RESEARCH ARTICLE

Formulation and Evaluation of Probiotics Properties of *Lactobacillus* with Antimicrobial Activities

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ABSTRACT

This study emphasizes exhibiting probiotics activities of *Lactobacillus*, formulation as granules, synthesizing nanoparticle by using *Lactobacillus* and demonstrating antimicrobial activity. Probiotics are not only microbes present in milk but also as are functional food to heal diseases. Four different isolates were obtained from sheep milk. These microbe's growth profile was observed in different animal milk, and some biochemical studies were conducted. All four isolates were tested against gram positive and negative microorganisms like *Salmonella typhi, Escherichia coli, Proteus vulgaris, Enterococcus faecalisis, Staphylococcus aureus* etc. The nanoparticles of TiO_2 was developed by using LAB via green synthesis. Granules of probiotics were prepared by using maltodextrin as agent by a lyophilization drying technique. The flow properties were studied for the prepared granule as formulation. The colony forming unit observed in case milk of buffalo was higher ($12^* \times 10^9$ CFU ± 2.5) which was observed in the other milking animals milk. The four isolates were identified as *Lactobacillus* (LAB) by Gram staining, motility catalase activity, along with other biochemical assays. The mimicking *in-vitro* simulation study showed higher survival rate in the intestine, demonstrated by bile salt studies. Microbes as a whole showed a good zone of inhibition as compared to the supernatant. Finally, these isolates have proven to be a good probiotic candidate that can be used as a natural antibiotic agent as a nutraceutical nominee. Granules exhibit good flow properties in case of all LAB formulations. The green synthesis of nanoparticles from LAB was observed at the absorption band at 405 to 410 nm using the UV method. LAB extracted from sheep milk was proven in the current investigation exhibiting as a potential candidate for the future probiotic antibacterial agent that resists GI environment.

Keywords: Formulation, Evaluation, Probiotics, *Lactobacillus*, Nanoparticle, Functional food, Antimicrobial

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INTRODUCTION

The developing countries after the COVID-19 outbreak are working on the development of some prophylactic agents. These may be vitamin tablets or some nutraceuticals. Nutraceuticals have gained the market due to their transmission-reducing effect on gastroenteritis infectious diseases.[1] This motivated the discovery of new microbes. Since ancient times microbes are used as part of food in diet due to its health benefits. This diet conferred some health benefits. These are like functional food helps even in healing many diseases.[2,3] Even they diminish the adverse effects of medications. These bacteria are found in the intestine of human, but they are inoculated in the host in dosage form to boost immunity.[4,5] milk and some plant extracts major source of probiotic, especially *Lactobacillus*.[2,6-8] Probiotics are nothing but are a group of good microbes that may help indirectly to fight intestinal pathogens. Probiotic bacteria produce chemicals such as bacteriocins, and reuterin that act as good antimicrobial agents. These chemicals can act as a new functional nature antimicrobial agent against many pathogenic strains exhibiting some health issues. The chemicals released by the microbes are in the form of some organic acids or polypeptides. The bacteriostatic effects are exhibited by organic acids released from bacteria which lower the pH of the microenvironment in host; damaging pathogenic DNA. Further, Lactobacillus release the chemicals like acidophilin, acidolin, lactocidin, and bulgarican.[9-13] These

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chemicals also act as good agents preventing pathogenic strains' growth to GIT.

The probiotics growth depends upon prebiotics and their different types, which act as growth media. Milk is the natural media that comes with growth factors necessary for probiotics. The study has shown that sheep milk contains a microbiome like *Lactobacillus.* These probiotics have shown the best growth in buffalo's milk as they contain ferrous ions with vitamins like B12, etc.[14] The functional properties of probiotics are due to the chemical content like organic acids and organism-specific mediators. These chemicals are difficult to isolate and synthesize at high concentrations. These microbes, if used as the part of formulation or formulated as dosage form, can be safe and confers health benefits.[7,15] The particular strains of *L. bulgaricus, L. brevis, and L. fermentum obtained* from various sources of natural origin, like milk and plant extract have been extensively studied and are in great demand in the market. Thus, the discovery of new strains exhibits various commercial importance that claims as nutraceutical food due to its pharmacological activities. Nanoparticles prepared by green synthesis are important in exhibiting biological activities like antimicrobial or biodegradation of sewage water. Green synthesis of silver nanoparticles is found as a good antimicrobial agent due to its biomolecule-nanoparticle organizations.[16]

Currently, the market also lacks new antimicrobial agents due to antibiotic resistance. These agents comes with many side effects. These probiotics as microbes, releases various chemical which shows antimicrobial properties. Thus, instead of extracting these chemical mediators, the microbes act as new antimicrobial agents based upon the various lab and animal studies.

