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Selection of probiotic candidate of lactic acid bacteria from *Hermetia illucens* larvae fed with different feeding substrates

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Abstract. *Siregar DJS, Julianti E, Tafsin M, Suryanto D. 2022. Selection of probiotic candidate of lactic acid bacteria from* Hermetia illucens *larvae fed with different feeding substrates. Biodiversitas 23: 6320-6326.* Exploration of probiotics candidate have been accelerated from unique and novel source, one of which is from black soldier fly (*Hermetia illucens*). The black soldier flies are known as omnivores which are commonly reared, harvested and utilized to convert organic wastes into biomass yet reducing the amount of abundant waste in nature. Lactic acid bacteria (LAB) is one of beneficial gut symbionts which can be found across animal taxa, including insect, however, limited information is currently reported from *H. illucens* as a potential harboring insect. This study aimed to determine the probiotics candidate from the gut of *H. illucens* fed with different feeding substrates to improve the gut microbiota. The larvae were nourished with chicken manure, tofu solid wastes and vegetable wastes prior isolation of LAB. The LAB isolates were tested for their antagonistic activity against enteric pathogens, pH and bile salt tolerance, and adherence to chicken intestine (ileum). Based on the growth performance under physiological stresses and multivariate analysis using principal component analysis (PCA), four LAB isolates were designated as putative probiotics. The four isolates were *Lactiplantibacillus. pentosus* 5P2i1, *Lacticaseibacillus. paracasei* 5P2i5, *Lactiplantibacillus. pentosus* 5P2i9, and *Lactiplantibacillus. plantarum* 6P1i1 was then declared as novel strains that could be developed further as poultry probiotics.

Keywords: Black soldier fly, *Hermetia illucens*, *Lacticaseibacillus paracasei*, vegetable waste

INTRODUCTION

Antibiotic use on livestock has been declared illegal in Indonesia through the Law on Livestock and Animal Health No. 18 of 2009 Article 22 Paragraph 4c (*UU No. 18 Tahun 2009 Pasal 22 ayat 4c*) issued by the Ministry of Agriculture. In addition to preventing and treating disease, antibiotics are also used in animal husbandry as an antibiotic growth promoter (AGP), which has been around since the 1940s and increases productivity and efficiency of animal feed. The use of antibiotics has the potential to endanger human health due to the development of antibiotic-resistant pathogenic bacteria and the accumulation of antibiotic residues in livestock meat (Zhang et al. 2021). Alternatives to antibiotics are urgently needed given the threat of adverse effects from antibiotics in order to maximize livestock productivity with goods that are safe for consumption in the form of probiotics (Zamojska et al. 2021).

Probiotics are viable microorganisms, mostly sourced from the lactic acid bacteria (LAB) group, that is administered into the host in sufficient amount (population density) to promote the growth and maintain health of their host (Quinto et al. 2014). Lactic acid bacteria is a class of facultative anaerobic bacteria that can increase intestinal acidity through production of lactic acid, yet preventing the growth of pathogenic bacteria in a variety of symbiotic environments, including animals and humans. This particular group can also produce other metabolites such as hydrogen peroxide, antimicrobials, and other bioactive products that enhance host productivity (Ibrahim et al. 2021). The genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* comprise a number of LAB groups (Bintsis 2018). It is possible to isolate LAB from the gastrointestinal tract because this bacterial community makes up the majority of the microflora in the intestine. An animal with a u digestive system is the black soldier fly (*Hermetia illucens*), which is likely to harbor LAB species and strains that have the potential to serve as probiotics (Osimani et al. 2021).

