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### ASSESSMENT OF PROBIOTIC PROPERTIES AND SAFETY OF LACTIC ACID BACTERIA ISOLATED FROM SOUTH SULAWESI ETHNIC CHEESE

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#### KEYWORDS

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#### ABSTRACT

Probiotic bacteria have been used widely as a functional food and health supplements. The functionality and safety of probiotics are the prerequisites given by WHO (World Health Organization) and FAO (Food Agricultural Organization) before utilizing probiotics. This study focuses on assessing probiotic properties and the safety of the lactic acid bacteria (LAB) isolated from dangke—traditional cheese of South Sulawesi. In the current study, the assessment of probiotic properties was carried by assessing its tolerance against low pH and bile salts. Safety assessments were divided into two assays viz., susceptibility testing and hemolytic activity. LAB from dangke demonstrated tolerance against low pH, bile salt and susceptibility against four types of antibiotics (ampicillin, cefotaxime, erythromycin, and tetracycline). Unfortunately, resistance towards gentamycin and an  $\alpha$ -hemolytic activity was observed. This bacterium met the functional criteria from probiotics but failed to meet the safety criteria for probiotic safety.

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## 1 Introduction

Probiotic incorporation in food and health supplement remains a trend ever since its first commercialization in 1903. Some probiotics used in the market today mainly originated from fermented foods or the human body and animal microflora (Zommiti et al., 2020). To ensure its beneficial action and its safety in our body, probiotic candidates must fulfil some requirements. The minimum criteria for probiotic candidates were emphasized in the International Scientific Association of Probiotics and Prebiotics (ISAPP) 2018 discussions based on joint FAO/WHO 2002 guidelines: 'a probiotic candidate must meet functionality and safety criteria, also be easy to use in technical terms' (FAO/WHO 2002; ISAPP 2018; Zommiti et al., 2020).

After being consumed, probiotic will be exposed to low pH and bile salts in the gastrointestinal tract; it must maintain specific viability to provide health benefits when consumed (Markowiak & Śliżewska 2017; Stasiak-Różańska et al., 2021). Though probiotics are usually encapsulated to protect their sustainability, some probiotic products suggest the protective capsule be removed before consumed by toddlers, which may affect its survival and efficacy. Furthermore, the varying incapability of several commercial probiotic supplements against the extreme condition in the stomach was reported in many studies (Zommiti et al., 2020; Stasiak-Różańska et al., 2021). Regarding its safety, the origin of the strain, its hemolysis activity, and resistance pattern to antibiotics become the requirements of a probiotic candidate must meet during the selection (Markowiak & Śliżewska 2017; Zommiti et al., 2020).

ISAPP suggested a wide distribution of mechanisms of probiotic health benefits in large taxonomic groups. Moreover, it was frequently found at the species level (Hill et al., 2014). Though not all health benefit effects might be observed in a single strain, multiple mechanisms including the production of strain-specific bioactive compounds might be expected (Hill et al., 2014). Each strain might possess different characteristics; therefore, assessments of its probiotic properties and safety to ensure the quality, functionality, and safety of a new probiotic candidate for commercial products are needed.

Dangke, an ethnic cheese of South Sulawesi, Indonesia, has been studied as the source of some potential probiotic candidates (Nur, 2012; Burhan et al., 2017). This staple food is made from buffalo or cow milk added with papaya sap to coagulate the protein then ripened; the compounds produced during the ripening process support the growth of LAB (Surono, 2015; Burhan et al., 2017; Djide et al., 2020). The current study managed to isolate and identify bacteriocin-producing lactic acid culture—*Lactobacillus fermentum* strain NBRC 15885—from Dangke; however, its probiotic properties and safety are yet to be determined (Djide et

al., 2020). Hence, this study focused on assessing probiotic properties and the safety of the strain to ensure its functionality and safety as a new commercial probiotic candidate.

## 2 Materials and Methods

To carry out this study, *L. fermentum* strain NBRC 15885 isolated from dangke was cultured in de Man Rogosa & Sharpe (MRS) Agar (Merck®) 24 hours before the test.

### 2.1 Assessment of Probiotic Properties

#### 2.1.1 Tolerance Against low pH

Tolerance against low pH was assessed following Sujaya et al. (2008) with some modification. LAB was cultured on MRS broth (Merck®) in aerobic condition at 37°C, for 48 hours. One ml of the media was transferred into 1.5 ml Eppendorf® tubes and centrifuged for 15 minutes at 3000 rpm. The filtrate was discarded, followed by adding a 500 µl saline solution (Otsuka®) into the tube. A total of 50 µl of the suspension was pipette into a new tube contained 1000 µl MRS Broth (Merck®) at pH 2 followed by incubation for 2.5 hours at 37°C. After 2.5 hours, 100µl of cell suspension was pipette into a new tube followed by 10-fold dilution using saline solution (Otsuka®). Fifty microliters of the dilution were pipetted into 5 ml broth MRS Broth (Merck®) (pH 6.5) then incubated at 37°C for 48 hours. The optical density of the media was measured at a wavelength of 660 nm using Spectronic-20 (Thermo-scientific®).

