Research Article

Moderate Antioxidant Activity of Exopolysaccharides from Dangke Derived *Enterococcus faecium*

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Abstract | Oxidative reactions contribute to food deterioration and are implicated in oxidative stress related disorders, motivating interest in natural antioxidants. This study evaluated the antioxidant activity of exopolysaccharides (EPS) produced by *Enterococcus faecium* strains isolated from Dangke, a traditional Indonesian dairy product. EPS were produced in a whey-based medium and recovered by heat treatment, enzymatic clarification, centrifugation, ethanol precipitation, dialysis, and freeze drying. Antioxidant capacity was quantified using a microplate-based 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. In the tested range (1-10 mg/mL), all EPS samples showed concentration-dependent scavenging but low potency at 10 mg/mL, inhibition reached 38.6-40.6%, whereas at 1 mg/mL it was 0.8-2.4%. Because 50% inhibition was not achieved within the tested range, EPS IC_{50} values are reported conservatively as IC_{50} > 10 mg/mL. Corresponding Trolox equivalent antioxidant capacity (TEAC) values are therefore reported as an upper bound (TEAC < 3.0×10^{-3} mg Trolox/mg EPS), indicating substantially lower activity than Trolox and ascorbic acid under the same assay conditions. Structural characterization and technological functionality tests were not performed. Therefore, mechanistic explanations and application claims remain beyond the scope of this work. Overall, this study provides a quantitative benchmark for EPS from a previously uncharacterized traditional dairy niche and supports prioritizing structural characterization and matrix-relevant assays in future screening.

Keywords | Antioxidant activity, Dangke, *Enterococcus faecium*, Exopolysaccharides, Traditional dairy product

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INTRODUCTION

Oxidative stress remains a central concept in both biomedical and food science. When the generation of reactive oxygen species exceeds endogenous antioxidant defenses, oxidative reactions can damage lipids, proteins, and nucleic acids, contributing to chronic diseases ranging from cancer and diabetes to cardiovascular and neurodegenerative disorders (Chooruk *et al.*, 2017; Kim *et al.*, 2021; Manzanares *et al.*, 2019). Similar processes occur in food matrices, where reactive species accelerate spoilage and reduce sensory quality (Fadlillah *et al.*, 2021). Free radicals such as hydroxyl radicals can initiate chain reactions that impair macromolecular integrity and promote oxidative deterioration (Jin *et al.*, 2025; Zhang *et al.*, 2015). As a result, the development of safe and effective antioxidant strategies is important both for maintaining food stability and for supporting health-related research

on oxidative mechanisms.

Naturally occurring antioxidants are attractive alternatives to synthetic compounds, partly because of safety considerations and consumer demand for cleaner ingredient labels (Lü *et al*., 2010; Manzanares *et al*., 2019). Lactic acid bacteria (LAB), long associated with fermented foods, have been studied as microbial sources of antioxidant-associated metabolites. Previous work indicates that LAB can scavenge radicals, reduce oxidative markers, or modulate antioxidant enzymes *In vitro* and *in vivo* (Chooruk *et al*., 2017; Kim *et al*., 2021; Yang *et al*., 2022). Among LAB products, exopolysaccharides (EPS) have attracted interest because they can be produced extracellularly and have been reported to show bioactivities that vary strongly by strain and preparation (Hakim *et al*., 2023; Kumar *et al*., 2022).

Importantly, antioxidant activity reported for LAB-derived EPS spans a wide range and is frequently modest, depending on chemical composition and structure (Adelekan *et al*., 2020; Ge *et al*., 2021; Zhou *et al*., 2022). Structural features including branching patterns, molecular weight, and the presence of hydroxyl, carboxyl, or sulfate groups are often discussed as influencing radical scavenging by affecting electron donation capacity and reactivity toward radicals (Adelekan *et al*., 2020; Jurášková *et al*., 2025). From a screening perspective, identifying weak activity is not a negative outcome, it provides a quantitative baseline, helps avoid overinterpretation of novelty as potency, and clarifies where a new source sits within the broader performance spectrum relative to standard antioxidants.

In dairy fermentations, EPS are also widely discussed for technological roles such as effects on viscosity, moisture retention, emulsification, and syneresis reduction (Zhang *et al*., 2020). However, such functionality must be demonstrated using rheological and stability measurements, and these technological properties were not evaluated in the present work. Here, the focus is on quantitative antioxidant benchmarking using a DPPH radical scavenging assay.

