INFLUENCE OF AGAR MEDIA ON THE ANTIBACTERIAL ACTIVITY ASSESSMENT OF *LACTOCOCCUS LACTIS* BY DIFFUSION ASSAY

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ABSTRACT
Successful application of techniques for quantitative or qualitative antibacterial determination (screening and effectiveness) relies not only on the sensitivity of the test-microorganisms, but also on the agar-medium used. The composition of agar media can significantly influence the outcome of diffusion-based antibacterial assays, affecting the interpretation of inhibition zone measurements. The aim of this study was to evaluate the influence of different agar media on the assessment of antibacterial activity of *Lactococcus lactis* strains using a diffusion assay. Four *L. lactis* strains were tested against selected *Bacillus* and *Pseudomonas* indicator strains using Nutrient Agar (NA), Mueller - Hinton Agar (MHA), and Luria - Bertani (LB) agar. The results demonstrated that inhibition zone diameters varied considerably depending on the medium used, with NA generally providing larger inhibition zones compared to MHA and LB. These differences highlight the strong dependence of antibacterial activity assessment on medium composition and emphasize the need for careful selection and standardization of assay conditions. The findings underline that comparisons of antibacterial activity data across studies should consider methodological differences, particularly the type of agar medium employed.
Keywords: culture media, diffusion assay, *Lactococcus lactis*, antibacterial activity, inhibition zones.

1. INTRODUCTION

Lactic acid bacteria (LAB) are among the most important microbial groups in the food industry, particularly *Lactococcus lactis*, a mesophilic LAB widely used as an active acidifier in the production of sour cream, fresh cheese, and brined cheese. It also contributes to the characteristic flavor and aroma of cheeses such as Cheddar and Camembert through diacetyl production by *Lactococcus lactis subsp. lactis* biovar *diacetylactis* [2].

Lactic acid and other metabolites produced by LAB improve organoleptic and textural properties and extend product shelf life. In addition, lactic acid and bacteriocins produced by some *Lactococcus* strains inhibit the growth of spoilage, pathogenic, and phytopathogenic Gram-positive and Gram-negative bacteria. Food spoilage caused by pathogenic or non-pathogenic microorganisms results in substantial food losses and potential health risks [5,8,14].

Pseudomonas fluorescens is a Gram-negative bacterium that causes food spoilage through the production of proteases, lecithinase, and lipases. It can grow over a wide temperature range, including in refrigerated foods [10, 17].

Bacillus species are Gram-positive bacteria that may contaminate products at all stages of storage. Their hydrolytic enzymes can cause rope spoilage in bread and deterioration of dairy products, leading to economic losses [4, 11, 16]. In addition, *Bacillus* spp. are wheat grain endophytes, and their heat-resistant spores can survive baking in the crumb core, representing a major concern as demand for preservative-free bread increases [15].

Excessive use of antibiotics to control food-spoilage and pathogenic bacteria has promoted microbial resistance, creating new risks to human health [9]. Consequently, the search for new

antibacterial compounds, such as bacteriocins, has attracted increasing scientific interest. Various methods are used to evaluate the antimicrobial activity of lactic acid bacteria, mainly through inhibition of pathogenic microorganisms by bacteriocins, organic acids, and hydrogen peroxide [18]. Antibacterial assays for lactic acid bacteria commonly employ agar plate and agar diffusion methods, which are effective for identifying probiotic strains with antimicrobial potential. These techniques assess bacteriocin production and organic acid secretion by measuring clear inhibition zones against indicator pathogens [12].

Dependence on different factors, including media composition and duration of cultivation has an important role and can affect the growth of microorganism, metabolic activity and the diffusion and activity of substances with antibacterial properties [7]. Most studies use standard, nutrient-rich media which provides enabling fast growth of indicator pathogens, and superior diffusion of antimicrobial compounds. The possibility of unspecific reaction between the active substance present in the tested culture and the agar medium should also be considered [3].

The aim of this study was to evaluate the influence of different agar media on the assessment of antibacterial activity of *Lactococcus lactis* strains using a diffusion assay, by analyzing variations in inhibition zone measurements against selected *Bacillus* and *Pseudomonas* indicator strains.

