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Probing of an Appreciable Antimicrobial Compound Producing Lactobacillus Strain from Milk Products of Thanjavur Region, Tamil Nadu and its Enhanced Production

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Lactobacillus is a heterogeneous class of lactic acid strains that synthesize bioactive compounds which contribute many health benefits to our mankind. Focusing this view, different Lactobacillus strains were collected from dairy products and were screened for their bioactive efficiencies against an infant diarrheal bacterial pathogen. 11 morphologically unique Lactobacillus strains were procured from curd, yoghurt and buttermilk purchased from the Thanjavur region, Tamil Nadu, India. While screening on a microtiter plate-based test, YMP3 revealed the best antimicrobial activity against a human pathogenic Vibrio cholerae with 67.8±2.1% inhibition. Further, the most appreciable strain was molecularly identified as Lactobacillus apis YMP3 based on 16S rRNA sequencing methodology. Based on the growth kinetics profile, this strain showed maximum production of antimicrobial compound between 72 to 108hrs of incubation. Furthermore, this strain evidenced the cultural conditions of pH 6.5 and 35°C temperature for the appreciable production of the antimicrobial compound. Based on these overall observations, the research stood as the promising baseline data for the enhanced antimicrobial investigation of this probiotic L. apis YMP3 against many human pathogenic strains and its possibilities for drug development.

Keywords: Bioactivity; Dairy products; Growth kinetics profile; Lactobacillus; Screening.

Microorganism produces numerous metabolites that have been determined to be either useful or harmful to human health[1]. Among these, Lactobacilli are widespread lactic acid bacteria (LAB) and they are found in diverse sources including plants, dairy products, mucosal surface of the human body and other food products[2,3,4]. The distinct characteristics of these species include gram-positive bacteria, thermophilic, homofermentative and non-spore-forming rods. In addition, they are also characterized by the formation of lactic acid during carbohydrate metabolism[5]. According to U.S. Food and Drug Administration (FDA), *Lactobacillus* is considered a "GRAS" status strain and hence can be utilized for various industrial purposes. In recent scenarios, LAB plays it vital role in pharma industries because the probiotic properties of these bacteria help to maintain gastrointestinal tract health by inhibiting penetration of pathogens and also help to promote a good immune system[6].

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The genus *Lactobacillus* can resist highly acidic conditions and it also can fight against virulent pathogens. This genus also produces promising biological compounds including fatty free acids, organic acids, ammonia, hydrogen peroxide, biosurfactant and bacteriocins which are well known as low molecular weight antibacterial peptides[7]. Bacteriocins can inhibit gram-positive and gram-negative pathogens, they utilize their antimicrobial property by an intrusive targeted organism cell wall and inhibit cell wall biosynthesis which resulted in the death of the targeted organism. Food industries were effective in utilizing these kinds of bioactive compounds as bio preservative ingredients in the food system to prevent microbial pathogens. Additionally, these genera reveal better viability in acidic pH medium which effectively ferment milk during fermentation and is frequently utilized as starters/ co-starters in the manufacturing of numerous dairy products[8]. A few studies also demonstrated the antimicrobial efficiency of *Lactobacillus*, a strain of *Lactobacillus fermentum* that has antibacterial activity against specific pathogenic organisms has been identified[9]. The antibacterial properties of *Lactobacillus fermentum* point to its potential application in starter cultures for conventionally fermented foods as a way to increase the cleanliness and safety of food products[10].

Lactobacilli demonstrates a wide range of bioactive substances, this method to develop eco-friendly methods for enhancing human situations as outlined by the UN 2030 Agenda and its Sustainable Development Goals can make use of Lactobacilli and their by-products. By enhancing present methods for food production, safety, and preservation, lactobacilli and their bioactive compounds may play a significant role in ensuring that people all over the world have access to healthy food. Major health issues, such as sexual, reproductive, neonatal, and environmental disorders, can be addressed by bioactive compounds of Lactobacilli in mere future. Accordingly, the focus of this paper will be the isolation of *Lactobacillus* from dairy products and explore its bioactive activity compound for pharmaceutical application against infant diarrheal bacterial pathogens.

