Original Research Article

Quantification of Antimicrobial Compounds Produced by Lactic Acid Bacteria Isolated from pulses

ABSTRACT:

In this study, 11 LAB isolates were isolated from the pulses (green gram and black gram) were tested for biochemical characteristics (catalase, MR-VP, citrate utilization,nitrate reduction) based on these testsfour isolates were evaluated for their antimicrobial activity against three prominent pathogens: *E. coli*,*Pseudomonas aeruginosa*, and *Aspergillus flavus*under different pH 9 (5.5, 6.5,7.5 and 8.5) and temperature (17^{O}C, 27^{O}C, 40^{O}C). Among thefour isolates, one isolate LB4 exhibited notable antimicrobial efficacyagainst all three pathogens under pH (6.5) 8.1± 0.6, 8.3 ± 0.5, and 6.6 ± 0.8 mm respectively. Optimum antimicrobial activity showed at temperature 27against same pathogens 10± 0.9, 5.0± 0.6, 8.3± 1.2 respectively. LB4 isolate was tested for quantification of key metabolites after three days of incubation, lactic acid, acetic acid, and hydrogen peroxide. Lactic acid (4.6g/l) and acetic acid (4.8g/l) were consistently produced in higher amounts, while hydrogen peroxide production remained relatively low (0.58g/l) by the LB4 isolate.The results indicatedthe intricate relationship between antimicrobial activity, environmental factors, and metabolite production, emphasizing the promising role of this isolate in combating microbial infections under diverse conditions.

Keywords: Lactic acid bacteria, Antimicrobial compounds, Pathogens, Antimicrobial activities.

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I.INTRODUCTION:

To meet billion's of people by 2050, a shift towards protein-rich, nutrient-dense foods is essential. The "Green Revolution" of the 1960s boosted cereal production but led the challenges like obesity and micronutrient malnutrition. Experts now advocate a second "green revolution" emphasizing nutritious foods like lentils and chickpeas to combat these issues(Thavarajah *et al.*, 2019).The Food and Agriculture Organization (FAO,

1994)classification included over 20 pulse species, of which most significant ones are the common bean, mung bean, mungo bean, chickpea, cowpea, lentil, pigeon pea, and pea.Pulses are both protein and fibre-rich food grains, along with substantial sources of essential vitamins and minerals, including iron, zinc, folate, and magnesium.Pulses are high in fiber and have a low glycemic index, making them particularly beneficial to people with diabetes by assisting in maintaining healthy blood glucose and insulin levels (Mudryj *et al.*, 2014).

Lactic acid and its by-products have been categorized as Generally Regarded as Safe (GRAS), when used as a food ingredient, the food industry is particularly interested in the bacteriocins produced by LABs(Altuntas *et al.*, 2010). In the context of food applications, bio-preservation involves employing antagonistic microorganisms or their metabolic products to prevent or eliminate pathogenic microorganisms in food items, thereby improving food safety and extending the shelf life of products(Lin and Pan, 2019). LABrenowned for their non-pathogenic nature, adaptability to industry, gastric resilience, and antimicrobial production, LAB has vital role in the food sector(Shehata *et al.*, 2016).In the past decade, LAB's use as probiotics has surged, accompanied by a search for novel strains with antimicrobial attributes, enhancing food safety (FAO/WHO 2006; (Leyva Salas *et al.*, 2017) ;(Olonisakin *et al.*, 2017). A focus has also emerged on deciphering the compounds behind LAB's antifungal proficiency, identification and quantification that antimicrobialcompounds.LAB's antifungal potency mainly arises from producing organic acids like acetic, lactic, phenyllactic acid, and cyclic dipeptides(Dalié *et al.*, 2010). LAB has a long history of safe application in fermentation processes. However, as bio-preservation agents they are still underutilized despite the aforementioned promising results on meat products(Barcenilla *et al.*, 2022).This positions them as promising preservatives, boasting lower toxicity than conventional agents. The current study aim is to quantification of the antimicrobial compounds production by lactic acid bacteria isolated from food pulses

2. Material and Method:

2.1Collection of seeds and Bacterial cultures:

The pulse seed samples were purchased from Department of Pulse,Centre for Plant Breeding and Genetics, TNAU, Coimbatore-641003. The Pathogenic bacterial cultures (*Escherichia coli*, *P.aeruginosa*)from the department of Microbiology, Tamil Nadu Agricultural University, Coimbatore-641003 and fungal culture (*A.flavus*NRRL3357) was received from

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Escherichia coli, P.aeruginosa
A.flavus

ARS Culture Collection, National Center for Agricultural Utilization Research 1815N.University St. Peoria, IL6160.

