BIODIVERSITAS
Volume 24, Number 9, September 2023
Pages: 5198-5206

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240964

A comparative assessment of *Lactiplantibacillus plantarum* isolated from chicken and humans as candidates for probiotics

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Manuscript received: 15 August 2023. Revision accepted: 30 September 2023.

Abstract. *Sunardi J, Purnama ET, Sugata M, Victor H, Jan TT, Jo J. 2023. A comparative assessment of* Lactiplantibacillus plantarum *isolated from chicken and humans as candidates for probiotics. Biodiversitas 24: 5198-5206. Lactiplantibacillus plantarum* is commonly analyzed as a potential probiotic. We hereby investigated two strains isolated from chicken crop (*Lpb. plantarum* F75) and human breast milk (*Lpb. plantarum* SU-KC1a). Ability to withstand osmotic stress (1.5%, 2.5% or 3.5% of NaCl) and phenol compounds (0.2% or 0.5%), ability to survive gastric juices for a maximum of 120 minutes and bile salt for a maximum 3 hours, as well as susceptibility to 25 antibiotic discs, were compared between both strains. Whole genomes of both strains were sequenced and analyzed *in silico* to determine the availability of antibiotic-resistance genes as well as the presence of mobile genetic elements and plasmid. Both strains were sensitive to increased concentrations of NaCl and phenol as well as to prolonged exposure to gastric juices. In contrast, both strains could withstand a prolonged exposure of 0.3% of bile salt. Both isolates had similar genome sizes and were susceptible to many tested antibiotics. The detected resistance genes were observed within the chromosomal genomes but no mobile genetic element nor plasmid was found. In conclusion, both strains of *Lpb. plantarum* displayed several characteristics of beneficial bacteria and could be used as probiotic candidates for poultry and human beings, respectively.

Keywords: Antibiotic resistance gene, characteristics, *Lactiplantibacillus plantarum,* probiotics, whole-genome sequencing

INTRODUCTION

There is an increasing awareness and interest in food quality and the associated health benefits (Bigliardi and Galati 2013). The COVID-19 pandemic indeed heightened societal acceptance and interest in healthy eating behaviors and a variety of healthy foods (Di Renzo et al. 2020). The concept of healthy foods suggests that foods are not merely nutrition but medicine as well to prevent and treat diseases (Nature Medicine 2023). Healthy foods containing substances beyond basic nutrition that might benefit health are also known as functional foods, which are defined as novel foods that have been formulated to contain substances or live microorganisms at a concentration that is both safe and sufficiently high to achieve the intended (Temple 2022). Therefore, fortification of food with beneficial living microorganisms is of interest.

Lactic acid bacteria are a group of microorganisms that are generally recognized as safe and can ferment carbohydrates to produce lactic acid. Furthermore, lactic acid bacteria have been widely used as probiotics because they could confer health benefits to the host if administered in adequate amounts (Hill et al. 2014). There are approximately 60 genera within this classification, several of which are widely used in food fermentation, including *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Lactobacillus* species (Mokoena 2017).

One of the most promising lactic acid bacteria as probiotics is *Lactobacillus plantarum*, now known as *Lactipantibacillus plantarum*. *Lpb. plantarum* is a gram-positive bacterium that can inhabit a wide range of ecological niches, including mammalian gastrointestinal tracts. The ability to inhabit the gastrointestinal tract is associated with the abilities of *Lpb. plantarum* to tolerate acid stress and to withstand bile salt exposure (Fidanza et al. 2021). Pertaining to the acid resistance, *Lpb. plantarum* could increase the ratio of saturated to unsaturated fatty acids in its cellular membrane upon exposure to low pH, resulting in a reduction in its membrane fluidity (Huang et al. 2016). In addition, Lpb. plantarum could increase the expression of phosphofructokinase and pyruvate-kinase as well as of ATP-synthase genes *atpA* and *atpC* to produce more ATP that allows a higher activity of proton pump, resulting in a maintenance of intracellular pH (Heunis et al. 2014; Seme et al. 2015; Huang et al. 2016). Furthermore, *Lpb. plantarum* could increase its intracellular concentration of alanine and arginine upon exposure to acid stress, resulting in tolerance to low pH (Heunis et al. 2014; Seme et al. 2015). Regarding the bile salt tolerance, *Lpb. plantarum* at least used four important mechanisms, i.e., (i) up-regulating activities of its four bile salt hydrolase (*bsh*) genes; (ii) altering composition and fluidity of cellular membrane; (iii) protecting against oxidative stress; and (iv) maintain the proton motive force (Gu et al. 2014; Fidanza et al. 2021). Interestingly, while other *Lactobacillus* species

exhibit specific genetic signatures that reflect adaptations to particular niches, *Lpb. plantarum* does not exhibit an adaptational signature, suggesting that it retains a diverse genome to adapt to various niches (Martino et al. 2016; Filannino et al. 2018; Inglin et al. 2018).