MATERIALS AND METHODS

Sampling and isolation of *Lactobacillus* strains

In the current investigation, diverse milk products including curd, yoghurt, and butter were gathered from the local market of the Thanjavur region, Tamil Nadu, India, for the isolation of *Lactobacillus* strains. For isolation, each of the collected samples underwent unique aseptic processing. Using pre-sterilized bacteriological saline water, one gramme of the sample was serially diluted before being spread out on MRS agar (HiMedia, Cat. No. M641) plates. Using the quadrant strike plate technique on freshly made MRS agar plates, morphologically separate colonies were purely grown after 48hrs of incubation at 37°C. The composition of the MRS agar is as follows: 10g proteose peptone, 10g HM peptone B, 5g yeast extract, 20g dextrose, 10g tween 80, 20g ammonium citrate, 5g sodium acetate, 0.1g magnesium sulphate, 0.05g manganese sulphate, 2g dipotassium hydrogen phosphate and 15g agar at final pH (at 25°C) 6.5 ± 0.2. A visual inspection for the distinctive colony morphology followed by the Gram's staining process, the purity of the strains was determined[11]. For later usage, all of the axenic strains were kept in lyophilized conditions.

Infant diarrheal bacterial pathogens for antimicrobial study

Vibrio cholerae, a harmful bacterium that causes newborn diarrhoea, was kindly donated by Thanjavur Medical College in Thanjavur, Tamil Nadu. For thorough antibacterial testing, this pathogenic strain was kept under lyophilized conditions. Tryptone soya broth (HiMedia, Cat. No. LQ508) was used to culture the pathogen and the OD of the culture was standardized at 10^8 CFU/ml inoculum concentration during the antimicrobial study. The composition of the Tryptone soya broth was as follows: 17g Tryptone, 3g Soya peptone, 5g Sodium chloride, 2.5g dextrose (Glucose) and 2.5g Dipotassium hydrogen phosphate at final pH (at 25°C) 7.3±0.2. The isolated *Lactobacillus* strains were tested for their significant antibacterial efficacy against this indicator pathogen.

Screening of extracellular or intracellular antimicrobial compound production

In 30ml screw-cap tubes with a 10ml working volume of MRS broth, each axenic

Lactobacillus strain was grown separately (HiMedia, Cat. No. M369). After 96hrs incubation, each cultured broth was spun down separately for 15min. at 3000 rpm to separate the cell pellet and supernatant. With an ultrasonicator, the cell pellet was suspended in 50ml of phosphate buffer at pH 7 and sonicated for 45 sec. at 20 KHz (Hielscher, USA) whereas the supernatant was immediately applied for the antimicrobial investigation. Further, the sonicated cell debris was removed utilizing the centrifugal conditions as indicated before. This study provides evidence for the presence of external antimicrobial molecules, intracellular antimicrobial compounds, or both in the test bacterial strains.

Antimicrobial assay

By measuring the isolated *Lactobacillus* strains' percentage of antibacterial activity against the study's indicator pathogen, *Vibrio cholerae*, bioactive compound synthesis was calculated. The pathogenic strain was cultivated in tryptic soy broth with the optical density of this broth culture adjusted to 0.1, which is standardized to 10^8cfu/ml inoculum concentration as per McFarland turbidity 0.5 standards). Using a microtiter plate-based test, the antibacterial activity was evaluated[12]. The test was carried out in 96-well polystyrene microtiter plates with flat bottoms and covers (Tarsons, India).

125ml of sterile, double-strength tryptone soy broth and 125ml of test sample solution were added to the column of the well plates. The effectiveness of each test sample well's antimicrobial defences was evaluated separately.

