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In silico analysis of antibiotic resistance genes in *Lactiplantibacillus plantarum* subsp. *plantarum* Kita-3

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ABSTRACT The absence of transferable antibiotic resistance genes is required for the safety of commercial probiotics. Previous studies have found that antibiotic resistance genes on plasmids in *Lactobacilli* make them unsafe for food purposes due to the genes' ability to transfer to pathogenic microorganisms. In contrast, bacteria from the Lactobacillaceae family are widely used as a probiotic. This study assessed the antibiotic susceptibility of *Lactiplantibacillus plantarum* subsp. *plantarum* Kita-3 (previously known as *Lactobacillus plantarum* K-3) isolated from Halloumi cheese using eight antibiotics. Genome sequencing was performed using the Illumina NovaSeq 6000 sequencing platform to detect the presence of antibiotic resistance genes on chromosomes and plasmids. *L. plantarum* subsp. *plantarum* Kita-3 was resistant to clindamycin, streptomycin, and chloramphenicol but susceptible to tetracycline, ampicillin, kanamycin, erythromycin, and ciprofloxacin. Genome sequencing of *L. plantarum* subsp. *plantarum* Kita-3 verified the presence of tetracycline, fluoroquinolones, β-lactamase resistance genes, and multidrug resistance efflux. Kita-3 had no transposable elements, gene transfer agents, plasmid-related functions, or intact prophages. Overall, this study produced the antibiotic resistance profile of *L. plantarum* subsp. *plantarum* Kita-3 to assess the risk of transferring antibiotic resistance genes to other bacteria. The study provides essential data on the safe use of *L. plantarum* subsp. *plantarum* Kita-3 as probiotics.

KEYWORDS Antibiotic resistance; Genome sequencing; In silico analysis; *Lactiplantibacillus plantarum* subsp. *plantarum*

1. Introduction

Probiotics are living microorganisms beneficial to the host when given adequate amounts (FAO/WHO 2002). Probiotics mainly belong to lactic acid bacteria (LAB) and have been widely applied in the food industry as starters for fermented and functional foods (Florou-Paneri et al. 2013). Lactic acid bacteria from the family Lactobacillaceae have been included in the GRAS (Generally Recognized as Safe) status (Monahan 2011). Therefore, probiotics used in food products must be considered carefully for food safety concerns. The Food and Agriculture Organization has developed requirements for guidelines to evaluate the safety of probiotics before they can be used commercially in food, including viability during processing and storage, survivability in conditions of acid and bile salts in the gastrointestinal tract, non-pathogenicity, assessment for antibiotic resistance since the probiotics should not possess transferable antibiotic resistance genes (FAO/WHO 2002).

Antibiotic resistance has become a global concern since the long-term use of antibiotics led to the evolution and spread of antibiotic resistance in bacteria associated with humans, animals, and the environment (Hernando-Amado et al. 2019). Antibiotic resistance can be acquired by horizontal gene transfer through plasmids or transposons (Carattoli 2013; Babakhani and Oloomi 2018), and recent findings suggest that antibiotic resistance may also be transferred via prophages in the transduction process (Wendling et al. 2021; Colavecchio et al. 2017).

Lactobacillaceae have phenotypically and genotypically diverse antibiotic resistance properties (Campedelli et al. 2019). The identification of resistant genes in several *Lactiplantibacillus plantarum* subsp. *plantarum* (previously known as *Lactobacillus plantarum*) strains was carried out using Polymerase Chain Reaction (PCR) on genes related to chloramphenicol (cat) and erythromycin (erm); the results identified cat resistant genes in the plasmids and chromosomes (Sukmarini et al. 2014). Guo et al. (2017) used 15 antibiotics to be tested with 33 *Lactobacillus* strains from different species, such as *L. helveticus*, *L. casei*, and *L. plantarum*; *L. plantarum* strains were resis-

tant to kanamycin, streptomycin, and ciprofloxacin. *Lactobacillus* strains resistant to the antibiotics ciprofloxacin and tetracycline carried the *gyrA* and *tet(M)* genes, but no antibiotic resistance gene transfer was observed (Guo et al. 2017). The research on *L. plantarum* ATCC 14917 using the Whole Genome Sequencing (WGS) method revealed no antibiotic resistance gene located in the mobile element in the genome, indicating that horizontal gene transfer to other bacteria is impossible (Feng et al. 2019). Based on Andriani et al. (2021), *L. plantarum* Dad-13 from dadih (fermented buffalo milk), *L. plantarum* Mut-7 from gatot (fermented dry cassava), *L. plantarum* T3 from growol (fermented fresh cassava) showed the absence of transferable antibiotic resistance genes.