We recently isolated two strains of *Lpb. plantarum* from chicken crop *(Lpb. plantarum* F75) and human breast milk (*Lpb. plantarum* SU-KC1a). It was of interest to assess whether both isolates exhibit similar genotypes and phenotypes as well as whether both isolates display certain characteristics as probiotic candidates, including abilities to survive against the presence of salt in the food products (Valdés et al. 2015), phenol in the gastrointestinal tract (Pacheco-Ordaz et al. 2018), the acidic conditions within the stomach and bile acids in the small intestine (Fidanza et al. 2021). Another criterion of interest is to assess whether the isolated *Lpb. plantarum* strains harbor transmissible resistance genes encoding resistance to common antibiotics (Fidanza et al. 2021). This could be done by assessing the antibiotic susceptibility phenotype, the relevant antibiotic resistance genes as well and the capacity to transfer those genes (Binda et al. 2020). In this study, several characteristics of beneficial bacteria (i.e., ability to resist osmotic stress and phenol compounds, ability to survive gastric juices and bile acids, as well as sensitivity to commonly used antibiotics) were compared between *Lpb. plantarum* F75 and SU-KC1a. Subsequently, the whole genomes of both strains were sequenced and analyzed to determine the presence of relevant antibiotic resistance genes as well as of mobile genetic elements and plasmid.

MATERIALS AND METHODS

Bacterial isolates

Lactiplantibacillus plantarum F75 and *Lactiplantibacillus plantarum* SU-KC1a were isolated from chicken crop and human milk samples and processed at the Department of Biology of Universitas Pelita Harapan, Tangerang, Indonesia. Both isolates were cultured on De Man, Rogosa, and Sharpe (MRS) agar (Merck, Germany) until further antibiotic testing.

Salt and phenol tolerance

Overnight culture of *Lpb. plantarum* F75 or SU-KC1a was inoculated at 10^7 cells/mL in MRS broth with the addition of 1.5%, 2.5%, or 3.5% (w/v) of NaCl. In parallel, the isolates were inoculated at 10^7 cells/mL in MRS broth with the addition of 0.2% or 0.5% (v/v) of phenol. Cultures grown in MRS broth without NaCl or phenol were used as respective controls. The cultures were incubated at 37ºC for 24 hours. The OD_{600} measurement was obtained to assess the survivability of each culture and the results were presented as relative abundance in percentage (Parlindungan et al. 2021) according to the formula:

Relative abundance (%) = OD test sample/OD control sample x 100%

Simulated gastric fluid and bile salt tolerance

For simulated gastric fluid tolerance, overnight culture of *Lpb. plantarum* F75 or SU-KC1a was inoculated at 10^7 cells/mL in MRS broth, supplemented with 2 g/L of NaCl and 6 g/L of pepsin, in which the pH was adjusted to 2. The cultures were incubated at 37ºC for 120 minutes. At 0, 30, 60 and 120 minutes, each culture was sampled and plated on MRS agar (MRSA) for enumeration. For bile salt tolerance, overnight culture of *Lpb. plantarum* F75 or SU-KC1a was inoculated at 10^7 cells/mL in MRS broth, supplemented with 0.3% (w/v) of bile salt. The cultures were incubated at 37ºC for 3 hours. At 1-hour interval, each culture was sampled and plated on MRS agar for enumeration (Parlindungan et al. 2021)

Antibiotic susceptibility test

Antibiotic susceptibility was determined via the Kirby-Bauer disk diffusion method (Jorgensen and Turnidge 2015). Mueller Hinton Agar (MHA) (Himedia, India) was used for the susceptibility testing of both isolates. Prior to testing, *Lpb. plantarum* F75 and SU-KC1a were inoculated in MRS broth and incubated at 37°C to obtain turbidity of 0.5 McFarland, which was equivalent to 1.5 x 10^8 viable cells. The liquid culture was prepared by using sterile cotton swabs that were pressed against the wall of the test tube to remove excess liquid and streaked on both mediums. The Petri dish was incubated at room temperature for 15 minutes before antibiotic discs were dispensed. Fifteen minutes after the bacteria were inoculated, 25 antibiotic discs (Liofilchem, Italy), including aminopenicillins (Amoxicillin [2 µg] and Ampicillin [10 µg]), polypeptide (Bacitracin [10 IU]), cephalosporin (Cefoxitin [30 µg]), penicillinase-resistant penicillin (Methicillin [5 µg] and Oxacillin [1 µg]), glycopeptide (Vancomycin [30 µg]), aminoglycoside (Gentamicin [10 µg], Kanamycin [30 µg], Neomycin [30 µg] and Streptomycin [10 µg]), tetracycline (Tetracycline [30 µg]), phenicols (Chloramphenicol [30 µg]), lincosamide (Clindamycin [2 µg] and Lincomycin [2 µg]), macrolides (Erythromycin [15 µg] and Tylosin [30 µg]), pleuromutilin (Tiamulin [30 µg]), folate antagonists (Sulfonamide [300 µg]), quinolones (Ciprofloxacin [5 µg], Nalidixic Acid [30 µg] and Ofloxacin [5 µg]), rifampin (Rifampicin [5 µg]), as well as monocarboxylic acid (Mupirocin [5 µg & 200 µg]), were placed individually on the surface of each agar plate. All plates were incubated in an inverted position at 37°C for 24 hours in a microaerophilic condition. The clear zone surrounding each disc was then measured. Measurement included the diameter of the antibiotic disc (6 mm). Each antibiotic disc was tested thrice. The measurement of the clear zone was expressed in terms of susceptible, intermediate, and resistance. Susceptible (S) signifies that the tested bacteria can be inhibited by the usual concentration of the antimicrobial agent upon application. Intermediate (I) signifies isolates in which the antimicrobial activity is lower than the susceptible isolates upon application of drugs with a certain concentration. Resistance (R) indicates bacteria that are completely resistant to an achievable concentration of antibiotics. While a microorganism was described as susceptible if its

diameter was within the category of S or I, a microorganism was identified as resistant if its result was within the category of R (Clinical and Laboratory Standards Institute 2023). The interpretive criteria for many antibiotic discs were mainly based on *Lactobacillus* spp. (Charteris et al. 1998) or on *Staphylococcus* spp. (Clinical and Laboratory Standards Institute 2023).

