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Research Article
Probiotic Testing of *Lactobacillus brevis* and *Lactobacillus plantarum* from Fermented Cabbage Waste Juice

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Abstract
Objective: The aim of this study was to test the ability of *Lactobacillus brevis* and *Lactobacillus plantarum* derived from fermented cabbage waste juice to acts as probiotics. **Materials and Methods:** Tests of probiotic ability included tests of bile salt resistance and pH resistance and tests of sensitive inhibition of *Escherichia coli* and *Salmonella pullorum* growth. **Results:** *Lactobacillus brevis* and *Lactobacillus plantarum* derived from fermented cabbage waste juice were able to grow and develop at pH values from 2.5-5.5 and bile salt concentrations of 1-5%. *Lactobacillus brevis* was able to strongly inhibit *Escherichia coli* and *Salmonella pullorum* growth, while *Lactobacillus plantarum* showed very potent inhibition of *Escherichia coli* growth and potent inhibition of *Salmonella pullorum* growth. **Conclusion:** *Lactobacillus brevis* and *Lactobacillus plantarum* derived from fermented cabbage waste juice are suitable for use as poultry probiotics.

Key words: Fermentation, cabbage waste, probiotic, *Lactobacillus brevis*, *Lactobacillus plantarum*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cabbage is a type of vegetable that grows in the highlands of Indonesia. Cabbages wilt, get damaged and decay easily, leading to a foul odor that causes environmental problems. Cabbage waste is commonly found in traditional, untapped markets. The use of cabbage waste as probiotics had not been previously studied, which inspired researchers to test *Lactobacillus brevis* (*L. brevis*) and *Lactobacillus plantarum* (*L. plantarum*) as poultry probiotics. *L. brevis* and *L. plantarum* which were tested as probiotics are derived from the juice of fermented cabbage waste. Utama *et al*.[1] and Plengvidhya *et al*.[2] stated that cabbage processed by fermentation contains microbes such as *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*. Cabbage waste is a waste product from vegetable markets and consists of outer shells of cabbages damaged by impact and collision, which make these cabbages not worth selling. The amount of waste generated is as much as 3-5% of the total weight of cabbage[3]. Cabbage waste can cause pollution, so there is a need to develop methods to handle and process this waste in order to use it as a probiotic.

Probiotics are live microbes that are administered into the gastrointestinal tract and provide benefits to the digestive tract of the host. Fuller[4] and Mateova *et al*.[5] explained that probiotics supplementary foods that are composed of microbes that are beneficial to and affect the host by improving the microbial balance in the digestive tract. Probiotic bacteria usually belong to the genera *Lactobacillus* and *Bifidobacterium*. Probiotics must exhibit the following characteristics: Ability to live at low pH, resistance to bile salts, production of toxins, live cell density of more than 10^6 CFU mL^{-1}, ability to survive and perform metabolic activities in the digestive tract and ability to survive during storage, in addition probiotics should not trigger an immune response[6,7].

Some types of probiotics can produce natural antibiotics that can inhibit the growth of pathogenic microbes. Probiotics can help stabilize intestinal microbes, improve the permeability of the intestinal barrier and enhance systems and responses of mucosal IgA that prepare the intestinal mucosal barrier against harmful microbial infections and infectious agents. Wolfenden *et al*.[8] stated that administering probiotics in the form of an effective competitive exclusion (CE) culture can reduce colonization by the pathogenic microbe *Salmonella enteriditis* (SE) in the digestive tracts of broiler chickens. The use of probiotics in chickens is reported to decrease urease activity and as a result, the formation of ammonia is reduced[9]. The use of probiotics in chickens increases the rate of growth and use of nitrogen, enhances immunity to infection and increases egg production[10,11]. The results of this study are consistent with the research by Mateova *et al*.[5], who stated that the use of probiotics increases the weight of broiler chickens. Several researchers have reported positive *in vivo* effects such as strengthened mucus production, activated macrophages in the presence of *Lactobacillus*, secretory IgA stimulation, proinflammatory enhancement and cytokine production[12]. Thus, further studies are needed to create safe probiotics for poultry and to enhance the production of antibiotic-free livestock. To this end the ability of the probiotics *L. brevis* and *L. plantarum* derived from fermented cabbage waste juice was tested to act as poultry probiotics.