MATERIALS AND METHODS

Bacterial Strains

The milk obtained from sheep milk was randomly screened for various cultures using the selective media. By examining their physical traits and using a number of biochemical assays, bacteria were recognized.[17] The four distinct lactic acid bacteria (LAB) that were separated from sheep milk were raised on MRS medium and repeatedly sub-cultured to produce pure strains. These microbes were incubated at 37ºC for a period of 40 hours in a micro-aerophilic jar. Following the above, selected zones were moved into agar medium at 4ºC pH adjusted to 7 before further other biochemical investigations such as sugar fermentation, organic acid concentration, pH determination, NaCl and bile tolerance.[18]

Sugar Fermentation Test

In a test tube, media was added to with phenol red (0.01 g per L) at pH 6.5. The prepared medium was autoclaved for 20 minutes at 121°C. The medium was subsequently inoculated with 1-mL of various sugar solutions that had been filtered and sterilised before the operation. A 500 µL overnight bacterial culture was also incubated anaerobically at 37°C for 20 hours. The test culture is observed for broth acid production, which is verified by a change in the color to yellow. The confirmatory test is carried out in Durham tubes to observe gas production due to the fermentation process by the microbes.[18]

NaCl Tolerance Test

To determine the NaCl tolerance, the MRS broth with the probiotics microbes test was introduced in the 10 test tube with different concentrations of NaCl (1-10%). A new overnight culture of LAB with 1% was added to these sterilized tubes. This culture was incubated for overnight. After one whole day of incubation, the microbes' growth was observed through medium turbidity. Bromecresol purple was used as the indicator in the tubes to get confirmatory results. The results were verified by the change in color as per reported. The cell growth was estimated by color code as:[19]

dark yellow = very turbid growth,
yellow = turbid growth,
faint yellow = slight turbid growth,
no change in color = zero growth with clear solution[20]

Calculation of pH Value and Measurement of Organic Acid

Buffalo milk that had been sterilized was diluted by 10% and added to the broth culture. The initial pH maintained by using pH meter was 6.71. As said above, the sample estimation was carried out at each interval time period. These samples were analysed with titration with 0.1 N NaOH in aseptic conditions. All the readings were recorded by using the digital electrode pH meter.[21]

Bile Tolerance Activity

To mimic the intestinal condition, the bile tolerance study was carried out using all four LAB strains. To carry out this study, the bile salts aliquots were added for 24 hours at 37ºC in active cultures of LAB grown in MRS broth. The procedure was carried out by adjusting the pH to 4.5 in the sterile 1 normality solution of NaOH and HCl solutions. To complete the procedure, concentrated bile solution extracted from the powdered bile was extracted at sterilized condition using 5 µ filter paper. The concentration range used for the test was from 1.0% to 1.5% compared with 0.0% culture considered a control. The simulated study mimicked the intestine transit period of three hour of food in gastrointestinal tract. Before administering the bile solution, the sample was analyzed every hour for the following three hours. The experiment was analysed in triplicate for accuracy.[22,23]

Lyophilisation and Flow Properties

The four selected bacteria were inoculated in MRS at 37°C for 20 hours with MRS broth (Sifin Pharma, Germany). These cells were cultivated in a microaerophilic environment with UHT skim milk in order to produce the microbial load. A semi-solid mass was generated after all the cells were cultivated at 37°C for 21 hours till early stationary phase. The resulting semi-solid masses were homogenised in a REMI homogenizer at a speed of 5500 rpm for 10 minutes at 4°C. The combinations of excipients maltodextrin and cell suspensions were frozen. The granules generated from the LAB strains A,B,C and D were

considered as formulation F1, F2, F3 and F4, respectively and their individual flow properties are studied.[5,23]