Whole-genome analyses

The whole genome of *Lpb. plantarum* F75 was sequenced by the Novogene Company Limited (Hong Kong, China) using the Illumina technology platform (USA). The whole genome of *Lpb. plantarum* SU-KC1a was sequenced by the PT. Genetika Science (Tangerang, Indonesia) using the Oxford Nanopore Technology (UK). The in-house bioinformatic analyses were subsequently performed to assemble the whole genomes and identify antibiotic-resistance genes within both genomes. The whole-genome sequence of Lpb. plantarum F75 was assembled according to the prior publication (Dikson et al. 2022). Briefly, the whole-genome sequence of *Lpb. plantarum* F75 and SU-KC1a were checked for their quality by FastQC. For *Lpb. plantarum* F75, the contig assembly was subsequently performed using SPAdes. The contig coverage was checked using Qualimap (Okonechnikov et al. 2016). The remaining contigs were re-ordered using Mauve with *Lpb. plantarum* SK151 (NZ_CP030105.1) as the reference genome. Unmapped contigs were separated from the mapped contigs, in which the mapped contigs were re-ordered and merged into one FASTA sequence using Artemis. In parallel, de novo genome assembly for *Lpb. plantarum* SU-KC1a was performed by the EPI2ME Labs workflows, comprising the Flye assembler and Medaka programs (Epi2Me 2020). The contigs were analyzed through the same process as the ones of *Lpb. plantarum* F75. The complete genome was finally submitted into dFAST for annotation (Tanizawa et al. 2018). Antibiotic resistance genes from both genomes were subsequently selected using the Comprehensive Antibiotic Resistance Database (CARD). Results obtained from the CARD were confirmed by the dFAST annotation. The unmapped contigs were analyzed individually using BLAST to determine whether each sequence was a part of the whole genome.

Data analysis

Descriptive statistics were presented as median, minimum, and maximum values. Data from salt tolerance, simulated gastric fluid tolerance or bile salt tolerance of each bacterial strain were analyzed individually using the Kruskal-Wallis test, in which a significant result ($p<0.05$) would be continued with the Dunn's multiple comparison test. Data from the phenol tolerance of each bacterial strain were analyzed individually using the Mann-Whitney test. Statistical analyses and data visualization were obtained by using the GraphPad Prism ver. 10.0.2 (California, USA).

RESULTS AND DISCUSSION

As gram-positive lactic acid bacteria, *Lactiplantibacillus plantarum* has been extensively studied and widely consumed due to its probiotic properties (Fidanza et al. 2021). It was of interest to assess whether *Lpb. plantarum* F75 and SU-KC1a, isolated from different hosts, exhibited characteristics as beneficial bacteria within the gastrointestinal tract. Three characteristics i.e., (i) able to survive against presence of salt in the food products (Papadimitriou et al. 2016) and phenol in the gastrointestinal tract (Pacheco-Ordaz et al. 2018) ; (ii) able to survive the acidic conditions in the stomach and to tolerate exposure to bile salt in the small intestine (Fidanza et al. 2021); and (iii) sensitive to common antibiotics (Fidanza et al. 2021), were tested during the present study.

During industrial processing and fermentation, probiotics might encounter osmotic stress, causing a decrease in cell productivity and inactivity (Papadimitriou et al. 2016). As expected, Figure 1 depicts an inverse relationship between the salt concentration and the cell viability. As compared to the growth of isolates in the control medium, *Lpb. plantarum* F75 and SU-KC1a had a relative abundance of 80.20% and 72.27%, respectively, in the presence of 1.5% of NaCl. In the presence of 2.5% of NaCl, the relative abundance of *Lpb. plantarum* F75 and SU-KC1a decreased to 78.33% and 68.44%, respectively. In the presence of 3.5% of NaCl, the relative abundance of *Lpb. plantarum* F75 and SU-KC1a further reduced to 35.78% and 53.85%, respectively. The relative abundance among three concentrations for either *Lpb. plantarum* F75 or SU-KC1a were not significantly different. This finding was in line with a previous study, showing that *Lpb. plantarum* had a relatively high tolerance to 1.5% of NaCl and a moderate tolerance to 3.5% of NaCl (Parlindungan et al. 2021).

Next, probiotics require the ability to survive phenolic compounds because this compound, present within the gastrointestinal tract, would inhibit microbial growth (Pacheco-Ordaz et al. 2018). Of note, the phenolic content in the intestinal system varies among humans, as it is influenced by various factors, particularly an individual's diet (Valdés et al. 2015). Figure 2 shows an inverse relationship between the phenolic concentration and the cell viability. As compared to the growth of isolates incubated in the control medium, *Lpb. plantarum* F75 and SU-KC1a had a relative abundance of 68.01% and 65.24%, respectively, in the presence of 0.2% of phenol. In the presence of 0.5% of phenol, the relative abundance of *Lpb. plantarum* F75 and SU-KC1a decreased to 7.40% and 5.99%, respectively. The relative abundance between both concentrations for either *Lpb. plantarum* F75 or SU-KC1a were not significantly different. It was noted that both *Lpb. plantarum* F75 and SU-KC1a were very susceptible to the presence of 0.2 and 0.5% phenol, as compared to other strains of *Lpb. plantarum* tested in a published study (Parlindungan et al. 2021).