MATERIALS AND METHODS

The materials used in this study were *L. brevis* and *L. plantarum* isolates from fermented cabbage waste, de Man, Rogosa and Sharpe (MRS) medium (Oxoid, UK#CM 0361), de Man, Rogosa and Sharpe (MRS) medium (Oxoid, UK #CM 0359), Aquades, physiological saline, HCl, bile salts, Mueller-Hinton agar (MHA) medium (Oxoid,UK #CM 0337) and *Escherichia coli* and *Salmonella pullorum* isolates. The tools and instruments used in this study were the following: Electric benchtop autoclave sterilizer (All American, USA), incubator (Memmert, Germany), digital scales (Ohaus, USA), glass Erlenmeyer flasks (Schott Duran, Germany), measuring cup (Schott Duran, Germany) and pH meter (Crison, Spain).

Lactobacillus brevis and *Lactobacillus plantarum* were obtained from the fermented cabbage waste juice. Fermented cabbage waste juice was produced by cutting of the cabbage waste as smoothly as possible. The cabbage waste was then blended and added with 8% of salt (without iodine) and 6.7% of molasses. Subsequently, the mixture was spontaneously fermented in a closed container for 6 days. The fermented products were then used as a source of the above mentioned bacteria. The study started by reviving the *Lactobacillus brevis* and *Lactobacillus plantarum* isolates, which were ordered as agar slants. The ability of the lactic acid bacteria to acts as probiotics was tested by pH and bile salt resistance tests and by testing the sensitivity of inhibition of *Escherichia coli* and *Salmonella pullorum* growth. The tests were conducted as follows:

- **pH resistance test:** The pH resistance was tested done by using the method described by Taheri *et al*.[13] with some modifications. The aim of this test was to determine the

resistance of *L. brevis* and *L. plantarum* isolates grown in acidic media or at various pH levels. A total of 1 mL of *L. brevis* or *L. plantarum* cultures was grown in 10 mL of MRS broth medium at pH 2.5, 3.5, 4.5 and 5.5 at 37°C for 12 h. The growth of the microorganisms was detected as the presence of turbidity in the medium and the absorbance of the cultures at a wavelength of 600 nm was quantitatively measured by using a spectrophotometer. Optical density (OD) was determined by the following formula:

$$OD = (OD_{600} \text{ grown cultures-} OD_{600} \text{ blank})$$

- **Bile salt resistance test:** Bile salt resistance was tested by using the method described by Taheri *et al.*[13] with some modifications. This aim of this test was to determine the resistance of *L. brevis* and *L. plantarum* isolates to the level of bile salts in the growth medium. The resistance of *L. brevis* and *L. plantarum* in bile-salt-containing medium was tested by adding bile salts at concentrations of 1, 2, 3, 4 and 5% into tubes containing MRS broth. A total of 1 mL of *L. brevis* or *L. plantarum* cultures was added to 10 mL of medium and the cultures were incubated at 37°C for 12 h. The growth of the microorganisms was detected as the presence of turbidity in the medium and the absorbance of the cultures at a wavelength of 600 nm was quantitatively measured by using a spectrophotometer. Optical density (OD) was determined by using the following formula:

$$OD = (OD_{600} \text{ grown cultures-} OD_{600} \text{ blank})$$

Sensitivity test of *Escherichia coli* inhibition: This test was conducted to determine the ability of *L. brevis* and *L. plantarum* isolates to inhibiting the growth of *Escherichia coli*. The method used was a modified dual-culture method. *Escherichia coli* was grown in petri dishes on MHA and the agar was then perforated using a thin hole tool to make a 1 cm diameter incision. *L. brevis* or *L. plantarum* isolates were inoculated into the hole at a volume of 250 µL and the plates were incubated for 24 h at 37°C. After incubation, the observed barrier zone was measured using a ruler[14].

Sensitivity test of *Salmonella pullorum* inhibition: This test was performed to determine the ability of *L. brevis* and *L. plantarum* isolates to inhibit the growth of *Salmonella pullorum*. The method used was a modified dual-culture method. *Salmonella pullorum* was grown in petri dishes on MHA and the agar was then perforated using a thin hole tool to make a 1 cm diameter incision. *L. brevis* and *L. plantarum* isolates were inoculated into the hole at a volume of 250 µL and the plates were incubated for 24 h at 37°C. After incubation, the observed barrier zone was measured using a ruler[14].

