In Vitro screening of ammonia and nitrite-degrading bacteria isolated from broiler chicken (*Gallus gallus domesticus*) intestines and pond sediment of nile tilapia (*Oreochromis niloticus*): A preliminary study

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In Vitro screening of ammonia and nitrite-degrading bacteria isolated from broiler chicken (*Gallus gallus domesticus*) intestines and pond sediment of nile tilapia (*Oreochromis niloticus*): A preliminary study

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Abstract. The high level of ammonia and nitrite is a toxic factor for both poultry and aquaculture animals that directly lead to lower economic benefits. Thus, reducing ammonia and nitrite levels is an essential key for successful culture and is also important to reduce the amount of ammonia and nitrite released into the environment. This study aimed to screen bacteria having a capacity to degrade either ammonia or nitrite *in vitro*. Five bacterial strains previously isolated from broiler chicken (*Gallus gallus domesticus*) intestine and pond sediment of Nile Nilapia (*Oreochromis niloticus*) were used in this study, namely IBP-1, IBP-2, IBP-3, IBP-4, and IBP-5 strains. The screenings were performed using either NH_4Cl containing medium or $NaNO_2$ containing medium to determine the ability of bacteria to reduce ammonia or nitrite respectively. The ammonia and nitrite levels were afterwards measured at the beginning (day 0: before bacterial inoculation), 24h (day 1), 48h (day 2), and 72h (day 3) after the addition of 1 ml of the bacterial suspension. The results showed that the five bacterial isolates were able to degrade the ammonia and nitrite content. The greatest reduction of ammonia was achieved by IBP-4 strain (0.00 mg/l), followed by IBP-5 strain (0.04 mg/l), IBP-1 strain (0.05 mg/l), IBP-3 strain (0.14 mg/l) and IBP-2 strain (0.19 mg/l). IBP-1 and IBP-2 strains showed the highest reduction of nitrite levels with values of 0.01 mg/l and 0.02 mg/l after 72h of bacterial inoculation. These results suggest that the five bacterial strains are potentially used for degrading toxic ammonia and nitrite.

1. Introduction

The demand for poultry, fish, and shrimp products has increased rapidly along with the increase in the world's population and their awareness of consuming healthy protein sources. The maintenance of intensive production for poultry, shrimp, and fish plays an essential role in the capacity to meet the growing demand for animal protein source products, and it also represents a major challenge, since intensive production generates large amounts of waste. The waste from poultry, shrimp, and fish could be a source of water, air, and soil pollution [1].

The feces resulting from poultry contains ammonia especially in the form of ammonium ions (NH_4^+) and free ammonia (NH_3). A chicken with 2 kg of body weight is able to produce 0,15 kg of manure containing ± 2,94% of nitrogen, where it could be a source of ammonia. The highest quantity of ammonia is yielded on poultry farms, where annual production ranges from 0,26 to 0,32 kg per bird [2]. According to Wlazlo *et al* [3], poultry excreted a high amount of nitrogen, ranging from 1,01 to 4,80 g/day, depending on the age of the poultry. In poultry houses, the concentration of ammonia in the air (>25 ppm) reduced the body weight gain, feed conversion, survival ability, carcass conviction rate, and immune system of birds [4].

In aquaculture, ammonia, nitrites, and nitrates (nitrogenous species) are usually the major sources of water pollutants in that they are toxic to aquatic life and aquatic ecosystems including shrimp and fish

[5]. Ammonia, nitrites, and nitrates are by-products of protein metabolism which is not digested completely [6]. Fish can generate fecal waste ranging from 0,2 to 0,5 kg dry matter per kg of feed, where it is implicated in water pollution in aquaculture [5].