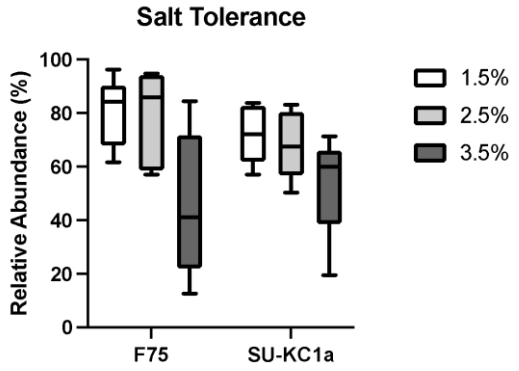

Figure 1. The salt tolerance on *Lpb. plantarum* strain F75 and SU-KC1a. Both isolates were cultured at 37ºC for 24 hours in MRS broth supplemented with 1.5%, 2.5% or 3.5% of NaCl. The relative abundance was displayed as a box and whiskers plot, in which whiskers represent minimum and maximum data. The horizontal line within each box indicates the median. Results of each bacterial strain were analyzed individually using the Kruskal-Wallis test with a p-value less than 0.05 would be considered as a statistically significance. The values were obtained from five experiments

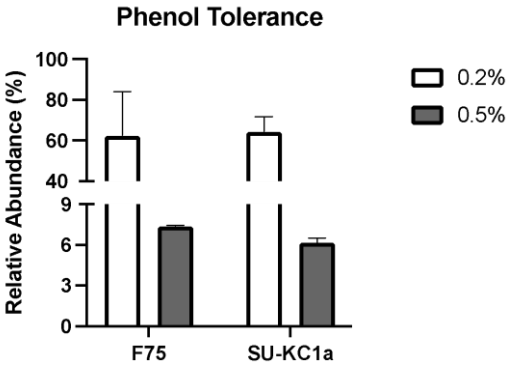

Figure 2. The phenol tolerance of *Lpb. plantarum* strain F75 and SU-KC1a. Both isolates were cultured at 37ºC for 24 hours in MRS broth supplemented with 0.2% or 0.5% of phenol. The median of relative abundance was displayed as bar graph. The error bar indicates the maximum data. Results of each bacterial strain were analyzed individually using the Mann-Whitney test with a p-value less than 0.05 would be considered as statistically significant. The values were obtained from three experiments

Simulated gastric fluid and bile salt tolerance assays were performed to estimate the viability of both isolates in the harsh conditions of the gastrointestinal tract. The duration of gastric emptying among Caucasian and Asian healthy volunteers had been measured by using scintigraphy (Abell et al. 2008; Vasavid et al. 2014). The results suggested that at the first hour of intake, the meal retention could vary between 30% to 90% and that at the fourth hour of intake, the meal retention was less than or equal to 10% (Abell et al. 2008). Thus, this experiment chose time points of 30, 60 and 120 minutes of exposure to the simulated gastric fluid. As shown in Figure 3, both *Lpb. plantarum* F75 and SU-KC1a were sensitive to the simulated gastric fluid, but still able to withstand it after 30-and 60-minute exposure. Of note, after exposure for 120 minutes, while *Lpb. plantarum* F75 was completely undetected, *Lpb. plantarum* SU-KC1a was still detected although at a very low concentration, i.e., 6 x 10^1 CFU/mL. The only significant difference was observed in a comparison between 0-and 120-minutes exposure for *Lpb. plantarum* SU-KC1a (p=0.0389). This finding was in line with a published study, showing that in contrast to other *Lactobacillus* species, several strains of *Lpb. plantarum* were able to tolerate gastric juice to a certain degree (Parlindungan et al. 2021).

Furthermore, *Lpb. plantarum* F75 and SU-KC1a were able to survive the exposure to 0.3% of bile salt for 3 hours (Figure 4). It was indeed observed that *Lpb. plantarum* F75 and SU-KC1a remained viable at 3.6 x 10^8 and 2.4 x 10^8 CFU/mL, respectively, after exposure to bile salt for 3 hours. Of note, only the difference between 0-and 2-hour exposure for *Lpb. plantarum* F75 was statistically significant (p=0.0395). Indeed, it has been reported that various *Lactobacillus* species, including *Lpb. plantarum*, were tolerant to 0.3% of bile salt (Yang et al. 2019; Parlindungan et al. 2021; Wang et al. 2021). This phenotype is attributed to the ability of various *Lpb. plantarum* strains to produce bile acid hydrolase, catalyzing the conversion of conjugated bile salts into free bile salts (Yang et al. 2019). Taken together, our findings suggested that both strains of *Lpb. plantarum* could tolerate the gastrointestinal conditions.