Diet is known to alter the structure and composition of gut microbiota of an organism (David et al. 2013). The successful biovalorization of organic wastes by *H. illucens* could be promoted and enhanced through stable microbial community thriving in the insect gut (Clariza-Samayoa et al. 2016). Numerous reports have used conventional and high throughput identification techniques to show the presence of gut bacteria including lactic acid bacteria from the digestive tract of *H. illucens*, which is highlighted using modified rearing conditions and variations in feed. The gut microbiome in *H. illucens* fed with chickenfeed, mixed fruits and vegetables, and grass-cuttings was predominated by *Actinomyces* spp., *Dysgonomonas* spp., and *Enterococcus* spp (Klammsteiner et al. 2020). Modification

of rearing temperature of *H. illucens* larvae showed a dominance of the *Providencia* and other Enterobacteriaceae groups when exposed to higher temperatures (Raimondi et al. 2020). The dominant count of viable *Lactobacillus*, *Leuconostoc*, and other enteric bacteria from the digestive tract was reported by Osimani et al. (2021) while studying the microbial dynamics of *H. illucens* fed with coffee silverskin and microalgae. A different study by Campbell et al. (2020) found that Enterobacteriaceae and lactic acid bacteria (LAB) predominated when *H. illucens* larvae were fed with brewer's byproducts. The larva, *H. illucens* can be utilized to transform and valorize any byproducts of the agroindustry, livestock manures, or urban solid wastes, including as a source of potential LAB that could be further investigated for probiotics (Nguyen et al. 2015). In this study, we attempted to rear *H. illucens* larvae using different feeding substrates as a source of prebiotics, which would later be harvested to assess the presence of lactic acid bacteria in their digestive tract. The results of this study should be helpful for the development of *H. illucens* larvae and for evaluating the likelihood of LAB cross-application from insects to livestocks.

MATERIALS AND METHODS

Insect rearing

The main component of the feeding substrates consisted of chicken manure for P0 treatment, a mixture of chicken manure and tofu solid wastes for P1 treatment, and a mixture of vegetable wastes and tofu solid wastes for P2 treatment. The feeding substrates (1:1) were further grinded using a food blender to yield particles of ± 0.5 mm in diameter and moistened with water (1 L). Six-days-old larvae with a total of 150 larvae were reared in plastic boxes and filled with 3 kg of feeding substrates. The boxes were covered with fine-mesh cotton gauze and maintained at a temperature of 25-27ºC, relative humidity of 70%, and 24 h in the dark. After two weeks, the larvae were collected for LAB isolation.

Isolation and identification of LAB isolates

The body surface of *H. illucens* larvae was disinfected with 70% EtOH for 3 min. Approximately 10 g of randomly collected *H. illucens* larvae (10 ind) were dissected from the base of the head to the tip of the abdomen and ground using a mortar. One mL of the slurry was transferred into nine mL of sterile physiological saline and vortexed. Ten-fold dilution of homogenates was then inoculated on de Man Rogosa Sharpe Agar (MRSA) supplemented with 1% of $CaCO_3$ (w/v). The medium was incubated at 37ºC for 72 h. Indications of lactic acid bacteria isolates were indicated by the presence of a halo zone around the colony (Figure 1). Each colony was purified and characterized using standard procedures to screen lactic acid bacteria isolates, especially in terms of catalase (-), gram positive, and acid-producers (homofermentative/heterofermentative). Genomic DNA isolation, PCR amplification (785F, 907R), and 16S rRNA sequencing was performed commercially by Macrogen inc., Singapore. The sequences were submitted to the BLASTn process at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and searched for the best hit for species-to-strain delimitation.

Antagonistic test

Disk diffusion method was used to assess the antimicrobial activity of LAB isolates against pathogenic bacteria such as *Escherichia coli* ATCC® 8739, *Salmonella enterica* serotype (ser.) Typhimurium ATCC® 14028, and *Salmonella enterica* ser. Pullorum. Direct suspension of colonies from LAB isolates and pathogenic bacteria was prepared in physiological salines to yield a standardized inoculum load (≈10^8 CFU/mL). Sterile cotton swabs were dipped into each pathogen suspension and swabbed entirely on Mueller Hinton Agar (MHA) and let to dry. Fifty µL of LAB suspension was inserted into disk along with ciprofloxacin as a tested antibiotic and sterile distilled water as a negative control on top of MHA. The media were incubated at 37ºC for 24 h. The presence of clear zone around disk indicated the antimicrobial activity of LAB and the diameter was measured in mm using a digital caliper.