Table 1. Antimicrobial activities of axenic bacteria isolated from different milk products

Strains Voucher No.	Antimicrobial activities Intracellular	Extracellular
CMP1	-	-
CMP2	-	41.7±1.3%
CMP3	-	-
CMP4	32.3±0.9%	-
YMP1	-	-
YMP2	-	-
YMP3	-	67.8±2.1%
YMP4	-	-
BMP1	-	-
BMP2	-	-
BMP3	-	-

Additionally, a set of wells had no antimicrobial test samples added to them, serving as growth controls; a set of wells had known antibiotic Chloramphenicol (200mg/L) added to them, serving as a positive control; and a set of wells had neither an inoculum nor any antimicrobial test samples or antibiotic compounds added to them, serving as a negative control. The well plates were injected with 2.5 ml of prepared test pathogenic bacterium, except for the negative control wells. The well plate was incubated at 37°C for two days. After incubation, the evaluations were performed in triplicate and calculated with a microplate reader (Biotek Elx808, WI, USA) to measure the well broth's absorbance at 600nm for each well. The following calculation was used to evaluate the percentage growth inhibition of the tested strain

$$\text{Percentage growth inhibition} = [(1 - (A_b/A_d)] \times 100$$

A_b = Absorbance of well plates with test samples
A_d = Absorbance of growth control well.

Molecular identification of potential strain

The *Lactobacillus* strain used for the molecular identification investigation has the strongest antibacterial activity. The Roche Kit (Germany) was used to collect a 2ml bacterial culture at the mid-exponential growth phase, and DNA was extracted using this culture. A universal set of the Eubac primers includes the following: 1492R - 52 - TACG GYTA CCTT GTTA CGAC TT and 27F - 52 - AGAG TTTG ATCM TGGC TCAG -32 . Using a 5µl reaction mixer, the PCR was carried out on an Eppendorf thermal cycler with the 5µl of 10x amplification buffer, 5µl of 1.5 mM $MgCl_2$, 1µl of each forward and reverse primer, 1µl of dNTP as well as 0.25µl of Taq polymerase. During the amplification procedure, 35 cycles of 35s at 94°C, the 40s at 55°C, 2min at 72°C, and finally an 8-minute extension at 72°C were used. Further, 1.2% agarose gel was used for the electrophoresis analysis of the PCR results (Genei). Using a Qiagen PCR purification kit, the PCR product was purified before being sequenced with ABI Prism 377 automated sequencer (Applied Biosystems, CA, USA). The tree topologies of the potential strain were performed with bootstrap analyses using a thousand replicates, the evolutionary distances were evaluated based

on the maximum neighbour-joining method[13] as well as phylogenetic trees were inferred with the neighbour-joining technique. The evolutionary analysis was done with MEGA X software[14].

Growth kinetics profile of the potential bacterium on antimicrobial compound production

The selected strain's best period for producing the most bioactive compounds about the development of cell biomass at regular intervals of 12hrs from 0 to 144hrs was investigated. The standardisation procedure was carried out in a 1000ml conical flask using 400ml of MRS broth as the base fermentation medium and 37°C and pH 7 as the culture conditions. The inoculum was made utilising prospective strains in their exponential growth phase in the same basal fermentation medium conditions, and its OD620nm was standardized to 0.1 following McFarland turbidity standards of 0.5 and is equal to 1×10^8cfu/ml of bacterial cell concentration.

A part of cultured broth was used to monitor the assessments, and the cell biomass and supernatant were splited with centrifugation at 3000rpm for 15min. The dry weight of cell biomass resulted from centrifugation that was hot air oven dried for 30min at 60% which was used to quantify bacterial cell growth, and the generation of bioactive compounds was directly estimated from the cell-free supernatant. Additionally, the microtiter plate-based test was used to calculate the bioactive compound's activity against the indicator pathogen *Vibrio cholerae* as reported in the screening trials. The data were highlighted as mean ± standard deviation after triplicate evaluation.

Recapitulation of pH and temperature

Optimizing parameters like pH and temperature conditions play a vital role in fermentation progress for enhanced bioactive molecule production. The potential strain was standardized for maximum bioactive compound production by adopting search techniques varying one parameter at a time. The standardized parameters were fixed for the subsequent evaluations. The effect of different pH conditions between pH 6 to 10 was estimated for the effective medium optimization, similarly, various temperature conditions from 20 to 50°C were applied to the production medium for the evaluation of enhanced production.