Bulk and Tapped Densities

Bulk and tapped density are categories of density that were identified. A suitable quantity of granules was weighted and added to a 100 mL measuring cylinder, after which the original volume was noted which is observed as shown in Table 1 and Table 2 as flow properties character. After then, until there was no longer any change in the volume, the apparatus tapped. Bulk and tapped density were estimated from the equation below[5] BD = granules weight/packing volume, TD= granules weight/ tapped volume.[5]

Carr's Index (CI)

Granules' CI was calculated to determine their bulk and tapped densities. The results of a comparison between the values of CI are shown in Table 1

$$CI = [(TD - BD) * 100]/TD$$

Hausner's Ratio

The following equation was used to get Hausner's probiotics granule ratio (Table 8).

$$Hausner'ratio = tapped\ density/bulk\ density$$

Angle of Repose (AR)

AR was measured using the funnel technique to evaluate the probiotic granules' flow characteristics. A conical pile will form when granular materials are deposited onto a horizontal plane. AR calculated as per given formula (Table 2).

$$Tan\ \theta = H/\ 0.5 * D$$

Green Synthesis and UV Characterisation of TiO_2 nanoparticles using LAB in MRS Broth

A total of 20 mL of TiO2 (0.025 m) was added into broth containing the isolated all LAB at 37°C for 48 hours. After 48 hours, the mixture was centrifuged for 10 minutes at 5000 rpm before being cleaned with distilled water. The final content was dried in an oven at 60°C for one hour and nature of the particles were estimated using UV. The content was evaluated at the range from 200 to 900 nm.[24-26]

Comparative Antimicrobial Activity of LAB Metabolites With and Without TiO_2

Using microorganisms in their whole and cell-free extract the antibacterial activity of the isolated LAB was assessed. Muller and Hinton's plates were seeded with indicator organisms before the sterilized porcelain beads were put on them and charged with CFE. After that, plates underwent an inversion-free 18 hours incubation period at 37°C. It was assumed that the formation of a distinct inhibition surrounding the beads constituted proof of antibacterial action against the indicator microorganisms. If there was an inhibition surrounding the beads, the diameter was measured in mm.[1,22] The same above protocol is again followed by taking TiO_2 nanoparticles inoculating with all LAB to evaluate the antimicrobial activity.

Table 1: Standard format of flow properties and compressibility index

Flow characters	*CI*
Excellent	1.0-10.0
Good	11.0-15.0
Fair	16.0-20.0
passable	21.0-25.0
poor	26.0-31.0
Very poor	32.0-37.0
Very, very poor	>38.0

Table 2: Standard format of flow properties of the angle of repose

AR value	*Flow properties*
<20.0	Excellent
20.0-30.0	good
30.0-34.0	passable
>40.0	Very poor

RESULTS

Identification of Isolates

By looking at the colony shape, physiological and biochemical characteristics of the isolated microorganisms from sheep milk, *Lactobacillus* spp. could be recognized. Small, round, convex, opaque, white-creamy isolates were what were seen (Figure 1).

Sugar Fermentation Pattern

The probiotics were identified by basic biochemical analysis up to the species level. The sugar fermentation tests was demonstrated as per Patil *et al.* (2019).[10] The four chosen isolates demonstrate the sugar fermentation test. No gas generation was seen in the sugar fermentation patterns shown in Table 3.

NaCl Test

Recognised LAB in sheep milk were tolerant to 1–10% NaCl. Table 4 displays the findings.

Measurement of Organic Acid and Calculation of pH Value

Organic acids coagulated the pasteurized buffalo milk due to the presence of identified LAB from sheep milk. Table 5 presents the outcomes.

Bile Test

The survival investigations of four distinct LAB strains were observed for three hours in the presence of 0.0% (as control), 1.0% (test-1) and 1.5% bile solution (test-2) in Table 6:

Figure 1: Plate of *Lactobacillus* on MRS media with dilution

Table 3: Sugar fermentation test

Name of the bacterial isolate	*Ri*	*So*	*MA*	*Su*	*Fr*	*Ce*	*Sa*	*La*	*Gas Production*
1	+	+	-	+	+	+	+	+	+
2	+	-	-	-	+	-	-	+	nil
3	+	+	-	+	+	+	+	+	+
4	+	-	-	-	+	-	-	+	nil

Ri- Ribulose, So- Sorbitol, Su=Sucrose, Fr=Fructose, Ce=Cellobiose, Sa=Salicin, La=Lactose, Ma- Mannitol (+) presence, (-) absence.

