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SHORT COMMUNICATION

Extracellular antimicrobial activity of thermophilic lactic acid bacteria isolated from traditional fermented milk in Moldova

ANATOLI CARTASEV – RAVI JADEJA

Summary

The aim of this study was to screen autochthonous thermophilic lactic acid bacteria isolates from traditional fermented milk in Moldova for extracellular antimicrobial activity against *Escherichia coli* and *Listeria monocytogenes*. Cell-free supernatants showed reproducible inhibition zones of 6.6–7.3 mm against *E. coli* and 5.5–6.3 mm against *L. monocytogenes* in the agar diffusion assay. Activity persisted after pH neutralisation and changed only slightly after catalase treatment, indicating that organic acids and hydrogen peroxide were not the main inhibitory factors. Protease treatment significantly reduced inhibition to 4.0–4.6 mm for *E. coli* and 3.0–3.5 mm for *L. monocytogenes*, although residual activity remained detectable ($p < 0.05$). These findings support a multifactorial inhibitory mechanism involving a proteinaceous (bacteriocin-like) fraction together with non-protein antimicrobial metabolites. The studied isolates are promising candidates for further purification and chemical characterisation of extracellular inhibitory compounds for food biopreservation.

Keywords

lactic acid bacteria; autochthonous thermophilic isolates; antimicrobial activity; agar diffusion assay; cell-free supernatant; food safety

Interest in clean-label food preservation has intensified due to consumer-driven reduction of synthetic additives and the persistent burden of food-borne pathogens. Lactic acid bacteria (LAB) are established components of fermented foods and can inhibit undesirable microorganisms through the production of organic acids, hydrogen peroxide, diacetyl, cyclic peptides, and bacteriocin-like inhibitory substances. The yoghurt starter species *Lactobacillus delbrueckii* subsp. *bulgaricus* is primarily exploited for rapid acidification and proteolysis, yet several reports indicate that certain strains can suppress pathogenic bacteria via diffusible metabolites and, in some cases, proteinaceous antimicrobials. For example, the antimicrobial effect of *L. bulgaricus* cell-free supernatant has been shown to be strongly pH-dependent, suggesting a prominent contribution of organic acids in many systems [1].

Chemical characterisation of LAB antimicrobial activity commonly proceeds by stepwise discrimination of activity drivers (pH neutralisation to minimise organic-acid effects; catalase treatment to exclude hydrogen peroxide; proteolytic enzymes to probe proteinaceous components), followed by targeted analytical workflows such as high-performance liquid chromatography (HPLC) or ion chromatography for organic acids, enzyme-based colourimetric assays for peroxide, and peptide/protein enrichment with subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) for bacteriocins and bioactive peptides. A bacteriocin-like inhibitory substance produced by *L. bulgaricus* has also been recovered

Anatoli Cartasev, Department Food Technology and Microbiology, National Institute for Applied Research in Agriculture and Veterinary Medicine, Ialoveni str. 100, MD-2070 Chisinau, Moldova.
Ravi Jadeja, Department of Animal Science, Robert M. Kerr Food and Agricultural Products Center, Oklahoma State University, 148 FAPC, Stillwater, Oklahoma 74078, USA.

Corresponding author:
Anatoli Cartasev, e-mail: anatolicartasev@gmail.com

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using resin-based fractionation, underscoring the feasibility of downstream isolation once active strains are identified [2]. Because the antimicrobial activity of lactic acid bacteria is typically multifactorial, stepwise discrimination tests (pH neutralisation, catalase, and protease treatment) are commonly used as a first-line approach to distinguish acid-, peroxide- and protein-associated effects prior to targeted chemical identification [3, 4].

From a food-chemistry perspective, the inhibitory activity of LAB reflects the presence of extracellular, diffusible compounds produced during growth and fermentation [5]. Such compounds represent a chemically heterogeneous mixture that may include organic acids, reactive oxygen species, and proteinaceous bacteriocin-like inhibitors, all of which are relevant to clean-label strategies of food biopreservation [3]. Therefore, even preliminary bioassay screening provides a rational starting point for subsequent physicochemical attribution and identification of active fractions.

In Moldova, local dairy ecosystems represent an underexplored reservoir of autochthonous thermophilic LAB with potential for bioprotective applications. Here, we report a concise screening of Moldovan autochthonous thermophilic lactic acid bacteria isolates for antagonism against *E. coli* and *L. monocytogenes* using a standardised diffusion assay, as a first step toward chemical identification of the active metabolite(s).