RESULT AND DISCUSSION

Lactobacillus isolated from naturally fermented milk products has displayed numerous applications and it has a long history[15]. In this study, different dairy products such as curd (CMP), yoghurt (YMP) and butter Milk (BMP) were used to isolate bacteria with their efficiency in bioactivity against infant diarrheal bacterial pathogens. During the isolation process through serial dilution on the MRS agar plate, 11 distinct bacterial isolates were isolated of which 4 were isolated from curd and the culture was initially labelled as CMP1, CMP2, CMP3 and CMP4. Followed by 4 isolates from yoghurt (YMP1, YMP2, YMP3 and YMP4) and 3 isolates from buttermilk including BMP1,

Fig. 1. Pure culture of potential antimicrobial compound producing *L. apis* YMP3

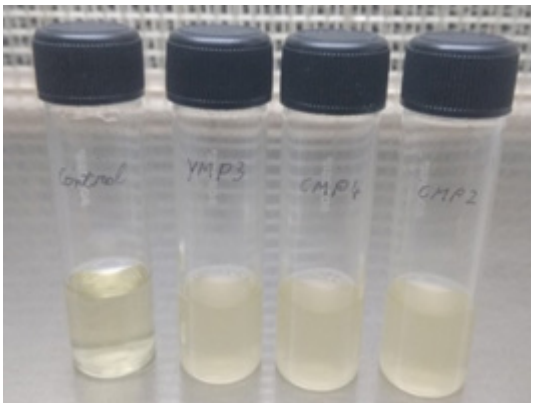

Fig. 2. Pure cultured *Lactobacillus* strains cultured in 30ml screw cap tube containing MRS broth for the screening of antimicrobial compound

BMP2 and BMP3. Using distinctive morphological features under visual inspection and the Gram's staining process, the purity of the strains was determined and axenic strains were maintained. Further preliminary analysis for antimicrobial activity was tested against infant diarrheal bacterial pathogens. All the bacterial broth of 11 axenic bacterial strains were subjected to antimicrobial screening and this confirmed selected bacterial strains which produced extracellular or intracellular antimicrobial compounds (Table 1). Abdelgadir et al.[16] isolated *Lactobacillus fermentum* from the surface of traditional fermented dairy products. Aroutcheva et al.[17] and Park and Oh[18] stated that these kinds of *Lactobacilli* strains were predominant in the human intestine and clinical studies on *L. fermentum* RC-14 proved that this strain shows a significant role in the microflora regulation in the human intestine[19] and this reported to a dominant *Lactobacilli* in the vaginal tract and human intestine[17].

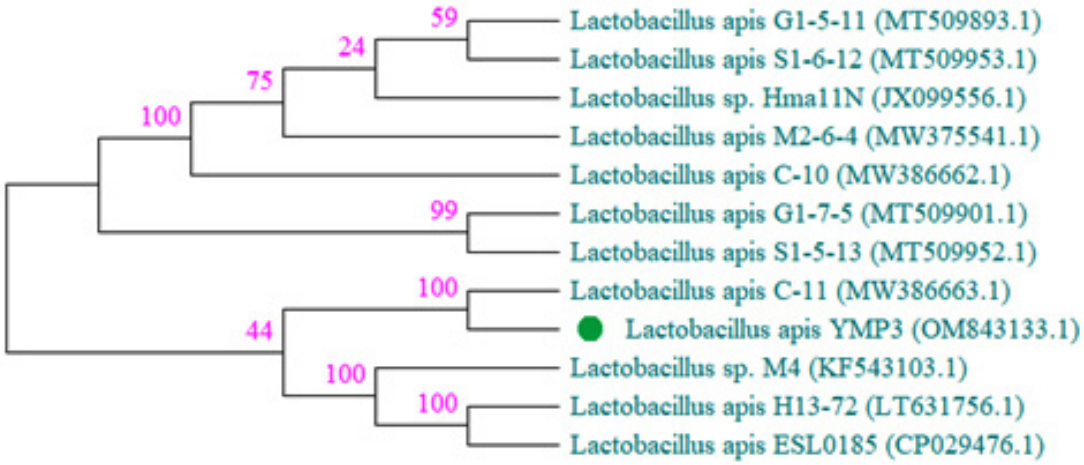

Fig. 3. The evolutionary history of *L. apis* YMP3 using the Neighbor-Joining method