2.2 Isolation of lactic acid bacteria:

Lactic acid bacteria Isolated from three different sources (groundnut, green gram and maize) with out any contamination as per method described by (Poornachandra Rao *et al.*, 2015). Ten grams from each sample were surface sterilized with 0.1% sodium hypochlorite and 70% ethanol. Then, fine ground with sterile pestle and mortar, this fine ground powder mixed in 100 mL of de Man Rogosa and Sharpe (MRS) broth incubated for 3-4 daysat room temperature. After 4 days the samples were serially diluted upto 10^8 then spread on (MRS) plates and incubated for two days at 37^OC. After incubation,the plates were observed for colonies exhibiting typical characteristics of lactic acid bacteria (LAB). These colonies were isolated and purified in fresh MRS agar plates.

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2.3Morphological and Physio-biochemical characterization of LAB isolates:

The purified LAB isolates observed for colony morphology, encompassing attributes such as form, size, shape, surface, texture, color, elevation, and margin according to (Kunchala et al., 2016). The phenotypic characterization procedures like Gram staining (Hamed, 2021). Furthermore, the isolates were subjected to biochemical tests, including catalase, MR-VP, citrate utilization and nitrate reduction, were conducted following methods outlined in the 14th edition of Mackie and Ma Cartney. The sugar fermentation patterns of the LAB isolates were tested for various sugars, such as glucose, fructose, arabinose, sucrose, sorbitol and xylose(Ismail *et al.*, 2018). Salt resistance of isolates was tested per (Nath *et al.*, 2021) with modified NaCl levels (4.5%, 6.5%, 8.5% and 10.5%).

3. The influence of temperature and pH on antimicrobial activity of bioactive compounds:

3.1 Cell free supernatant preparation for antimicrobial activity:

As per the method followed by (Liasi *et al.*, 2009) with some changes, the LAB strain *W*grown on MRS (De Man Rogosa Sharpe) with different values from 4.5 to 8.5and incubated for 24 hrs, centrifuged at 6000 rpm for 15mins and the cell free supernatant filter

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through 0.2 micron syringe filter. The indicator strains *E.coli* and *P. aeruginosa* cultured on LB broth for overnight and then swabbed on already prepared NA plates allowed to dry then filled 40 µL supernatant after formation of wells. Similarly followed for antifungal activity against *Aspergillus flavus* NRRL3357 on potato dextrose agar media. Incubated the plates at three different temperatures ($17^{O}C$, $27^{O}C$, $40^{O}C$) and observed for zone of inhibition around the well.

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4 Estimation of antimicrobial compounds of the lactic acid bacteria:

4.1 Quantitative estimation of lactic acid and acetic acid:

As the method described by (Kim *et al.*, 2023). Lactic acid production of four isolates was estimated after three days of inoculation. Transfer the 15 ml of culture broth into 100 ml flasks. Samples were titrated using 0.1M NaOH and 1 ml phenolphthalein indicator (0.5% in 5% alcohol). Titrated acidity, representing lactic and acetic acid (% w/v), was calculated. Notably, 1 ml of 1N NaOH equals 90.08 mg lactic acid and 60.05 mg acetic acid. Titrate acidity was then determined.

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Formula: $= \frac{\text{vol .of NaOH} \times \text{N.of NaOH} \times \text{ME of NaOH}}{\text{vol .of sample}}$

ME = Molar equivalent weight of NaOH is equal to 90.08 mg of Lactic acid productionand 60.08 mg of Acetic acid production.

