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Genotypic and Phenotypic Analyses of Antibiotic Resistance in Indonesian Indigenous Lactobacillus Probiotics

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Abstract

Background and Objective: In the authors' previous study, four unique *Lactobacillus* strains (*Lactobacillus plantarum* Dad-13, *Lactobacillus plantarum* Mut-7, *Lactobacillus plantarum* T-3 and *Lactobacillus paracasei* SNP-2) from Indonesian fermented foods and healthy feces have been studied as probiotic agents. In the current study, antibiotic resistance phenotypes of the highlighted *Lactobacillus plantarum* and *Lactobacillus paracasei* against eight antibiotics (amoxicillin, tetracycline, erythromycin, clindamycin, chloramphenicol, streptomycin, kanamycin, ciprofloxacin) and antibiotic resistance genes of these strains were investigated.

Material and Methods: The bacterial antibiotic susceptibility to eight antibiotics was assessed using disk diffusion method. Genome sequencing was carried out using NovaSeq 6000 sequencing platform. Genome was annotated using Rapid Annotation using Subsystem Technology v.2.0. Each group of the predicted products of resistance genes was further aligned using multiple sequence comparison by log-expectation and their functions were verified using comprehensive antibiotic resistance database 2020.

Results and Conclusion: All strains showed resistance to aminoglycoside and ciprofloxacin but sensitive to amoxicillin, clindamycin and erythromycin. Resistance to chloramphenicol and tetracycline varied within the strains. Two strains were sensitive and others were intermediate resistance to chloramphenicol. One strain was resistant to tetracycline, while the other three strains demonstrated intermediate resistance to the antibiotic. Genome sequence of the four strains verified the presence of the tetracycline, β-lactamase and ciprofloxacin resistance genes as well as multidrug resistance efflux systems. Occurrence of the resistance genes was correlated to the phenotype results, except for amoxicillin and aminoglycosides. Rapid Annotation using Subsystem Technology annotation showed that all *Lactobacillus* strains did not include transposable elements, gene transfer agents and plasmid linked functions; thus, horizontal transfer of the antibiotic resistance genes unlikely occurred.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Lactic acid bacteria (LAB) are prevalent in nature, food products and human and animal intestines. The bacterial roles in food processing can be traced back to thousands of years ago, providing people with numerous types of fermented vegetables, dairy products and meat/fish products [1]. *Lactobacillus* genus is considered as generally recognized as safe in the USA due to its extensive involvement in food fermentation [2]. In the latest years, use of LAB as probiotics has increased to give meaningful health benefits to humans and animals [3]. In contrast, increased use of LAB has alarmed people about safety issues such as spread of antibiotic resistance [4]. Excessive and misuse of antibiotics in agriculture and animal husbandry have triggered circulation of antibiotic-resistant bacteria, threatening health of humans and animals [5,6]. In medical fields, significant increase of multiple-drug-resistant infectious diseases causes thousands of deaths each year, activating global threats to public health as well as large financial burdens [6].

The current reports have shown that LAB, widely known as conventional starters of the fermented foods and probiotics, possess antibiotic resistance potencies. These bacteria can contribute to transmission of antibiotic resistance genes

[4,7], especially in fermented products that are not previously heated. Furthermore, LAB can be vehicles for the transmission of antibiotic resistance genes from the animal microbiota to human gastrointestinal tract bacteria [8]. *Lactobacillus* spp. are usually resistant to aminoglycoside antibiotics (e.g. streptomycin, kanamycin, neomycin and gentamycin) and are mostly susceptible to chloramphenicol, erythromycin, tetracycline and clindamycin [9]. Strains with acquire resistance to chloramphenicol, erythromycin, tetracycline and clindamycin have been identified in *Lactobacillus* spp. from food fermentation [8]. Based on the European Food Safety Authority (EFSA) recommendations, bacteria that possess moveable antibiotic resistance genes are not permitted in animal feeds and fermented and probiotic foods consumed by humans [10].