Non-parametric statistical analysis: The data from the pH resistance test, bile-salt resistance test and sensitivity tests of *Escherichia coli* and *Salmonella pullorum* inhibition were analyzed by non-parametric statistical analysis[15].

RESULTS AND DISCUSSION

Resistance of *Lactobacillus brevis* and *Lactobacillus plantarum* to pH: The resistance of *L. brevis* and *L. plantarum* to pH is shown by the data in Table 1. Table 1 shows the ability of *L. brevis* and *L. plantarum* to survive in pH values from 2.5-5.5. Overall, the isolation and identification of lactic acid bacteria from the fermented cabbage waste juice yielded 8 isolates but upon reinoculation, only 2 isolates exhibited consistent growth. This observation was a result of the addition of salt as much as 8% of the fresh weight of cabbage juice, during the fermentation process. The addition of salt to the media was intended to serve as selection method and inhibit the growth of pathogenic bacteria during fermentation. The amount of lactic acid bacteria in the fermented cabbage waste juice was 2×10^{10} CFU mL^{-1} and the total fungal growth was 29×10^{8} CFU mL^{-1}. The secondary metabolite content and pH were as follows: pH, 3.46 total acid 1.10%, acetic acid 0.02%, butyric acid 0.002% and lactic acid 0.80%.

Lactobacillus brevis and *L. plantarum* isolates have the ability to survive in low-pH environments. The *L. brevis* and *L. plantarum* isolates showed resistance to a pH of 2.5 for 12 h (Table 1). The populations of the *L. brevis* and *L. plantarum* isolates were greater than 10^{8} CFU mL^{-1} (Table 1), which proved that the ability of *L. brevis* and *L. plantarum* isolates to grow at low pH values is very good, indicating that these bacteria could survive in the digestive tracts of poultry. Saarela *et al.*[16] stated that resistance to low pH is an important characteristic of probiotics. The recommended concentration of lactic acid bacteria as poultry probiotics is 10^{8} CFU kg^{-1} of feed in order for the bacteria to survive in the digestive tract[17]. Table 1 shows that the higher the pH concentration is the more the microbes grow. Purwoko[18] stated that bacterial growth can be determined by measuring the difference in absorbance before and after

Table 1: Optical density (OD) of *Lactobacillus brevis* and *Lactobacillus plantarum* isolates at various pH values*

		Optical density (OD)								
	McFarland standard	0 h				12 h				
Isolates	0.5 (10^8 CFU)	2.5	3.5	4.5	5.5	2.5	3.5	4.5	5.5	SEM
Lactobacillus brevis	0.04	0.08	0.09	0.11	0.18	0.50	1.34	1.93	2.43	0.01
Lactobacillus plantarum	0.04	0.09	0.09	0.10	0.16	0.09	1.10	2.11	2.55	0.02

*Growth in MRS broth medium with an incubation period of 12 h, SEM: Standard error of the treatment means

Table 2: Optical density (OD) of *Lactobacillus brevis* and *Lactobacillus plantarum* isolates in various bile salt concentrations*

		Optical density (OD)									
	McFarland standard	0 h					12 h				
Isolates	0.5 (10^8 CFU)	1	2	3	4	5	1	2	3	4	5	SEM
Lactobacillus brevis	0.04	0.15	0.29	0.26	0.24	0.32	2.22	2.33	2.35	2.35	2.38	0.02
Lactobacillus plantarum	0.04	0.15	0.29	0.25	0.20	0.31	2.36	2.40	2.41	2.42	2.44	0.03

*Growth in MRS broth medium with an incubation period of 12 h, SEM: Standard error of the treatment means

incubation. The number of bacterial cells can be measured by determining the turbidity of the culture, the more turbid a culture is, the greater the number of cells.

Resistance of *Lactobacillus brevis* and *Lactobacillus plantarum* to bile-salt concentration: The resistance of *L. brevis* and *L. plantarum* to various concentrations of bile salts is shown by the data in Table 2. The results showed that all isolates were able to grow in media containing bile salts at concentrations from 1-5% for 12 h.

Bile salts are amphipathic compounds, one end is soluble in water (polar/hydrophilic) and the other end is not water soluble (nonpolar/hydrophobic). This amphipathic structure causes the bile salts to emulsify fat and directly affect the life of microorganisms in the digestive tract, especially in the small intestine. The *L. brevis* and *L. plantarum* isolates had a life span of 12 h at a bile salt concentration of 5% (Table 2). The higher the concentration of bile salts is, the greater the population sizes of *L. brevis* and *L. plantarum*. Prasad *et al.*[19] stated that bile salts are secreted by the small intestine and are environmental stress factors for microbial growth. Bile salts inhibit microbial growth by weakening microbial cell membranes, the main components of which are fats and fatty acids that can be damaged by bile salts.

