Lactosmart: A Novel Therapeutic Molecule for Antimicrobial Defense

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The problem of antibiotic resistance has prompted researchers around the globe to search for new antimicrobial agents. Antimicrobial proteins and peptides are naturally secreted by almost all the living organisms to fight infections and can be safer alternatives to chemical antibiotics. Lactoferrin (LF) is a known antimicrobial protein present in all body secretions. In this study, LF was digested by trypsin, and the resulting hydrolysates were studied with respect to their antimicrobial properties. Among the hydrolysates, a 21-kDa basic fragment of LF (termed lactosmart) showed promise as a new potent antimicrobial agent. The antimicrobial studies were performed on various microorganisms including *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* as well as fungal pathogens such as *Candida albicans*, *Candida tropicalis*, and *Candida glabrata*. In addition, the lipopolysaccharide (LPS)-binding properties of lactosmart were studied using surface plasmon resonance technique *in vitro*, along with docking of LPS and molecular dynamics (MD) simulation studies. The results showed that lactosmart had better inhibitory effects against pathogenic microorganisms compared to LF. The results of docking and MD simulation studies further validated the tighter binding of LPS to lactosmart compared to LF. The two LPS-binding sites have been characterized structurally in detail. Through these studies, it has been demonstrated that in native LF, only one LPS-binding site remains exposed due to its location being on the surface of the molecule. However, due to the generation of the lactosmart molecule, the second LPS-binding site gets exposed too. Since LPS is an essential and conserved part of the bacterial cell wall, the pro-inflammatory response in the human body caused by LPS can be targeted using the newly identified lactosmart. These findings highlight the immense potential of lactosmart in comparison to native LF in antimicrobial defense. We propose that lactosmart can be further developed as an antibacterial, antifungal, and antibiofilm agent.

Keywords: lactoferrin, antifungal, antibacterial, LPS, SPR, biofilm

INTRODUCTION

The emergence of multidrug resistance (MDR) in microorganisms against antibiotics has become a global problem (Aminov, 2009; Willers et al., 2016; Exner et al., 2017). Various conventional drugs with promised efficacy and specificity are unable to withstand the threat of antibiotic drug resistance (Cohen, 2000; Yoneyama and Katsumata, 2006; Fair and Tor, 2014).

The rising crisis of MDR bacteria has led to the channelization of relevant research in the direction of antimicrobial molecules from natural sources as potential novel antibiotics. The spectrum of natural innate immune proteins and their potent fragments herald a promising approach to fight the problem of drug resistance. Among the natural antimicrobial proteins, lactoferrin (LF) has been identified as a potent host defense system based on its wide spectrum of bactericidal and bacteriostatic activities (Arnold et al., 1977; Yamauchi et al., 1993; Bolscher et al., 2009; Leon-Sicairos et al., 2009; Ochoa and Cleary, 2009; Yen et al., 2011; Sinha et al., 2013; Leon-Sicairos et al., 2014). In the past, several studies have demonstrated the antibacterial and antifungal effects of LF and its derivative peptides, for instance, lactoferricin B (Jones et al., 1994; Strom et al., 2000; Gifford et al., 2005; Sharma et al., 2013; Sun et al., 2018; Ligtenberg et al., 2021) and lactoferrampin (van der Kraan et al., 2004, 2006; Haney et al., 2009).

Structurally, LF consists of two iron-bound lobes, N-lobe (1–333) and C-lobe (345–692) (Anderson et al., 1987, 1989; Baker and Baker, 2004, 2009; Gonzalez-Chavez et al., 2009). Among the two lobes, the highly cationic properties of N-lobe are responsible for membrane disruption by interacting with anionic components present on bacterial surface (Bellamy et al., 1992; van der Kraan et al., 2004; Puddu et al., 2010). It has been established that the lipid A component of the LPS is a known drug target for antimicrobial therapeutics (Morrison et al., 1976; Cohen et al., 1992; Cutone et al., 2014). One of the mechanisms by which LF acts as an antimicrobial agent is through binding to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), thereby disrupting the bacterial membrane integrity and activating the chemical signaling pathway (Ellison et al., 1988; Appelmelk et al., 1994; Brandenburg et al., 2001). This leads to the secretion of pro-inflammatory responses that downregulates the release of cytokine production (Farnaud and Evans, 2004; Legrand and Mazurier, 2010). In the past, it had been reported that LF binds to LPS with its hexameric sequence present in the 18-loop region of the lactoferricin (Elass-Rochard et al., 1995; Odell et al., 1996; Farnaud and Evans, 2004; Drago-Serrano et al., 2012).