The presence of nitrogenous species at high concentrations in both poultry and aquaculture has shown adverse effects. Furthermore, the release of a high level of ammonia and nitrite from poultry farms and aquacultures is harmful to human health. The waste containing ammonia also inflicted complaints from nearby residents due to unpleasant odor [1, 12]. Thus, reducing ammonia and nitrite in poultry and aquaculture production is an essential key for successful culture and is also important to reduce the amount of ammonia and nitrite released to the environment [7]. The use of several bacteria can reduce nitrogenous species (ammonia, nitrite, and nitrate levels) and had been reported to be successful in poultry farms [8], and aquaculture systems [9].

The removal of nitrogenous species in poultry feces and aquaculture involves several stages of nitrogen cycles namely ammonification, nitrification, and denitrification. The initial form of nitrogen from chicken, shrimp, and fish and its waste products is organic matter. The organic matter containing nitrogen is revamped to ammonium (NH_4^+) or ammonia (NH_3) by bacteria or fungi [5]. The ammonia is then converted to nitrite (NO_2^-) and then to nitrate (NO_3^-) mainly by *Nitrosomonas* and *Nitrobacter* species respectively in a process called nitrification. The process of conversion of nitrate to nitrogen gases (N_2) is called denitrification and nitrogen gas returns to the atmosphere [10].

The use of *Bacillus subtilis* (10^8 CFU/ml^{-1}) directly had been reported to maintain the ammonia, nitrite, and nitrate levels [11]. Mi *et al.* [12] reported that *Bacillus coagulans, Lactobacillus plantarum,* and *Bacillus substilis* can decrease the concentrations of nitrogenous species. The study aimed to perform in vitro screening of several bacteria as ammonia and nitrite-degrading bacteria.

2. Materials and Methods

2.1. Preparation of media

Luria Bertani (LB) agar (pH 7.0): 10 g of bacto-peptone; 10 g of sodium chloride; 0.5 g of yeast extract; and 15 g of bacto-agar suspended in 1000 ml of distilled water (dH_2O). Luria Bertani (LB) liquid medium (pH 7.0): 10 g of bacto-peptone; 10 g of sodium chloride; and 0.5 g of yeast extract suspended in 1000 ml of distilled water (dH_2O). Ammonia media: 0.5 g of glucose, and 0.1 g of NH_4Cl suspended in 1000 ml of distilled water. Nitrite media: 0.5 g of glucose, and 0.1 g of $NaNO_2$ suspended in 1000 ml of distilled water.

2.2. Bacterial collection, culture condition and identification

Five strains of bacteria were collected from the Laboratory of Microbiology and Biotechnology, Faculty of Animal Science, University of Mataram (Indonesia). These bacteria had previously been isolated from broiler chicken (*Gallus gallus domesticus*) intestines by Nurbaiti *et al.* [13] and pond sediment of Nile Tilapia (*Oreochromis niloticus*). All bacteria (IBP-1, IBP-2, IBP-3, IBP-4, and IBP-5 strains) from glycerol stock were streaked on Luria Bertani Agar (LB agar) and incubated for 20 hours at 37 ºC. The single colony grown was randomly taken into LB medium (liquid) and incubated for 20 hours at 37 ºC with 120 rpm. Identification of bacteria was carried out by following a few steps including morphological colony properties (form, elevation, and margin), Gram's staining, and catalase test.

2.3. Measurement of ammonia levels

Ammonia media containing 0.5 g of glucose and 0.1 g of NH_4Cl were dissolved in 1000 ml of distilled water. The ammonia level was detected using Hanna Instruments Handheld Colorimeter LR HI700 (resolution 0.01 ppm). Ammonia media were diluted to a detectable range (0.97 ppm) and divided into 20 ml for each treatment. The ammonia level was measured before inoculating bacteria to determine the initial content of ammonia. 1 ml of overnight bacterial culture namely IBP-1, IBP-2, IBP-3, IBP-4, and IBP-5 strains was inoculated to ammonia media for each treatment, excluding control groups. The control group consisted of the same medium without the addition of bacterial suspension. Each treatment measured the ammonia levels at 24h, 48h, and 72h after bacterial inoculation.