Table 4: NaCl tolerance exhibited by LAB

NaCl (%)	*Isolate Sample*			
	1	*2*	*3*	*4*
1	+++	+++	+++	+++
2	+++	+++	+++	+++
3	+++	+++	+++	+++
4	+++	+++	+++	++
5	+++	+++	+++	++
6	++	++	++	++
7	+	++	++	++
8	+	+	+	+
9	-	+	+	-
10	-	-	-	-

(+++) High growth, (++) Moderate growth, (+) low growth, (-) no growth. Based upon visual inspection: Dark yellow (+++) = very turbid growth, yellow (++) = turbid growth, faint yellow (+) = slight turbid growth and no change in color = Nil growth with clear solution.

Table 5: Measuring acid content and calculating pH

Isolates	*Waiting time (Hour)*	*Temp. (C)*	*Acid (%)*	*Before pH*	*After pH*
1	24	37°	2.18 ± 0.45	6.7	6.1
	48		5.12 ± 0.84		5.7
	72		9.27 ± 0.45		4.7
2	24	37°	2.81 ± 0.64	6.7	6.9
	48		5.61 ± 0.84		5.5
	72		9.84 ± 0.55		4.7
3	24	37°	2.58 ± 0.94	6.7	6.0
	48		4.42 ± 0.55		5.5
	72		5.61 ± 0.78		4.6
4	24	37°	2.81 ± 0.28	6.7	5.3
	48		6.81 ± 0.45		4.6
	72		8.57 ± 0.78		4.0

± reflect the standard error of the means, n=3, and the statistical significance ($p<0.05$) of the data in the same column.

At 0% bile content, all four LAB cultures demonstrated modest activity for the first three hours of incubation. At 1% bile content, strains 2, 3, and 4 were followed by isolate 1, which demonstrated the maximum growth and survival. Only a few LAB strains have displayed acceptable growth up to 3 hours of incubation.

Growth of the LAB in Various Animal Milk

The serial dilution study was carried out in the form of CFU count till 10 X 10^9 times. The purpose of this investigation was to determine the actual microbial load. The findings for various kinds of milk, measured in CFU/mL for milk from cows, goats, sheep, and buffalo, are shown in Table 7.

The culture plated from MRS broth for all the LAB were white colored dots which was pure without any contaminations by other microbes, as seen in the above figure.

Table 6: Bile tolerance test for isolated LAB

(Log) cfu / g^1 0.0 % bile solution				
Bacterial isolates	*0h*	*1h*	*2h*	*3h*
1	200 ± 2	191 ± 7	181 ± 5	181 ± 2
2	197 ± 6	195 ± 4	195 ± 1	192 ± 2
3	201 ± 4	201 ± 5	199 ± 2	194 ± 4
4	188 ± 1	181 ± 1	178 ± 8	177 ± 9
(Log) cfu / g^1 1.0 % bile solution				
	0h	1h	2h	3h
1	205 ± 8	181 ± 1	171 ± 4	166 ± 8
2	195 ± 5	182 ± 2	174 ± 4	155 ± 2
3	201 ± 7	204 ± 5	188 ± 5	174 ± 1
4	188 ± 1	177 ± 4	165 ± 1	156 ± 6
(Log) cfu / g^1 1.5 % bile solution				
Bacterial strain	0h	1h	2h	3h
1	204 ± 1	161 ± 4	150 ± 2	100 ± 1
2	191 ± 2	157 ± 5	145 ± 6	99 ± 2
3	192 ± 2	121 ± 5	88 ± 8	65 ± 5
4	171 ± 6	141 ± 1	90 ± 4	55 ± 4

± error, n=3, [1] signify denotes a viable number (CFU/g).
Values statistically significant ($p<0.05$)

Table 7: CFU/mL count

Sr. No	*Animal*	*count* (a,b,c,d)*
1	Cow	10* X 10^8 CFU ± 4.1
2	Buffalo	12* X 10^9 CFU ± 2.5
3	Sheep	14* X 10^8 CFU ± 1.9
4	Goat	04* X 10^8 CFU ± 2.4

Isolated microorganisms from sheep milk are shown in (a,b,c,d), and the asterisk (*) denotes statistical significance (n=3) at ($p<0.05$).