Our laboratory had isolated *Lactiplantibacillus plantarum* subsp. *plantarum* Kita-3 (previously identified as *Lactobacillus plantarum* K3), a probiotic candidate isolated from Halloumi cheese. *L. plantarum* K3 showed the capacity as probiotics due to the tolerance to gastric acid at pH 2.0-2.5 and bile salts; it possesses moderate antibacterial activity against *Shigella dysenteriae* and strong antibacterial activity against *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus* (Ratna et al. 2021). In this study, we evaluated the antibiotic susceptibility of *L. plantarum* subsp. *plantarum* Kita-3 using the standard microdilution method and identified the corresponding resistant genes. The minimum inhibitory concentration (MIC) was determined against eight antibiotics: ampicillin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, and ciprofloxacin. Genome sequencing was performed to detect the presence of genes that potentially confer antibiotic resistance in *L. plantarum* subsp. *plantarum* Kita-3.

2. Materials and Methods

2.1. Bacterial isolate, culture media, and growth conditions

Strain *Lactiplantibacillus plantarum* subsp. *plantarum* Kita-3 (previously known as *Lactobacillus plantarum* K3) was obtained from Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Culture media and growth conditions are described in the ISO10932/IDF223 standard (International Organization for Standardization 2010). The isolates were activated in de Man Rogosa and Sharpe (MRS) medium (MRS; Merck™) incubated at 37 °C for 24 h (International Organization for Standardization 2010). This protocol was developed by Lawalata et al. (2011) with modifications.

2.2. Preparation of antibiotics

Eight antibiotics consisting of ampicillin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, and ciprofloxacin, were obtained from Universitas Gadjah Mada Health Homes and Pharmacies. Each antibiotic was dissolved in the appropriate solvent (Table 1) as antibiotic stock.

2.3. Determination of the minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined using the 96-well microtitration plate method consisting of 12 columns and eight rows described previously (Clinical and Laboratory Standards Institute 2012). In the first step, 50 µL of MRS broth was put into each well in the second to twelfth columns. Subsequently, 100 µL of the antibiotics that had been prepared was put into each well in the first column. A two-fold dilution was performed by transferring 50 µL from each well in the first column to each well in the second column and so on. Wells in the eleventh and twelfth columns contained MRS broth with culture as positive control and MRS broth without culture and antibiotics used as negative controls, respectively. *L. plantarum* subsp. *plantarum* Kita-3 suspension in 0.85% NaCl was adjusted to the standard on a scale of 0.5 McFarland (1.5×10^8 CFU/mL), and a 50 µl of cells suspension was inoculated to each well. The 96-well plates were incubated at 37 °C for 18 h. The culture media's optical density (OD) was determined with a microplate reader at the wavelength of 620 nm. The MIC test was carried out in triplicate. Based on the EFSA-FEEDAP (2018) guidelines (Table 2), a strain was recorded as resistant to antibiotics if its MIC value was higher than the reference cut-off value. Con-

TABLE 1 Antibiotic working concentration range for MIC determination.

Antibiotic	Concentration range (µg/mL)	Solvent
Ampicillin	0.032 – 16	Distilled Water
Kanamycin	2 – 1024	Distilled Water
Streptomycin	0.5 – 256	Distilled Water
Erythromycin	0.016 – 8	95% ethanol
Clindamycin	0.032 – 16	Distilled Water
Tetracycline	0.125 – 64	Distilled Water
Chloramphenicol	0.125 – 64	95% ethanol
Ciprofloxacin	0.25 – 64	0.05M HCl

TABLE 2 *Lactobacillus* species cut-off values (µg/mL) (EFSA-FEEDAP 2018) and (Commission 2002).

Antibiotic	*Lactobacillus plantarum*
Ampicillin	2
Kanamycin	64
Streptomycin	16
Erythromycin	1
Clindamycin	4
Tetracycline	32
Chloramphenicol	8
Ciprofloxacin	4

versely, if the MIC value is equal to or lower than the reference cut-off value, the strain is susceptible to particular antibiotics.

