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Effectiveness of predatory bacterium *Bdellovibrio bacteriovorus* in combination with *Pseudomonas fluorescens* and *Lactobacillus acidophilus* as candidates for *in vitro* anticolibacillosis

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ABSTRACT

Background: *Bdellovibrio bacteriovorus* has stood out since its initial discovery because of its exceptional capacity to feed on other Gram-negative bacteria. Since this specific "predatory bacterium" may be used as both a probiotic and an antibiotic, research on it has expanded in response to the growing issue of Antimicrobial Resistance (AMR). It is necessary to investigate the relationship between *B. bacteriovorus* and other Gram-negative bacteria, as well as the presence of Gram-positive bacteria, in the same environment to determine whether or not these factors have an impact on their ability as predators.

Aim: This study aimed to assess the effectiveness of the combination of the predatory bacterium *B. bacteriovorus* with *Pseudomonas fluorescens* and *Lactobacillus acidophilus* as potential candidates for *in vitro* anticolibacillosis.

Methods: The method employed *Escherichia coli* ATCC 15144 as the prey, while *B. bacteriovorus* 109 J ATCC 15143 was used as the predator, combined with *P. fluorescens* and *L. acidophilus* as the nutrient sources of the predator. In the challenge experiment, a ratio of the bacterial combination was used to optimize predation to *E. coli* of 10^7 PFU/10^5 CFU per ml.

Results: The study showed that the combination of *B. bacteriovorus* with *P. fluorescens* and *L. acidophilus* after 24 hours of *in vitro* incubation at 37°C increased the predatory bacteria count by tenfold, effectively reducing the *E. coli* population. However, in the absence of Gram-negative bacteria as a nutrient source, the predator population gradually declined.

Conclusion: The combination of *B. bacteriovorus* as a predatory bacterium with *P. fluorescens* and *L. acidophilus* is an effective candidate for *in vitro* anticolibacillosis.

Keywords: *B. bacteriovorus*, *P. fluorescens*, *L. acidophilus*, Anticolibacillosis, *in vitro*.

Introduction

Colibacillosis is a major cause of mortality (up to 20%) and morbidity in poultry, resulting in significant losses in meat production (2% live weight decrease, 2.7% decrease in feed conversion ratio), decreased egg yield (up to 20%), decreased hatchability rates, and decreased carcass percentage (up to 43%) by the time it is slaughtered (Kathayat *et al.*, 2021). This disease is caused by the pathogenic Gram-negative *Escherichia coli* bacterium, which is a facultative anaerobic bacterium that poses a persistent and economically detrimental threat to poultry farmers (Abalaka *et al.,* 2017; Rybak *et al.*, 2022; Kika *et al*., 2023). Although *E. coli* is frequently found in the avian digestive system as a commensal bacteria, not all strains are pathogenic (Rybak *et al.*, 2017). Environmental variables, host immune response, and pathogen virulence interact in a complicated manner to affect the clinical manifestation of colibacillosis (Weerts *et al*., 2021), causing significant morbidity and mortality, ranging from gastrointestinal and respiratory infections to serious systemic diseases. Avian pathogenic *E. coli* (APEC) is responsible for mortality rates as high as 53.5% in young poultry (Mellata, 2013). With losses estimated to be hundreds of millions of dollars every year, APEC has a huge financial impact on the world's poultry sector (Ghunaim *et al.*, 2014). In the United States alone, APEC-related carcass condemnations cost the broiler industry approximately $40 million per year (De Brito *et al*., 2003). According to recent studies, APEC, namely

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isolates from sequence types ST95 and ST131, or serogroup O1, O2, and O18, is linked to colibacillosis in chickens and can serve as a source or reservoir of extraintestinal illnesses in humans in addition to being a zoonotic foodborne pathogen (Koutsianos *et al.*, 2022). The virulence characteristics of these serogroups may vary, such as their capacity to adhere to the intestinal epithelium and infiltrate host organs (Koutsianos *et al*., 2022). Pathogenic strains acquire specific virulence factors, such as adhesions, toxins, and invasions (Sora *et al*., 2021), allowing bacteria to cling to the tissues of the host, colonize different organs, and avoid the host's immune system. Fimbriae (pili), outer membrane proteins, and toxins, including cytotoxins, hemolysins, and endotoxins are examples of common virulence factors (Meena *et al*., 2019).