Probiotics should be sensitive to common antibiotics (Binda et al. 2020). As shown in Table 1, both *Lpb. plantarum* F75 and SU-KC1a were susceptible to many antibiotics (n=15 for both F75 and SU-KC1a). However, two differences were observed between both strains. The first difference indicated that *Lpb. plantarum* SU-KC1a was susceptible to Tiamulin 30 µg. This finding was in line with the published data of *Lactobacillus* species isolated from chickens, which had reported that 90% of tested *Lactobacillus* isolates were resistant to Tiamulin (Dec et al. 2017). This suggests an intensive usage of Tiamulin in poultry husbandry caused the development of Tiamulin resistance within *Lpb. plantarum* isolated from chicken (Rolain 2013). The second difference indicated that while *Lpb. plantarum* F75 was resistant to Mupirocin 5 µg, *Lpb. plantarum* SU-KC1a was resistant to Mupirocin 200 µg. Thus, *Lpb. plantarum* F75 and SU-KC1a were classified as low-level and high-level Mupirocin resistance, respectively (Wattal and Oberol 2014). High-level Mupirocin resistance is an intrinsic characteristic of *Bifidobacterium* species because this antibiotic is commonly used to isolate *Bifidobacterium* species from environmental samples (Bunesova et al. 2015; Bunesova et al. 2015). *Lpb. plantarum* SU-KC1a, isolated from human milk by using mupirocin as a selective agent, also demonstrated this phenotype, suggesting that this high-level resistance might be acquired by several *Lpb. plantarum* strains. This hypothesis was supported by a published study that a Mupirocin-based selective medium could allow the growth of a few Gram-positive bacteria, in addition to *Bifidobacterium* species (Bunesova et al. 2015).

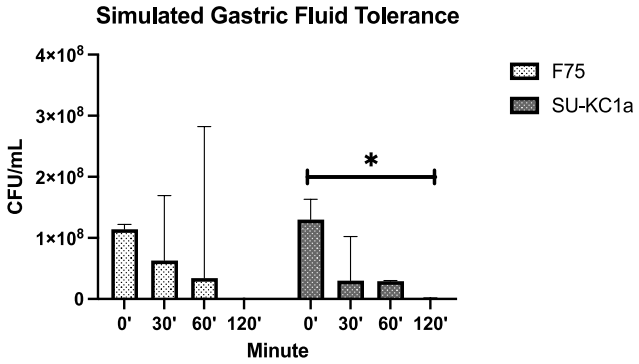

Figure 3. The simulated gastric fluid tolerance of *Lpb. plantarum* strain F75 and SU-KC1a. Both isolates were cultured at 37°C in MRS broth supplemented with 2 g/L of NaCl and 6 g/L of pepsin, in which the pH was adjusted to 2. At 0, 30, 60 and 120 minutes, each culture was sampled and plated on MRS agar for enumeration. The median of CFU/mL was displayed as bar graph. The upper error bar indicates the maximum data. The results of each bacterial strain were analyzed individually using the Kruskal-Wallis test with a p-value less than 0.05 would be considered as statistically significant. Results of *Lpb. plantarum* strain SU-KC1a was subsequently analyzed using the Dunn's multiple comparison test to determine which comparison was significantly different. An asterix sign indicates p-value less than 0.05. The values were obtained from three experiments

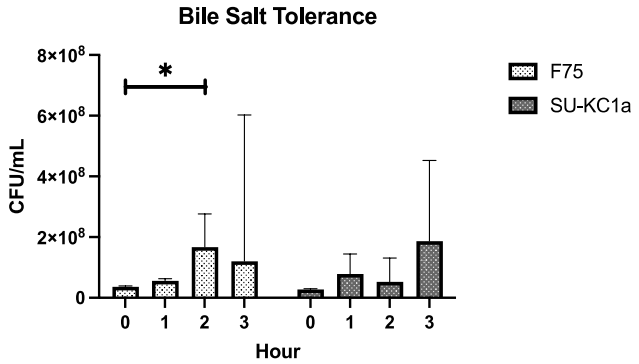

Figure 4. The bile salt tolerance of *Lpb. plantarum* strain F75 and SU-KC1a. Both isolates were cultured at 37°C in MRS broth supplemented with 0.3% (w/v) of bile salt. At 1-hour intervals, each culture was sampled and plated on MRS agar for enumeration. The median of CFU/mL was displayed as a bar graph. The upper error bar indicates the maximum data. Results of each bacterial strain were analyzed individually using the Kruskal-Wallis test with p-value less than 0.05 would be considered as statistically significant. Results of *Lpb. plantarum* strain F75 were subsequently analyzed using the Dunn's multiple comparison test to determine which comparison was significantly different. An asterisk sign indicates p-value less than 0.05. The values were obtained from three experiments

Table 1. Antibiotic disc diffusion results on *Lactiplantibacillus plantarum* F75 and SU-KC1a