2.4. Genomic DNA extraction, library construction, sequencing, and genome assembly

The Genomic DNA extraction, library construction, sequencing, and genome assembly of *L. plantarum* subsp. *plantarum* Kita-3 followed protocols described elsewhere (Suroto et al. 2021). A total of 1 µg of DNA was used as input material for the DNA sample preparations. Following the manufacturer's recommendations, sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA), and index codes were added to attribute sequences to the sample.

2.5. Bioinformatic analyses

Genome annotation was performed by the online program Rapid Annotation using Subsystem Technologies (RAST) SEED (http://rast.nmpdr.org/) (Overbeek et al. 2014). The annotation of antibiotic resistance by RAST was further confirmed by The Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca) (Alcock et al. 2020). The PHAge Search Tool Enhanced Release (PHASTER) (https://phaster.ca) (Arndt et al. 2016) was used to detect the presence of prophage.

3. Results and Discussion

3.1. Results

L. plantarum subsp. *plantarum* Kita-3 was susceptible to tetracycline (MIC value 0.5 µg/mL), ampicillin (MIC value 0.25 µg/mL), kanamycin (MIC value 16 µg/mL), erythromycin (MIC value 0.25 µg/mL), and ciprofloxacin (MIC value 0.5 µg/mL). Nevertheless, it was resistant to clindamycin (MIC value 8 µg/mL), streptomycin (MIC value 128 µg/mL), and chloramphenicol (MIC value 32 µg/mL) (Table 3).

Analysis at the genomic level was performed to detect antibiotic resistance genes in the genome of *L. plantarum* subsp. *plantarum* Kita-3 by the RAST webserver. In the RAST system, 19 genes related to antibiotic resistance were detected and classified into tetracyclines, fluoroquinolones, β-lactamases, and multidrug resistance efflux pumps (Figure 1a).

Antibiotic resistance genes detected by RAST were confirmed using CARD (Table 4). For tetracycline resistance in *L. plantarum* subsp. *plantarum* Kita-3, there are two genes responsible for its resistance. *Tet(M)* encodes ribosomal protection proteins, and Tet(O) encodes translational elongation factor G [EF-G, EF-G-para]. Fluoroquinolones resistance was determined by DNA gyrase subunits A, B (EC 5.99.1.3) encoded by *gyrA*, *gyrB*, and topoisomerase IV subunits A, B (EC 5.99.1.-) encoded by *parC* and *parE*, respectively. Several genes encode the β-lactamase resistance found in the *L. plantarum* subsp. *plantarum* Kita-3 genome. The β-lactamase class A is encoded with one *blaF*, and β-lactamase class C is encoded by two genes, *ampC1*, *ampH*, and the *Exo-1* encoding exo β-lactamase. In addition to the genes encoding resistance to tetracycline, fluoroquinolones, and beta-lactamase antibiotic, four multidrug resistance efflux pumps genes were also detected from the Major Facilitator Superfamily (MFS) and Multidrug and Toxic Compound Extrusion (MATE). Thus, based on the genotype analysis, only three groups of antibiotic resistance were detected.

In contrast, the results showed that *L. plantarum* subsp. *plantarum* Kita-3 was resistant to clindamycin, streptomycin, and chloramphenicol, but no genes encoding the resistance against those three antibiotics were detected. The RAST also predicted prophages-related sequences, but no plasmids and transposable elements were detected (Figure 1b). Analysis of *L. plantarum* subsp. *plantarum* Kita-3 using PHASTER showed three incomplete prophages (Table 5) without a region containing antibiotic resistance genes (Figure 2). The incomplete prophages had been associated with the incapability to enter the lytic cycle (Nepal et al. 2022). Taken together, no antibiotic-resistance genes can be transferred to other bacteria via transduction.