4.2 Quantitative estimation of hydrogen Peroxide:

According to (Wakil and Osamwonyi, 2012) procedure, the estimation of hydrogen peroxide production of four isolates after three days of inoculation was performed. Broth culture of 15 ml were mixed with 15ml of 0.1M H_2SO_4 for acidity. Titration with 0.1M potassium permanganate ($KMnO_4$) was used to react with hydrogen peroxide, knowing 1.701 mg H_2O_2 equal to 1 ml of 0.1 N $KMnO_4$. The endpoint, marked by sample discoloration, indicated reaction completion. Hydrogen peroxide volume wascalculated from $KMnO_4$ molar equivalent weight.

Formula:

$$= \frac{\text{vol .KMn O}_4 \times \text{N of KMn O}_4 \times \text{ME of KMn O}_4}{\text{Vol .of Sample} \times \text{Vol .of H}_2\text{O}_2}$$

ME = Molar equivalent weight of $KMnO_4$ is equal to 1.701mg of H_2O_2

5. Statistical analysis:

The experiments wereperformed in three replicates, the data analyzedusing Origin Pro software,Tukey test. The obtained p-value was less than 0.05, indicating statistical significance.

6. Results

6.1Morphological and biochemical characterization of LAB isolates:

In this experiment, 40 isolates were purified from pulses (green gram, and black) based on colony morphology. The colony morphology of the isolates appeared as round shaped with elevated, flat and entire colonies and gram-positive rods. Among the 40 isolates, 11 were catalase negative as a presumptive test for LAB isolates. Those catalase negative LAB isolates were to other biochemical tests, among the 11 isolates4 were negative for the MR-VP test and citrate reduction test, 7 were nitrate reduction negative. All 11 isolates showed optimum growth at pH 6.5 and temperature 27^{O}C. 4 isolates resistant to salt concentration at 8.5 % results showed in **Table 1** and 9 isolates fermented glucose, 8 isolates fermented fructose and xylose, 4 isolates fermented arabinose and sucrose 4 isolates fermented sorbitol mentioned in the **Table 2**. Based on all these biochemical tests 4 isolates were selected and observed for their antimicrobial activity under different pH and temperature (17^{O}C, 27^{O}C, 40^{O}C). Among the 4 isolates LB4 showed good antimicrobial activity. Quantification of antimicrobial compound production by LB4 isolate.

6.2 The influence of Temperature and pH on the antimicrobial activity of bioactive compounds:

Isolate LB4 demonstrated optimumantimicrobial effects against pathogenic bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and aflatoxin producing fungal pathogen *Aspergillus flavus*under different temperature and pH. The isolate LB4 showed good antimicrobial activity at a temperature 27°C, with inhibition values of 10.3 ± 0.9, 5.0 ± 0.6, and 8.3 ± 1.2 for *E. coli*, *P. aeruginosa*, and *A. flavus* NRRL3357 respectively. The highest antimicrobial activity was observed at pH 6.5, where the inhibition values were 8.1 ± 0.6, 8.3 ± 0.5, and 6.6 ± 0.8 against the same pathogens.The results showed in **table 3 and 4**

6.4 Quantification of bioactive compounds:

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Throughout the experiment, the production of these antimicrobial compounds by the 4 isolates measured at different time points: 24 hours, 48 hours, and 72 hours. Among the 4 isolates only LB4 isolate showed substantial production of antimicrobial compounds as compared to other 3 isolates. The findings indicated that isolate LB4 produced varying quantities of these metabolites. Specifically, in the span of 24 hours, the strain generated 4.0 g/l of lactic acid and 4.2 g/l of acetic acid, while only producing 0.6 g/l of hydrogen peroxide. Within 48hrs, the quantities of these compounds increased except H_2O_2, lactic acid 4.8 g/l, and acetic acid production reached 5.07 g/l, whereas hydrogen peroxide production remained relatively low at 0.46 g/l respectively. Finally, after 72 hours, the strain continued to produce significant amounts of lactic acid (4.6 g/l) and acetic acid (4.8 g/l), with hydrogen peroxide production slightly increasing to 0.58 g/l. Comparing these results, it becomes evident that LB4 exhibits a substantial production of these secondary metabolites. Lactic acid and acetic acid were consistently produced in higher quantities across all time points, whereas hydrogen peroxide showed levels of production. For further insights, the detailed results of this study were presented in **Table 5,** offering a comprehensive overview of the quantities of these secondary metabolites produced by LB4 at different time intervals.