Traditional fermented dairy products are rich reservoirs of EPS-producing LAB. Microbial surveys of artisanal cheeses and fermented milks highlight how local ecosystems can select for strains with distinct technological and bioactive traits (Fguiri *et al*., 2016; Taj *et al*., 2022; Terzic-Vidojevic *et al*., 2009). Dangke is a fresh, high-protein cheese traditionally produced from bovine or buffalo milk using simple acid coagulation in small scale settings in Indonesia, typically without standardized starter cultures (Malaka *et al*., 2022). Under these conditions, the milk and whey matrix composition and processing environment (e.g., tropical temperatures, limited refrigeration, and repeated heating/holding steps) may shape the composition and stress adaptation of resident LAB and could influence EPS yield and properties. However, EPS from Enterococcus faecium isolates associated with Dangke remain largely uncharacterized.

Enterococcus faecium is frequently isolated from artisanal dairy products and has been associated with flavor development and antimicrobial activity in certain fermented-food contexts (Ozturkoglu-Budak *et al*., 2023; Pieniz *et al*., 2015). At the same time, *E. faecium* also includes lineages with virulence factors and antibiotic resistance determinants. Therefore, while Dangke is a food-associated products, any future application involving live strains would require strain-level safety assessment. The present study evaluates EPS preparations produced by Dangke isolates and does not propose direct use of the isolates as starter or probiotic cultures.

Although EPS from several LAB, including *E. faecium,* have been reported to possess antioxidant activity in traditional dairy products, such as EPS from *E. faecium* BDU7 isolated from Ngari (Abdhul *et al*., 2014) and EPS MC-5 from *E. faecium* strains associated with traditional Iranian Kishk (Vasough *et al*., 2021), the range of products and ecological contexts that have been quantitatively characterized remains limited. Many studies focus on whole cells or crude culture supernatants rather than purified EPS fractions (Abduxukur *et al*., 2023; Pieniz *et al*., 2015) and some do not benchmark against standardized reference antioxidants such as ascorbic acid and Trolox, which is important for interpreting relative potency (Jiménez *et al*., 2008; Ozcan *et al*., 2017). In particular, no data are currently available on the antioxidant properties of dialysis-purified EPS produced by *E. faecium* strains isolated from Dangke.

Accordingly, the present study quantifies DPPH radical scavenging activity of EPS produced by Dangke-derived E. faecium strains across a defined concentration range and benchmarks activity using IC_{50} and Trolox equivalent antioxidant capacity (TEAC) relative to ascorbic acid and Trolox. The aim is to position these EPS within the broader spectrum of LAB-derived EPS activity, including the possibility that they fall toward the weak end and to provide a quantitative baseline for this previously unexplored traditional-dairy niche. Further work should prioritize EPS structural characterization, together with matrix-relevant antioxidant assays.

MATERIALS AND METHODS

A schematic overview of the experimental workflow, from Dangke sampling and isolation of *E. faecium* strains through EPS extraction, and antioxidant evaluation is presented in Figure 1.