Class	Antibiotic	Zone diameter interpretive standard (mm)			Results (mm) mean (min-max)	
		R	I	S	F75	SU-KC1a
Aminopenicillins	Amoxicillin 2 µg[b]	≤12	13-15	≥16	32 (31-34)	27 (25-29)
	Ampicillin 10 µg[a]	≤12	13-15	≥16	24 (22-24)	22 (20-25)
Polypeptide	Bacitracin 10 IU[a]	≤15	16-17	≥18	34 (33-34)	24 (23-24)
Cephalosporin	Cefoxitin 30 µg[a]	≤14	15-17	≥18	6 (6-6)	6 (6-6)
Penicillinase-resistant-penicillin	Methicillin 5 µg[c]	≤14	15-19	≥20	6 (6-6)	6 (6-6)
	Oxacillin 1 µg[c]	≤10	11-12	≥13	6 (6-6)	6 (6-6)
Glycopeptide	Vancomycin 30 µg[a]	≤14	15-16	≥17	6 (6-6)	6 (6-6)
Aminoglycoside	Neomycin 30 µg[c]	≤14	15-19	≥20	26 (23-27)	24 (23-25)
	Streptomycin 10 µg[a]	≤11	12-14	≥15	18 (17-18)	16 (15-17)
	Gentamicin 10 µg[a]	≤12	-	≥13	28 (26-30)	24 (22-16)
	Kanamycin 30 µg[a]	≤13	14-17	≥18	22 (20-23)	20 (20-20)
Tetracycline	Tetracycline 30 µg[c]	≤14	15-18	≥19	13 (12-13)	12 (12-14)
Phenicols	Chloramphenicol 30 µg[a]	≤13	14-17	≥18	33 (32-24)	27 (24-29)
Lincosamide	Clindamycin 2 µg[a]	≤ 8	9-11	≥12	10 (10-10)	9 (9-10)
	Lincomycin 2 µg[d]	≤14	-	≥21	9 (8-10)	9 (8-10)
Macrolides	Erythromycin 15 µg[a]	≤13	14-17	≥18	38 (35-40)	36 (34-39)
	Tylosin 30 µg[f]	≤13	14-17	≥18	30 (30-31)	30 (28-32)
Pleuromutilin	Tiamulin 30 µg[e]	≤14	15-19	≥20	11 (10-11)	16 (14-18)
Folate antagonists	Sulphonamide 300 µg[a]	≤12	13-16	≥17	20 (20-20)	19 (18-20)
Quinolones	Ciprofloxacin 5 µg[a]	≤13	14-18	≥19	16 (15-17)	17 (16-19)
	Nalidixic acid 30 µg[a]	≤13	14-17	≥18	6 (6-6)	6 (6-6)
	Ofloxacin 5 µg[a]	≤13	14-18	≥19	11 (11-12)	11 (10-12)
Rifampin	Rifampicin 5 µg[a]	≤14	15-17	≥18	22 (21-23)	18 (18-19)
Monocarboxylic acid	Mupirocin 5 µg[g]	-	-	≥14	10 (10-10)	6 (6-6)
	Mupirocin 200 µg[g]	-	-	≥14	22 (20-23)	8 (7-10)

Note: All measurement includes the diameter of the disc (6 mm). S: susceptible, I: intermediate; R: resistance. Numbers within grey boxes indicated the resistance phenotype. Each antibiotic was tested thrice. [a]Clear zone were interpreted based on standard from (Clinical and Laboratory Standards Institute 2023). [b]Standard for Amoxicillin (2 µg) was unavailable, thus the standard for Ampicillin (10 µg) was adopted. [c]Interpretation for antibiotics Neomycin (30 µg), Oxacillin (1 µg), and Tetracycline (30 µg) were based on *Staphylococcus* spp. (Clinical and Laboratory Standards Institute 2023). interpretation for antibiotic Lincomycin (2 µg) was based on (Chukiatsiri et al. 2012). [e]Interpretation for antibiotic Tiamulin (30 µg), Neomycin (30 µg) and Methicillin (5 µg) were based on common resistance standard set by CLSI (Clinical and Laboratory Standards Institute 2023). [f]Standard for Tylosin (30 µg) was unavailable, thus the standard for Erythromycin (15 µg) was adopted. [g]Interpretation for antibiotic Mupirocin was based on *Staphylococcus aureus* (Hetem and Bonten 2013).

In contrast**,** both isolates were resistant to the same eight antibiotics, i.e., Cefoxitin 30 µg, Methicillin 5 µg, Oxacillin 1 µg, Vancomycin 30 µg, Tetracycline 30 µg, Lincomycin 2 µg, Nalidixic acid 30 µg and Ofloxacin 5 µg. It was important to note that the resistance profiles of *Lpb. plantarum* F75 and SU-KC1a were not unique because the antibiotic resistance among *Lactobacillus* species towards β-lactam antibiotics (Cefoxitin, Methicillin and Oxacillin), glycopeptide, tetracycline, lincosamides and quinolones (Nalidixic acid and Ofloxacin) had been reported, in which some of those were even classified as an intrinsic resistance of *Lactobacillus* species (Dec et al. 2017; Campedelli et al. 2019; Das et al. 2020; Li et al. 2020). Of note, the resistance of *Lpb. plantarum* F75 and SU-KC1a to several tested β-lactam antibiotics and quinolones should not raise a health concern because both strains were still susceptible to other β-lactam antibiotics and Ciprofloxacin (Table 1). This was further supported by the susceptibility profiles of both strains to all tested aminoglycosides (Table 1), which contrasted with other published findings (Campedelli et al. 2019; Li et al. 2020).

Next, the whole genomes of both isolates were subsequently analyzed. It was revealed that the genomic size of *Lpb. plantarum* F75 and SU-KC1a were similar, in which their sequence lengths were 3,205,093 and 3,369,538 bp, respectively (Table 2). These sequence lengths were within the reported genomic size of several strains of *Lpb. plantarum*, ranging from 2.9 to 3.7 million bp (Karaseva et al. 2023; National Library of Medicine 2023). However, as depicted in Figure 5, there were several differences within the genomic annotation between two strains. The BLAST search revealed that several unmapped contigs from both genomes were similar to the reported plasmid sequences from other species of *Lactiplantibacillus*. However, it was yet unclear whether those unmapped contigs were plasmid or whether they were parts of the chromosomal DNA because the search for plasmid in both genomes through the PlasmidFinder database did not reveal any plasmid. Furthermore, the search for transposons and integrons via the BacAnt and Mobile Element Finder web-based tools indicated that there was no mobile element in both strains.