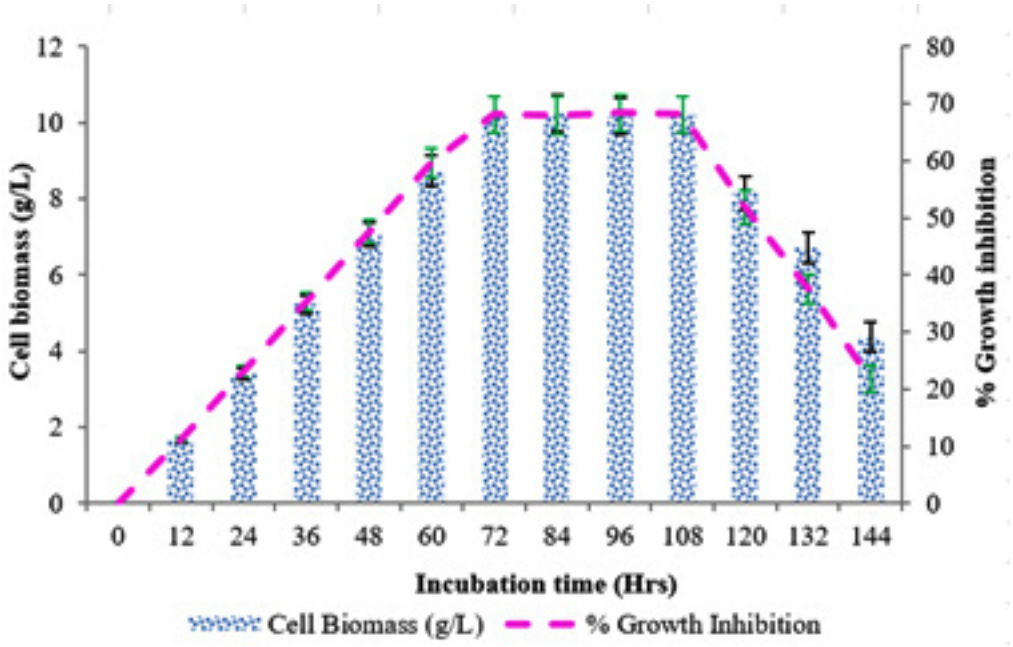

Fig. 4. Growth kinetics profile as a function of time on bioactive compound production using *L. apis* YMP3

Lactobacilli against various clinical pathogens are noted to be a promising role in the health-promoting properties[20]. In this investigation, the antimicrobial activity results showed that the maximum inhibition activity of infant diarrheal bacterial pathogen was YMP3 with 67.8±2.1% inhibition activity and this observation was recorded at the extracellular site. Similarly, the extracellular bioactive compound of CMP2 showed 41.7±1.3% inhibition activity. CMP4 showed 32.3±0.9% inhibition activity and this was at the intracellular site (Table 1). Thus, the pure stock culture of YMP3 was maintained on agar (Fig. 1) and axenic strains broth was also maintained in strew cap bottles (Fig. 3). There are few studies which highlighted bioactive compounds of *Lactobacilli* bacteria towards its efficiency on antimicrobial activity. Owusu-Kwarteng et al.[21] emphasized that bacteria