Flow Properties Studies

Table 8 displays the findings of the flowability investigation. The bulk density values ranged from 0.45 to 0.51. TD values were between 0.52–0.59. AR values were observed in the range of 25.2–28.98. The produced granules' CI result was in the range of 12.1 to 14.03, though. The values of Hausner's ratio were found to be between 1.13 and 1.16. According to the findings,

all four formulae exhibited good flow characteristics. The effective method of preparation of granules by lyophilization may be responsible for the superior flow characteristics.

Evaluation of TiO_2 using UV Spectrophotometer

The extracellular biosynthesis TiO_2 nanoparticles was successfully evaluated using the UV method. UV spectrum recorded at 405 nm for LAB-1, 410 nm for LAB-2, 408 nm for LAB-3, and 407 nm for LAB-4, respectively which proves the presence of TiO_2 nanoparticles (Figure 2).

Antimicrobial Activity (Inhibition zone mm)

It was observed that all isolates showed positive zone of inhibition against different selected microorganisms.

It was found that probiotics as the whole organism shows more antimicrobial activity as compared to the cell-free extract. Further, the TiO_2 nanoparticles along with LAB show enhanced antibacterial activity (Tables 9 and 10).

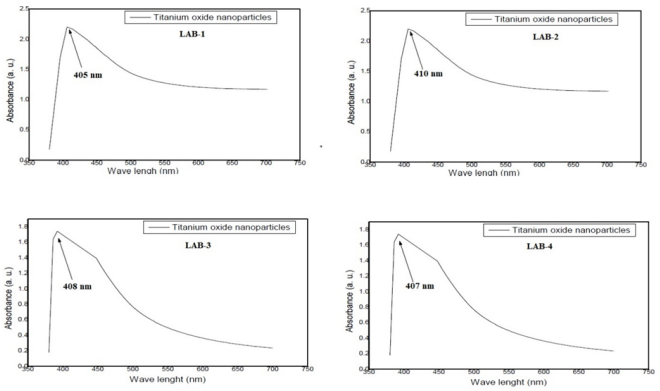

Figure 2: UV-spectrum of TiO_2 nanoparticle synthesized using LAB

DISCUSSION

Study aimed to produce an optimal growing environment for four distinct LAB isolates with their encapsulation. The results shown 4 LAB isolates were with a good survival rate at simulated mimicking study of the gastrointestinal tract by exhibiting extreme changes in pH and GI condition studies *in-vitro*. Additionally, these LAB inoculations resulted in a considerable increase in the milk of cows, buffaloes, goats, and sheep, with a particularly high growth rate in buffalo milk. This is due to the iron and folic acid level in buffalo milk, which is significantly higher than that in milk from other animals and is crucial for the growth of LAB.[14] However, our investigations have demonstrated that employing pasteurized or tyndallized milk can work as an excellent natural flourishing agent. Previous studies have noted the importance of prebiotics for the growth of LAB. The flow properties exhibited by the LAB by using maltodextrin by lyophilization exhibited good flow granule flow properties. Formation of nano delivery is feasible by LAB and incubating for period of 48 hours. This becomes one of easy method of green synthesis of nanoparticles. LAB as the whole organism had shown a higher inhibition zone than cell-free extract, indicating a major contribution of these microbes as good nutraceutical agent. Further, the TiO_2 nanoparticles exhibited more antibacterial activity in combination of all LAB as compared to its absence. These microbes with some periclinal trails can be considered the ideal antimicrobial candidate to treat broad-spectrum pathogenic infection and prevent antibiotic resistance.