2.4. Measurement of nitrite levels

Nitrite media were used to determine the ability of bacteria for reducing nitrite content. Nitrite media containing 0.5 g of glucose and 0.1 g of $NaNO_2$ were suspended in 1000 ml of distilled water. The nitrite level was detected using Hanna Instrument Handheld Colorimeter Nitrite LR. Nitrite media were diluted to a detectable range (236.64 ppb) and divided into 20 ml for each treatment. The measurement of nitrite level was performed before inoculating bacteria to determine the initial content of nitrite. The overnight culture of bacteria (1 ml) namely IBP-1, IBP-2, IBP-3, IBP-4, and IBP-5 strains was inoculated into nitrite media respectively, excluding control groups. The measurement of nitrite level was conducted at 24h, 48h, and 72h after bacterial inoculation.

2.5. Data analysis

The data were collected and tabulated in Microsoft Excel 2010. The data of ammonia and nitrite level were descriptively tested. The reduction percentage of ammonia and nitrite for each period measurement was presented in a bar-plot which was made using R Studio.

3. Results and Discussion

3.1. Identification of bacteria

The initial identification schemes were conducted with biochemical tests as suggested by Bergey's manual of determinative bacteriology [14]. The profiles of each bacterium are shown in Table 1.

Table 1. The profile of bacterial strains.

Bacterial strains	Colony Morphology			Cell shape	Gram test	Catalase test
	Form	Elevation	Margin			
IBP-1	Round	Flat	Entire	Rod-shape	+	+
IBP-2	Round	Convex	Entire	Rod-shape	+	+
IBP-3	Round	Convex	Entire	Rod-shape	-	+
IBP-4	Round	Umbonate	Entire	Rod-shape	+	+
IBP-5	Round	Flat	Entire	Coccus	-	+

3.2. Effect of several bacteria on the ammonia level

The use of several bacterial strains for degrading toxic ammonia had been reported by the previous study in broiler chickens [8,12,15], and aquaculture animals, including fish [16] and shrimp [7]. The level of ammonia was reduced after inoculating several bacteria compared to the control (without bacterial inoculation) **(**Table 2**)**.

Table 2. Level of ammonia on each period measurement.

Measurement Time	Ammonia Levels (mg/L)					
	Control	IBP-1	IBP-2	IBP-3	IBP-4	IBP-5
0h	0.97 ± 0.00	0.97 ± 0.00	0.97 ± 0.00	0.97 ± 0.00	0.97 ± 0.00	0.97 ± 0.00
24h	0.97 ± 0.00	0.32 ± 0.09	0.41 ± 0.08	0.72 ± 0.09	0.29 ± 0.04	0.30 ± 0.07
48h	0.97 ± 0.00	0.19 ± 0.07	0.21 ± 0.11	0.24 ± 0.10	0.14 ± 0.07	0.19 ± 0.04
72h	0.87 ± 0.06	0.05 ± 0.04	0.19 ± 0.03	0.14 ± 0.00	0.00 ± 0.00	0.04 ± 0.04
Reduction (%)	10.31 ± 0.59	94.50 ± 0.45	80.58 ± 0.31	86.57 ± 0.00	100.0 ± 0.00	95.46 ± 0.43

All bacterial strains showed a consistent decline in ammonia levels during the measurement time. All samples initially contained 0.97 mg/l of ammonia and were reduced in the range of 80.58% to 100%.

The greatest reduction of ammonia level compared with control was noted at 72h after inoculating bacteria using IBP-4 strains, followed by IBP-5, and IBP-1 strains (Figure 1).

