effect on health is 10^6–10^8 colony-forming units (CFU) per gram.

Table 1. TPC count of *Pediococcus pentosaceus* and *Lactobacillus plantarum*.

Lactat Acid Bacteria	Dilution (NaCl)			Number of Microbes
	10^{-6}	10^{-7}	10^{-8}	CFU/g
Pediococcus pentosaceus	367 x 10^6	153 x 10^7	83 x 10^8	3,7 x 10^8
Lactobacillus plantarum	117 x 10^6	28 x 10^7	3 x 10^8	1,2 x 10^8

Table 1 indicates that the TPC calculation of *P. pentosaceus* is 3.7 x 10^8 CFU/g. In comparison, *L. plantarum* has a bacterial count of 1.2 x 10^8 CFU/g. The calculation of bacteria is in accordance with the standards or regulations set forth by Fardiaz (1992). In the TPC results, *P. pentosaceus* was observed to select colonies at the 10^{-6} dilution, as this resulted in colonies of ≥ 300. In contrast, *L. plantarum* selected colonies at a 10^{-6} dilution. As one of the probiotics, it is hypothesized that LAB has a significant effect at a minimum concentration of 10^6 CFU/g. It is thought that higher numbers of live cells in the sample result in increased biological effects [18].

Table 2. Characteristic of EBNs.

Number	Characteristic	Before Spraying		After Spraying		
				L1	L2	L3
1	Colour	White yellowish	P1	White	White	White
			P2	White	White	White
2	Texture	Rough	P1	Rough	Rough	Rough
			P2	Slightly rough	Slightly rough	Slightly rough
3	Fragrance	Fish smell	P1	Fish smell and soft	Fish smell and soft	Fish smell and soft
			P2	Fish smell and soft	Fish smell and soft	Fish smell and soft

The observed characteristics, including color, texture, and aroma, of EBNs are presented in Table 2. Prior to bacterial spraying and incubation, the color of the EBNs was yellowish-white. Following treatment, however, they exhibited a pure white coloration. The color of EBNs is typically white to pale brown. The most common colour is white, although some nests exhibit slight shades of brown or yellow. As stated by [19], EBNs are typically white, transparent, and crystalline. This phenomenon can be attributed to the manner in which the nests are constructed. When the building process is executed effectively, the resulting nest will exhibit a white, transparent, and crystalline composition. Furthermore, the color of EBNs can be influenced by the surrounding environment in which these birds construct their nests.

EBNs exhibit a distinctive texture that varies considerably depending on the species of swallow that constructs them, the surrounding environment, and the method of nest building employed. As illustrated in Table 2, the texture of EBNs prior to bacterial spray treatment and incubation is characterized by a rough and hollow texture. Following the bacterial spray treatment and incubation period, the texture of the EBNs remained noticeably rough in treatment 1 (P1) of 1 ml bacterial spray. However, a slight degree of roughness was observed after the treatment.

Table 3. Results for Nitrite Levels in EBNs Samples[1].

Treatment	Before Treatment	Nitrite (ppm) After Treatment		
		L1	L2	L3
P1	10,75± 0,02	$1,65 \pm 0,17^a$	$1,94 \pm 0,02^a$	$2,27 \pm 0,2^b$
P2		$2,47 \pm 0,13^b$	$1,78 \pm 0,22^a$	$2,45 \pm 0,12^b$

P1L1: Volume of suspension 1 ml and incubation for 1 hour
P1L2: Volume of suspension 1 ml and incubation for 3 hours
P1L3: Volume of suspension 1 ml and incubation for 6 hours
P2L1: Volume of suspension 2 ml and incubation for 1 hour
P2L2: Volume of suspension 2 ml and incubation for 3 hours
P2L3: Volume of suspension 2 ml and incubation for 6 hours
[1]Data were presented as mean ± standard deviation
[a-b] differences in superscript indicate a significant difference ($P < 0.05$)

Nitrite is a byproduct of the ammonia breakdown process, which is known as nitridation. The ammonia breakdown process is divided into two stages : firstly, the process of ammonia breakdown into nitrite, and secondly, the process of nitrite breakdown into nitrate. As stated by [20], the nitrification process comprises two distinct stages : the formation of nitrite (nitrification) and the conversion to nitrate (nitrification). The reduction of nitrite levels can be achieved through the utilization of the nitrite-degrading lactic acid bacteria, which possess the enzyme nitrite reductase. Lactic acid bacteria are capable of producing the enzyme nitrite reductase (NiR), which is involved in the degradation of nitrite (21). Nitrite reductase from nitrate-reducing organisms, including staphylococci, micrococci, and certain lactic acid bacteria, can convert nitrite (NO_2) into nitrous oxide (NO), which has bactericidal properties and is non-toxic to humans (22). The results of the nitrite level tests on EBNs are presented in Table 3.