Key factors in modern animal production include disease prevention, improved growth rates, and enhanced feed conversion efficiency (Kostadinović and Lević, 2018). One approach to achieving these goals has been the use of antibiotics. Antibiotics are frequently used in chicken nutrition as a preventive measure to boost growth, improve feed intake and utilization, and reduce clinical illness mortality (Kostadinovic *et al*., 2001). However, since 2006, the European Union has prohibited the use of antibiotic growth promoters (AGPs) in livestock due to rising concerns about the spread and transmission of antibiotic-resistant bacteria through the food chain (Kostadinović and Lević, 2018). In early 2018, Indonesia also began prohibiting the use of AGPs. This ban is crucial because of the high risk of the emergence and spread of new diseases.

The primary treatment for colibacillosis is antibiotics; however, recent research indicates the global scale of the appearance of *E. coli* strains resistant to antibiotics (Pires *et al*., 2022). Even in flocks of chickens that have not been exposed to antibiotics, multidrug resistance in *E. coli* has grown to be a serious concern (Zhang *et al*., 2017; Han *et al*., 2020; Hess *et al*., 2022). One of the most significant bacterial infections resistant to antibiotics in poultry in Europe is *E. coli*. Therefore, rather than depending only on antimicrobial treatments, investigating preventive strategies like vaccination and biosecurity measures (Paudel *et al*., 2024).

A new therapy for multidrug-resistant bacteria has been investigated, including the use of predatory bacteria as "living antibiotics" that can combat a variety of infectious bacteria (Cavallo *et al.*, 2021). *Bdellovibrio bacteriovorus* is the predatory species that has been examined the most. It exhibits a predatory lifestyle and can attack a range of species connected to endobiotic predation and human infections, especially those caused by Gram-negative bacteria (Summers and Kreft, 2022). Another therapeutic approach has been suggested: applying *Bdellovibrio* spp. either alone or in conjunction with bacteriophages or antibiotics, or using Bdellovibrio enzymes as antimicrobials (Bratanais *et al*., 2020; Pérez *et al*., 2020). However, to overcome resistance in target organisms and enhance effectiveness, a combination with other bacteria is required (Wu *et al*., 2017). Hobley *et al*. (2006) explored the interaction of *B. bacteriovorus* HD100 with mixed cultures containing both Gram-negative and Gram-positive bacteria, demonstrating its ability to reduce *E. coli* concentrations from approximately ~10^8 to ~10^5 cells/m. A class of predatory bacteria known as Bdellovibrio and like organisms obtain their energy and biosynthetic ingredients from live Gram-negative bacteria that they prey on (Odooli *et al*., 2021). Some bacteria that can be used in combination include *Pseudomonas* spp. and *Lactobacillus* spp. (Waso-Reyneke *et al*., 2022; Mulvey *et al*., 2023). Several Gram-negative pathogenic bacteria can be preyed upon and killed by *B. bacteriovorus in vitro* (Raghunathan *et al*., 2019).

Therefore, this study assessed the effectiveness of combining the predatory bacterium *B. bacteriovorus* with *Pseudomonas fluorescens* and *Lactobacillus acidophilus* as potential new candidates for *in vitro* anticolibacillosis therapy.

Materials and Methods

This study aimed to assess the effectiveness of combining *B. bacteriovorus*, *P. fluorescen,* and *L. acidophilus* isolates in inhibiting the growth of *E. coli* bacteria *in vitro*. The research was conducted from July to October 2024 at the Institute of Tropical Disease, Universitas Airlangga.

Bacterial isolates

Escherichia coli ATCC 15144 was used as the prey of *B. bacteriovorus* 109 J ATCC 15143 and *B. bacteriovorus* 109 J ATCC 15143, which combined with *P. fluorescens* and *L. acidophilus* as nutrient sources. The challenge test against *E. coli* and the predator attack phases were prepared following standardized methods (Ottaviani *et al*., 2019).