#### 2.1.2 Tolerance Against Bile Salt (0.3%)

Tolerance against low pH was assessed by using the same method mentioned above. MRS broth supplemented 0.3% bile was used in this test.

### 2.2 Safety Assessment of the Strain

#### 2.2.1 Hemolytic Activity

Hemolytic activity was assessed by inoculating LAB culture in Blood Agar (Merck®) added with 5% sheep blood followed by 24 hours incubation at 37°C. The change in media colour was observed (Singh et al. 2014).

#### 2.2.2 Antimicrobial Resistance

MRS Agar media was spread (Merck®) with previously diluted LAB culture—adjusted to the standard McFarland 0.5 (equivalent to  $1.5 \times 10^8$  cfu/ml). Antibiotic discs (ampicillin, erythromycin, tetracycline, and gentamicin (Oxoid®) were aseptically placed on the media. The plates were incubated 24 hours at 37°C. The diameter of the inhibitory zone was measured. CLSI M100-S22 (Clinical Laboratory Standard Institute 2012) was used as a reference to determine the susceptibility of test antibiotics.

### 3 Results

#### 3.1 Assessment of Probiotic Properties

According to Sujaya et al. (2008), if LAB shows an OD<sub>660</sub> > 0.01 after being grown at a low pH of 0.3% bile salt, the bacteria might survive in the gastrointestinal tract, and it can be able to withstand the extreme condition of the gastrointestinal tract. The OD<sub>660</sub> value of dangke LAB after being grown in MRS Broth at pH 2 and MRS Broth added with 0.3% bile salts were shown in Table 1.

#### 3.2 Safety Assessment of the Strain

Safety assessments of dangke LAB were divided into two assays viz., hemolytic activity and antimicrobial resistance. Hemolysis

activity was studied on blood agar media. Cultures of dangke LAB exhibit  $\alpha$ -hemolysis activity characterized by forming a greenish zone with a clear outer area around the streak (Figure 1).

Assessment of antimicrobial resistance was carried out against the five antibiotics i.e. ampicillin, cefotaxime, erythromycin, gentamycin, and tetracycline. According to the CLSI standard (Clinical Laboratory Standard Institute 2012), there are three categories of resistance in assessment: >14 mm inhibition zone is considered resistant (R), inhibition zone diameter ranges from 15 - 19 mm is deemed to be intermediate (I), and a diameter < 20 mm is considered to be susceptible (S). The results showed LAB were sensitive to ampicillin, cefotaxime, erythromycin, tetracycline while showing resistance to gentamicin antibiotics (Table 2).

Table 1 The OD<sub>660</sub> value of dangke LAB grown in extreme GIT condition

Condition	OD <sub>660</sub>
pH 2	1.620 ± 0.053
Bile salts 0.3%	1.627 ± 0.012

R = resistant, I = intermediate, S = susceptible

Table 2 Antibiogram Profile of *L. fermentum* NBRC 15885

Antibiotic ( $\mu$ g)	Diameter of inhibition zone (mm)	Interpretation
Ampicillin (10)	41.33 ± 1.61	S
Cefotaxime (30)	26.00 ± 1.80	S
Erythromycin (15)	29.56 ± 3.96	S
Gentamycin (10)	7.78 ± 0.42	R
Tetracycline (30)	21.89 ± 1.83	S

R = resistant, I = intermediate, S = susceptible



Figure 1 Hemolytic Activity of *L. fermentum* NBRC 15885 Isolated from Dangke.