3.2. Discussion

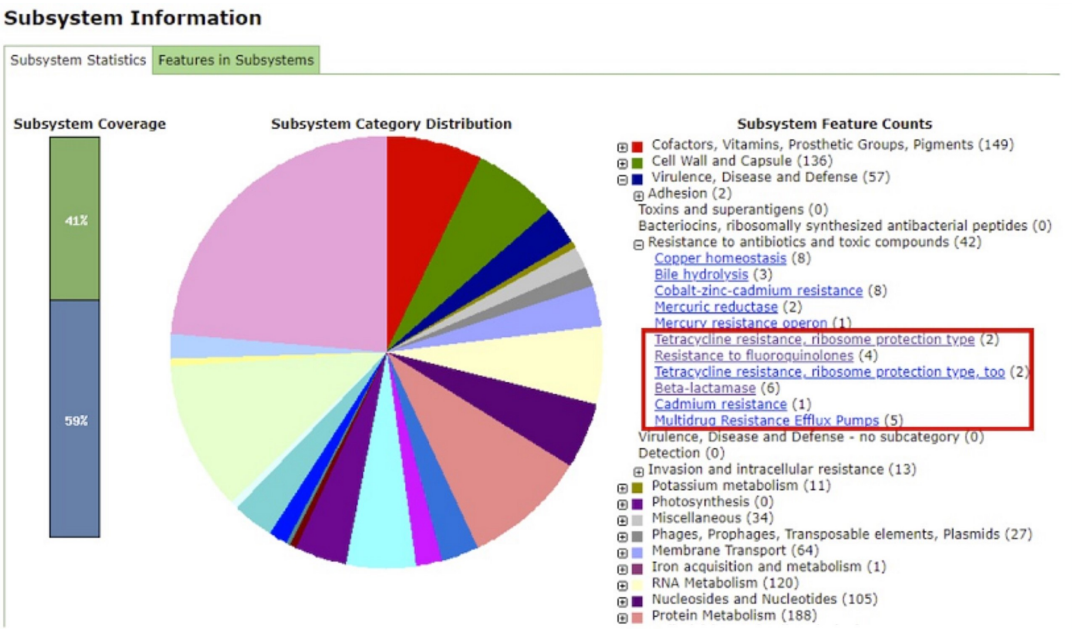
The phenotypic and genotypic resistance in *L. plantarum* subsp. *plantarum* Kita-3 does not correspond to several cases since *L. plantarum* subsp. *plantarum* Kita-3 had a higher MIC- value than the microbiological cut-off values of chloramphenicol, clindamycin, and streptomycin but did not have corresponding resistance genes. These results are consistent with other findings (Stefańska et al. 2021; Rozman et al. 2020; Dec et al. 2017).

Chloramphenicol resistance can occur due to the pres-

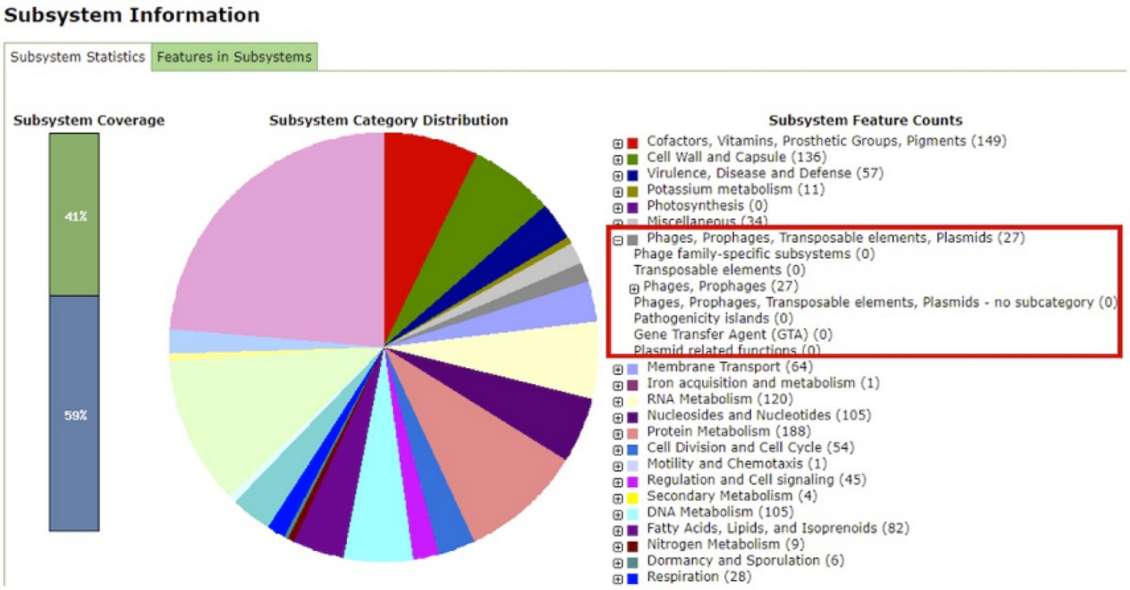
TABLE 3 Comparison of the antibiotic susceptibility assessments with RAST predictions for strain *L. plantarum* K3.