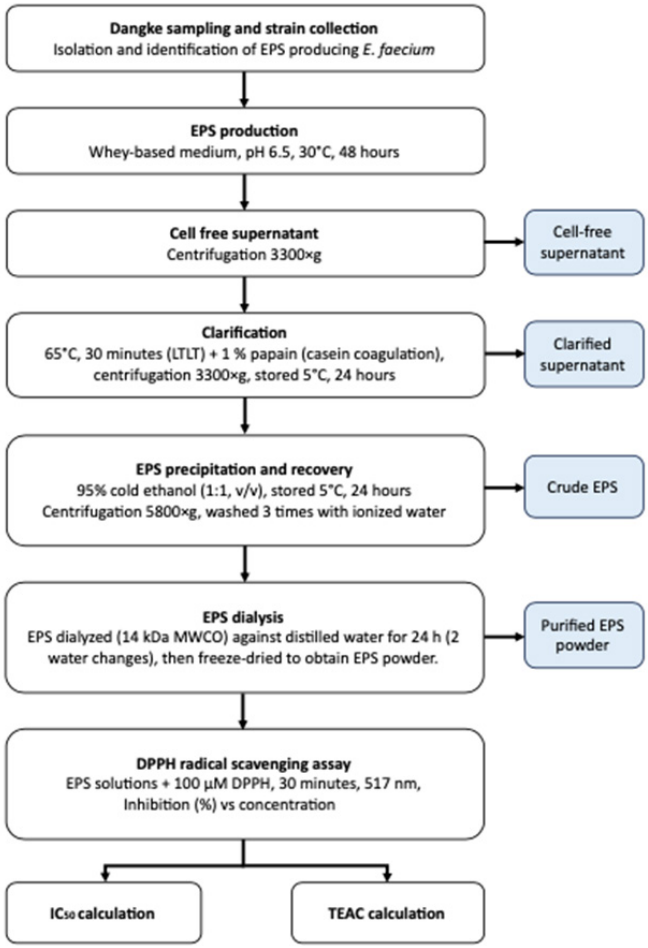

Figure 1: Schematic overview of the experimental design.

Bacterial strains, culture media, and EPS production

Enterococcus faecium strains used in this study were previously isolated from Dangke using phenotypic and molecular approaches commonly applied in surveys of LAB from artisanal dairy products (Kadir *et al*., 2025). Cultures were maintained on standard LAB media at 4 °C, with long-term stocks stored at -80 °C (Hakim *et al*., 2023; Torino *et al*., 2015). EPS production was carried out in a whey-based medium enriched with glucose and nitrogen sources to support polysaccharide synthesis. The pH of the production medium was adjusted to 6.5 before inoculation, and cultures were incubated at 30 °C for 48 hours under static conditions, consistent with reported optimal ranges for LAB EPS biosynthesis (Alhudhud *et al*., 2014; Malaka *et al*., 2020).

Exopolysaccharides extraction

EPS from Dangke-derived *E. faecium* isolates were extracted with modifications to Malaka *et al* (2020). Cells were removed by centrifugation (3300×g), and the resulting cell-free supernatant was heated at 65 °C for 30 min (LTLT) to inactivate endogenous enzymes. To remove co-extracted proteins (e.g., casein and other peptides), a 1% (w/v) papain solution was added and the mixture was incubated under conditions sufficient for proteolysis, followed by centrifugation (3300×g) to remove insoluble material. To reduce potential interference from nucleic acids and other low-molecular-weight compounds that may contribute to apparent antioxidant activity, the clarified supernatant was subjected to ethanol precipitation (95% cold ethanol, 1:1 v/v) and stored at 5 °C for 24 h before centrifugation (5800×g). The resulting precipitate was re-dissolved in distilled water and washed three times (each wash followed by centrifugation) to remove residual medium components. The washed EPS fraction was then dialyzed to remove small metabolites and free sugars: the precipitate was dissolved in distilled water (1:20 w/v) and placed in a 14 kDa MWCO dialysis membrane, dialyzed against distilled water for 24 h with two water changes. Dialyzed material was freeze-dried to obtain EPS powder. No structural characterization was performed. Therefore, structure activity relationships and mechanistic interpretations of antioxidant activity are explicitly considered outside the scope of this study and are treated as a limitation when interpreting the DPPH results.

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Antioxidant activity was evaluated using a a microplate based DPPH assay following established protocols (Navajas-Porras *et al*., 2020; Zhang *et al*., 2019). EPS were dissolved in distilled water at the desired concentrations immediately before analysis. A 100 µM DPPH solution in methanol was prepared, and equal volumes of sample solution and DPPH solution were mixed and incubated in the dark for 30 min. Absorbance was measured at 517 nm (Wu *et al*., 2023). Trolox and ascorbic acid were used as reference antioxidants.

The percentage of DPPH radical scavenging (inhibition) was calculated according to the following equation:

$$\text{Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where, A_0 is the absorbance of the control (DPPH solution mixed with solvent) and A_1 is the absorbance of the reaction mixture containing DPPH and the EPS or standard antioxidant solution (Navajas-Porras *et al*., 2022).

IC_{50} and Trolox equivalent antioxidant capacity (TEAC)

Dose-response curves (percent inhibition vs. concentration) were generated for each EPS preparation and for Trolox and ascorbic acid. IC_{50} was defined as the concentration required to achieve 50% inhibition. For Trolox and ascorbic acid, IC_{50} values were determined by linear interpolation between the two concentrations bracketing 50% inhibition using $y = ax + b$ (not forced through the origin) and solving for $y = 50$. For EPS preparations, because 50% inhibition

was not achieved at the highest tested concentration (10 mg/mL), IC_{50} values were conservatively reported as IC_{50} > 10 mg/mL. TEAC was calculated using the IC_{50} ratio approach (Xiao *et al*., 2020):

$$\text{TEAC (mg Trolox/ mg sample)} = IC_{50}\text{ (Trolox)} / IC_{50}\text{ (sample)}$$

Because EPS IC_{50} values are reported as >10 mg/mL, TEAC values for EPS are reported as an upper bound, TEAC < IC_{50}(Trolox) / 10.