Experiment procedure

Cultivate and incubate *B. bacteriovorus* on YE-NB agar at 37°C for 24 hours. *Lactobacillus acidophilus* was cultured on Methicillin-resistant Staphylococcus aureus (MRSA), while *P. fluorescens* was grown on Mueller–Hinton agar. Prepare test tubes containing 3 ml of Trypticase Soy Broth (TSB) medium. Five bacterial colonies of *B. bacteriovoruses*, *P. fluorescens*, and *L. acidophilus* were selected and transferred into individual sterile tubes containing TSB. The tubes were vortexed until homogeneous. The turbidity of each bacterial suspension was measured using a nephelometer, and the turbidity was adjusted to a 0.5 McFarland standard (1.5 x 10^8 CFU/ml). Prepare another sterile tube containing 1 ml of TSB, then add five colonies from each bacterium (*B. bacteriovorus*, *P. fluorescens,* and *L. acidophilus*) with their adjusted McFarland values into the tube. Vortex until homogeneous. The turbidity of the bacterial mixture was measured, and then incubated at 37°C for 24 hours. After incubation,

the tube was vortexed to homogenize, and turbidity was measured again using a nephelometer. In the next step, an *E. coli* suspension with a known McFarland value was added into the tube containing the mixture of *B. bacteriovorus*, *P. fluorescen,* and *L. acidophilus*. Vortex to ensure homogeneity and incubate again at 37°C for 24 hours. After incubation, the tube was vortexed again and turbidity was measured using a nephelometer. For the best predation activation in the challenge experiment, we used a predator-prey ratio of 10^7 PFU/10^5 CFU per ml (PFU = Plaque-Forming Unit) (Ottaviani *et al*., 2019).

Results

The culture media are nutrient-rich gels or liquids used to cultivate microorganisms, such as bacteria. Another name for this medium is growth media. Different types of media were used to grow different types of cells. Agar plates and broths are the most commonly used growth media for microorganisms. Certain bacteria or germs require specific media to thrive. In this study, each of the bacteria that were the objects of research was grown in media that were in accordance with the characteristics and nature of the bacteria so that they could grow optimally (Fig. 1). The expected research results at this initial stage provided evidence that each bacteria was truly a pure isolate whose potential and impact on activity were explored (Fig. 1). Several types of bacteria used in this study grew optimally and could be clearly observed according to their respective media and characteristics, including *B. bacteriovorus* on YE-NB agar, *P. fluorescens* on Mueller–Hinton agar, and *L. acidophilus* on MRSA.

After all the bacteria had grown well, all the bacteria to be combined, including *B. bacteriovorus*, *P. fluorescens*, and *L. acidophilus*, were first adjusted to a 0.5 McFarland standard using a nephelometer based

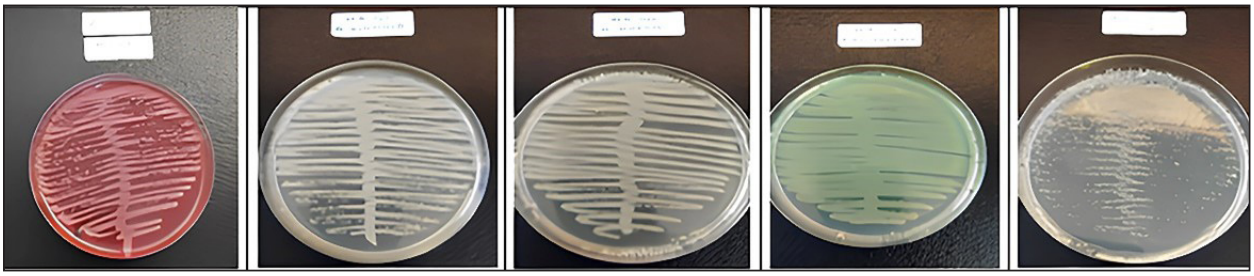

Fig. 1. Results of bacterial isolate cultivation. (A) *Escherichia coli*, (B) *Bacillus subtilis*, (C) *Bdellovibrio bacteriovorus*, (D) *Pseudomonas fluorescens*, and (E) *Lactobacillus acidophilus*. The bacteria of the objects of research were grown in media that were in accordance with the characteristics and nature of the bacteria.