Species	Strain	Antibiotics[a] (MIC as µg/mL)							
		TET	CLI	AMP	KAN	STR	ERY	CHL	CIP
Lactobacillus plantarum[b]	Kita-3	0.5	8	0.5	16	128	0.25	32	0.5
Cut-off values (µg/mL)		32[d]	4[d]	2[d]	64[d]	16[d]	1[d]	8[d]	4[e]
RAST		+	-	+	-	-	-	-	+

[a] TET, tetracycline; CLI, clindamycin; AMP, ampicillin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CHL, chloramphenicol; CIP, ciprofloxacin. [b] Strains Indonesian Indigenous probiotic isolates. [c] Red-text highlight color shows MIC of antibiotics higher than the corresponding cut-off values, considering those of the [d] (EFSA-FEEDAP 2018) and [e] EUC (2002), +: detected, -: not detected.



(a) Subsystem virulence, disease, and defense



(b) Subsystem phage, prophage, transposable elements, plasmids

FIGURE 1 RAST Analysis on antibiotics resistance genes and mobile genetic elements in *L. platarum* K3.

ence of acetyltransferases that add an acetyl group to the antibiotic, which causes chloramphenicol to be unable to bind to the 50s subunit of the bacterial ribosome (Kapoor et al. 2017). However, the encoding genes of such activities are not present in Kita-3. The chloramphenicol resistance phenotypes in *L. plantarum* were also observed in the study conducted by Campedelli et al. (2019) and Sukmarini et al. (2014). Chloramphenicol resistance may not only be associated with the presence of specific genes encoding antibiotic-modifying enzymes. However, it may also consequence in reduced expression of genes related to efflux pumps and oxidative stress, as well as genes encoding outer membrane proteins (Rojo-Bezares et al. 2006).

Clindamycin resistance can be occurred due to modification of the target site resulting in ribosomal mutations that prevent the binding of the antibiotic to its target ribosome (Leclercq 2022). The clindamycin resistance phenotype in *L. plantarum* subsp. *plantarum* Kita-3 was in line with the studies published elsewhere (Stefańska et al. 2021; Campedelli et al. 2019; Flórez et al. 2006). *L. plantarum* subsp. *plantarum* Kita-3 showed resistance against streptomycin; this phenotype is typical in Lactobacillaceae (Stefańska et al. 2021; Andriani et al. 2021; Campedelli et al. 2019; Gueimonde et al. 2013). Lactobacillaceae have low cell membrane impermeability to the aminoglycoside group due to the absence of an electron transport system

TABLE 4 Annotation of antibiotic resistance genes in *Lactobacillus plantarum* K3 by CARD.