Acid tolerance test

To assess the acid tolerance, 1 mL of overnight LAB cultures was centrifuged, then the pellets were resuspended into 5 mL of sterile buffer solution with different pH (2.0, 3.0, 6.0, 7.0), and adjusted to 10^8 CFU/mL. The cultures were incubated for 90 min at 37ºC under agitation. The viability of LAB isolates as expressed in log CFU/mL was determined after 0, 30, 60, and 90 min incubation by serial dilution and pour plate onto MRS agar. The seeded medium was incubated at 37ºC for 24 hr and counted thereafter. The percentage survival of LAB isolates was calculated according to the following equation:

$$\% \text{ viability} = 100 \times Af/Bf$$

The variable *Af* represents the viable colony on MRS agar (log CFU/mL) after tolerance test and *Bf* represents the initial cell count or colonies (log CFU/mL) before test.

Bile tolerance test

To assess the bile tolerance, the fresh culture of each isolate was prepared as earlier explained. The pellets were resuspended into bile salt solutions (0.1%, 0.3%) and then incubated for 90 min at 37ºC under agitation. At different time intervals (0, 30, 60, 90 min), the samples were taken and serially diluted until 10^8 and spread onto MRS agar. The seeded medium was incubated and counted for the viable colonies (log CFU/mL) while the percentage of viability was calculated using previous equation.

Adhesion test

To assess the adherence possibility of LAB isolates, some chicken epithelial cells were used as growing substrates. The entire gastrointestinal tract of chicken was removed immediately from a local market and transported in cold condition to the laboratory. The gut contents were removed and ileal segments were opened, washed

repeatedly using phosphate buffer saline (PBS) and held in PBS at 4°C for 90 min. The cleansed ileum was cut into four fragments (1 cm^2) and each was incubated in LAB suspension (10^9 CFU/mL) at 37°C for 90 min. At different time intervals (0, 30, 60, 90 min), the samples were taken, macerated and serially diluted until 10^8 and spread onto MRS agar. The seeded medium was incubated and counted for the viable colonies (log CFU/cm^2).

Selection of LAB isolates as putative probiotics

All graphical images were built using GraphPad Prism ver. 8.0.2. All experiments were conducted in duplicate and the results were expressed as mean and analyzed descriptively. A principal component analysis (PCA) was performed using Minitab ver. 17.0 to visualize the clustering of multivariate data as a basis of selection to potential LAB isolates as putative probiotics.

RESULTS AND DISCUSSION

These isolates were randomly selected based on their emergence of diluted larval gut samples within 10^5-10^8 CFU/mL. Two isolates were recovered from P0 treatment (larvae fed with chicken manure), four isolates were recovered from P1 treatment (larvae fed with chicken manure feces and tofu solid wastes), and five isolates were recovered from P2 treatment (larvae fed with vegetable wastes and tofu solid wastes). All isolates showed typical characteristics of lactic acid bacteria, to cite: Gram-positive, catalase-negative, non-motile, non-spore-forming rods, and the appearance of clear zones around colonies as the indication of soluble $CaCO_3$ on isolation medium. The DNA sequencing based on 16S rRNA region reached a species and strain-level identification of these isolates, which were later identified as *Lactiplantibacillus pentosus*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus paracasei* based on BLAST search results (Table 1).

The species, *L. plantarum* was being the dominant species with five isolates followed by *L. paracasei* and *L. pentosus*, each with 3 isolates. Remarkably, *L. paracasei* was not found in the P0 or P1 treatments, whereas *L. plantarum* was not present in the P2 treatment. This suggests that the different type and composition of feeding substrates consumed by *H. illucens* larvae may have an impact on the gut bacteria. These results are also supported by Jeon et al. (2011) who reported differences in bacterial communities by using a metagenomic approach in *H. illucens* larvae fed with three different types of feed, namely calf forage, cooked rice, and food waste. Furthermore, *H. illucens* larvae fed with coffee roasting wastes also showed a higher level of genera diversity which included *Lactobacillus*, *Leuconostoc*, and *Weissella* which was more diverse than our findings (Osimani et al. 2021). Currently, there have been no report on *L. paracasei* found in vegetable wastes. The species, *L. paracasei* is notably known for its high adaptability and ability to thrive in a variety of habitats (Martino et al. 2016). Yet, the contribution of *L. paracasei* as one of the gut microbiota in *H. illucens* larvae remains unknown. However, a study reported that *H. illucens* larvae immunized with a strain of *L. paracasei* (syn. *Lactobacillus casei*) produced peptides with antibacterial properties (Choi et al. 2017). In general, the occurrence of LAB and probiotics may increase the growth performance of *H. illucens* larvae through effective digestion of feeding substrate and delayed developmental stage into pupae (De Smet et al. 2018).