In order to complement the experimental results using the Kirby-Bauer disk diffusion method, *in silico* analyses of both genomes were subsequently performed to investigate genes that may contribute to the antibiotic resistance profile observed in *Lpb. plantarum* F75 (i.e., resistant to Cefoxitin, Methicillin, Oxacillin, Vancomycin, Tetracycline, Lincomycin, Tiamulin, Nalidixic acid and Ofloxacin) and SU-KC1a (i.e., resistant to Cefoxitin, Methicillin, Oxacillin, Vancomycin, Tetracycline, Lincomycin, Nalidixic acid, Ofloxacin and Mupirocin). Of note, the *in silico* analyses were unable to locate putative resistance genes within both genomes towards penicillinase-resistant penicillin (Methicillin and Oxacillin) as well as quinolones (Nalidixic acid and Ofloxacin). The observed antimicrobial genes towards the other antibiotics were located within the chromosomal genome of *Lpb. plantarum* F75 and SU-KC1a (Table 3).

Table 2. Genome annotation statistics for *Lactiplantibacillus plantarum* F75 and SU-KC1a

Isolate	Total sequence length (bp)	Predicted CDS	Predicted rRNA	Predicted tRNA	Predicted GC content (%)	Predicted coding ratio (%)
F75	3,205,093	3,021	5	70	44.6	84.4
SU-KC1a	3,369,538	3,379	16	70	44.4	83.0

Note: CDS, coding sequence; rRNA, ribosomal ribonucleic acid; tRNA, transfer ribonucleic acid

Table 3. Antibiotic resistance genes within the genome of *Lactiplantibacillus plantarum* F75 and SU-KC1a

Strain	Gene	AMR gene family	Antibiotic
F75	*CMA-2*	*CMA beta lactamase*	Cefoxitin
	ddl	*Van ligase*	Vancomycin
	rrp11	*Glycopeptide resistance gene cluster, vanR*	
	hpk11	*VanS, glycopeptide resistance gene cluster*	
	aad	*VanX, glycopeptide resistance gene cluster*	
	vanH gene in vanF cluster	*VanH, glycopeptide resistance gene cluster*	
	drrA	*major facilitator superfamily (MFS) antibiotic efflux pump*	Tetracycline
	drrB	*major facilitator superfamily (MFS) antibiotic efflux pump*	
	lmrB	*ABC antibiotic efflux pump*	Lincomycin
	TaeA	*ABC antibiotic efflux pump*	Tiamulin
SU-KC1a	*SRT-1*	*SRT beta lactamase*	Cefoxitin
	ddl	*Van ligase*	Vancomycin
	rrp11	*Glycopeptide resistance gene cluster, vanR*	
	hpk11	*VanS, glycopeptide resistance gene cluster*	
	aad	*VanX, glycopeptide resistance gene cluster*	
	vanH gene in vanF cluster	*VanH, glycopeptide resistance gene cluster*	
	drrA	*major facilitator superfamily (MFS) antibiotic efflux pump*	Tetracycline
	drrB	*major facilitator superfamily (MFS) antibiotic efflux pump*	
	lmrB	*ABC antibiotic efflux pump*	Lincomycin
	ileS	*Antibiotic resistant isoleucyl-tRNA synthetase*	Mupirocin

Note: AMR, antimicrobial resistance

The following antimicrobial resistance genes were observed within the both genomes: (i) two genes responsible for the Cefoxitin resistance, i.e., *CMA-2* and *SRT-1* (Müller et al. 2014; Tamma et al. 2019; He et al. 2020); (ii) genes that confer resistance towards Vancomycin, i.e., *vanH in vanF cluster, ddl, aad, rrp11 and hpk11* (Filannino et al. 2016; Floch 2017; Stogios and Savchenko 2020; Selim 2022); (iii) two Tetracycline resistance genes, i.e., *drrA* and *drrB* (Rahman and Kaur 2018; Peterson and Kaur 2018); and (iv) the *lmrB* gene that confers resistance to Lincomycin (Hirooka 2014). In contrast, the Tiamulin resistance gene, *TaeA* gene (Ciucă et al. 2023), was observed only within the genome of *Lpb. plantarum* F75. Tiamulin is primarily used in veterinary medicine, which could explain the Tiamulin resistance profile only observed in *Lpb. plantarum* F75.