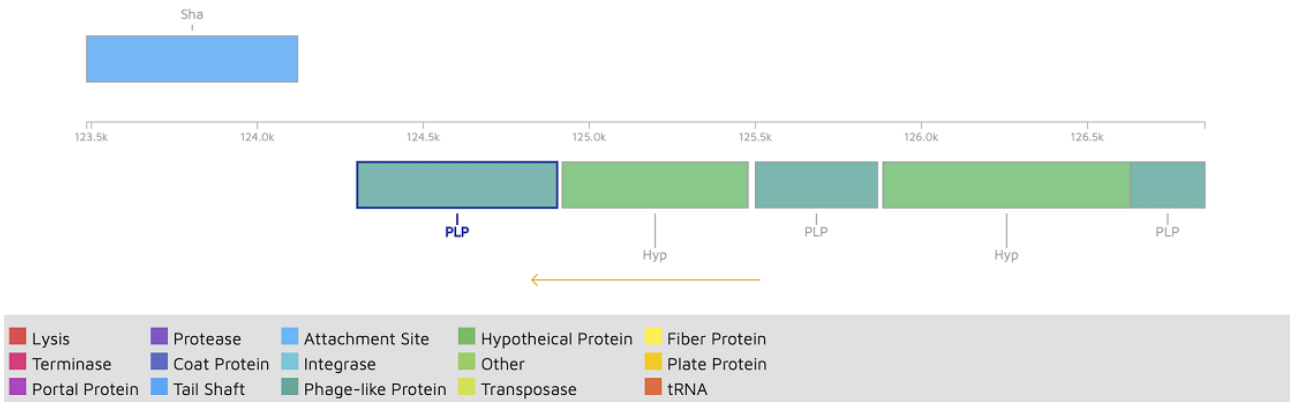
Subsystem	Size of amino acid	Homolog and origins	Identity (%)	Functional Role	Resistance mechanism
Tetracycline resistance	651	*tet(T)* *Streptococcus pyogenes*	27.64	Translation elongation factor G	Antibiotic target protection
	663	*otr(A)* *Streptomyces rimosus*	39.63	Ribosome protection-type tetracycline resistance related proteins, group 2	Antibiotic target protection
Floroquinolones resistance	808	*gyrA* *Clostridiodes difficile*	55.82	DNA Gyrase subunit A	Antibiotic target alteration
	637	*gyrB* *Clostridium ljungdahlii* DSM 13528	56.57	DNA Gyrase subunit B	Antibiotic target alteration
	808	*parC* *Clostridiodes difficille*	40.3	Topoisomerase IV subunit A	Antibiotic target alteration
	643	*parE* *Morganella morganii*	65.13	Topoisomerase II subunit B	Antibiotic target alteration
β-lactamase resistance	224	*blaF* *Mycolicibacterium fortuitum*	30.00	β-lactamase class A	Antibiotic inactivation
	434	*ampC1* *Escherichia coli* ETEC H10407	31.51	β-lactamase class C	Antibiotic inactivation
	434	*ampC1* *Escherichia coli* ETEC H10407	27.88	β-lactamase class C	Antibiotic inactivation
	385	*ampH* *Escherichia coli* O157:H17 str. Sakai	26.98	β-lactamase class C	Antibiotic inactivation
	314	*EXO-1* *Streptomyces albus*	31.87	EXO β-lactamase	Antibiotic inactivation
Multidrugs resistant efflux	193	*tetR* *Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18	31.58	Major facilitator superfamily (MFS) antibiotic efflux pump	Antibiotic efflux
	462	*cdeA* *Clostridioides difficile*	19.9	Multidrug and toxic compound extrusion (MATE) transporter	Antibiotic efflux
	444	*mepA* *Staphylococcus aureus*	25.95	Multidrug and toxic compound extrusion (MATE) transporter	Antibiotic efflux
	407	*mdtG* *Escherichia coli*	46.72	Major facilitator superfamily (MFS) antibiotic efflux pump	Antibiotic efflux
	410	*mdtG* *Escherichia coli*	46.05	Major facilitator superfamily (MFS) antibiotic efflux pump	Antibiotic efflux

TABLE 5 Putative prophages predicted by PHASTER.

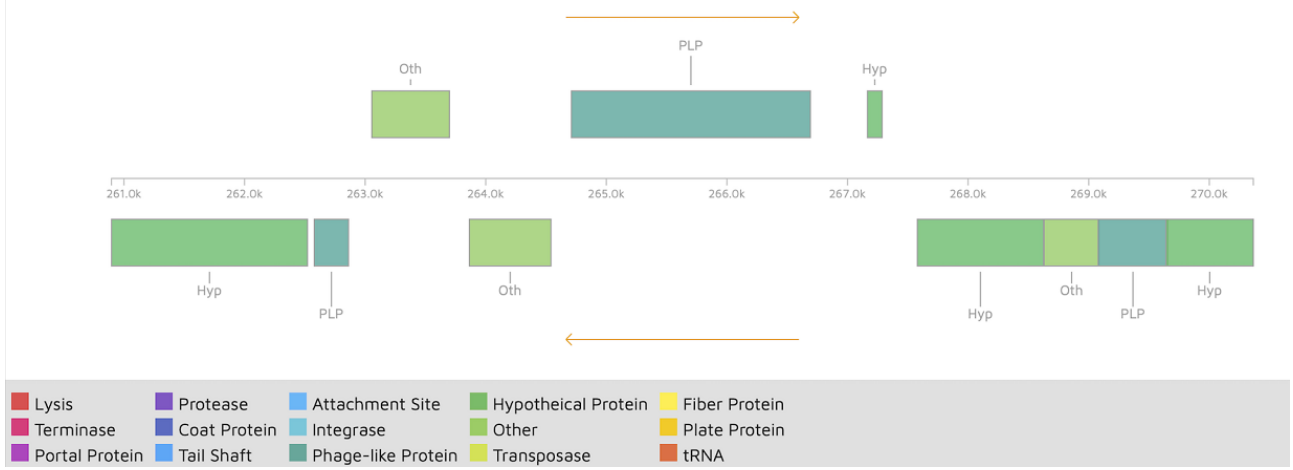
Region	Region length	Completeness	Score	Total proteins	Region position (nt)	GC content (%)
1	3.4 kb	incomplete	20	6	123,485-126,854	49.07
2	9.4 kb	incomplete	20	10	260,897-270,365	44.78
3	9.7 kb	incomplete	30	30	279,617-289,251	44.82