Antagonistic activity against three indicator strains (pathogen) including *E. coli*, *S. enterica* ser. Typhimurium, and *S. enterica* ser. Pullorum was tested with 11 LAB isolates. Using the disk diffusion test, all LAB isolates displayed inhibitory activities against all the tested pathogens at different degrees (Table 2).

The range of inhibition zones produced by LAB isolates against *E. coli* was between 11.3 to 20.1 mm, *S. enterica* ser. Typhimurium between 13.3 to 21.5 mm, and *S. enterica* ser. Pullorum between 13.4 to 20.8 mm. The most antagonistic isolate was *L. pentosus* 5P2i1 against *E. coli*, followed by *L. plantarum* 6P1i1 against both *S. enterica* ser. Typhimurium and Pullorum. Overall, the antagonistic test results demonstrated that all LAB isolates from *H. illucens* larvae displayed a broad antibacterial spectrum of activity against pathogenic bacteria and were regarded as prime candidates for probiotics. Infections by zoonotic and foodborne enteric pathogens result in significant financial loss to the poultry industry as well as high morbidity and mortality (Nallala et al. 2017). It is anticipated that the use of probiotic bacteria, particularly those that are antagonistic to pathogenic bacteria, will be able to reduce the pathogen population either through competitive exclusion or direct antibiosis in the host's digestive tract (Vieco-Saiz et al. 2019). The LAB group secrete significant amounts of organic acids, bacteriocins, hydrogen peroxide (H_2O_2), hydrogen cyanide (HCN), and other antibacterial metabolites that can prevent the growth of other microbes known as antibiosis (Reis et al. 2012). However, in this study, we did not conduct any additional research on the antibacterial compounds produced by the antagonistic isolates. When compared to other LAB investigations that are more concentrated on honey bee groups, the discovery of antagonistic LAB isolates from *H. illucens* larvae is considerably promising (Vásquez et al. 2012).

The viability of LAB isolates in simulated gastric juice or acidic solution and bile salt was examined. The simulation was designed to match the typical pH condition in the GI tract of a broiler chicken such as pH 2.0 and pH 3.0 in the gizzard or proventriculus, pH 6.0 in the duodenum, and pH 7.0 in the jejunum and ileum. In addition, the tolerance test was also meant to mimick the transit time of feeds during digestion in a broiler chicken which took at least 30 to 90 min in each segment of GI tract (Ravindran 2013). The dynamics of population density from each isolate is presented in Figure 1. Following the duration of time observed during the tolerance test, the majority of the LAB isolates displayed a decline in viable cells with varying density. After 90 min exposure to the simulated environment, the percentage of viable cells were measured to signify the differences among isolates (Figure 2).

Table 1. Species identification of LAB isolates by 16S rRNA sequencing and BLAST search