Recently, LAB from Indonesian fermented foods and fecal materials have been studied by the current authors for the potentials of these bacteria as probiotic agents. These LAB included *Lactobacillus* (*L.) plantarum* Dad-13 from *dadih* (fermented buffalo's milk), *L. plantarum* Mut-7 from *gatot* (fermented dried cassava), *L. plantarum* T3 from growol (fermented fresh cassava) [11] and *L. paracasei* SNP-2 from feces of healthy infants [12]. These LAB tolerated gastric juices with low pH and bile salts and inhibited microbial pathogens of *Escherichia coli* and *Shigella dysentriae* [13]. These characteristics are essential for the probiotics agents. Consumption of fermented glutinous rice (*tape*) supplemented with *L. paracasei* SNP2 was linked to increased number of *Lactobacillus* spp. in feces of healthy individuals [14]. Moreover, a safety assessment of *L. plantarum* Mut -7 and *L. plantarum* Dad- 13 has been carried out in animal models [15,16]. Regardless of the properties of these probiotics candidates that have been intensively studied, information on the antibiotic resistance schemes of these indigenous probiotics and the presence of antibiotic-resistance genes in their genomes are not still available. Therefore, the major aim of this study was to investigate the occurrence of antibiotic resistance schemes and their associated genes in *L. plantarum* and *L. paracasei* as potential indigenous probiotics.

2. Materials and Methods

2.1 Bacterial strain and growth conditions

Four *Lactobacillus* strains of *L. plantarum* Dad-13, *L. plantarum* Mut-7, *L. plantarum* T3 and *L. acidophilus* SNP-2 from Food and Nutrition Culture Collection, Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia, were used in this study. The four strain stocks were cultures in de Man, Rogosa, Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 24 h. Cultures were then stored at 5 °C and re-cultured every two weeks in MRS broth.

2.2 Antibiotic susceptibility assessment

Antibiotic susceptibility was assessed using disk diffusion method based on standard procedures [17] with some modifications [18]. *Lactobacillus* strains were cultured on MRS broth and incubated at 37 °C for 24 h and then diluted to prepare cell densities of 10^8 CFU ml^{-1}. Inocula were streaked back and forth on MRS agar surface three times using sterile cotton swabs and rotated approximately 60 ° each streak to ensure inoculum distribution. Agar plates were allowed to dry at room temperature for a maximum of 15 min; then, paper disks containing antibiotics were transferred onto the agar surface. Concentrations of the antibiotics per disk were as follows [18] amoxicillin, 10 µg (Hexpharm, Indonesia); tetracycline, 30 µg (Novapharin, Indonesia); erythromycin, 30 µg (Pharos, Indonesia); clind-amycin, 10 µg (Mersi, Indonesia); chloramphenicol, 30 µg (Pharos, Indonesia); streptomycin, 25 µg (Meiji, Indonesia); kanamycin, 30 µg (Meiji, Indonesia) and ciprofloxacin, 5 µg (Hexpharm, Indonesia). Each strain was incubated at 37 °C for 18-24 h and the diameter of the clear zone around the disk was recorded. The diameter of the clear zone was used as an suggestion for the boundary line between the susceptible/ resistant strains and the results were reported as resistant (R), intermediate resistant (IR) or susceptible (S) based on the standards by Clinical and Laboratory Standards Institute (CLSI, 2012) (Table 1).

Table 1. Standard diameters of the inhibition zones

Disk diffusion method	Zone diameter (mm)
Susceptible (S)	> 20
Intermediate resistant (IR)	15–19
Resistant (R)	≤ 14

2.3 Detection of antibiotic-resistance genes

Genome sequencing of the four *Lactobacillus* strains was carried out using NovaSeq 6000 sequencing platform. Genome was annotated using Rapid Annotation using Subsystem Technology (RAST) v.2.0 (https://rast.nmpdr.org/) to investigate number of subsystems and predict resistance genes in the virulence, disease and defense subsystem. Each group of the predicted products of resistance genes was further aligned using multiple sequence comparison by log-expectation (MUSCLE) (https://www.ebi.ac.uk/Tools/msa/muscle/) to identify amino acid sequences within the strains. Annotation of the antibiotic-resistance genes from RAST was further verified using comprehensive antibiotic resistance database (CARD), 2020 (https://card.mcmaster.ca) [19]. Transposable elements of the *Lactobacillus* strains were predicted using RAST in the subsystem of phages, prophages, transposable elements and plasmids.