7. DISCUSSION:

(Yelnetty *et al.*, 2014) isolated LAB from fermented local goat milk were rod chain shape, coccus chain shape and mostly dominated by rods. (Zayed *et al.*, 2022) reported that LAB showed salt resistant at low concentration (1 to 3%) as the salt concentration increases the growth of the isolates was decreased, in contrast the isolates in this current study showed good growth at concentration of (4.5 to 8.5%). (Hamed, 2021) isolated and characterized LAB as probiotic reported the different carbohydrate fermentation of the isolates, in this study isolates showed different carbohydrates fermentation indicated the varied metabolic pathways.

(Amarantini *et al.*, 2021) reported that the LAB strain Pr 4.3L displays more potent inhibitory effects against the indicator bacteria when tested at 30°C andwithin the pH range of 6 to 7. The most significant zone of inhibition was observed when the temperature was set at 30°C and the pH was at 7. Conversely, the smallest inhibitory zone, when the pH was 4. In this current study the antimicrobial activity was not found in extreme pH conditions, the highest antimicrobial activity was found at neutral pH(6.5) against bacterial pathogen *P. aeruginosa*(8.3 ± 0.5) and fungal pathogen *A.flavus*(6.6 ± 0.8) and the temperature at 27^{O}C.

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This present study reveals that the antimicrobial activity of isolates influenced by the pH and incubation temperature. Some authors reported the antimicrobial activity may effected by the method used for assessment.

The author (Mukisa *et al.*, 2017)in theirstudy reveals that besides producing lactic acid, LAB in cereal fermentations may also contribute to production of other flavour compounds notably acetate, ethanol, acetaldehyde, diacetyl, acetone, and acetoin. They reported that the strain *Weisellaconfusa* produced the higher amount of diacetyl compound compared to above mention compounds. Another (Pelyuntha et al., 2020) reported the strain *W. confusa* WM36 produced lactic acid and acetic acid up to 266 mm (2.6 %w/v) and 261mm (1.6%w/v), respectively in their work. Organic acids and 2, 4-Di-tert-butylphenol: Major compounds of *Weissellaconfusa* WM36 cell-free supernatant against growth, survival and virulence of *Salmonella Typhi*. In this present study comparatively the production of lactic acid and acetic acid (4.8 g/l and 5.07 g/l) respectively was more than H_2O_2 (0.46 g/l) by the isolate within 48 hrs of incubation, as time increases the production of organic acids decreased but the production of H_2O_2 (0.58 g/l) increased compared to initial days. This findings indicates the production of organic compounds higher as compared to previous reports (Pelyuntha et al., 2020) .

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8. Conclusion:

LB4 isolated from pulses displayed dynamic production of bioactive compounds over a 72-hour period. Lactic acid and acetic acid were consistently produced in higher amounts compared to hydrogen peroxide across all time points. The antimicrobial potential of these compounds was assessed under varying conditions. Notably, isolate LB4 exhibited good antimicrobial effects against bacterial and fungal pathogens with efficacy varying across temperatures and pH levels. Antimicrobial activity was notable at to neutral pH(6.5) values and temperature 27°C, while the absence of activity was observed at 40°C and extreme pH conditions. These findings emphasize the importance of temperatureand pH in influencing the antimicrobial compounds produced by LB4. The antimicrobial compounds produced by isolate may be use in food industries as a preservatives. The further investigation was needed to study influence of other factor on the production of antimicrobial compounds.

