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# Isolation, Phenotypic Characterization and Antagonistic Profiling of Bacteriocin-Producing Lactic Acid Bacteria from Fermented Foods

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## Abstract

Foodborne contamination and antimicrobial resistance have increased interest in naturally derived preservation systems. This study screened fermented and animal-derived food samples for lactic acid bacteria (LAB) with bacteriocin-like activity and evaluated the activity of crude cell-free preparations against selected indicator organisms. Thirty-five morphologically distinct colonies were recovered from curd, dosa batter, raw cow milk, kanji and meat. Eleven isolates were catalase-negative and were retained as presumptive LAB. All 11 isolates showed Gram-positive, oxidase-negative and urease-negative reactions, acid tolerance between pH 2.5 and 6.5, and salt tolerance up to 12% (w/v). Cell-free supernatants from four isolates (IS1, IS2, IS4 and IS6) produced the clearest inhibition against Bacillus subtilis and were examined further by broth microdilution. The lowest recorded MIC values were obtained for IS4 and IS6 against *Salmonella typhi* (0.070 and 0.081 mg/mL, respectively). IS4 also showed the lowest MIC against *Staphylococcus aureus* (0.310 mg/mL), whereas IS1 was most active against *B. subtilis* (0.119 mg/mL). These findings identify fermented foods as useful sources of antagonistic LAB.

**Keywords**: lactic acid bacteria; bacteriocin-like activity; fermented foods; foodborne pathogens; cell-free supernatant; minimum inhibitory concentration.

## 1. Introduction

Foodborne bacteria remain an important challenge for public health, food processing and household food preservation. Chemical preservatives can be effective, but consumer demand for minimally processed and clean-label products has encouraged investigation of biological preservation systems. LAB are particularly attractive because they are established members of fermented-food microbiota and can inhibit competing microorganisms through organic acids, hydrogen peroxide, carbon dioxide and ribosomally synthesised antimicrobial peptides (Cotter et al., 2013; Chikindas et al., 2018).

Bacteriocins are genetically encoded antimicrobial peptides or proteins. Their activity commonly involves interaction with the bacterial envelope, membrane permeabilisation, dissipation of the proton motive force and loss of intracellular metabolites. Activity is often strongest against Gram-positive bacteria, although some LAB preparations can affect Gram-negative organisms when the outer membrane is destabilised or when multiple inhibitory metabolites act together (Alvarez-Sieiro et al., 2016; Pérez-Ramos et al., 2021).

Fermented foods provide a practical reservoir for discovering locally adapted LAB. However, catalase negativity, Gram staining and biochemical reactions are screening-level evidence; they do not establish species identity. In view of the recent reclassification of the former *Lactobacillus* group, reliable identification should use 16S rRNA sequencing and, where required, whole-genome or MALDI-TOF confirmation (Zheng et al., 2020). The present work therefore uses the cautious term “presumptive LAB” for phenotypically screened isolates.

The objective was to isolate presumptive LAB from five food matrices, record their phenotypic and tolerance profiles, screen cell-free supernatants for antagonistic activity, and compare the MIC and IC50 values of crude preparations from the strongest isolates against four indicator organisms.


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## 2. Materials and Methods

### 2.1. Sample collection and isolation

Raw cow milk, curd, dosa batter, kanji and fresh meat were collected locally and processed aseptically. Samples were homogenised where necessary and serially diluted ten-fold. A 0.5-mL aliquot from an appropriate dilution was spread on MRS agar and incubated at 35 °C for 24–48 h under microaerophilic conditions. Colonies differing in size, colour, margin or elevation were purified by repeated streaking and maintained in MRS broth.

### 2.2. Primary screening and phenotypic characterization

Twenty-four-hour cultures were screened by Gram staining and catalase testing with 3% hydrogen peroxide. Oxidase and urease reactions were also recorded. Isolates showing the expected screening profile for LAB were preserved in MRS broth containing 25% (v/v) glycerol at −80 °C. Cell morphology, endospore formation and motility were examined by standard methods. Carbohydrate fermentation was assessed in phenol-red broth containing ribose, lactose, mannose, glucose, fructose, mannitol, sorbitol, L-arabinose, arbutin or citrate. Milk coagulation and sensitivity to trypsin and chymotrypsin were also recorded.