3. Results and Discussion

3.1 Phenotypic profile of the antibiotic resistance

Susceptibility assay of the four strains against eight antibiotics (amoxicillin, tetracycline, erythromycin, clindamycin, chloramphenicol, streptomycin, kanamycin and ciprofloxacin) showed that all the *Lactobacillus* strains were resistant to at least three antibiotics (Table 2). All strains demonstrated resistance to streptomycin, kanamycin and ciprofloxacin. In contrast, strains were sensitive to amoxicillin, clindamycin and erythromycin. Resistance levels against tetracycline and chloramphenicol varied within the bacterial strains. Only *L. plantarum* Mut-7 showed resistance to tetracycline while others showed intermediate resistance. The *L. plantarum* Dad-13 and *L. paracasei* SNP-2 were susceptible to chloramphenicol and *L. plantarum* Mut-7 and *L. plantarum* T-3 showed intermediate resistance to the drug (Table 2). In this study, antibiotic susceptibility assessment showed that the indigenous *Lactobacillus* strains included multiple-drug resistance patterns. All strains were resistant to aminoglycosides (streptomycin and kanamycin) and ciprofloxacin. Furthermore, strains demonstrated various resistant levels to tetracycline and chloramphenicol. However, all the strains were susceptible to amoxicillin, clindamycin and erythromycin. It has been reported that lactobacilli are highly susceptible to erythromycin and clindamycin [20], but they are highly resistant to aminoglycosides [21,22]; as seen in the current study. Resistance to aminoglycosides might be linked to their native and intrinsic resistance due to the bacterial cell wall structure and cell membrane impermeability. Membrane impermeability is considered as the primary mechanism behind LAB resistance to aminoglycosides due to the absence of cytochrome-related electron transport systems that are able to facilitate drug uptake [23]. The fact that aminoglycoside resistance occurs in those strains might be due to their nature of intrinsic resistance.

3.2 Antibiotic-resistance genes

The RAST platform detected 17-18 genes of antibiotic resistance in each strain, which could be classified into tetracycline, beta-lactam, fluoroquinolone and multiple-drug efflux resistances (Fig. 1). Amino acid sequences of the strains in the same group of resistance characteristics showed high identities (data not shown) using MUSCLE analysis. Based on the RAST and MUSCLE analyses, each subsystem encoding antibiotic resistance was further verified using CARD analysis (Table 3). Based on the gene predictions using RAST, all strains included tetracycline, beta-lactamase and fluoroquinolone resistance encoding genes. However, RAST did not detect presence of antibiotic resistance genes against aminoglycosides and chloramphenicol. Translation elongation factor G and ribosomal protection involved in tetracycline resistance were present in all strains. These demonstrated phenotypical resistance to tetracycline, although the degree of resistance varied between the *Lactobacillus* strains. Based on the RAST prediction, tetracycline resistance genes in all the strains played roles in elongation factor G and protect ribosomes that bound to tetracycline compounds. naturally, tetra-cycline binding to ribosomes alters ribosome conformation that disrupts the elongation cycle and stops protein synthesis [24].