Table 8: Granule flow characteristics of LAB as probiotics

Formula	*BD*	*TD*	*AR*	*CI*	*Hausner's ratio*	*Flow property*
F-1	0.41 ± 0.11	0.56 ± 0.11	27.1 ± 0.74	11.28 ± 0.11	1.19 ± 0.24	good
F-2	0.42 ± 0.22	0.56 ± 0.08	25.4 ± 0.44	13.03 ± 0.12	1.17 ± 0.12	good
F-3	0.43 ± 0.20	0.51 ± 0.16	26.7 ± 0.88	12.46 ± 0.14	1.12 ± 0.65	good
F-4	0.49 ± 0.17	0.54 ± 0.14	27.8 ± 0.12	14.55 ± 0.17	1.49 ± 0.77	good

Table 9: Antimicrobial activity of isolated LAB

LAB strains	*Antimicrobial activity (Inhibition zone mm) ZDI ± SD*				
	Cell free extract				
	A	*B*	*C*	*D*	*E*
1	15.4 ± 0.87	17.4 ± 0.41	19.7 ± 0.21	19.2 ± 0.51	15.7 ± 0.14
2	21.1 ± 0.81	25.4 ± 0.25	18.4 ± 0.22	14.1 ± 0.82	18.5 ± 0.85
3	13.2 ± 0.57	12.4 ± 0.54	19.2 ± 0.81	18.5 ± 0.41	17.2 ± 0.75
4	24.1 ± 0.45	11.4 ± 0.68	15.5 ± 0.87	14.8 ± 0.27	14.6 ± 0.54
	Whole microbes				
1	16.7 ± 0.25	18.5 ± 0.58	21.6 ± 0.53	21.5 ± 0.71	18.2 ± 0.54
2	24.5 ± 0.58	26.9 ± 0.47	19.2 ± 0.44	18.2 ± 0.38	19.6 ± 0.24
3	15.3 ± 0.36	15.2 ± 0.52	20.5 ± 0.14	19.4 ± 0.63	18.5 ± 0.65
4	26.9 ± 0.82	15.5 ± 0.39	18.7 ± 0.38	15.7 ± 0.54	15.5 ± 0.57

ZDI: Zone diameter inhibition; SD –Standard deviation, ± Statistically significant data were input ($p<0.05$) and the standard error of means for n = 3 is indicated. A,B,C,D,E- *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhi*, *Enterococcus faecalisis*, *Staphylococcus aureus* etc.

Table 10: Antimicrobial activity of isolated LAB with TiO2 Nanoparticles.

LAB strains	*Antimicrobial activity (Inhibition zone mm) ZDI ± SD*				
	Cell free extract				
	A	*B*	*C*	*D*	*E*
1	16.4 ± 0.17	18.4 ± 0.81	20.7 ± 0.41	21.2 ± 0.71	16.7 ± 0.74
2	22.1 ± 0.51	26.4 ± 0.55	19.4 ± 0.52	15.1 ± 0.52	19.5 ± 0.55
3	14.2 ± 0.87	13.4 ± 0.54	19.6 ± 0.51	19.5 ± 0.61	18.2 ± 0.45
4	25.1 ± 0.45	13.4 ± 0.48	18.7 ± 0.67	15.8 ± 0.57	15.6 ± 0.54
	Whole microbes				
1	17.7 ± 0.95	19.5 ± 0.38	25.6 ± 0.73	22.5 ± 0.21	19.2 ± 0.24
2	25.5 ± 0.78	27.9 ± 0.67	20.2 ± 0.74	19.2 ± 0.58	20.6 ± 0.14
3	16.3 ± 0.56	16.2 ± 0.62	21.5 ± 0.74	20.4 ± 0.43	19.5 ± 0.55
4	27.9 ± 0.82	17.5 ± 0.49	19.7 ± 0.28	16.7 ± 0.54	16.5 ± 0.87

ZDI: Zone diameter inhibition; SD –Standard deviation, ± Statistically significant data were input ($p<0.05$) and the standard error of means for n = 3 is indicated.

CONCLUSION

LAB extracted from sheep milk was proven in the current investigation as a potential candidate for the future probiotic antibacterial agent that resists GI's bile and pH condition. Gram-positive and gram-negative microorganisms are both susceptible to LAB, according to research. Further, this LAB has shown great potential to grow in milk from different milking animals. Buffalo milk has proven ideal natural media for flourishing LAB compared to other milch animal milk. Lyophilization helped in preparing a stable granule formulation with good flow properties in case of all LAB. Green synthesis of the nanoparticles is possible by using the LAB cultures. These nanoparticles and the whole organism or the cell free extract can show good antibacterial activities, proving to be new nutraceutical mediators against bacterial pathogens.

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