### 2.3. Physiological tolerance tests

Growth was examined at 10, 15, 20, 25, 30, 35, 40 and 45 °C; at pH 2.5, 4.5, 6.5 and 8.5; and in MRS broth containing 2, 4, 6, 8, 12 or 20% (w/v) NaCl. Growth was judged from visible turbidity after incubation under the same conditions used for routine cultivation.

### 2.4. Cell-free supernatants and antagonistic screening

Isolates were grown for 48 h in MRS broth. Cultures were centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was adjusted to pH 6.5 with 1 M NaOH and passed through a 0.22-µm membrane. This treatment reduced the contribution of acidity and removed intact cells; therefore, the observed inhibition is described as bacteriocin-like or proteinaceous activity rather than as purified bacteriocin activity. Indicator lawns were prepared at approximately 0.5 McFarland density. Sterile 2-mm discs loaded with 20–100 µL of supernatant were placed on inoculated agar and incubated at 37 °C for 24 h.

### 2.5. MIC and IC50 determination

Crude preparations from IS1, IS2, IS4 and IS6 were reconstituted at 50 mg/mL and tested by broth microdilution in 96-well plates. The evaluated concentrations were 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039 and 0.020 mg/mL. *Bacillus subtilis*, *Streptococcus mutans*, *Salmonella typhi* and *Staphylococcus aureus* were used as indicator organisms. Optical density at 600 nm was used to estimate growth inhibition. Values reported as NA indicate that an endpoint was not measurable within the tested range. The numerical MIC and IC50 values below are reproduced from the supplied dataset.

## 3. Results

### 3.1. Recovery of candidate LAB

Thirty-five distinct colonies were recovered from the five sample types. Curd and dosa batter yielded the largest numbers of colonies, whereas meat yielded the fewest. Eleven isolates were catalase-negative and were retained for further work. The isolation pattern is shown in Table 1 and Figure 1.

| Food source | Total colonies | Catalase-negative | Retained isolates |
|---|---|---|---|
| Curd | 10 | 4 | 4 |
| Dosa batter | 11 | 3 | 3 |
| Raw cow milk | 6 | 2 | 2 |
| Kanji drink | 5 | 2 | 2 |
| Meat | 3 | 0 | 0 |
| Total | 35 | 11 | 11 |