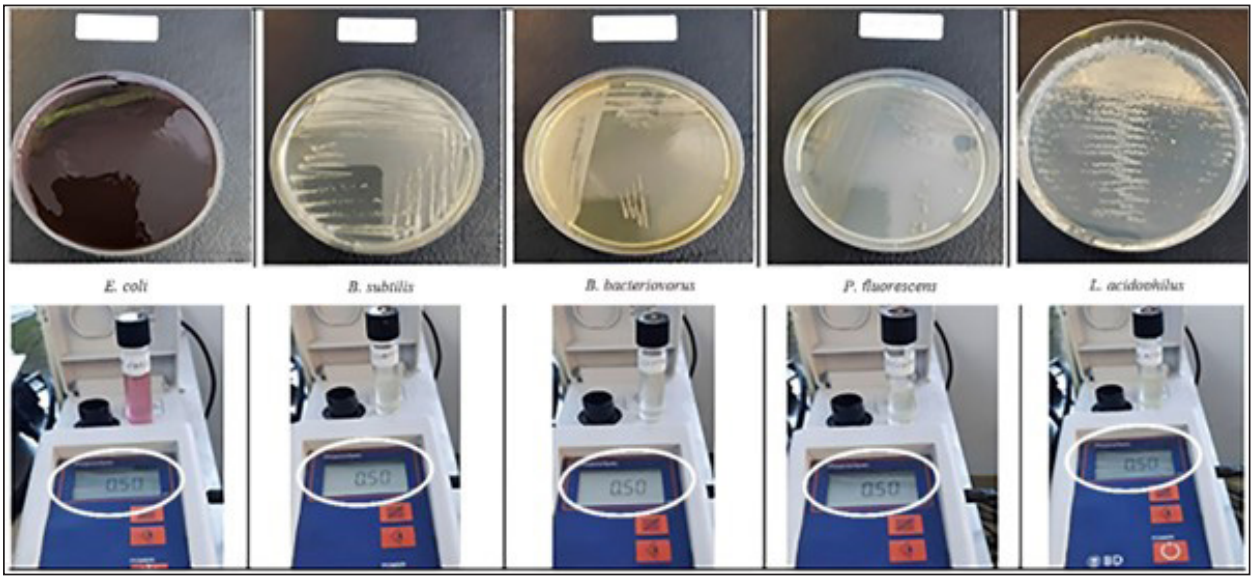

Fig. 2. All bacteria, including *Bdellovibrio bacteriovorus*, *Pseudomonas fluorescens*, and *Lactobacillus acidophilus* had grown well before being combined. These were first adjusted to a 0.5 McFarland standard using a nephelometer based on the turbidity values obtained from the three bacterial suspensions in the tubes.

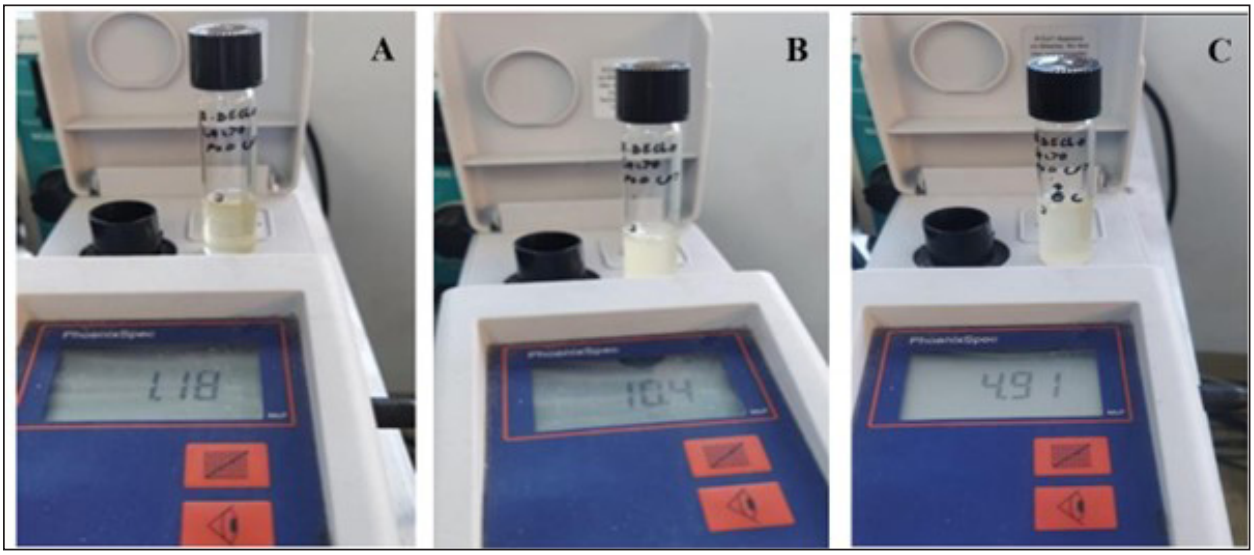

Fig. 3. The combination of *Bdellovibrio bacteriovorus*, *Pseudomonas fluorescens*, and *Lactobacillus acidophilus*. (A) Before incubation; (B) after incubation; (C) after challenge with *Escherichia coli*.