In the present study, we have performed the partial digestion of LF with trypsin, which generates a potent antimicrobial molecule with the size of about 21 kDa (85–284). We have proposed its name as lactosmart due to its higher potency against pathogens when compared to native LF as a whole protein. The lactosmart has been tested for antibacterial and antifungal properties along with its inhibitory potential of biofilm formation by *Pseudomonas aeruginosa* through established assays (Ramamourthy and Vogel, 2020). Our primary focus was on the comparison of LPS-binding properties of lactosmart with native LF using surface plasmon resonance (SPR) technique. Docking and molecular dynamics (MD) simulation studies with LPS have also been performed to further substantiate our claims. Through our studies, we have demonstrated that LF sequesters LPS through two binding sites that are situated on the N-lobe.

MATERIALS AND METHODS

Preparation and Purification of Lactosmart

Bovine lactoferrin (Blf) was provided by Morinaga Milk Industry Co., Ltd. (Japan). The powdered LF was solubilized in 50 mM *Tris*-HCl pH 7.8 with 0.5 M CaCl$_2$. The trypsin hydrolysis of LF was carried out at a protein:enzyme molar ratio of 50:1 for 30 min at 37°C. The hydrolysis was stopped by adding 30 mM benzamidine, and the hydrolyzed sample was stored at −20°C until further use. The hydrolysate was subjected to ion exchange chromatography using Diethylaminoethyl (DEAE) in the same buffer. The unbound fractions were collected as 3-ml fractions in the test tubes. The bound protein was eluted with a salt gradient of 0–0.5 M NaCl, while the unbound fractions were concentrated using centricons and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The bound fractions were further subjected to gel filtration chromatography using Sephadex G-75 column. The gel filtration profile showed three distinct peaks (**Figure 1A**). All the three peaks were again analyzed by SDS-PAGE. The peak corresponding to the molecular weight of 21 kDa was desalted and lyophilized for further use (**Figure 1B**).
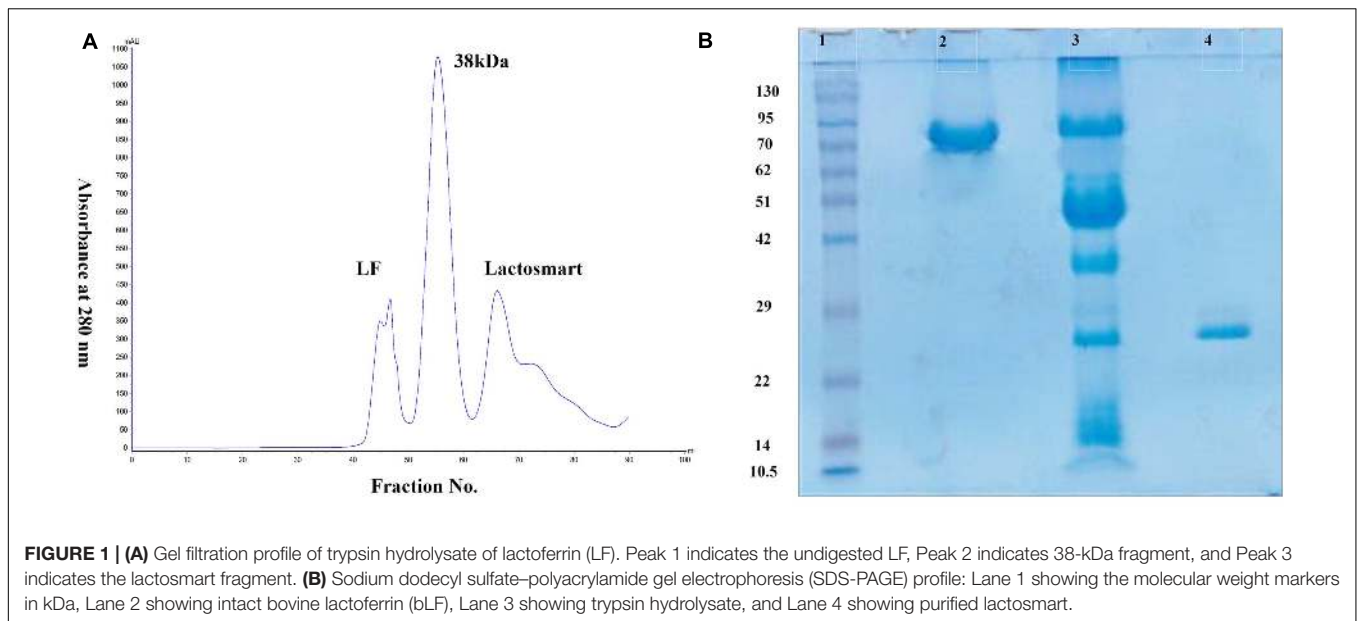
Binding of Lipopolysaccharide With Lactosmart