Data analysis

All experiments were performed in triplicate and results are presented as mean ± standard deviation (SD). All data were statistically evaluated using SPSS software, in which a two-way analysis of variance (ANOVA) applied to examine whether the differences observed among various treatment groups were statistically significant. When the ANOVA indicated significant effects, Tukey's honestly significant difference (HSD) post hoc test was used to identify which specific group means differed significantly.

RESULTS AND DISCUSSION

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity of exopolysaccharides (EPS) isolated from *E. faecium* strains was evaluated using the DPPH radical scavenging assay. All EPS samples showed a clear concentration-dependent increase in scavenging capacity, with higher concentrations yielding greater inhibition of DPPH radicals (Table 1). At the highest concentration tested (10 mg/mL), EPS from strain DCC1 showed the highest inhibition (40.61 ± 0.01%), followed by EPS DCB3 (40.17 ± 0.01%) and EPS DCC3 (38.56 ± 0.01%). In contrast, at 1 mg/mL, inhibition was low for all EPS (0.8-2.4%), indicating limited radical scavenging at concentrations that are closer to those of typical small-molecule antioxidants. Statistically analysis confirmed that both strain and concentration, as well as their interaction, had significant effects on DPPH inhibition ($p < 0.05$).

The concentration dependent trend observed here is consistent with previous reports that LAB-derived EPS generally display increasing DPPH scavenging as dosage rises, reflecting the need for relatively high polymer concentrations to provide enough reactive sites (Adebayo-Tayo and Fashogbon, 2020). However, the absolute magnitude of scavenging observed for the Dangke-derived EPS is modest. Even at 10 mg/mL, inhibition remains below 41%, which places these samples at the lower end of the activity range reported for LAB EPS in comparable DPPH assays. In contrast, the standard antioxidants Trolox and ascorbic acid exhibited very high scavenging at much lower concentrations (Table 2). Ascorbic acid inhibited DPPH radicals by >99% at 0.1 mg/mL, while Trolox achieved >90% inhibition at the same concentration. These results highlight the far greater potency of small, highly mobile electron donors compared with high-molecular-weight polysaccharides in the DPPH system. Consequently, although EPS from strains DCC1 and DCB3 showed the highest activity among the Dangke isolates, their overall radical-scavenging efficiency remains substantially lower than that of Trolox and ascorbic acid.

Table 1: DPPH Scavenging activity (%) of EPS samples produced by *E. faecium* isolated from Dangke cheese.

Concentration (mg/mL)	Inhibition (%)					
	EPS DCA2	EPS DCA3	EPS DCB3	EPS DCB5	EPS DCC1	EPS DCC3
1	0.81 ± 0.01^{a}	1.03 ± 0.01^{b}	1.23 ± 0.01^{c}	1.62 ± 0.02^{d}	2.04 ± 0.00^{e}	2.43 ± 0.02^{f}
2	4.27 ± 0.01^{g}	4.68 ± 0.02^{h}	5.70 ± 0.00^{i}	5.90 ± 0.00^{j}	6.52 ± 0.01^{k}	7.53 ± 0.02^{l}
4	11.20 ± 0.01^{n}	11.21 ± 0.01^{n}	10.80 ± 0.00^{m}	14.08 ±0.01^{q}	11.82 ± 0.01^{p}	11.62 ± 0.01^{o}
6	19.78 ± 0.02^{v}	19.17 ± 0.01^{u}	18.14 ± 0.03^{r}	21.03 ±0.01^{w}	18.76 ± 0.01^{s}	18.97 ± 0.01^{t}
8	28.34 ± 0.03^{x}	29.58 ± 0.02^{z}	30.38 ± 0.03^{aa}	31.41 ±0.01^{ab}	33.86 ± 0.01^{ac}	28.97 ± 0.00^{y}
10	37.93 ±0.02^{ad}	39.17 ± 0.01^{ag}	40.17 ± 0.01^{ah}	38.34 ±0.03^{ae}	40.61 ± 0.01^{ai}	38.56 ± 0.01^{af}
IC50 (mg/mL)	>10	>10	>10	>10	>10	>10

Data are shown as mean ± SD (n=3). Different superscript indicated a significant difference (Tukey test; $p<0.05$).