***Table 1. Distribution of colonies and catalase-negative isolates recovered from the tested food sources.***

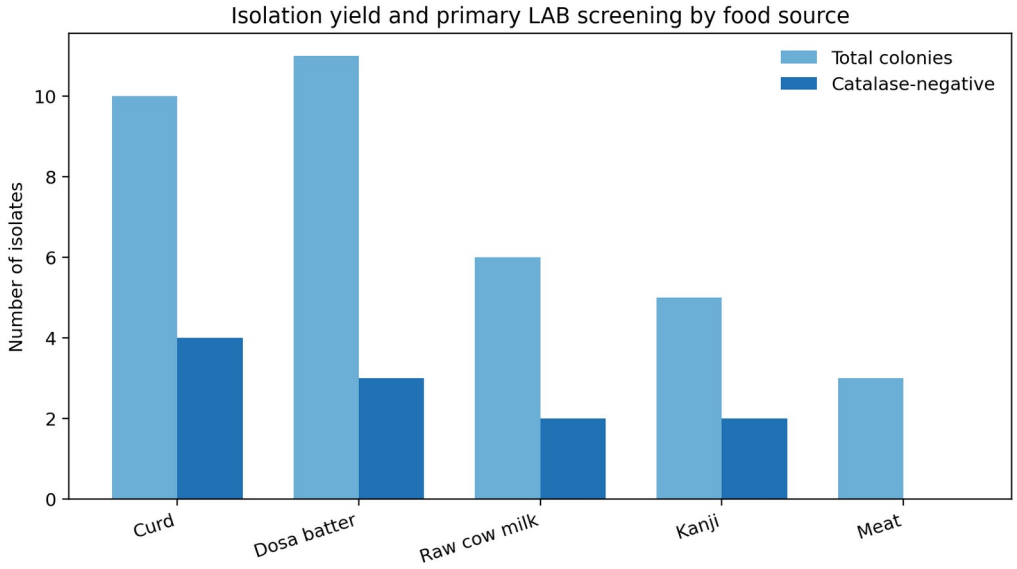



***Figure 1: Isolation yield and primary LAB screening by food source.***

### 3.2. Phenotypic and tolerance profile

All 11 retained isolates were Gram-positive and negative for catalase, oxidase and urease. The supplied observations described the cells as coccoid and non-spore-forming. They fermented glucose, fructose, lactose, mannose and ribose, but not mannitol, sorbitol or L-arabinose. Arbutin conversion and milk coagulation were positive, whereas citrate utilisation was negative. Trypsin and chymotrypsin sensitivity supported the proteinaceous nature of the active inhibitory material. These observations are consistent with LAB-like phenotypes but do not by themselves establish genus or species.

The isolates grew reliably from 20 to 40 °C, with the strongest growth at 30–35 °C. Growth was observed at 15 and 45 °C but not at 10 °C. All isolates tolerated pH 2.5–6.5, whereas pH 8.5 prevented growth. Growth continued in 2–12% NaCl and was inhibited at 20%.

### 3.3. Disc-diffusion screening

The cell-free supernatants differed in activity. IS1, IS2, IS4 and IS6 produced the most distinct zones against B. subtilis and were selected for quantitative testing. IS3, IS5, IS7, IS8 and IS9 produced little or no visible inhibition under the screening conditions.

### 3.4. MIC and IC50 profiles

| Pathogen | MIC IS1 | MIC IS2 | MIC IS4 | MIC IS6 | IC50 IS1 | IC50 IS2 | IC50 IS4 | IC50 IS6 |
|---|---|---|---|---|---|---|---|---|
| *S. typhi* | NA | 0.193 | 0.070 | 0.081 | NA | 0.159 | 0.069 | 0.078 |
| *B. subtilis* | 0.119 | 0.260 | 0.242 | 0.162 | 0.118 | 0.259 | 0.240 | 0.158 |
| *S. mutans* | 0.820 | 0.293 | 0.228 | 0.232 | 0.078 | 0.285 | 0.225 | 0.230 |
| *S. aureus* | NA | 0.573 | 0.310 | 0.383 | NA | 0.390 | 0.306 | 0.338 |

***Table 2. MIC and IC50 values (mg/mL) of crude preparations against the four indicator organisms. NA, no measurable endpoint within the evaluated range.***

IS4 and IS6 were the most active preparations against *S. typhi*, with MIC values of 0.070 and 0.081 mg/mL. Against *B. subtilis*, IS1 showed the lowest MIC (0.119 mg/mL), followed by IS6 (0.162 mg/mL). Against *S. mutans*, IS4 and IS6 showed similar MIC values (0.228 and 0.232 mg/mL), while IS1 was less active (0.820 mg/mL). Against *S. aureus*, IS4 showed the lowest measurable MIC (0.310 mg/mL), followed by IS6 (0.383 mg/mL) and IS2 (0.573 mg/mL). IS1 did not produce a measurable endpoint against *S. typhi* or *S. aureus* within the tested range. Figure 2 provides a visual comparison of the MIC dataset; lower values indicate stronger activity.