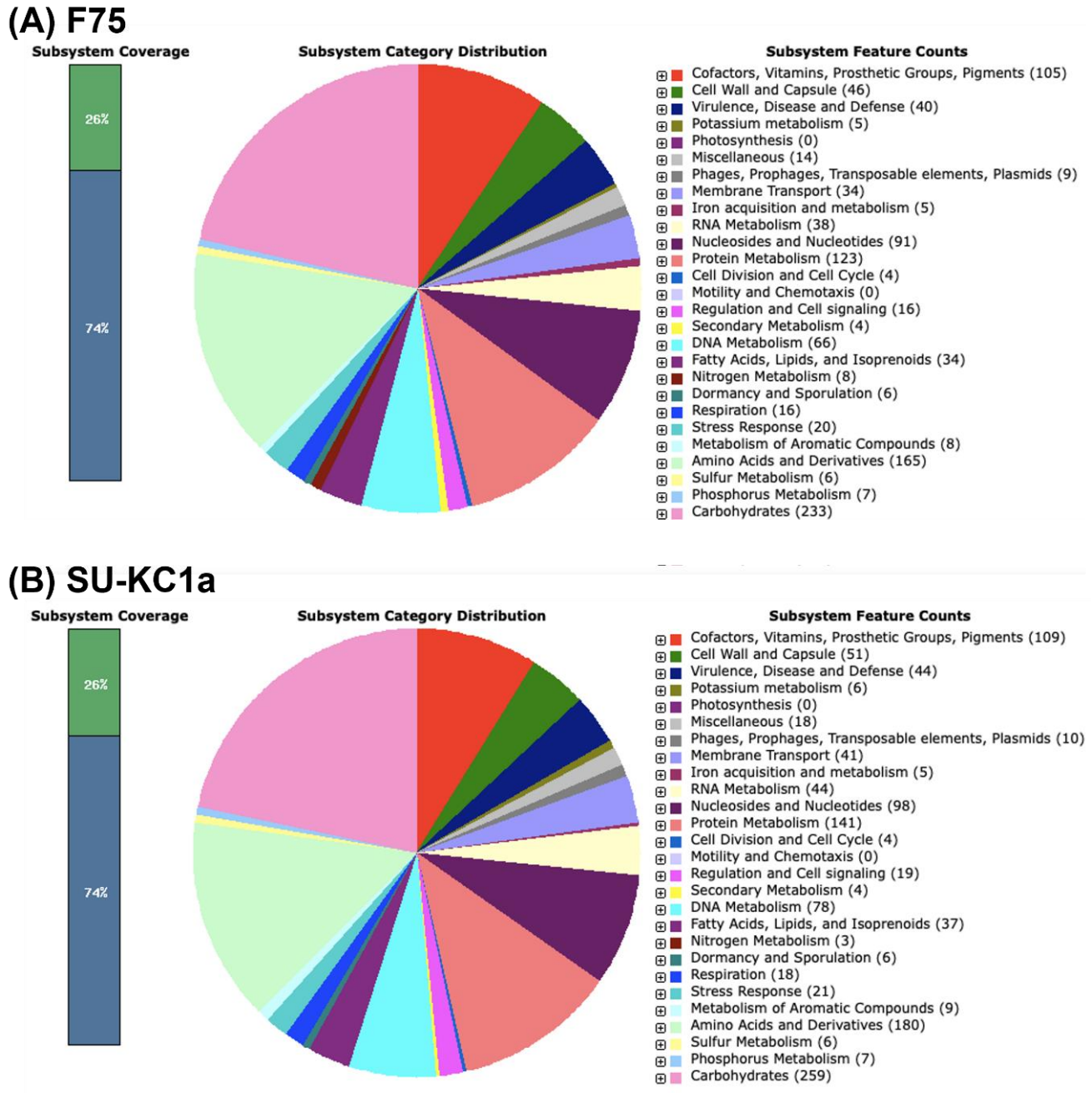

Figure 5. Overall genomic annotation of *Lpb. plantarum* strains. The genomic annotation of Lpb. plantarum F75 *(A)* and SU-KC1a *(B)* were obtained from dFAST. Subsystem categories were presented on the right side. Numbers within brackets indicate number of annotated genes in the respective categories

With regard to the Mupirocin resistance, this antibiotic binds to isoleucyl-tRNA synthetase (IleRS) and halts the protein synthesis by preventing an incorporation of isoleucine into the growing polypeptide chain. IleRS is encoded by the *ileS* gene, in which it has been reported that certain point mutations in *ileS* gene could mediate the low-level Mupirocin resistance. Two point mutations at the IleRS (V588F and V631F) that induce a low-level resistance to Mupirocin (Hetem and Bonten 2013; Singh et al. 2021) were observed within both genomes of *Lpb. plantarum* F75 and SU-KC1a. However, a gene that confers high-level Mupirocin resistance, *mupA* (Hetem and Bonten 2013), could not been detected within *Lpb. plantarum* SU-KC1a genome. Thus, it was elusive yet on which gene mediates the high-level resistance of this isolate towards Mupirocin.

Of note, capabilities of both strains were tested to produce short-chain fatty acids via gas chromatography-mass spectrometry, as those end-products of bacterial fermentation on non-digestible dietary fibers could modulate the host physiology (Morrison and Preston 2016; Silva et al. 2020). Both *Lpb. plantarum* F75 and SU-KC1a could ferment glucose within MRS broth to release organic acids, including acetic acid and butyric acid (data not shown). This qualitative finding was supported by a previous study, demonstrating that *Lactobacillus* species could produce short-chain fatty acids (Thananimit et al. 2022).

There are several limitations in our study. First, we only investigated several characteristics of probiotic candidates. Despite those are arguably the main characteristics, we acknowledge that other untested characteristics, e.g., ability to produce bacteriocin and to bind to intestinal mucus, are important as well. Second, the Kirby-Bauer disk diffusion method cannot determine the minimum inhibitory concentration of antibiotics towards both isolates. Third, we investigated the presence of plasmid *in silico*. Hence, the finding will need to be confirmed through molecular techniques.

In conclusion, despite being isolated from different species, *Lpb. plantarum* F75 and SU-KC1a exhibited similar characteristics as probiotics, including sensitivity to increased concentrations of NaCl and phenol as well as ability to survive the gastrointestinal condition. Furthermore, both isolates had similar genome sizes and exhibited similar antibiotic resistance profiles. In addition, all detected resistance genes were observed within the chromosomal genome. Taken together, our findings suggested that both isolates could be used as probiotic candidates in poultry and human foods, respectively.

ACKNOWLEDGEMENTS

This work was supported by the Institute of Research and Community Service of Universitas Pelita Harapan (P-14-FaST/VIII/2022 and P-01-FaST/XII/2021). The authors thank Dr. Reinhard Pinontoan for kindly providing the isolate of *Lpb. plantarum* F75.

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