Table 2: DPPH Scavenging activity (%) of ascorbic acid and trolox as the reference antioxidants.

Concentration (mg/mL)	Inhibition (%)	
	Ascorbic acid	Trolox
0.01	49.82 ± 0.01^{c}	32.44 ± 0.01^{a}
0.02	62.59 ± 0.00^{e}	44.47 ± 0.00^{b}
0.04	70.27 ± 0.02^{f}	55.91 ± 0.01^{d}
0.06	81.03 ± 0.02^{h}	71.41 ± 0.01^{g}
0.08	89.54 ± 0.03^{j}	83.27 ± 0.02^{i}
0.10	99.64 ± 0.27^{l}	92.85 ± 0.00^{k}
IC50 (mg/mL)	0.010	0.030

Data are shown as mean ± SD (n=3). Different superscript indicated a significant difference (Tukey test; $p<0.05$).

As summarized in Tables 1 and 2, inhibition increased steadily with EPS concentration, whereas ascorbic acid and Trolox achieved very high inhibition (>70%) even at 0.02 mg/mL. The relatively low inhibition of the EPS at 1-10 mg/mL therefore reflects genuinely weak radical-scavenging potency in the DPPH assay rather than high

experimental variability, which was small across triplicate measurements.

When compared with other studies of DPPH activity for LAB derived EPS, the Dangke EPS clearly fall at the lower end of the spectrum. For example, EPS from mixed LAB cultures in fermented coconut beverages have been reported to achieve 42-76% inhibition at concentrations similar to those used here (Adebayo-Tayo *et al*., 2024), and EPS from *Weissella cibaria, Lactobacillus plantarum*, and several dairy isolates often exceed 50% inhibition at 10 mg/mL (Adelekan *et al*., 2020; Adesulu-Dahunsi *et al*., 2018). Research by Vasough *et al*. (2021) showed that EPS extracted from *E. durans* K48, *E. faecium* R114, and *E. faecium* T52 exhibited 38-50% inhibition at 10 mg/mL, while Abdhul *et al*. (2014) reported 63.5% inhibition for EPS from *E. faecium* BDU7 at the same concentration. These comparisons indicate that the EPS from Dangke derived *E. faecium* strains display comparatively weak DPPH scavenging.

Previous work suggests that differences in EPS structure, including monosaccharide composition, molecular weight distribution, branching, and the abundance of electron-donating functional groups, can substantially affect radical-scavenging efficiency (Ahmed *et al*., 2020; Li *et al*., 2019; Liu *et al*., 2015; Zhang *et al*., 2020). In the present study, we did not carry out detailed structural characterization of the EPS, and parameters such as molecular-weight distribution, linkage pattern, and specific functional groups were not determined. As a result, our suggestion that structural features underlie the relatively low DPPH activity should be regarded as hypothesis-generating and based primarily on the broader EPS literature rather than on direct structural evidence from the present samples. Comprehensive structural analysis, including monosaccharide composition, molecular-weight profiling, and more detailed linkage information, will be an essential focus of future work and will be required to establish more definitive mechanistic links between EPS structure and antioxidant function.

IC50 AND TEAC VALUES

IC_{50} is defined as the concentration required to inhibit 50% of DPPH radicals. For all EPS preparations, 50% inhibition was not achieved within the tested range (1-10 mg/mL). Consistent with Table 1, the highest inhibition observed at 10 mg/mL was 38-41% (maximum 40.61%). Therefore, IC_{50} values for the EPS preparations could not be determined by interpolation and are reported conservatively as IC_{50} > 10 mg/mL. This interpretation is supported by Figure 2, which shows a shallow dose–response across 1-10 mg/mL, with curves remaining well below 50% inhibition and clustering closely, indicating only minor between-strain differences under the present assay conditions. In contrast, the reference antioxidants show substantially higher potency, achieving comparable or greater inhibition at much lower concentrations.