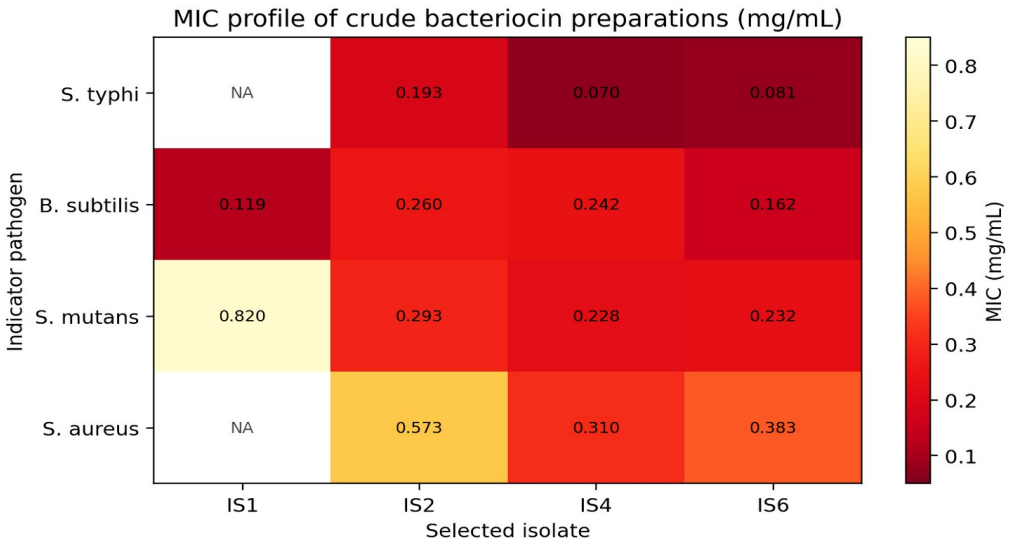



***Figure 2. Heatmap of MIC values for crude preparations. NA indicates no measurable endpoint within the tested concentration range.***

## 4. Discussion

Curd and dosa batter produced the greatest number of catalase-negative isolates in this dataset. This pattern is biologically plausible because both foods undergo lactic fermentation and therefore provide conditions that favour acid-tolerant LAB. The absence of retained isolates from meat should not be interpreted as evidence that meat cannot harbour LAB; it reflects the recovery and screening conditions used here.

The isolates showed a coherent LAB-like screening profile: Gram-positive reaction, catalase negativity, carbohydrate fermentation, acid tolerance and salt tolerance. Nevertheless, phenotypic tests cannot reliably distinguish closely related LAB taxa. The isolates should therefore be described as presumptive LAB until sequence-based identification is completed. This distinction is important because the former broad genus Lactobacillus has been divided into several genera, including Lactiplantibacillus, Lacticaseibacillus and related taxa (Zheng et al., 2020).

Neutralisation of the supernatants before diffusion testing was useful for reducing the direct contribution of organic acids. The remaining inhibition, together with sensitivity to proteolytic enzymes, supports the presence of proteinaceous antimicrobial material. However, neutralisation does not remove all non-peptide factors, and crude supernatants may contain hydrogen peroxide, residual metabolites or synergistic compounds. Purification, protease controls, heat stability testing and activity-unit calculations are therefore needed before the compounds can be classified as defined bacteriocins (Alvarez-Sieiro et al., 2016; Pérez-Ramos et al., 2021).

The strongest activity was observed for IS4 and IS6 against *S. typhi* and for IS4 against S. aureus. Activity against the Gram-negative *S. typhi* is noteworthy because the outer membrane can limit access of many bacteriocins. In a crude preparation, this result may reflect a peptide with membrane-disruptive activity, interaction with other metabolites, or changes in outer-membrane permeability. The result is promising but should be confirmed with purified material and appropriate controls. MIC values should also be interpreted alongside replicate number, growth curves, blank correction and the exact endpoint definition.

## 5. Conclusion

The supplied data show that fermented foods, particularly curd and dosa batter, yielded catalase-negative, acid-tolerant isolates with measurable antagonistic activity. Four preparations (IS1, IS2, IS4 and IS6) were the strongest in the screening assay. IS4 and IS6 showed the lowest MIC values against S. typhi, while IS1 was most active against B. subtilis and IS4 was most active against S. aureus. The findings support further investigation of these isolates as sources of food-compatible antimicrobial compounds. The present study is a phenotypic and crude-extract screening study; molecular identification, purification, structural characterisation, safety assessment and validation in real food matrices are necessary before practical application.

## 6. Limitations and future work

The study did not include 16S rRNA or whole-genome identification, purified peptide preparation, bacteriocin gene detection, replicate-level statistical analysis, or validation in a food matrix. Future work should include sequence-based identification, activity-unit reporting, protease and heat controls, peptide purification and mass spectrometric

characterisation. Stability should be tested across pH, temperature, salt and storage conditions, followed by challenge studies in selected foods.

**Author Contribution**: Each author contribute equally.

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