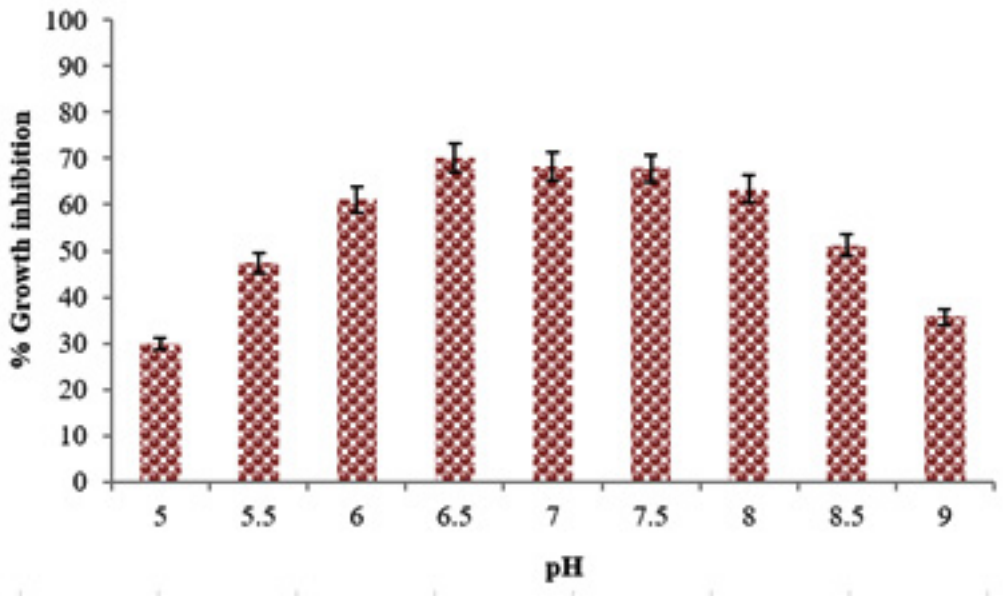

Fig. 5. Effect of various pH on the bioactive compound production using *L. apis* YMP3

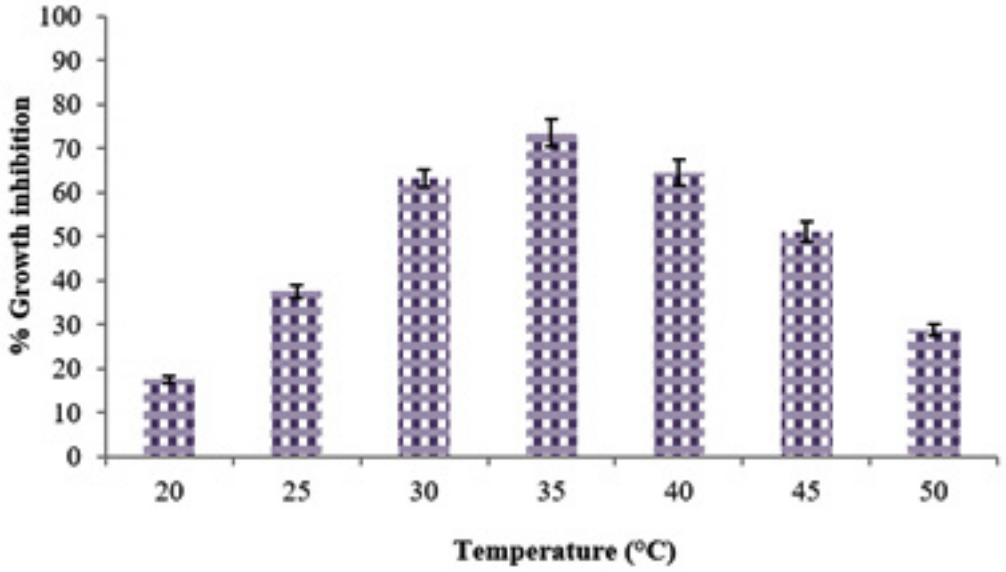

Fig. 6. Effect of different temperatures on bioactive compound production using *L. apis* YMP3

isolated from fermented milk products were usually noted as safe because they can withstand low pH and they can produce antimicrobial substances. Mathara et al.[22] reported the influence of *Lactobacillus* in the controlling proliferation of bacterial pathogens in fermented milk products. In addition, these bacteria are reported to produce a wide range of active compounds including lactic and acetic acid, hydrogen peroxide, bacteriocins, protein, fatty acids, phenyl lactic acid, retrocyclin and reuterin[23]. In correlation to the present findings, fermented food products showed maximum antagonistic activity in *L. plantarum* strains toward clinical pathogens[24, 25]. Few *in vitro* studies of antimicrobial activity against *C. difficile*, *E. coli*, *Shigella* spp., *S. mutans, S. aureus* and *P. aeruginosa* pathogen was also exhibited good results using *Lactobacillus* strains[26, 27].