The binding analysis of LPS with lactosmart was carried out in real time using SPR spectroscopy with Biacore-3000 (Biacore AB, Uppsala, Sweden). For the SPR studies, 100 ng of the protein was immobilized on CM-5 chip using the amine coupling kit provided by the manufacturer. All the binding studies were carried out in the 10-mM phosphate-buffered saline (PBS) pH 7.4 buffer. Various concentrations of the LPS were allowed to flow at a flow rate of 30 μl/min. Regeneration of the immobilized surface was achieved by 10 mM NaOH after each cycle. All the SPR sensograms were normalized against the PBS buffer. Association and dissociation phases of the binding were fitted in the 1:1 Langmuir binding model using Bia evaluation software 4:1 provided by the manufacturer. The dissociation constant (KD) was calculated by using the average values of rate of dissociation (kd) and the rate of association (ka) using the formula KD = kd/ka. All the results were subjected to fitting efficacy to check the accuracy of the results.

Antibacterial Studies
Medium, Antimicrobial Agent, and Bacterial Strains
The bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). A differential medium xylose-lysine deoxycholate agar (XLD agar) was used for the cultivation of *Shigella flexneri*. Luria–Bertani (LB agar) medium was used for the cultivation of *Escherichia coli*, *Staphylococcus aureus*, and *P. aeruginosa*. Mueller–Hinton broth (MHB) was used for antimicrobial susceptibility testing of proteins. Kanamycin was purchased from Sigma Aldrich (India). Media components were purchased from Himedia (India).

FIGURE 1 | (A) Gel filtration profile of trypsin hydrolysate of lactoferrin (LF). Peak 1 indicates the undigested LF, Peak 2 indicates 38-kDa fragment, and Peak 3 indicates the lactosmart fragment. **(B)** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) profile: Lane 1 showing the molecular weight markers in kDa, Lane 2 showing intact bovine lactoferrin (bLF), Lane 3 showing trypsin hydrolysate, and Lane 4 showing purified lactosmart.

Measurement of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined using a standard serial dilution broth method as per the guidelines of Clinical and Laboratory Standards Institute (CLSI) reference document M07-A10 (Wayne, 2015) for lactosmart fragment and bLF against *S. flexneri*, *E. coli*, *S. aureus*, and *P. aeruginosa*. These bacterial strains were cultured for a duration of 24 h. After the preparation of 0.5 McFarland bacterial suspension in MHB medium, lactosmart and bLF were added in each well of a 96-well microtiter plate from the highest to lowest concentration. The protein concentration in the wells ranged from 2 to 0.003 mg. After incubation at 37°C for 24 h, the absorbance at 600 nm was measured.

Growth Inhibition Curve

The effect of bLF and its hydrolyzed fragment lactosmart on the growth of *S. flexneri*, *E. coli*, *S. aureus*, and *P. aeruginosa* was examined. Bacteria were cultured at 37°C, with agitation 200 rpm. The culture was grown in MHB to 0.1 at OD_{600} and then equally distributed into 96-well microtiter plates (200 µl/well). Then, bLF and lactosmart were added to their final MICs. Kanamycin was used as a positive control, and the culture without the protein was used as a bacterial growth control. The absorbance was recorded at 600 nm using microplate spectrophotometer (Epoch, Biotek, United States) at 1-h interval (Zasloff, 2002).

Disc Diffusion Method

The method of Bauer et al. (1966) was used to check the antimicrobial activity of lactosmart fragment and bLF. Bacterial cultures of S. *aureus*, *E. coli*, *P. aeruginosa*, and *S. flexneri* were grown at their optimum temperature (37°C) overnight in an MHB medium. Thereafter, the bacteria were diluted to about 10^5 colony-forming units (cfu/ml). The protein MIC was loaded onto sterile papers (4 mm diameter) and placed on the Mueller–Hinton agar (MHA) surface. Kanamycin (30 µg/ml) was used as a positive control. Plates were incubated at the optimal temperature for each strain for 18–24 h. The diameter of the bacterial inhibition zone indicated the antibacterial activity.

Inhibition of Biofilm Formation

The antibiofilm activity of bLF and lactosmart (85–284) was assessed using the broth microdilution method with slight modification (Ramamourthy and Vogel, 2020). The bacterial suspension of *P. aeruginosa* (9027) was grown in MHB supplemented with 1% glucose adjusted to 0.5 Mcfarland standard at 37°C for 24 h. Here, 100 µl of cells was inoculated in a 96-well microtiter plate in the presence of lactosmart and LF at decreasing concentrations from 2 to 0.003 mg/ml. The wells containing sterile MHB supplemented with 1% glucose were considered the negative control and used as a blank. After 24 h of incubation, bacterial cells were discarded, and the plate was washed thrice with PBS pH 7.4. The fixation of the biofilm was done by adding 150 µl of methanol to the well for 20 min. Subsequently, it was kept at room temperature for 1 h. Then, 125 µl of 0.1% solution of crystal violet was added to each well and incubated at room temperature for 30 min. Excess dye was removed by submerging the plate thrice in PBS buffer and then left overnight to dry. Then, 125 µl of 30% acetic acid was added in each well, and the plate was read at 550 nm.