MATERIALS AND METHODS

Bacterial strains and cultivation

Autochthonous thermophilic lactic acid bacteria isolates ($n = 3$) were obtained from traditional fermented cow's milk products collected in the Comrat region, Moldova (small-scale household production; 2024) and maintained on de Man, Rogosa and Sharpe (MRS) broth (Neogen, Lansing, Michigan, USA) at 37–45 °C under microaerophilic conditions. Indicator strains were *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 19115 (American Type Culture Collection, Manassas, Virginia, USA), grown in brain heart infusion (BHI) broth (BD Difco, Franklin Lakes, New Jersey, USA) at 37 °C. Based on phenotypic characterisation, including microscopy (rod-shaped cells), Gram staining (Gram-positive), catalase test (catalase-negative), thermophilic growth at 37–45 °C, and dairy origin, the isolates were presumptively assigned to *Lactobacillus* spp. Because molecular identification was not performed within the scope of this short communication, the isolates are referred to as autochthonous thermophilic lactic acid bacteria; species-level confirmation by 16S rRNA gene sequencing is intended in follow-up work.

Preparation of cell-free supernatant

LAB were cultured in MRS broth (10 ml) at 37 °C for 12 h. Cultures were centrifuged at 5 000 ×*g* for 5 min using a Sorvall ST 8R benchtop centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The supernatant was collected and sterile-filtered through 0.2 μm Supor PES syringe filters (Pall, Port Washington, New York, USA) to obtain cell-free supernatant (CFS). The antimicrobial activity of the CFS was assessed by agar diffusion against the indicator strains on BHI agar (BD Difco) [6].

Agar diffusion assay

Agar diffusion was performed using a disk diffusion approach with a soft-agar overlay. The indicator inoculum was prepared from an overnight culture and adjusted to approximately 10^6–10^7 CFU·ml^{-1} by dilution in sterile saline. Molten soft agar (0.7–0.8% agar) prepared with BHI medium (BD Difco) was inoculated with the standardised indicator suspension (final ~10^6–10^7 CFU·ml^{-1}) and overlaid onto the corresponding agar base. Sterile paper disks (6 mm; Whatman Antibiotic Assay Discs, Cytiva, Marlborough, Massachusetts, USA) were placed on the surface and loaded with 10 μl of CFS (0.2 μm-filtered) [7]. Sterile uninoculated MRS broth, processed in the same way (including centrifugation and 0.2 μm filtration), was used as a negative control.

pH neutralisation test

To evaluate whether inhibition is primarily acidification-driven, aliquots of CFS were adjusted to pH 6.5–7.0 using sterile 1 mol·l^{-1} NaOH (Sigma-Aldrich, St. Louis, Missouri, USA), followed by re-testing in the same diffusion assay. Residual activity after neutralisation was compared with the untreated CFS [8].

Catalase sensitivity test

To assess the contribution of hydrogen peroxide, neutralised CFS was treated with catalase (Sigma-Aldrich) at 1 mg·ml^{-1} for 30–60 min at 25–37 °C prior to testing. A decrease or loss of inhibition after catalase treatment was interpreted as evidence of peroxide-mediated activity [9].

Protease sensitivity test

To probe proteinaceous inhibitors, neutralised

CFS was treated with proteinase K at 1 mg·ml^{-1} (Qiagen, Hilden, Germany) for 60 min at 37 °C. The enzyme was inactivated by heat treatment at 80–100 °C for 5–10 min using a water bath (Precision GP 02, Thermo Fisher Scientific) prior to diffusion testing. A marked reduction in inhibition following protease exposure was interpreted as evidence of peptide/proteinaceous bacteriocin-like inhibitory substances (BLIS) [10].

RESULTS AND DISCUSSION

All screened autochthonous thermophilic LAB isolates produced measurable inhibition halos against both indicator strains in the agar diffusion assay using cell-free supernatants, confirming the presence of extracellular diffusible inhibitory factors. No inhibition zones were observed with sterile uninoculated MRS negative-control disks. The observed inhibition was modest but reproducible across quadruplicate measurements, with inhibition zones ranging from 6.6 mm to 7.3 mm against *E. coli* (Tab. 1) and from 5.5 mm to 6.3 mm against *L. monocytogenes* (Tab. 2). Under the applied assay conditions, the mean inhibition was numerically higher against *E. coli* than against *L. monocytogenes*. This pattern may reflect strain-specific differences in the composition and diffusion of inhibitory metabolites in agar, as well as matrix-dependent sensitivity of the indicator organisms.

Although the inhibition zones were moderate, diffusion-based assays are strongly influenced by compound diffusivity, agar composition, and the physicochemical state of the supernatant. Therefore, small differences in zone diameter may still reflect biologically meaningful antagonism, particularly when supported by consistent replication and mechanistic discrimination tests.

For both indicator strains, one-way ANOVA showed significant treatment effects for all isolates ($p < 0.001$). Tukey's honestly significant difference (HSD) test indicated that pH neutralisation did not significantly change inhibitory activity, while catalase treatment produced only limited effects and reached significance only in a few isolate–indicator combinations. In contrast, proteinase K treatment significantly reduced inhibition in all isolates against both *E. coli* and *L. monocytogenes* ($p < 0.05$), although residual activity remained detectable. Residual inhibition after protease treatment suggests that non-protein metabolites may also contribute, including organic acids that remain active locally in agar microenvironments, as well as low-molecular-weight compounds such as phenyl lactic acid, fatty acids, or other bioactive metabolites reported for lactic acid bacteria.