## 4 Discussion

### 4.1 Assessment of Probiotic Properties

WHO/FAO defines probiotics as living microbes that provide a health benefit when given in adequate amounts (FAO/WHO 2002). Based on these definitions, probiotics must maintain specific viability to benefit our health, despite being formulated into an encapsulated product (Papadimitriou et al., 2016; Zommiti et al., 2020). The gastrointestinal tract creates various challenges for probiotic cultures, including acidity (pH 2-4), digestive enzymes (Papadimitriou et al., 2016), and bile salts (Ruiz et al., 2013), thus, resistance against these conditions becomes an essential requirement in the screening of new probiotics where a minimum decrease in viability under these critical conditions (Papadimitriou et al., 2016; Wang et al., 2018). Furthermore, acid resistance is also an important technological aspect since the accumulation of acid products from fermentation may lead to cell death (Papadimitriou et al., 2016). The acidic conditions of G.I. not only damage the cell membrane and cell walls, but also affect membrane potential, protein denaturation, and damage jeans (Papadimitriou et al., 2016), while bile salt can cause cell death through damaged lipid packaging or cytoplasm acidification (Ruiz et al., 2013). LAB tolerance against low pH can occur through several mechanisms, including production of alkali compounds that neutralize pH, biofilm formation, protection or repair of cell macromolecules, or the presence of F<sub>0</sub>F<sub>1</sub>-ATPase proton pump system (Jin et al. 1998; Wang et al., 2018). The mechanism of resistance of BAL against bile salts can occur through hydrolysis of bile salts, alterations in the components of cell walls and membranes, and the active efflux of bile acid and its salts (Ruiz et al., 2013). Based on the results, *L. fermentum* NBRC 15885 bacteria meet the functional criteria of probiotics.

### 4.2 Safety Assessment of the Strain

Haemolysis is one of the virulence factors. Haemolysis is a phenomenon that indicates the release of haemoglobin from red blood cells, it can be classified into three types: alpha ( $\alpha$ ), beta ( $\beta$ ), and gamma ( $\gamma$ , non-hemolysis) types. Among those three,  $\gamma$ -hemolysis is considered safe. FAO/WHO requires testing of hemolysis activity for a probiotic strain if the same species shows potential for hemolysis (FAO/WHO 2002). Owusu-Kwarteng et al. (2015) reported  $\alpha$ -hemolysis activity by *L. fermentum* strains of millet dough; therefore, hemolytic activity needs to be assessed. LAB culture of dangke shows  $\alpha$ -hemolysis activity. The first phenomenon of  $\alpha$ -hemolysis with true outer hemolysis was observed by Davis & Rogers in 1939, with hydrogen peroxide production as the possible mechanism. Several years later, another mechanism, such as the production of hemolysin or biosurfactants, was reported and eventually used to screen biosurfactant-

producing bacteria (Morán et al., 2002). The amphiphilic parts of surfactant molecules can disrupt cell membrane integrity through modification of lipid compounds and integral proteins, which leads to lysis of red blood cell membranes (Manaargadoo-Catin et al., 2016). Several strains of *Lactobacillus* spp. are also reported to produce biosurfactants and hydrogen peroxide (Sharma & Saharan, 2014; Sharma & Saharan, 2016; Cornea et al., 2016; Mann et al., 2021).

Another safety requirement of probiotic strains by FAO/WHO is the determination of antibiotic resistance patterns (FAO/WHO 2002). Based on the test result, LAB dangke shows resistance against aminoglycoside antibiotic gentamicin which has already been reported by previous researchers (Georgieva et al., 2015; Dec et al., 2017; Zhang et al., 2018; Dong et al., 2019). The mechanism of LAB resistance against the aminoglycoside group was first reported in 1998 as a loss of aminoglycoside uptake mediated by cytochrome-mediated electorial transport (Charteris et al., 1998). Several years later, intrinsic gene *aph(3')*-IIIa, *aac(6)-aph(2)*, and *ant(6)* were reported as the gene responsible for aminoglycosides resistance in *Lactobacillus* (Wong et al., 2015). Recently, *asp23* was reported to regulate the gentamicin resistance mechanism in *L. casei* (Zhang et al., 2018).

Although probiotics with resistant genes are not harmful, there is a potential to transfer the resistance gene from probiotics to indigenous microorganisms in the gut, or even opportunistic pathogens living in the gut that might cause severe infections (Zheng et al., 2017; Wang et al., 2020). Besides, several cases of difficult-to-treat bacteremia or endocarditis caused by *Lactobacillus* species which are used in probiotic products have been reported (Ashraf & Shah, 2011; Zheng et al., 2017). On the other hand, this resistance can be helpful in combination with antibiotic therapy because probiotics must survive in the influence of these antibiotics (Imperial & Ibane, 2016). The study of 43 commercial probiotic strains used as a probiotic-antibiotic combination in Japan shows that the amount used clinically with antibiotics is quite limited (Hammad & Shimamoto, 2010). These findings turn the usage of probiotics in the health field into a double-edged sword. As reviewed by Tong (2021), though some probiotic strains show resistant activity towards antibiotics, but these activities are sometimes intrinsic and have restricted transference to other genera (Tong 2021).

### Conclusion

In conclusion, *L. fermentum* strain NBRC 15885 isolated from dangke exhibits probiotic potential as it can tolerate bile salt and extreme acidity. However, it didn't meet the safety criteria of FAO/WHO. It is recommended to expand the study in various research fields to uncover more potentials of this strain.

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### Conflict of interest

The authors declare no conflict of interest in this study.

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