Based on the screening of antimicrobial activity in the present study, one promising bacterial isolate YMP3 was selected for further identification. Identification was done by molecular profiling using the 16s rRNA gene sequencing method. The molecular characterization revealed that selected bacterial isolates were identified as *Lactobacillus apis* and they were named *Lactobacillus apis* YMP3. The phylogenetic tree of *Lactobacillus apis* YMP3 is illustrated using the neighbour-joining method (Fig. 3). The Neighbor-Joining approach was used to infer the evolutionary history[13]. In the bootstrap test (1000 repetitions), the proportion of duplicate trees that linked taxa clustered together is displayed next to the branches[28]. The evolutionary distances were measured using the Maximum Composite Likelihood technique[29]. Twelve nucleotide sequences were subject to this investigation. For each sequence pair, all unclear places were eliminated (pairwise deletion option). The final dataset contained 1464 locations altogether. In MEGA X, evolutionary analyses were performed[14]. Based on FAO and WHO guidelines, microbial identification through 16SrRNA can be recorded as a more appropriate method than other molecular characterization[30]. According to Pogacic et al.[31] and Tulini et al.[32] 16S rRNA analysis proved as an effective method to identify bacteria which produce lactic and acetic acid; which was identified from fermented dairy products. Previous studies have supported the use of the current methodology as a practical, affordable, and appropriate method for separating *Lactobacillus* bacteria from conventional milk products.

In the present study, the growth kinetic profile of *L. apis* YMP3 was done against the growth inhibition percentage of the selected pathogen. The result elaborated that 72 to 108hrs of incubation time showed maximum growth of *L. apis* YMP3 and inhibition activity of selected pathogen. From the beginning of the lag phase's incubation period until the completion of the decrease growth phase under investigation, evidence of both bacterial growth and pathogen inhibition activities was seen (Fig. 4). Nearly 60 to 70% of selected pathogen growth was inhibited at 72 to 108hrs. of incubation time. In another study, Arques et al.[33] showed that 72hr incubation, the number of *Staphylococcus aureus* falls to 0.46 log cfu/ml. The maximum amount of active compound production was seen after the late-log phase, according to growth and bioactive compound production profiles. During the stationary period, the level of production remained constant; Ivanova et al. [34] obtained similar outcomes.

The influence of different pH ranges from 5 to 9 was tested against the growth inhibition percentage of the selected pathogen using a crude bioactive compound from *L. apis* YMP3. The result illustrates that pH 6.5 showed a maximum of 70% of inhibition of the selected pathogen and this was followed by pH 7 and 7.5. Based on the result, it is demonstrated that both alkaline and acidic pH doesn't favour maximum inhibition of selected pathogen using crude bioactive compound from *L. apis* YMP3. Similarly, the effect of different temperatures ranging from 20 to 50°C was tested against the growth inhibition percentage of the selected pathogen using a crude bioactive compound from *L. apis* YMP3. At 35°C, 70% growth inhibition was observed as the maximum reduced growth. A notable observation was also recorded at 30 and 40°C and the least inhibition was recorded at 20°C. Increased and declined temperature doesn't enhance the growth inhibition of selected pathogens. Vignolo et al.[35] demonstrated that a temperature of 25ÚC favoured maximum bioactive compound lactation production by *Lactobacillus casei*. According to the study, particularly low temperatures were unfavourable for growth-enhanced production. The isolated *Lactobacillus fermentum* M1 demonstrated

antimicrobial activity in a different investigation against E. coli ATCC 25922, which displayed the highest growth inhibitor percentage possible at a temperature of 60ÚC.

CONCLUSION

This study hypothesises that probiotic lactic acid bacteria with notable bioactivities may be isolated from conventional dairy products. The study also suggests that the isolated bacterium, *L. apis* YMP3 exhibited excellent antimicrobial activity against the clinical pathogen, *V. cholerae*. Further, the potential strain evidenced a good response regarding the enhanced production of its antimicrobial compound from the growth optimization studies. Considering all together, this strain can be used for large-scale production which also enhances the possibilities for its purification and structural characterization of the effective antibacterial substance and extensive studies have to be achieved for drug development.

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Conflict of Interest

The authors declare that they have no conflict of interest on publication of this article.

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