2. MATERIALS AND METHODS

The study included *Lactococcus lactis* CNMN-LB-06, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-07, *Lactococcus lactis* CNMN-LB-09, *Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14 lactic acid bacteria strains stored in National Collection of Non-pathogenic Microorganisms at the Institute of Microbiology and Biotechnology of TUM. LAB strains were maintained in MRS medium, cultivation was carried out at 30±1°C for 24 hours. The inoculum concentration was adjusted to 0.5 McFarland standard.

The antibacterial activities of LAB were tested against microorganisms *Bacillus subtilis* CNMN-BB-06, *Bacillus subtilis* CNMN-BB-09, *Bacillus cereus* var. *fluorescens* CNMN-BB-07, *Pseudomonas fluorescens* CNMN-PsB-02, *Pseudomonas fluorescens* CNMN-PsB-12, using the agar well diffusion method. Test cultures were maintained on nutrient agar (NA) and King B media. Cultivation was carried out at a temperature of 35±1°C for a period of 24-48 hours. The growth media used were: (1) Nutrient Agar (NA), containing (g/L): meat extract 1.0, yeast extract 2.0, peptone 5.0, sodium chloride 5.0, and agar 15.0; (2) Mueller–Hinton Agar (MHA), containing (g/L): meat infusion 2.0, casein hydrolysate 17.5, starch 1.5, and agar 13.0; and (3) Luria–Bertani agar (LB), containing (g/L): casein peptone 10.0, yeast extract 5.0, and sodium chloride 10.0. Test bacterial cultures were inoculated by the pour plate method to obtain a uniform bacterial lawn, and agar wells of 8.0 mm diameter were prepared using a cork borer. To ensure proper diffusion of the LAB suspension into the agar medium, plates were kept at room temperature for 1 h before incubation. Growth inhibition zones were measured after 24 h of incubation [2].

Statistical analysis of data was performed using Microsoft Excel 2016, there were three biological replications for each test.

3. RESULTS

The results obtained on the inhibitory effects demonstrated that *L. lactis* strains showed activity against *Bacillus* and *Pseudomonas* bacteria, being cultivated on the agarized media LB, MH or NA, the inhibition zones varying between 11,2 - 16,0 mm, 11,2 - 19,2 mm and 14,0 - 22,3 mm, respectively, the zones of inhibition being dependent on the strain or tested medium. (Table 1).

Table 1. Antibacterial activity of lactic acid bacteria strains at different culture media against *Bacillus* and *Pseudomonas* strains

Strain	Media	*B. subtilis* CNMN-BB-06	*B. cereus* var. *fluorescens* CNMN-BB-07	*B. subtilis* CNMN-BB-09	*P. fluorescens* CNMN-PsB-02	*P. fluorescens* CNMN-PsB-12
Zone of inhibition (mm)						
L. lactis ssp. *lactis* CNMN-LB-06	LB	12,0±1,1	16,0±1,1	13,3±0,7	15,3±0,7	13,7±0,7
	MHA	11,7±1,7	17,3±0,7	17,3±0,7	15,5±0,6	19,2±0,3
	NA	19,3±0,7	21,0±1,1	21,7±0,7	22,3±0,7	15,7±0,7
L. lactis ssp. *lactis* bv.*diacetylactis* CNMN-LB-07	LB	Ni	12,3±1,7	Ni	11,3±0,7	11,2±0,3
	MHA	11,2±0,3	12,3±1,3	Ni	Ni	12,7±1,3
	NA	18,7±0,7	18,7±0,7	19,3±0,7	22,0±1,1	16,0±1,1
L. lactis ssp. *lactis* CNMN-LB-09	LB	13,3±0,7	11,3±0,7	14,3±0,7	12,2±0,3	14,7±0,7
	MHA	Ni	17,0±1,1	Ni	17,3±0,7	16,3±1,7
	NA	17,3±0,7	18,0±1,1	18,7±0,7	22,0±1,1	17,0±0,0
L. lactis ssp. *lactis* bv.*diacetylactis* CNMN-LB-14	LB	11,7±0,7	11,2±0,3	13,3±1,7	11,2±0,3	14,3±0,7
	MHA	Ni	14,3±0,7	Ni	16,7±0,7	12,7±1,3
	NA	14,0±1,1	14,0±1,1	14,7±1,7	14,0±0,0	15,0±0,0