Isolate	BLAST hit (species/strain)	Query cover (%)	*E*-value	% Sequence identity	Genbank acc. no.	Source	Submitted accession number
5P0i4	*Lactiplantibacillus plantarum* subsp. *plantarum* JCM8341	81	0.0	99.93	LC633333.1	FP	OP905664.1
6P0i3	*Lactiplantibacillus plantarum* strain DM083	97	0.0	99.93	OP579188.1	H	OP905670.1
5P1i1	*Lactiplantibacillus pentosus* strain BMOBR061	99	0.0	99.61	MN880129.1	DP	OP905665.1
5P1i3	*Lactiplantibacillus plantarum* strain DM083	97	0.0	99.73	OP579188.1	H	OP905666.1
6P1i1	*Lactiplantibacillus plantarum* strain MRKAK14	99	0.0	100.00	OP218361.1	DP	OP905671.1
6P1i3	*Lactiplantibacillus plantarum* strain MRKAK4	97	0.0	99.93	OP218348.1	DP	OP905672.1
5P2i1	*Lactiplantibacillus pentosus* strain BSR3	100	0.0	99.80	KY203913.1	FP	OP905667.1
5P2i5	*Lacticaseibacillus paracasei* strain RV-M192	100	0.0	99.93	MK966341.1	P	OP905668.1
5P2i9	*Lactiplantibacillus pentosus* strain BMOBR061	99	0.0	100.00	MN880129.1	DP	OP905669.1
6P2i1	*Lacticaseibacillus paracasei* strain 32X	97	0.0	99.87	MK774610.1	FP	OP905673.1
6P2i2	*Lacticaseibacillus paracasei* strain LLC-E96	98	0.0	100.00	MW664018.1	H	OP905674.1

Note: DP: Dairy products, FP: Fermented products, H: Human. P: Plants.

Table 2. Antagonistic activity of gut LAB isolates from *H. illucens* larvae against pathogenic bacteria by disk diffusion technique

Strain	Zone of inhibition (mm)		
	Escherichia coli	*Salmonella enterica* ser. Typhimurium	*Salmonella enterica* ser. Pullorum
5P0i4	14.8	15.6	13.4
5P1i1	16.2	13.3	13.4
5P1i3	15.5	13.5	14.8
5P2i1	20.1	19.6	19.1
5P2i5	19.7	19.3	18.7
5P2i9	19.9	19.2	18.6
6P0i3	13.7	13.4	14.4
6P1i1	17.3	21.5	20.8
6P1i3	11.3	15.8	16.6
6P2i1	13.5	14.1	13.2
6P2i2	13.3	13.7	14.2

In pH 2.0, the most tolerant strain was obtained from *L. pentosus* 5P2i1 with the remaining cells at log 6.23 CFU/mL or 68.29% while the least was obtained from *L. plantarum* 5P0i4 with log 2.60 CFU/mL or 29.03% of viable cells. In pH 3.0, the most tolerant strain was obtained from *L. paracasei* 5P2i5 (log 6.90 CFU/mL, 75.80%) while the least was *L. plantarum* 5P0i4 (log 3 CFU/mL, 33.47%). Approaching neutral pH conditions, most of the growth of LAB isolates was decreasing. In pH 6.0, the highly surviving isolate was observed from *L. paracasei* 6P2i2 with log 5.26 CFU/mL or 57.75% of viable cells while the least was *L. pentosus* 5P1i1 (log 2.04 CFU/mL, 22.59). In pH 7.0, only five LAB isolates were known to survive with the highest population from *L. paracasei* 6P2i2 with log 4.20 CFU/mL or 46.20% (Table 3).

Similar to the trend of growth under bile salt stress, the population of LAB isolates also showed a decreasing trend following duration of exposure and increased concentration of oxgall. The most tolerant strain to 0.1% and 0.3% bile salt was observed from *L. pentosus* 5P1i1 and *L. paracasei* 6P2i2. In general, all tested strains showed less resistance to intestinal conditions than gastric juice environment. The most significant factors affecting the survival and performance of probiotics after ingestion by animals are their high acidity (pH<3) in the gastric environment and high concentration of bile salt components in the proximal intestine (Verón et al. 2017). The majority of probiotics may display variable resistance to acidic conditions, which is a trait that depends on the species, isolate, and strain origins (Gupta et al. 2017). Furthermore, strains that were able to endure this environment may also be capable of surviving the dynamical environment in the avian gut and attaching to the intestinal cells while exerting beneficial effects (Reuben et al. 2019). The production of exopolysaccharides and digestive enzymes, such as bile salt hydrolase, by lactic acid bacteria was the mechanism underlying their ability to survive under harsh conditions (Nguyen et al. 2020, Shehata et al. 2016).