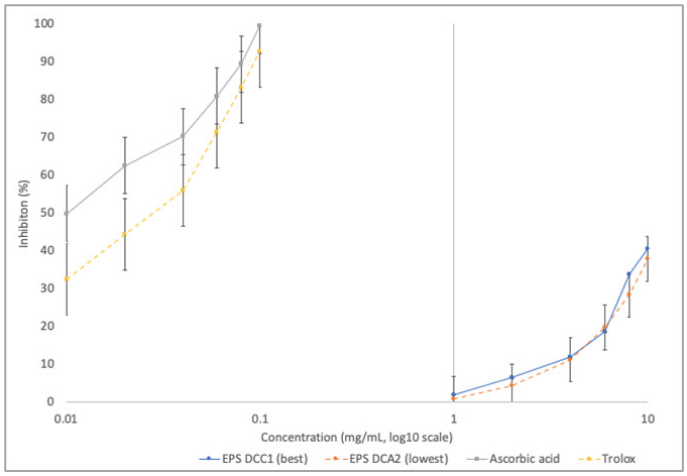

Figure 2: Dose–response curves for DPPH inhibition by EPS from *E. faecium* strains DCC1 (best) and DCA2 (worst) compared with ascorbic acid and Trolox. Concentration is plotted on a log10 scale. Values are mean ± SD (n = 3).

For Trolox, 50% inhibition occurred within the tested range (44.47% at 0.02 mg/mL and 55.91% at 0.04 mg/mL), allowing IC_{50} estimation by linear interpolation (0.03 mg/mL). Because EPS IC_{50} values are reported as >10 mg/mL, TEAC for EPS cannot be reported as a single point estimate and is instead reported as an upper bound: $TEAC(EPS) < IC_{50}(Trolox)/10$, corresponding to $TEAC (EPS) < 3.0 \times 10^{-3}$ mg Trolox/mg EPS under the present assay conditions.

To contextualize this low TEAC, we estimated the EPS level required to match a typical food addition of ascorbic acid (0.01%, w/w = 100 mg/kg). As ascorbic acid showed >70% scavenging at 0.02 mg/mL, its IC_{50} is <0.02 mg/mL and TEAC 1.5 mg Trolox/mg. Thus, 0.01% ascorbic acid corresponds to >150 mg Trolox-equivalents per kg, whereas achieving the same Trolox-equivalent contribution with EPS would require >50 g EPS/kg (>5% w/w), which is likely technologically and economically impractical for most foods. This disparity is typical when comparing small, rapidly diffusing antioxidants with high-molecular-weight polysaccharides in the DPPH assay (Hernández-González *et al*., 2021; Zhang *et al*., 2015), and reinforces that Dangke-derived EPS are weak direct radical scavengers relative to Trolox and ascorbic acid.

CONCLUSIONS

This study establishes a quantitative DPPH benchmark for EPS preparations produced by Dangke-derived *E. faecium*. The EPS exhibited reproducible concentration-dependent

scavenging but low potency compared with Trolox and ascorbic acid; 50% inhibition was not reached at 10 mg/mL, so EPS IC_{50} is conservatively reported as >10 mg/mL and TEAC as an upper bound (< 3×10^{-3} mg Trolox/mg EPS). These findings place the Dangke EPS preparations toward the weak end of LAB-EPS performance in the DPPH assay and support interpreting them as poor direct radical scavengers under these assay conditions. Because EPS structural characterization were not performed, structure-activity explanations cannot be made from the present dataset. Future work should combine compositional characterization with matrix-relevant oxidation models.

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NOVELTY STATEMENT

This study is novel in that it reports and benchmarks the antioxidant activity of exopolysaccharides (EPS) produced by Enterococcus faecium isolated from Dangke, a traditional Indonesian fermented dairy product for which EPS bioactivity has been scarcely documented. It also contributes novelty by using a standardized in vitro antioxidant assay framework with appropriate reference antioxidants, enabling clearer comparison and positioning of Dangke-derived EPS for future functional and structural investigations.

AUTHOR'S CONTRIBUTION

Conceptualization: RWK, RM. Methodology: RWK, RM. Formal analysis: RWK. Validation: RWK, RM, NN, MO, WW. Supervision: RM, NN, MO, NN. Writing original draft preparation: RWK, RM, NN. Writing review and editing: RWK, RM, NN, WW.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI or AI-assisted technologies were used to generate, analyze, or interpret the research data. Generative AI tools were used only to assist in improving the grammar and readability of the manuscript during the revision stage, and all scientific interpretations and conclusions are entirely the authors' own.

Conflict of interest

The authors have declared no conflict of interest.

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