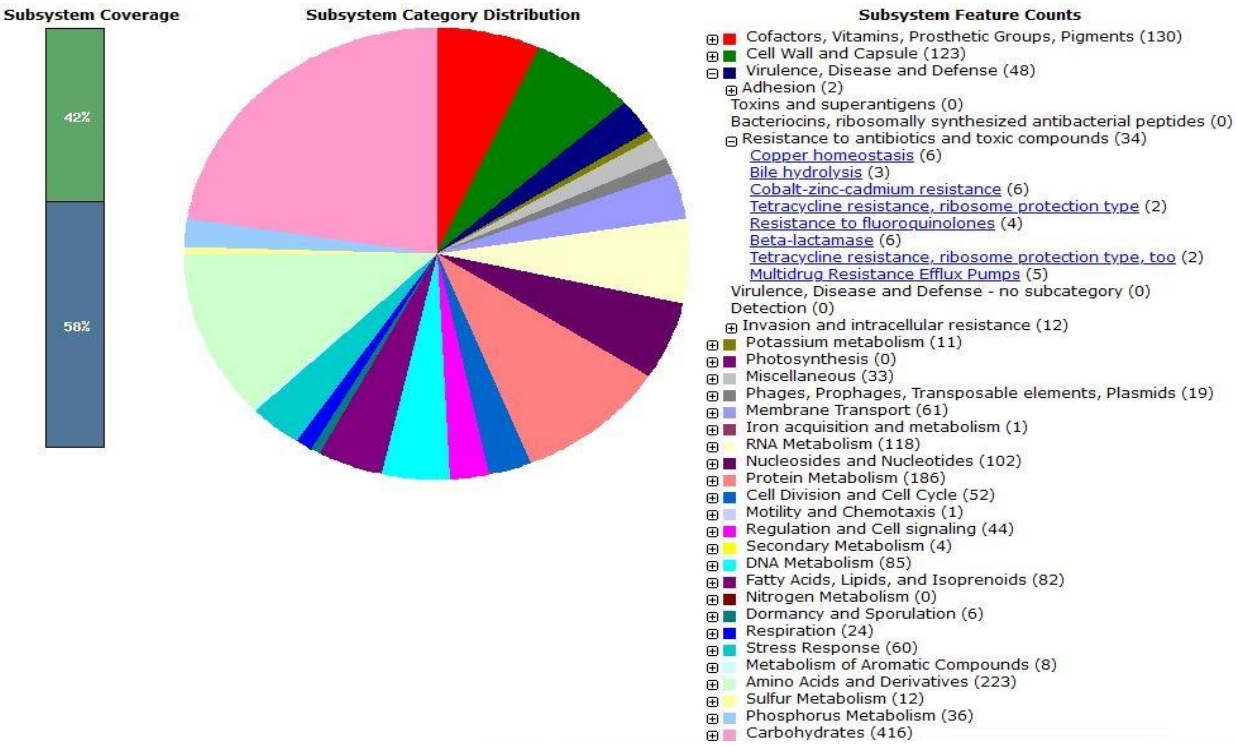

Figure 1. Subsystems of the antibiotic resistance and toxic compounds analyzed by RAST

Table 2. Comparison of the antibiotic susceptibility assessments with RAST predictions for *Lactobacillus* strains

Strains	Penicillin		Lincosamide		Macrolide		Aminoglycoside				Others				Fluoroquinolone	
	AMX		CLN		ERY		STR		KAN		TET		CHL		CIP	
	Disk Test	RASTT	Disk Test	RAST	Disk test	RAST	Disk test	RAST	Disk test	RAST	Disk test	RAST	Disk test	RAST	Disk test	RAST
L. plantarum Dad-13	S	+	S	-	S	-	R	-	R	-	IR	+	S	-	R	+
L. plantarum Mut-7	S	+	S	-	S	-	R	-	R	-	R	+	IR	-	R	+
L. plantarum T-3	S	+	S	-	S	-	R	-	R	-	IR	+	IR	-	R	+
L. paracasei SNP-2	S	+	S	-	S	-	R	-	R	-	IR	+	S	-	R	+

R: resistant, IR: intermediate resistant, S: susceptible, +: detected, - : not detected, L: Lactobacillus, AMX: Amoxicillin, CLN: Clindamycin, ERY: Erythromycin, STR: Streptomycin, KAN: Kanamycin, CHL: Chloramphenicol, CIP: Ciplofloxacin, TET: Tetracycline

Table 3. Annotation of antibiotic resistance genes in *Lactobacillus* strains using CARD

Subsystem	Size of amino acid	Homolog and origins	Identity (%)	Proposed function	Resistance mechanism
Tetracycline resistance	698	FusA *Staphylococcus aureus subsp. aureus* MRSA252	70.00	Translation elongation factor G	Antibiotic target alteration
	672	TetT *Streptomyces pyogenes*	29.33	Ribosome protection-type tetracycline	Antibiotic target protection
Fluoroquinolone resistance	853	GyrA *Clostridiodes difficile (Clostridium ijungdahlii DSM 13528)*	55.82	DNA Gyrase sub Unit A Conferring resistance to fluoroquinolones	Antibiotic target alteration
	648	Gyr B *Clostridiodes difficile (Clostridium ijungdahlii DSM 13528)*	61.00	DNA Gyrase sub Unit B Conferring resistance to fluoroquinolones	Antibiotic target alteration
	816	ParC *Streptococcus pneumonia R6*	54.00	Topoisomerase IV subunit A Conferring resistance to fluoroquinolones	Antibiotic target alteration
	668	ParE *Staphylococcus aureus subs aureus RN2240*	66.00	Topoisomerase IV subunit B Conferring resistance to fluoroquinolones	Antibiotic target alteration
Beta-lactamase	274	BlaF *Mycolicibacterium fortuitum*	30.00	Beta-lactamase class A	Antibiotic inactivation
	391	AmpH *Escherichia coli DH1*	26.98	Beta-lactamase class C	Antibiotic inactivation
	391	Mir4 *Escherichia coli*	27.07	Beta-lactamase class C	Antibiotic inactivation
	343	AmpC1 *Escherichia coli* ETEC H10407	27.56	Beta-lactamase class C	Antibiotic inactivation
	343	AmpC1 *Escherichia coli* ETEC H10407	31.51	Beta-lactamase class C	Antibiotic inactivation
	376	Exo1 *Streptomyces albus*	31.87	Exo beta-lactamase	Antibiotic inactivation
Multidrug resistance efflux	188	TetR *Salmonella enterica* subsp. *enterica* serovar *Typhi* str. CT18	40.00	Major facilitator superfamily (MFS) antibiotic efflux pump	Antibiotic efflux
	444	MepA *Staphylococcus aureus*	30.18	Multiple-drug and toxic compound extrusion (MATE) transporter	Antibiotic efflux
	463	CdeA *Clostridioides difficile*	23.00	Multiple-drug and toxic compound extrusion (MATE) transporter	Antibiotic efflux
	410	MdtG, *Escherichia coli* O139:H28 str. E24377A	46.05	Major facilitator superfamily (MFS) antibiotic efflux pump	Antibiotic efflux
	407	MdtG, *Escherichia coli* O139:H28 str. E24377A	46.00	Major facilitator superfamily (MFS) antibiotic efflux pump	Antibiotic efflux