Table 3. Population dynamics (CFU/mL) and % viability of LAB isolates from *H. illucens* larvae after 30, 60, and 90-hr exposure to different bile salt concentration (0.1%, 0.3%)

Strain	Log CFU/mL at 0.1%			% Viability (Final)	Log CFU/mL at 0.3%			% Viability (Final)
	30 hr	60 hr	90 hr		30 hr	60 hr	90 hr	
5P0i4	7.20	6.30	4.85	53.47	3.43	2.48	-	-
5P1i1	7.04	5.70	5.85	64.91	3.95	3.18	1.70	18.87
5P1i3	3.85	3.00	2.70	30.06	2.04	-	-	-
5P2i1	6.48	5.04	4.00	44.13	3.85	3.04	2.48	27.33
5P2i5	6.11	5.90	3.95	43.42	4.60	3.11	-	-
5P2i9	7.70	6.70	4.85	53.32	5.78	3.70	2.95	32.51
6P0i3	7.48	5.18	3.48	38.24	2.78	1.30	-	-
6P1i1	5.11	3.90	3.00	32.95	3.85	2.30	-	-
6P1i3	7.18	6.26	4.70	52.09	3.95	2.85	1.48	16.37
6P2i1	6.90	5.11	3.78	41.50	5.48	4.95	2.85	31.25
6P2i2	7.48	5.95	5.00	55.16	6.85	5.04	3.23	35.64