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Table 1: Morphological and biochemical characterization of lactic acid bacteria

Isolates	Colony shape	Gram's staining	Catalase	MR-VP test	Citrate utilization	Nitrate reduction	Osmotic resistance		
							4.5%	6.5%	8.5%
LB1	Round, elevated	+	-	-	-	-	+++	++	++
LB2	Round, elevated	+	-	-	-	-	+++	++	++
LB3	Round, elevated	+	-	-	-	-	+++	++	++
LB4	Round, elevated	+	-	-	-	-	+++	++	++
LB5	Round, convex	+	-	+	+	+	+++	++	++
LB6	Round, convex	+	-	+	+	-	+++	++	-
LB7	Round, entire	+	-	+	+	+	+++	++	-
LB8	Round, entire	+	-	+	+	-	+++	++	-
LB9	Round, entire	+	-	+	+	-	+++	++	-
LB10	Round, flat	+	-	+	+	-	+++	++	-
LB11	Round, flate	+	-	+	+	-	+++	++	-

(-) negative and (+) positive to biochemical tests and gram's staining (+++) optimum growth (++) medium growth in salt concentrations

Table 2 : Carbohydrate fermentation of LAB isolates

Isolates	Carbohydrates fermentation					
	Glucose	Fructose	Arabinose	Sucrose	Sorbitol	Xylose
LB1	+	+	+	+	-	+
LB2	+	+	+	+	+	+
LB3	+	+	+	+	-	+
LB4	+	+	+	+	+	+
LB5	+	+	+	-	-	-
LB6	+	-	-	-	+	-
LB7	+	+	-	-	+	-
LB8	+	-	-	-	-	+
LB9	+	+	-	-	-	+
LB10	-	+	-	-	-	+
LB11	+	+	-	-	-	+

(-) Carbohydrate negative, (+) Carbohydrate fermentation positive.

Table 3:Influence of temperature on antibacterial activity of antimicrobial compounds

Isolates	pathogens	Temperature*		
		17^{O}C	27^{O}C	40^{O}C
LB1	*E.coli*	-	-	-
	P. aeruginosa	-	-	-
	A. flavus NRRL3357	-	3.5 ± 0.6	-
LB2	*E. coli*	-	-	-
	P .aeruginosa	-	-	-
	A. flavus NRRL3357	-	3.3 ± 0.5	-
LB3	*E. coli*	-	-	-
	P. aeruginosa	-	-	-
	A. flavus NRRL3357	-	2.7±0.3	-
LB4	*E. coli*	1.8 ±0.4	10.3± 0.9	-
	P. aeruginosa	3.2±2.4	5.0 ± 0.6	-
	A. flavus NRRL3357	-	8.3 ±1.2	-

*The inhibition zone values were mentioned as Mean and SD

Table 4:Influence of pH on antimicrobial activity of antimicrobial compounds

Isolates	Pathogen	pH*				
		4.5	5.5	6.5	7.5	8.5
LB1	*E.coli*	-	4.1± 1.0	4.4 ± 0.9	-	-
	P. aeruginosa	-	4.0 ± 0.9	2.9 ± 1.0	-	-
	*A.flavus*NRRL3357	-	-	2.8 ± 0.6	-	-
LB2	*E. coli*	-	3 ± 0.8	2.6 ± 0.4	2.5 ±0.3	-
	P .aeruginosa	-	3.3 ± 0.9	3.7 ± 0.2	-	-
	*A.flavus*NRRL3357	-	-	3.2 ± 0.5	-	-
LB3	*E. coli*	-	2.5 ± 0.4	2.1 ± 0.1	-	-
	P. aeruginosa	-	2.3 ± 0.6	3 ± 0.3	-	-
	*A.flavus*NRRL3357	-	-	3.6 ± 0.7	-	-
LB4	*E. coli*	-	6.1 ± 1.0	8.1 ± 0.6	6.5 ± 0.9	-
	P. aeruginosa	-	6.6 ±1.0	8.3 ± 0.5	5.9 ± 1.0	-
	*A.flavus*NRRL3357	-	-	6.6 ± 0.8	4.1 ±1.2	-

*The inhibition zonevalues were mentioned as Mean and SD

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Table 5: Quantification of antimicrobial compounds

Antimicrobial compounds	Quantity of antimicrobial compounds of LB4 isolate*		
	24 h (g/l)	48 h (g/l)	72 h (g/l)
H_2O_2	0.6 ± 2.1	0.46 ±2.6	0.56 ± 2.7
Lactic acid	4 ± 0.0	4.8 ± 0.7	4.6 ± 8.8
Acetic acid	4.2 ± 0.3	5.07 ± 1.3	4.8 ± 6.6

* Quantified antimicrobial compounds values were mentioned as mean and SD

Reference:

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