Bile salts in the small intestine can also be said to affect microorganisms as "biological detergents", i.e., fluids that have the ability to dissolve phospholipids, cholesterol and proteins. Most of these compounds can rearrange cell membranes, thus causing microbial cell lysis. At high concentrations, bile salts are very toxic antimicrobial substances[20]. Table 2 shows that the *L. brevis* and *L. plantarum* isolates were able to grow well in bile salt concentrations of up to 5%, with the population size staying above the McFarland standard of 10^8 CFU mL^{-1}.

Table 3: Results of sensitivity test of *Escherichia coli* and *Salmonella pullorum* growth inhibition*

	Clear zones (mm)	
Isolates	*Escherichia coli*	*Salmonella pullorum*
Lactobacillus brevis	16.0±1.0	19.3±1.1
Lactobacillus plantarum	21.3±1.1	17.0±1.0

*The method used was a modified dual-culture method in Mueller-Hinton agar medium with an incubation period of 24 h

Bezkorovainy[21] stated that bile in the small intestine inhibits the growth of existing microbes, therefore, for *L. brevis* and *L. plantarum* isolates to be probiotics, these bacteria should be able to withstand bile salts in order to survive and perform probiotic function in poultry intestines. Resistance to bile salts is an important characteristic of probiotics.

Sensitivity test of *Escherichia coli* and *Salmonella pullorum* growth inhibition: Davis and Stout[22] stated that in terms of antibacterial potency, clear zones of 20 mm indicates very strong inhibition, 10-20 mm indicates strong inhibition, 5-10 mm indicates moderate inhibition and 5 mm or less indicates weak inhibition. The *L. brevis* isolate showed strong inhibition of *Escherichia coli* and *Salmonella pullorum* growth, while *L. plantarum* showed very strong inhibition of *Escherichia coli* growth and strong inhibition of *Salmonella pullorum* growth (Table 3). *L. brevis* produces a natural antibiotic called lactobrevin and *L. plantarum* produces lactolin, which can inhibit the growth of pathogenic bacteria.

Lactobacillus sp. ferment carbohydrates to produce lactic acid, which can lower the pH. Acidic pH can inhibit the growth of microbes, especially microbial pathogens[23]. Murry *et al.*[24] showed that pure cultures of *L. plantarum* produce lactic acid at high quantities *in vitro*, which can decrease the pH, thus inhibiting the growth of pathogenic

bacteria. *Lactobacillus* sp. can inhibit the growth of pathogenic bacteria by blocking receptors from pathogenic bacteria and preventing these bacteria from colonizing the intestines or by removing toxic metabolites produced by the pathogenic microbes[25]. Lactic acid bacteria produce a wide variety of antimicrobial components: hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (butane-2,3-Dione) and bacteriocin. All of these components are antagonistic to the growth of pathogenic bacteria[26]. Therefore, the use of *L. brevis* and *L. plantarum* isolates derived from fermented cabbage waste juice as poultry probiotics is very feasible as these bacteria exhibited endurance at low pH and in bile salts and exhibited sensitive inhibition of *Escherichia coli* and *Salmonella pullorum*.

In this study, a new type of probiotic was found, derived from fermented cabbage waste juice that can be beneficial to poultry. This study can be used as a scientific reference by researchers as there has been no previous research on probiotics derived from cabbage waste. For the poultry industry, this study describes the innovation of technology to derive probiotics from sources are readily available, making the process cheap and environmentally friendly. Thus, this invention can be exploited by all parties to create poultry farms that are free of antibiotics.

CONCLUSION

Lactobacillus brevis and *Lactobacillus plantarum* are suitable for use as poultry probiotics, which is evident from the ability of these bacteria to grow and develop at low pH (2.5-5.5) and in bile salts at concentration of 1-5%. *L. brevis* demonstrated strong inhibition of *Escherichia coli* and *Salmonella pullorum* growth, while *L. plantarum* showed very strong inhibition of *Escherichia coli* growth and strong inhibition of *Salmonella pullorum* growth.

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