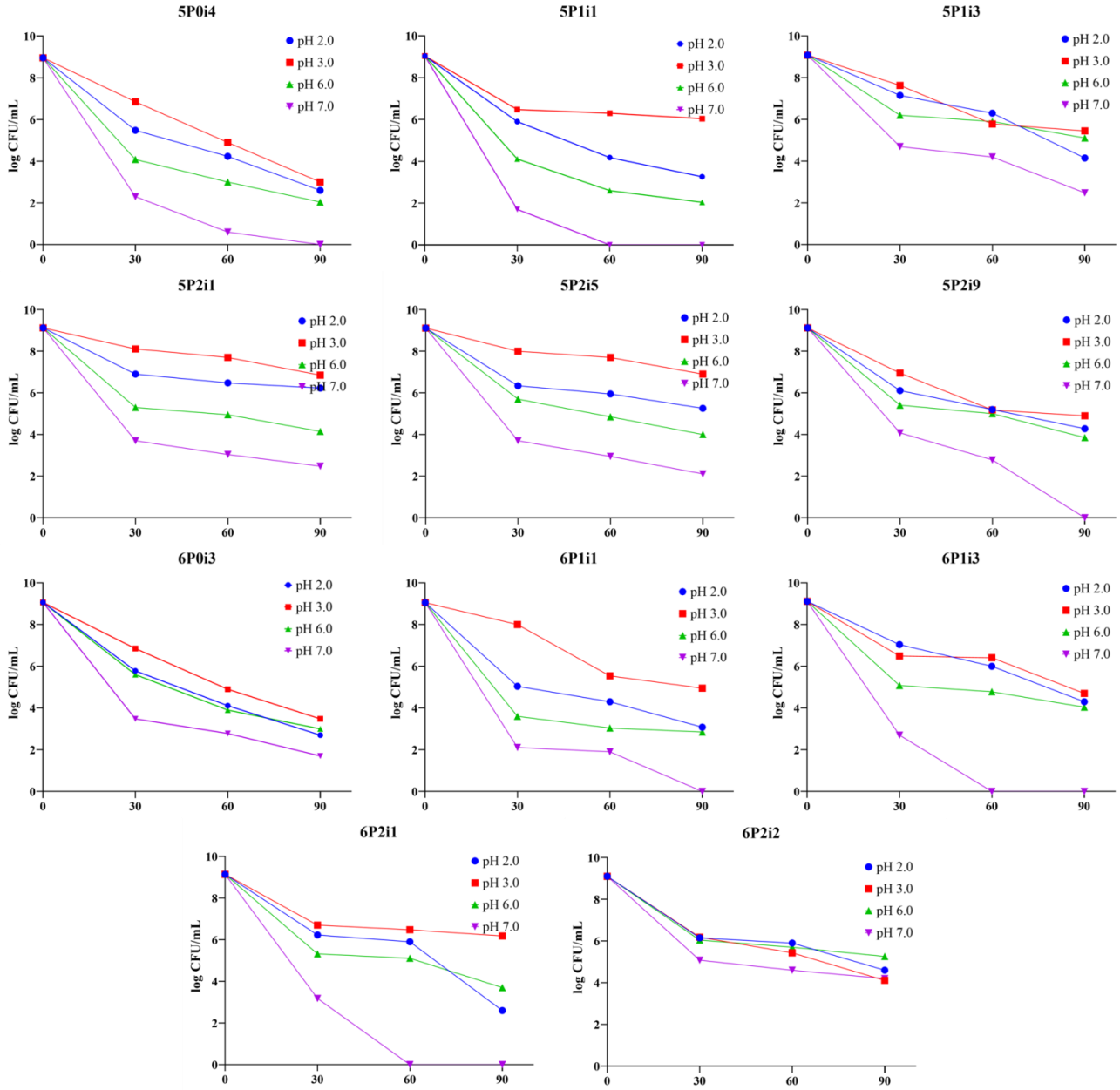

Figure 1. Survival of LAB isolates from *H. illucens* larvae under simulated gastric solution at different pH (2.0, 3.0, 6.0, and 7.0). X axis represents duration of simulation (min)

The adherence of LAB to host's intestinal tissue is also a supporting feature of probiotics for successful colonization (Setyawardani et al. 2014). In this study, chicken intestine or ileum were used as growth substrates to assess the adherence capacity of each LAB isolate. The results showed that all isolates showed adherence ability which was indicated by the increasing number of viable cells in the intestinal tissue tested along with the duration of observation which indicated success in spatial colonization (Figure 3). The highest count was observed from *L. pentosus* 5P219 with log 7.48 CFU/cm^2, followed by *L. paracasei* 5P2i5 (log 6.34 CFU/cm^2), *L. pentosus* 5P2i1 (log 6.08 CFU/cm$_2$), *L. plantarum* 5P0i4 (log 6.00 CFU/cm^2), and so on. Furthermore, when exposed to chicken intestines, the majority of LAB isolates exhibited stable growth and a gradual increase in population count from 30 to 90 min, which at least indicating that they would be resistant to physical removal during gut contraction and peristaltic flow (Peres et al. 2014). Hamida et al. (2015) also reported that LAB isolates showed different adhesion abilities which is similar to our findings with a viable population range between 7.65 to 9.40 log CFU/mL after exposure to chicken ileal cells in pH 7.2 for 30 min. In addition, the efficacy of LAB as probiotics may be investigated further through competitive adherence to chicken ileal cells together with the inoculation of enteric pathogens which is not covered in this study (Reuben et al. 2020). The adhesion capability depends on the physiology of the cell and the composition of the cell wall of different bacterial strains and species (Ranadheera et al. 2012).

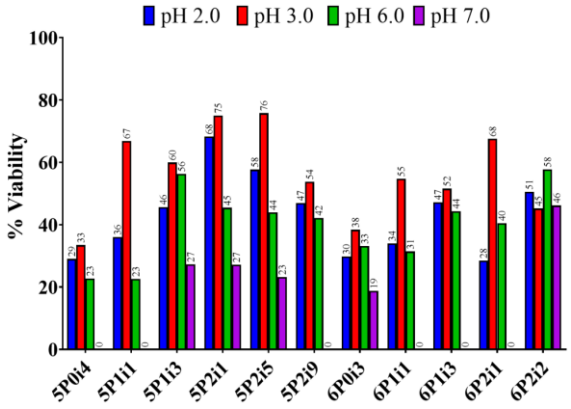

Figure 2. Viability of LAB isolates from *H. illucens* larvae after 90-min of exposure to different pH

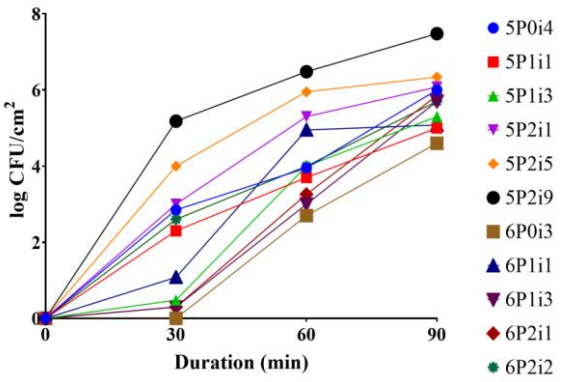

Figure 3. Adherence of LAB isolates from *H. illucens* larvae to poultry ileum epithelial cells