related to cytochromes (Kirtzalidou et al. 2011; Anisimova and Yarullina 2019). Since the resistance of these three antibiotics was not encoded by particular genes, it may imply that the resistant phenotypes are natural or intrinsic.

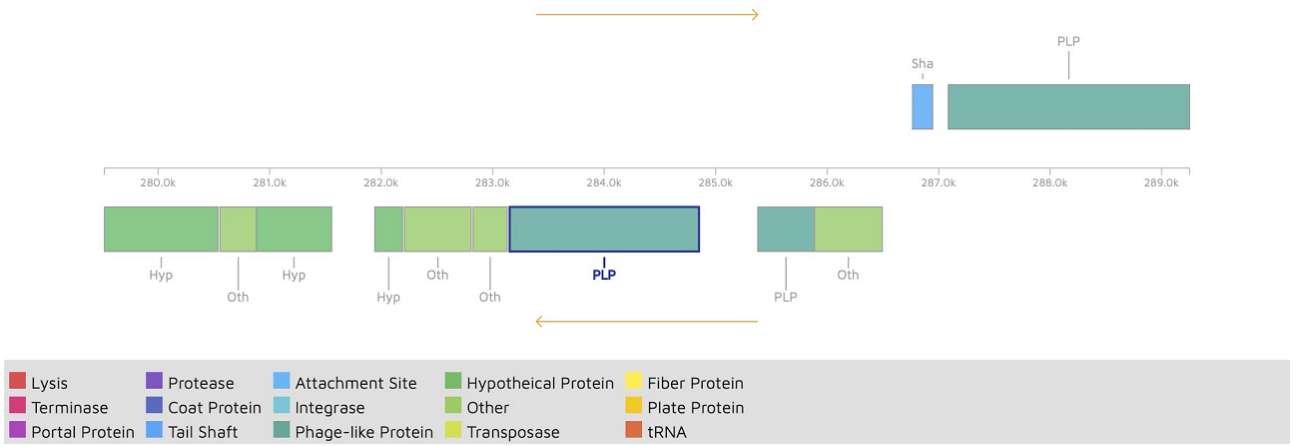
The β-lactamases in *L. plantarum* subsp. *plantarum* Kita-3 are classified into β-lactamases class A, β-lactamases class C belonging to penicillin-binding proteins, and EXO β-lactamases. In contrast, the Kita-3 strain is susceptible to penicillin, a β-lactam group of antibiotics. The susceptibility of *L. plantarum* subsp. *plantarum* Kita-3 to ampicillin suggests that the gene encoding the β-lactamase may not function. The susceptibility of *L. plantarum* to ampicillin has been reported by (Stefańska et al. 2021; Anisimova and Yarullina 2019; Shao et al. 2015).



(a) Detail ORFs organization in putative prophage region 1



(b) Detail ORFs organization in putative prophage region 2



(c) Detail ORFs organization in putative prophage region 3

FIGURE 2 Detail Open Reading Frames (ORFs) of putative prophages in *L. platarum* K3 predicted by PHASTER.

L. plantarum subsp. *plantarum* Kita-3 has fluoroquinolone resistance genes classified into DNA gyrase subunit A, B and topoisomerase IV subunit A, B. The enzymes work together in DNA replication, transcription, recombination, and repair. Fluoroquinolone resistance can occur due to mutations in DNA gyrase and topoisomerase IV, thereby causing amino acid changes and modifying the structure of the target protein (Redgrave et al. 2014; Hooper and Jacoby 2016). Mutations occur in the "quinolone resistance determining regions" (QRDR) in each gene (Li et al. 2015). The QRDR in DNA gyrase is close to tyrosine 122, covalently bound to a phosphate group on DNA in the initial strand-breaking reaction (Ng et al. 1996). The *gyrA* gene is influenced by mutations in the quinolone resistance-determining region, resulting in topoisomerase changes (amino acid substitution in enzymes) that cause genotypic resistance in *L. plantarum* (Ogbolu et al. 2012). In contrast, Kita-3 showed sus-

ceptibility to ciprofloxacin, indicating that those fluoroquinolones genes might also not be functional.