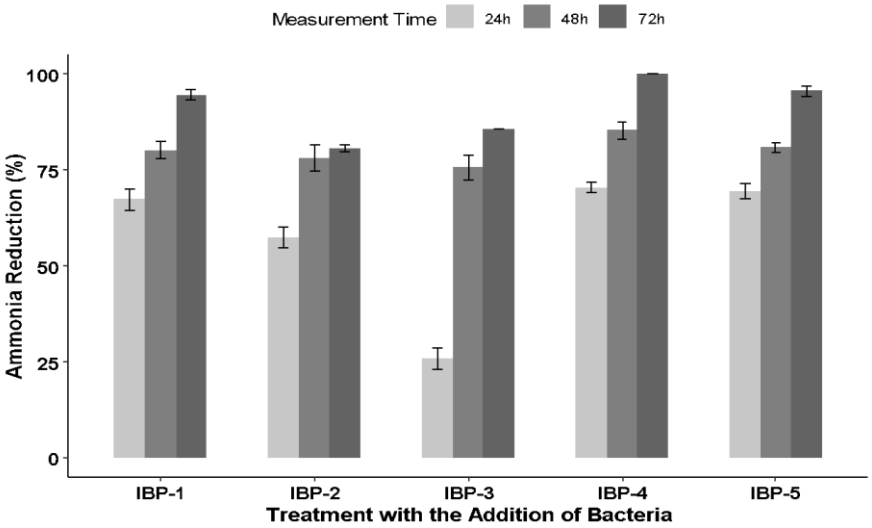

Figure 1. Reduction of ammonia each time of measurement (24h, 48h and 72h). Ammonia reduction was calculated by subtracting the initial ammonia content with the ammonia content on the period of measurement.

The percentage of ammonia reduction is the amount of ammonia degraded by the bacteria after the incubation. Mahardika *et al* [15] reported that the use of bacteria (*Lactobacillus sp.* and *Bacillus sp.*) in broiler chickens was able to reduce the ammonia levels ranged 36.2% to 68.6%. In aquaculture, the addition of *Nitrosomonas* dan *Nitrobacter* decreased the ammonia concentration ranged from 19.6% to 25.5% compared with control groups [16]. Nitrification is the aerobic oxidation of ammonia to nitrite continued by the aerobic oxidation of nitrite to nitrate [17]. Following the chemical process of ammonia oxidation according to Andriani *et al* [18]:

Nitritation: $NH_4^+ + 1.5O_2 \rightarrow 2H^+ + 2H_2O$ + 84 kcal/mole ammonia

Most bacteria that conduct the nitrifying process originated from *Nitrobacter*, *Nitrosomonas*, and *Nitrococcus* [16,17]. According to Mahardika *et al.* [15] that *Lactobacillus sp.* and *Bacillus sp.* can also inhibit the chemical reaction of the ammonia formation between uric acid and water and the uricase enzyme from gram-negative bacteria. The toxic ammonia had been reported to be degraded by *Bacillus substilis* [19], *Bacillus amyloliquefaciens* [20], *Bacillus coagulans*, and *Lactobacillus plantarum* [12]. The process of ammonia overhaul is influenced by the environment, pH, temperature, water content, and humidity [21].

3.3. Effect of several bacteria on the nitrite level

The next nitrification step is to convert nitrite to nitrate. The nitrite level was reduced from an initial 0.24 mg/l to 0.01 mg/l (IBP-1 strains) and 0.02 mg/l (IBP-2 strains). It was found that these IBP-1 and IBP-2 strains showed the best capacity to reduce the nitrite contents on the samples compared to the other three strains (Table 3).