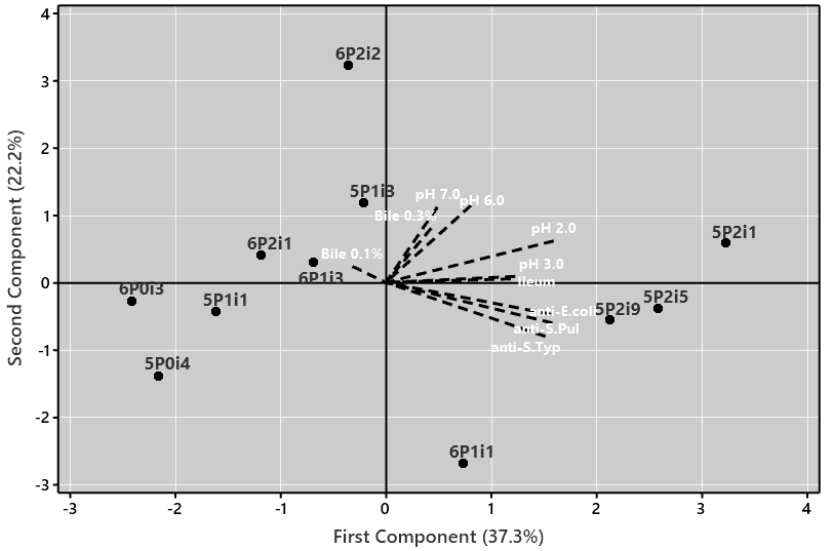

Figure 4. Biplot constructed from the principal component analysis (PCA) for 11 LAB isolates from *H. illucens* larvae showing the variable projection to acid tolerance, adherence to ileum tissue, antagonistic activity, and bile salt tolerance

The interaction of various glycoproteins, lipid-anchored proteins, and proteins on the bacterial cell surface layer with certain receptors for molecules in the extracellular matrix layer of epithelial cells and the intestinal mucosal layer resulted in adhesive activity (Schillinger et al. 2005). The following step is to identify which isolates are suitable for use as poultry symbionts based on the parameters that have been observed as probiotic candidates. There are two methods for selecting suitable probiotic candidates: giving weight values and ranking them based on the probiotic parameters studied, or using multivariate methods to find the most optimal probiotic properties. Based on the results of the tested parameters, a multivariate analysis using PCA was used to identify and categorize the best candidate as probiotics. Based on Figure 4, four LAB isolates namely *L. pentosus* 5P2i1, *L. paracasei* 5P2i5, *L. pentosus* 5P2i9, and *L. plantarum* 6P1i1 are located at the upper and lower right quadrants and showing the best results, especially regarding to the pH tolerance, antagonistic activity against pathogenic bacteria and adherence to ileum. Other researchers have used a similar multivariate approach to identify putative probiotics from different sources that may be complementary or antagonistic depending on the physiological performance of species and strains. Sampaio et al. (2021) selected four LAB strains identified as *L. paracasei* and *L. casei* using PCA that showed the consistent results in terms of antagonistic activity, auto-aggregation, acid and bile salt tolerance, co-aggregation, and hydrophobicity. In addition, Vijayalakshmi et al. (2020) combined PCA with other statistics such as heat map and network analyses to determine the most promising LAB strains (*L. casei, L. plantarum,* and *L. rhamnosus*) with different bioactivities similar to the previous report.

As a result, it is anticipated that the LAB isolates from *H. illucens* larvae, which have been characterized as probiotics, may be applied to poultry as one of the productive and functional symbionts in enhancing its growth performance and maintaining the health condition under risk of enteric pathogenic bacterial infection. Additional in vivo studies are required to determine the exact health-

promoting benefits of the LAB isolates and to validate their apparent suitability for usage as new probiotics.

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