The tetracycline resistance gene in *Lactobacillus* protects the ribosome and elongates factor G since tetracycline can inhibit protein synthesis (Van Hoek et al. 2011). Therefore, strains with tetracycline-resistant ribosomal protection genes can protect ribosomal proteins from binding to tetracycline compounds. Furthermore, the interaction between the ribosomal protective protein and helix 34 in 16S rRNA contributes to allosteric disruption of the main tetracycline binding site, causing tetracycline to detach from the ribosome (Schedlbauer et al. 2015). As a result, ribosomes return to the required confirmation, and protein synthesis continues (Li et al. 2013). In contrast to the present resistance of tetracycline in the genome of Kita-3, the phenotype of *L. plantarum* subsp. *plantarum* Kita-3 seems to be the opposite.

Further analysis using CARD showed resistance mediated by efflux pumps. In multidrug resistance (MDR) efflux pumps, Major facilitator superfamily (MFS) and Multidrug and toxic compound extrusion (MATE) play a role in releasing drugs that cross the bacterial cell membrane (Kumar et al. 2020). Efflux pumps can be associated with multidrug resistance (MDR) and have clinical significance due to their importance for drug design. Based on sequence similarity, the MDR efflux pumps consist of five prominent families: the ATP-binding cassette (ABC) family, the multidrug and toxic compound exporters (MATE) family, the small multidrug resistance (SMR) family (part of the much larger drug/metabolite transporter superfamily), the resistance-nodulation-division proteins (RND), and the major facilitator superfamily (MFS) (Nishino et al. 2009). This study found two transporters of MDR efflux pumps, MFS and MATE. Both transporters are known to have been implicated in the mechanism of antibiotic resistance (Blanco et al. 2016).

Generally, two sequences are homologous if they are more than 30% identical throughout the protein sequence (Pearson 2013). In this study, tetracycline, beta-lactamase, and multidrug resistance had a low identity of amino acid sequences compared to those in the database. This low identity may indicate that those gene products are not homologous and contribute to different functions than predicted or false-positive results. Peptide antibiotic resistance proteins with amino acid identity between 40% to 60%, such are fluoroquinolones resistance proteins in *L. plantarum* subsp. *plantarum* Kita-3 are considered distant homologs (Rozman et al. 2020). Moreover, Gibson et al. (2015) used only protein, which showed 80% amino acid identity over >85% of the target sequence length, to identify that protein may have a definite role in antibiotic resistance.

The antibiotic resistance genes are frequently found on plasmids, transposons, and prophages, increasing the risk of dispersal resistance among bacteria (Carattoli 2013; Babakhani and Oloomi 2018; Wendling et al. 2021; Colavecchio et al. 2017). *L. plantarum* subsp. *plantarum* Kita-3 did not have transposable elements, gene transfer agents, intact prophages, and plasmid-associated functions. The results implied that antibiotic-resistant genes might be present in the chromosome of *L. plantarum* subsp. *plantarum* Kita-3, and those genes may not be transferable.

4. Conclusions

Our research showed that *Lactiplantibacillus plantarum* subsp. *plantarum* Kita-3 is phenotypically resistant to clindamycin, streptomycin, and chloramphenicol while susceptible to tetracycline ampicillin, kanamycin, erythromycin, and ciprofloxacin. No corresponding resistance genes are related to clindamycin, streptomycin, and chloramphenicol. The resistant genes to tetracycline, fluoroquinolones, beta-lactamase, and multidrug efflux pump are detected and considered low or distant homologs with similar proteins in the database. *L. plantarum* subsp. *plantarum* Kita-3 did not have transposable elements, gene transfer agents, plasmid-related functions, and intact prophages. It suggests that horizontal gene transfer may not occur. These results would provide comprehensive data to support safety evaluations and recommendations for the safe use of *L. plantarum* subsp. *plantarum* Kita-3.

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Authors' contributions

AW, DAS, ESR designed the study. AW carried out the laboratory work. AW, DAS, ESR, TU, RW analyzed the data. AW wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

All authors report that there are no conflicts of interest.

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