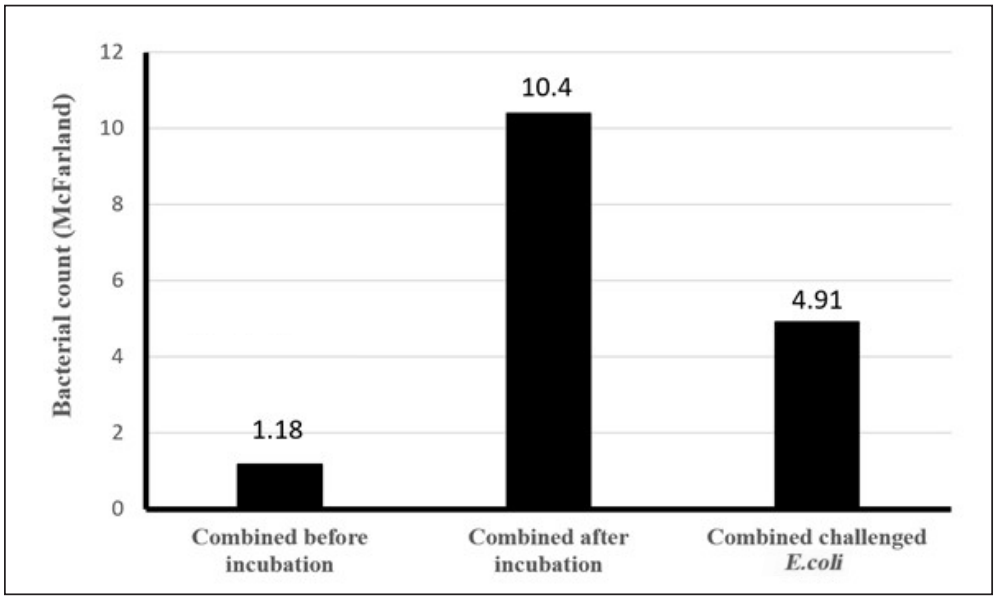

Fig. 4. Pattern of predation activity of *Bdellovibrio bacteriovorus* toward the bacteria that comprise its prey *in vitro*. There was a very significant increase in the population of the combination of bacteria (*B. bacteriovorus*, *Pseudomonas fluorescens* and *Lactobacillus acidophilus*) (tenfold) after incubation, and then after being challenged with *Escherichia coli*, there was a significant decrease in.

on the turbidity values obtained from the three bacterial suspensions in the tubes (Fig. 2).

In Figure 3, it is observed that the combination of *B. bacteriovorus*, *P. fluorescen,* and *L. acidophilus* resulted in a bacterial count of 1.18 McFarland (1.5×10^{16}) before incubation (Fig. 3A). After incubation at 37°C for 24 hours, the count increased to 10.4 McFarland (1.5×10^{160}) (Fig. 3B), and following the challenge with *E. coli*, it decreased to 4.91 McFarland (1.5×10^{90}) (Fig. 3C).

The pattern of predation activity of *B. bacteriovorus* toward the bacteria that comprise its prey is illustrated in Figure 4. The increase in the combined bacterial population after incubation (tenfold) then a significant decrease in the bacterial population after being challenged with *E. coli.*

Discussion

The most extensively studied predatory bacterium is *B. bacteriovorus*. Numerous other Gram-negative bacteria are the prey of this particular bacterium. According to the outcomes shown in Figure 3, following a 24 hours incubation period at 37°C, the mixture of the three bacteria predatory bacterium *B. bacteriovorus*, *P. fluorescens*, and *L. acidophilus showed* a tenfold increase in bacterial count, from 1.18 McFarland (approximately 1.5×10^{16}) to 10.4 McFarland (1.5×10^{160}). This increase was attributed to the existence of Gram-negative bacteria such as *P. fluorescens*, which serve as a nutrient source for *B. bacteriovorus* (Waso *et al*., 2019), as Bdellovibrio utilizes the nutrients found in the cytoplasm of its prey as a carbon source and energy source for growth (Herencias *et al*., 2024).