*Ni – no inhibition

The results showed that the tested *B. subtilis*, *B. cereus* var. *fluorescens* and *P. fluorescens* strains were more sensitive to LAB bioactive compounds when cultivated on Nutrient Agar (NA) medium. The most sensitive strain was *P. fluorescens* CNMN-PsB-02, with inhibition zones ranging from 14.0 to 22.3 mm. Selection of the growth agar medium for antibacterial assay is important, as it directly influences bacterial growth and the measured inhibition effect. As expected, both the appearance and diameter of inhibition halos varied according to the agar medium used. Among the four LAB strains tested, only *L. lactis* ssp. *lactis* CNMN-LB-06 showed activity against all four *Bacillus* and *Pseudomonas* strains, regardless of cultivation medium. *L. lactis* biovar *diacetylactis* CNMN-LB-07 inhibited all bacterial strains on NA medium, with zones up to 22.0 mm. *L. lactis* ssp. *lactis* biovar *diacetylactis* CNMN-LB-14 showed antibacterial activity against tested strains cultivated on LB and NA media, with inhibition zones of 11.2–14.3 mm and 14.0–15.0 mm, respectively. For *L. lactis* ssp. *lactis* CNMN-LB-09, higher antagonistic activity was recorded on the same media, with inhibition zones of 11.3–14.7 mm and 17.0–22.0 mm, respectively. On Mueller–Hinton Agar, *B. subtilis* CNMN-BB-06 and *B. subtilis* CNMN-BB-09 did not show sensitivity to *L. lactis* ssp. *lactis* CNMN-LB-09 and *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-14.

4. DISCUSSION

According to the literature, the antimicrobial activity of exometabolites synthesized by lactic acid bacteria may vary considerably. Differences in culture media directly influence LAB growth, metabolic activity, and antimicrobial effectiveness. However, the inhibition zone is only a qualitative indicator and is also affected by factors such as agar depth, well size, and compound solubility. Analysis of media composition has shown major differences in the content of organic and inorganic compounds among commonly used media [3]. Similar effects of salts and nutrient concentration on inhibition zones were reported by Bhattacharjee et al. for rhubarb stalk extracts tested against pathogens [1].

Analysis of the obtained results demonstrated that studied *L. lactis* CNMN-LB-06, *L. lactis* ssp. *lactis* biovar. *diacetylactis* CNMN-LB-07 and *L. lactis* CNMN-LB-09 possessed capacity to produce antibacterial metabolites active against *B. subtilis*, *B. cereus* var. *fluorescens* and *P. fluorescens* bacteria cultivated on NA, being more efficient for the growth of all tested strains. For testing the antibacterial activity of lactic acid bacteria against Gram-positive and Gram-negative microorganisms, Nutrient agar supports growth and can be used, but in contrast to Mueller-Hinton Agar, is not standardized for diffusion assays. Nutrient agar, Mueller-Hinton and Luria-Bertani are an enriched culture media, are widely used by researchers, supports fast growth of many bacterial species, including *Pseudomonas* and *Bacillus*, but differ through nutrient composition and concentration which influence the growth of microbial strains. High nutrient level in LB, due to peptone and yeast extract, interfere with diffusion of antimicrobial compounds, and are recommended better for maintaining strains before testing, while nutrient level in MHA and NA is more consistent for diffusion of antimicrobial compounds, more preferred for antibacterial assay [6].

5. CONCLUSIONS

The results of this study demonstrate that the composition of culture media significantly influences the assessment of antibacterial activity of *Lactococcus lactis* when evaluated by diffusion assay. The diameter of inhibition zones varied considerably depending on the medium used, confirming that the measured antibacterial effect is strongly dependent on assay conditions.

Among the tested media, Nutrient Agar generally supported the largest inhibition zones, followed by Mueller - Hinton Agar, while Luria - Bertani medium showed lower sensitivity for detecting antibacterial effects. These differences are likely related to variations in nutrient composition and their impact on both microbial growth and diffusion of bioactive compounds.

The findings highlight that inhibition zone measurements should be interpreted with caution, as they reflect not only the intrinsic antibacterial potential of the tested strains but also the properties of the culture medium. Therefore, the selection and standardization of agar media are critical for obtaining reliable and comparable results in antibacterial assays.

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DECLARATIONS

The author confirms that this manuscript is original, has not been published previously, and is not under consideration elsewhere. Author contributed to research, result analysis, and manuscript writing.

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