Table 3. Level of nitrite on each period measurement

Measurement Time	Nitrite Levels (mg/L)					
	Control	**IBP-1**	**IBP-2**	**IBP-3**	**IBP-4**	**IBP-5**
0h	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.00
24h	0.20 ± 0.04	0.14 ± 0.07	0.11 ± 0.09	0.24 ± 0.04	0.22 ± 0.06	0.14 ± 0.09
48h	0.18 ± 0.08	0.10 ± 0.04	0.02 ± 0.07	0.12 ± 0.08	0.13 ± 0.04	0.14 ± 0.04
72h	0.19 ± 0.06	0.01 ± 0.00	0.02 ± 0.03	0.10 ± 0.06	0.12 ± 0.04	0.14 ± 0.05
Reduction (%)	19.44 ± 1.27	95.83 ± 0.00	91.39 ± 0.59	58.33 ± 1.18	51.39 ± 0.90	40.28 ± 1.09

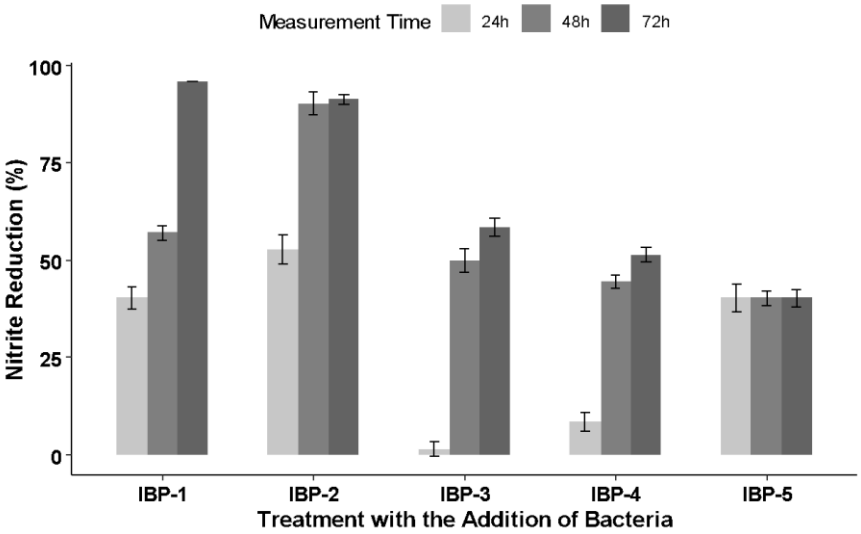

Figure 2. Reduction of nitrite each time of measurement (24h, 48h and 72h). Nitrite reduction was enumerated by subtracting the initial nitrite content with the nitrite content on the period of measurement.

Decreases in nitrite were observed at 24h after the addition of bacterial suspension at five bacterial strains with values ranged 1.39% to 52.78% removal. Low degradation activity was found at 24h at the IBP-3 and IBP-4 strain. On the second day, it was noted that the nitrite reduction reached almost half of the initial content, excluding the IBP-2 strain which reduced nitrite levels to 90.28%. The percentage of nitrite reduction reached its peak after 72h inoculating bacteria at IBP-1 and IBP-2 strains with 95.83% and 91.39% removal respectively. The chemical reaction of nitrite reduction is as follows according to Andriani *et al* [18]:

Nitratation: $NO_2^- + 0.5O_2 \rightarrow NO_3^- + 17$ kcal/mole nitrite

The nitrite conversion into nitrate is commonly done by *nitrite-oxidizing bacteria* (NOB) using nitrite oxidoreductase enzyme that converts nitrite to nitrate. A previous study had reported that *B. Substilis M7-1* degraded nitrite content reached 99.7% in 24h [14]. Zhang *et al.* [22] reported that nitrification is influenced by pH, temperature, salinity, nitrite accumulation, and dissolved oxygen.

4. Conclusion

Five bacterial strains had different capacities to degrade ammonia and nitrite, ranging from 80.58% to 100%. Among the tested strains, IBP-1 and IB4 strains showed the best capacity to reduce nitrite and ammonia respectively. Further *in vivo* studies in poultry, and aquatic animals are required to investigate the capacity of the bacteria to degrade ammonia and nitrite concentration. Also, identification using molecular techniques such as amplification and sequencing 16s rRNA needs to be done to have a more accurate bacterial identity.

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