Conversely, when challenged with *E. coli*, the bacterial count of the mixture comprising the predatory bacteria *B. bacteriovorus*, *P. fluorescens*, and *L. acidophilus* also decreased in parallel with the reduction in the number of *E. coli*, from 10.4 McFarland (1.5×10^{160}) to 4.91 McFarland (1.5×10^{90}). This finding is consistent with the behavior of *Bdellovibrio*, which takes turns between two phases in its life cycle. This bacteria does not develop during the free-living assault phase; instead, it seeks out prey. It can reach up to 160 μm s^{-1}, or roughly 100 body lengths s^{-1} in swims, which is faster than most bacteria (Summers and Kreft, 2022). Because it depends on its prey for nutrition, its quick movement requires a high metabolic rate, which causes it to lose viability within 10 hours if no prey is found (Hobley *et al*., 2020). Typically, after locating its prey, Bdellovibrio assesses its suitability for several minutes (Hobley *et al*., 2006). If the prey is deemed suitable, the bacterium enters the periplasmic space through pores in the peptidoglycan layer and outer membrane, losing its flagellum in the process (Starr and Baigent, 1966). Bdellovibrio kills the prey as it enters the periplasm, allowing the cytoplasm contents to spill into the periplasmic space (Romo *et al*., 1992). Additionally, the bacterium alters the peptidoglycan of the prey, causing the prey cell to round into a "bdelloplast." Instead of division during the bdelloplast phase, Bdellovibrio uses the released nutrients to grow as a long filament (Lambert *et al*., 2006). The filament divides into new cells after the nutrients run out, creating as many offspring as the resource permit. This usually results in three to six new predators for every *E. coli* cell (Seidler and Starr, 1969).

Under normal binary fission, this bacterium would only be capable of producing offspring in powers of two (2^n), which could potentially result in unused prey resources if those resources are sufficient for five but not eight progeny. To escape and find new prey, freshly created Bdellovibrio cells lyse the remains of the prey cell and develop flagella. Additionally, according to Gao *et al*. (2022), because *L. acidophilus* lowers intestinal pH and produces metabolites, it can help manage the balance of gut flora (Tegegne and Kebede, 2022). Many gut pathogens prefer a pH of neutral or slightly alkaline. The metabolism of *L. acidophilus* produces lactic acid, which lowers pH and prevents pathogenic bacteria from growing and reproducing (Gao *et al.*, 2022).

Furthermore, enzymes like azo reductase, nitro reductase, and β-glucosidase are produced by pathogenic microbes and catalyze the transformation of procarcinogenic precursors into carcinogens (Goldin and Gorbach, 1980, 1984). *Lactobacillus acidophilus* decreases the enzymatic activity of these harmful microbes in addition to preventing their proliferation and lowering the synthesis of these enzymes (Goldin and Gorbach, 1984; Chang *et al*., 2012). Additionally, a crucial mechanism by which *L. acidophilus* competes with pathogenic bacteria for attachment sites impairs pathogenic bacterial functions, thereby preventing their invasion of host cells (Singh *et al.*, 2013). Surface-layer proteins, extracellular polysaccharides, and lipoteichoic acids on the surface of many *L. acidophilus* strains can compete with pathogens for adhesion (Kopp-Hoolihan, 2001; Tegegne and Kebede, 2022).

Conclusion

The predatory bacterium *B. bacteriovorus,* when combined with *P. fluorescens* and *L. acidophilus*, has been shown to increase the population of the predator bacteria tenfold at 37°C within 24 hours and effectively reduce the number of *E. coli*. However, if these predatory bacteria do not obtain nutrients and energy from Gram-negative bacteria, their population will gradually decline over time.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Author's contribution

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by all authors: laboratory processing of samples (EBA, MY, ISH, GMS). The first draft of the manuscript was written by EBA and

MY, and subsequently revised by MY and EBA. All authors read and approved the final manuscript.

Data availability

Escherichia coli ATCC 15144 and *B. bacteriovorus* 109 J ATCC 15143 specimens were cultured and maintained in Microbiology Laboratory of Faculty of Veterinary Medicine, Universitas Airlangga. The *P. fluorescens* and *L. acidophilus* specimens obtained in this study are available from the same laboratory. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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