The results of the analysis of variance (ANOVA) factorial, as presented in Table 3, indicate that the spraying of *L. plantarum* and *P. pentosaceus* at a concentration of 1.2 x 10^8 CFU/g with fermentation has a statistically significant impact (p<0.05) on the reduction of nitrite levels in swallow nest samples fermented with nitrite-degrading bacteria. Table 3 illustrates that the reduction in nitrite levels varies between individual test samples. In Treatment 1 (P1), the swallow's nest was inoculated with a volume of 1 ml of *L. plantarum* and *P. pentosaceus* at an incubation time of 1 hour (L1), resulting in 84.65% reduction in nitrite levels (1.65 ± 0.17 ppm), 82% reduction (1.94 ± 0.02 ppm) in Treatment 3 hours incubation time (L2), and a 78% reduction (2.27 ± 0.2 ppm) in Treatment 6 hours incubation time (L3). Moreover, in treatment 2 (P2), the swallow

nest inoculated with a volume of 2 ml of *L. plantarum* and *P. pentosaceus* at an incubation time of 1 hour (L1) exhibited a reduction in nitrite levels by (77%, 2.06 ± 0.47 ppm), 83% (1.78 ± 0.22 ppm) in the treatment of 3 hours incubation time (L2), and 77.2% (2.45 ± 0.12 ppm) in the treatment of 6 hours incubation time (L3).

The results of Duncan's test demonstrate that the mean nitrite concentration of swallow's nests treated with a spray containing *L. plantarum* and *P. pentosaceus* at a volume of 1 ml (P1) and an incubation period of 1 hour (L1) is markedly lower ($p < 0.05$) than that observed at the 6-hour incubation period (L3). However, the incubation period of one hour (L1) was not found to be significantly different ($p > 0.05$) from the incubation period of three hours (L2). The mean nitrite concentration of the swallow nest in treatment 2, which involved spraying *L. plantarum* and *P. pentosaceus* at a volume of 2 ml (P2) with an incubation period of 3 hours (L2), was found to be significantly lower ($p < 0.05$) than that of the incubation period of 6 hours (L3). Nevertheless, the incubation period of six hours (L3) was not statistically different ($p > 0.05$) from the incubation period of one hour (L1). This is attributable to the high water content of the swallow's nest sample. Our results indicated that the lower concentration and short incubation time was obtained in combined of L1 and P1 treatment. These result strongly suggest to reduce nitrite concentration in EBN. Moreover, both combined treatments were efficient in inoculum and timeless for incubation. So, these condition lead to cost-effective suitable for large-scale nitrite processing in EBNs factories.

Bacteria typically require water in order to engage in metabolic processes and to maintain viability. However, an excess of water in the sample can have an impact on the metabolic activity of the bacteria. As posited by [23], the quantity of water present within the substrate is a significant variable to be taken into account during the fermentation process, given that the water content exerts a profound influence on the growth of the microorganisms that play a pivotal role in the fermentation process. A smaller ratio of substrate to water is conducive to optimal enzyme production, as it facilitates enhanced contact between the substrate and microbes. A deficiency in water content will result in the microbes entering a dormant state, whereas an excess will impede the movement of air within the substrate. The low nitrite level observed in EBNs treated with 1 ml of *L. plantarum* and *P. pentosaceus* for one hour (1.65 ppm) can be attributed to the ability of these bacteria to reduce nitrite levels. This is consistent with the function of the nitrite reductase enzyme in *L. plantarum* and *P. pentosaceus*, which plays a pivotal role in reducing nitrite levels. As stated by [24], *P. pentosaceus* is capable of producing nitrite reductase (NiR), which plays a role in the degradation of nitrite. [25] reported that *L. plantarum* exhibits an exceptional capacity for nitrite degradation.

The EBNs were of a domestic origin. The nests exhibited elevated nitrite levels. This is attributed to the confined and stuffy condition of the house, coupled with inadequate ventilation, which is postulated to accelerate the oxidation of nitrate to nitrite. In conditions of low light, the activity of nitrifying bacteria serves to accelerate the nitrification process. The storage of EBNs that have the potential to inhibit the increase in nitrite levels is at a temperature conducive to the preservation of biological matter, namely in a freezer and a vacuum-sealed package. In addition, suboptimal cultivation practices employed by swallow farmers have been identified as a contributing factor to elevated nitrite levels in EBNs [26]. In light of the findings of this study, the use of lactic acid

bacteria, including *L. plantarum* and *P. pentosaceus*, to reduce nitrite levels represents a potential strategy for ensuring that EBNs are ready for direct trade with consumer countries.

4   Conclusion

These findings demonstrate that nitrite levels in EBNs can be effectively reduced using *L. plantarum* and *P. pentosaceus*. The method is cost-effective, safe, easy to implement, leaves no residue, and yields excellent results, making it suitable for large-scale nitrite processing in EBN factories.

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