Tab. 1. Inhibition zones of autochthonous thermophilic lactic acid bacteria isolates against *Escherichia coli* in the agar diffusion assay.

Isolate code	Inhibition zone [mm]			
	CFS	CFS neutralised to pH 7.0	Neutralised CFS + catalase	Neutralised CFS + protease K
LB-0224MD	6.60 ± 0.22 ^{a}	6.25 ± 0.31 ^{a}	5.90 ± 0.22 ^{b}	4.60 ± 0.29 ^{c}
LB-0324MD	7.30 ± 0.42 ^{a}	7.25 ± 0.30 ^{a}	6.70 ± 0.36 ^{a}	4.40 ± 0.29 ^{b}
LB-0424MD	6.85 ± 0.31 ^{a}	6.55 ± 0.26 ^{a}	6.00 ± 0.36 ^{b}	3.95 ± 0.24 ^{c}

Values are mean ± standard deviation ($n = 4$). Within each row (same isolate), values followed by different superscript letters differ significantly (one-way ANOVA with Tukey's honestly significant difference post-hoc test, $p < 0.05$).
CFS – cell-free supernatant.

Tab. 2. Inhibition zones of autochthonous thermophilic lactic acid bacteria isolates against *Listeria monocytogenes* in the agar diffusion assay.

Isolate code	Inhibition zone [mm]			
	CFS	CFS neutralised to pH 7.0	Neutralised CFS + catalase	Neutralised CFS + protease K
LB-0224MD	5.50 ± 0.36 ^{a}	5.30 ± 0.29 ^{a}	5.00 ± 0.26 ^{a}	3.50 ± 0.48 ^{b}
LB-0324MD	6.20 ± 0.32 ^{a}	5.90 ± 0.14 ^{a}	5.10 ± 0.12 ^{b}	3.00 ± 0.08 ^{c}
LB-0424MD	6.30 ± 0.50 ^{a}	6.10 ± 0.08 ^{a}	5.75 ± 0.29 ^{a}	3.35 ± 0.30 ^{b}

Values are mean ± standard deviation ($n = 4$). Within each row (same isolate), values followed by different superscript letters differ significantly (one-way ANOVA with Tukey's honestly significant difference post-hoc test, $p < 0.05$).
CFS – cell-free supernatant.

In many studies, neutralisation of cell-free supernatants to near-neutral pH leads to a pronounced loss of inhibitory activity, indicating that organic acids are often the dominant driver of antagonism in diffusion assays [11]. In contrast, the persistence of inhibition after pH neutralisation in the present work resembles reports where inhibition is maintained under neutral conditions and is partly sensitive to proteolytic treatment, consistent with the presence of a proteinaceous fraction alongside other antimicrobial metabolites [12]. Moreover, the residual activity observed after proteinase K treatment supports a mixed (multifactorial) mechanism, as reviewed for lactic acid bacteria antagonism and bacteriocin-like inhibitory substances [13].

These results indicate that the antagonistic effect was not driven primarily by organic acids or hydrogen peroxide [4], but included a substantial proteinaceous fraction consistent with bacteriocin-like inhibitory substances [11]. At the same time, the persistence of residual inhibition after protease treatment supports the contribution of additional non-protein antimicrobial metabolites, such as low-molecular-weight bioactive compounds [12]. Therefore, the studied LAB isolates produce extracellular inhibitory factors with a multifactorial mode of action, and further purification and chemical characterisation are required to define the identity and relative contribution of the active components.

This short communication reports the screening of three autochthonous isolates; therefore, the results should be interpreted as preliminary and require confirmation on a larger isolate set.

The innovativeness of this work lies in the exploration of autochthonous Moldovan thermophilic LAB isolates as a poorly studied source of extracellular antimicrobial compounds. The originality of the study consists in the preliminary discrimination of acid-, peroxide-, and proteinaceous-related inhibitory effects in cell-free supernatants and in the evaluation of their antagonistic activity against relevant foodborne indicator bacteria. These findings contribute to the search for native LAB-based solutions for food biopreservation and safety enhancement.

CONCLUSIONS

The tested autochthonous thermophilic LAB isolates (LB-0224MD, LB-0324MD, and LB-0424MD) showed extracellular inhibitory activity against *E. coli* and *L. monocytogenes*. Activity persisted after CFS neutralisation (pH 7.0) and changed only slightly after catalase treatment, indicating that organic acids and hydrogen peroxide were not the main inhibitory factors. Proteinase K treatment markedly reduced, but did not completely eliminate, inhibition, supporting the presence of a proteinaceous (bacteriocin-like) fraction together with non-protein antimicrobial metabolites. These results indicate a multifactorial inhibitory mechanism and support further purification and identification of the active extracellular compounds.

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