Therefore, strains with tetracycline resistance ribosomal protection genes can protect ribosomal proteins from binding to tetracycline compounds, causing tetracycline resistance [25]. Interactions between the ribosomal protection proteins and helix 34 on 16S rRNA molecules contribute to allosteric disruptions at the major tetracycline-binding sites that cause detach of the tetracycline molecules from the ribosomes [26].

Ribosomes restore their necessary conformation and protein synthesis continues [27].

Fluoroquinolone resistance assessed by DNA gyrase subunits A and B (EC 5.99.1.3) and topoisomerase IV subunits A and B (EC 5.99.1) were present in all strains, which supported resistance to ciprofloxacin and quinolones (Table 3). Moreover, the phenotypic profile showed resistance to ciprofloxacin. In fact, DNA gyrase and topoisomerase IV are fluoroquinolone targets by inhibiting their functions during supercoiling, disrupting DNA replication and even cell death at lethal concentrations [28]. High resistance to fluoroquinolones in bacteria is due to mutations in genes encoding DNA gyrase and topoisomerase [29-30]. These mutations cause amino acid changes and modify target protein structures, especially in regions of each subunit enzyme called quinolone-resistance-determining-regions (QRDR) that make the enzyme less sensitive by fluoroquinolone inhibition [28,31]. Prominent resistance of *Lactobacillus* spp. to ciprofloxacin has previously been reported [20,31]. In this study, all strains possessed beta-lactamases classified into beta-lactamase Class A, beta-lactamase Class C and other penicillin binding proteins as well as EXO beta-lactamases (EC 3.5.2.6). In contrast, all strains showed susceptibility to amoxicillin, indicating that those beta-lactamase genes might not be functional. Each class of beta-lactamases (A, B, C and D) is characterized by the presence of a specific conserved active substance. Class A beta-lactamase includes three conserved motifs of S-X-X-K, S-DN and K-T-G at positions of 70, 130 and 234, respectively [32]. The Class C beta-lactamase sequence has conserved motifs of S-X-S-K, Y-S[A]-N and K-[TS]-G at positions of 64, 150 and 314, respectively [32,33]. All conserved motifs were present in those beta-lactamases in the current strains. Therefore, susceptibility mechanism of the four *Lactobacillus* strains against amoxicillin in this study are still unclear. Two groups of multiple-drug resistance efflux were detected in all the strains. Further analysis by CARD indicated that the majority of the facilitator superfamily (MFS) antibiotic efflux might involve fosfomycin resistance and multiple-drug and toxic compound extrusion (MATE) antibiotic efflux might be involved in tetracycline, glycylcycline and fluoroquinolones resistance phenotypes.

In this study, multiple-drug resistance efflux was seen in all *Lactobacillus* strains. Either MFS or MATE type has been known to involve in antibiotic resistance mechanisms [34]. Their expression levels contribute to levels of antibiotic resistance; thus, higher expression levels correlated to higher resistance levels [35]. Interestingly, the efflux systems vary in mechanisms to resist antibiotics, including intrinsic, acquired and transient-induced phenotypic resistances. As stated in Results section, the multiple-drug resistance efflux in the current strains may involve tetracycline, glycylcycline and fluoroquinolone resistance phenotype. Multiple-drug resistance effluxes are encoded in prokaryotic chromosomes and considered primordial elements showing highly conservations in microorganisms [36].

All *Lactobacillus* strains were resistant to aminoglycosides; however, none of them included kanamycin and streptomycin resistance genes. Mechanism of resistance to aminoglycosides in LAB involves alteration in ribosome binding sites by mutations in *rpsL* and *rsmG,* leading to amino acid replacements in specific gene positions [37]. However, detailed analysis of those genes from the four strains did not show mutations. Results of the RAST predicted bacterial cell components such as the phages, prophages, transposable elements and plasmids subsystem. However, the current *Lactobacillus* strains did not include transposable elements, gene transfer agents and plasmid-related functions (Fig. 2). In previous studies, resistance genes could horizontally be transferred to other microorganisms if located in plasmids or transposons [38]. Based on the RAST analysis, the *Lactobacillus* strains did not include transposable elements, gene transfer agents and plasmid-related functions. It can be concluded that the antibiotic resistance genes were not located in plasmids or transposable elements and horizontal transfer of the antibiotic resistance genes unlikely occurred, according to EFSA [10]. Thus, the *Lactobacillus* strains in this study can be considered safe.

4. Conclusion

In this study, all *Lactobacillus* strains showed multiple-drug resistance patterns. All strains were resistant to aminoglycosides and ciprofloxacin and showed various resistance levels to tetracycline and chloramphenicol. However, the bacteria were susceptible to amoxicillin, clindamycin and erythromycin. Occurrence of the resistance genes was correlated to phenotype results, except for amoxicillin and aminoglycosides. The RAST subsystem prediction demonstrated that the *Lactobacillus* strains did not include transposable elements, gene transfer agents and plasmid-associated functions; therefore, possibility of horizontal gene transfer could be ignored. Thus, all *Lactobacillus* strains of this study were considered safe for human consumption.

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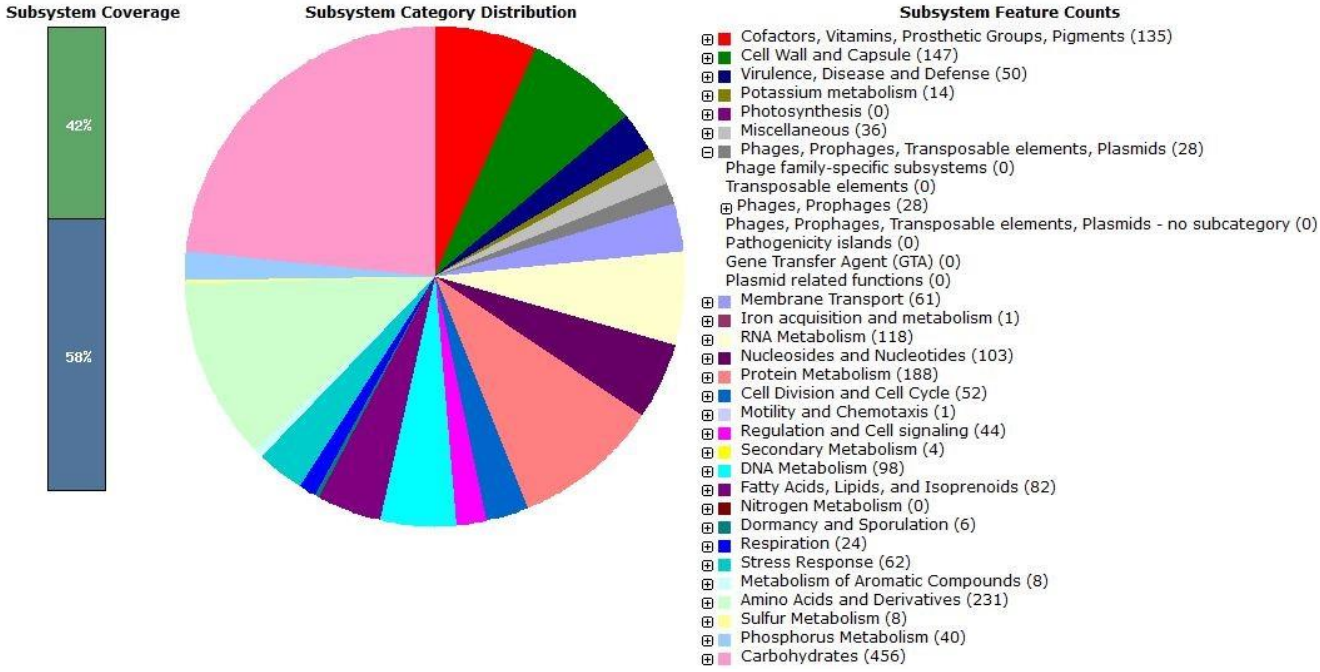

Figure 2. Subsystems of the phages, prophages, transposable elements, plasmids analyzed by RAST

6. Conflict of Interest

The authors report no conflicts of interest.

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بررسی ژنوتیپ و فنوتیپ مقاومت آنتی‌بیوتیکی زیست‌یار *لاکتوباسیلوس* بومی اندونزی

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چکیده

سابقه و هدف: در مطالعات قبلی نویسندگان، چهار سوش منحصربه‌فرد لاکتوباسیلوس (*لاکتوباسیلوس پلانتاروم* Dad-13، *لاکتوباسیلوس پلانتاروم* Mut-7، *لاکتوباسیلوس پلانتاروم* T-3 و *لاکتوباسیلوس پاراکازیی* SNP-2) به‌دست آمده از غذاهای تخمیری و مدفوع افراد سالم اندونزی، به‌عنوان عوامل زیست‌یار[1] مورد مطالعه قرار گرفتند. در مطالعه حاضر، فنوتیپ‌های مقاوم به آنتی‌بیوتیک *لاکتوباسیلوس پلانتاروم* و *لاکتوباسیلوس پاراکازیی* مورد اشاره در برابر ۸ آنتی‌بیوتیک (آموکسی‌سیلین، تتراسایکلین، اریترومایسین، کلیندامایسین، کلرامفنیکل، استرپتومایسین، کانامایسین، سیپروفلوکسین) و ژن‌های مقاومت به آنتی‌بیوتیک این سوش‌ها مورد مطالعه قرار گرفتند.

مواد و روش ها: حساسیت به آنتی‌بیوتیک باکتری‌ها به ۸ آنتی بیوتیک با استفاده از روش انتشار دیسک مورد بررسی قرار گرفت. توالی‌یابی ژنوم با استفاده از پلتفرم تعیین توالی NovaSeq 6000 انجام شد. ژنوم با استفاده از فناوری زیرسیستم رمزگشایی سریع v.2.0 رمزگشایی شد. پس از آن‌که هرگروه فرآورده‌های پیش‌بینی شده ژن‌های مقاوم با استفاده مقایسه توالی چندگانه به‌وسیله (MUSCLE) در یک گروه قرار داده شدند، و عملکردشان توسط پایگاه داده جامع مقاومت آنتی‌بیوتیکی (CARD) ۲۰۲۰ مورد تایید قرار گرفت.

یافته‌ها و نتیجه‌گیری: تمام سوش‌ها به آمینوگلیکوزید و سیپروفلوکسین مقاوم، اما نسبت به آموکسی‌سیلین، کلیندامایسین و اریترومایسین حساس بودند. مقاومت در برابر کلرامفنیکل و تتراسایکلیندر این سوش‌ها کتفاوت بود. دو سوش حساس و بقیه مقاومت متوسطی به کلرامفنیکل داشتند. یک سوش به تتراسایکلین مقاوم بود، درحالی‌که سه سوش دیگر مقاومت آنتی‌بیوتیکی متوسطی نسبت از خود نشان دادند. توالی ژنوم چهار سوش، حضور ژن‌های مقاوم به تتراسایکلین، بتا-لاکتاماز و سیپروفلوکسین و نیز سیستم‌های شارش مقاومت چند دارویی را تایید کرد. وجود ژن‌های مقاوم با نتایج فنوتیپ ارتباط داشت، بجز برای آموکسی‌سیلین و آمینوگلیکوزیدها. رمزگشایی سریع با استفاده از فناوری زیرسیستم-نشان داد که تمام سوش‌های لاکتوباسیلوس حاوی عناصر قابل جابجایی ، عوامل انتقال ژن و توابع مرتبط با پلاسمید می‌باشند؛ انتقال غیرمحتمل افقی ژن‌های مقاوم به آنتی‌بیوتیک روی می‌دهد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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