Antifungal Studies
Medium, Antimicrobial Agent, and Fungal Strains

Three *Candida* species were used in the present study, namely, *C. albicans* ATCC 5314, *C. glabrata* ATCC 90030, and *C. tropicalis* ATCC 750. *Candida* cells were maintained on yeast extract-peptone-dextrose (YEPD) in the ratio 1:2:2 along with 2.5% agar at 4°C. Fluconazole was procured from Sigma Aldrich (Germany). The chemicals used were of analytical grade and

were procured from Merck (India). Media components were purchased from Himedia (India).

Minimum Inhibitory Concentration

The MIC of the lactosmart against *Candida* strains was determined by broth dilution method as per the guidelines of CLSI reference document M27-A3 (Wayne, 2008) and was defined as the lowest concentration that causes 90% decrease in absorbance in comparison to that of the control (without protein).

Growth Pattern

The *Candida* cells were inoculated into fresh YEPD media. Varying concentrations of lactosmart were added to the culture and incubated at 37°C with agitation (200 rpm). The aliquots were removed after every 2 h, and growth was recorded in terms of absorbance at 595 nm using Labomed Inc. spectrophotometer (United States) for each concentration and plotted against time in hours.

Agar Disc Diffusion Assay

The *Candida* cells (10^5 cells/ml) were inoculated into molten YEPD agar at 40°C and poured into 90-mm Petri plates. Sterile filter discs (4 mm) were loaded with different concentrations of lactosmart and placed on agar plates (Sharma et al., 2016). For higher concentrations, wells were prepared with the help of a sterile syringe. The average diameter of zones of inhibition (ZOI) was measured after 48 h. Fluconazole (10 μg/disc) was used as a positive control.

Docking of Lipopolysaccharide With Lactosmart

The docking studies were performed using Schrödinger software. The target protein lactosmart was selected and prepared for docking by removing water and adding hydrogen in the protein molecule. The ligand was downloaded from the PDB server as a PDB file, and it was directly used for docking. Since no prior information was available about the active binding site, blind docking method was used to dock the LPS into lactosmart.

Molecular Dynamics Simulations

The MD simulations of protein–ligand complexes were performed using the AMBER software suite (Case et al., 2014). The LPS parameters were adopted from the latest version of AMBER-compatible GLYCAM_06 force field, which has been thoroughly developed for LPS and related systems (Cieplak et al., 1995; Cornell et al., 1995; Tessier et al., 2008; Vanquelef et al., 2011). The protein parameters were taken from the modified ff99SB library (Maier et al., 2015) in AMBER. The protein–ligand complexes were immersed in a cubic TIP3P water box with sufficient counterions to maintain the electroneutrality of the system. The ion parameters were taken from the literature (Joung and Cheatham, 2008). We used Particle Mesh Ewald (for computing the long-range electrostatics) (Cheatham et al., 1995) and periodic boundary conditions along with the SHAKE algorithm (to constrain hydrogen). The systems thus created were first minimized to remove any close contacts or atomic clashes in the complexes, if any. The minimization was performed in two steps: first, by applying harmonic restraint of

50 kcal/mol on the protein–ligand complexes (minimizing the ions and solvent molecules), and in the next step, the restraint was removed entirely. We used 10,000 conjugate gradients and 10,000 steepest descent cycles in the minimization steps. The minimized systems were then heated to room temperature (300 K of NVT MD for 50 ps) followed by equilibrating the assemblies for 10 ns. Finally, 100-ns-long MD simulations were performed on the systems under consideration. We utilized the CPPTRAJ tool (Roe and Cheatham, 2013) in AMBER to process the MD simulation trajectories [to monitor the fluctuations in root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), the radius of gyration, and the number of hydrogen bonds as a function of run length]. The energetics of the protein–ligand binding was computed using the MM-PBSA methodology (Onufriev et al., 2000; Gohlke and Case, 2004).

RESULTS

Antibacterial Activity and Growth Pattern

The antibacterial activity of lactosmart was studied and compared with the intact LF. MIC was calculated against four bacterial species, namely, *S. flexneri*, *E. coli*, *P. aeruginosa*, and *S. aureus*. We found that the MIC value of lactosmart against *S. flexneri* was 0.5 mg, 1 mg for *P. aeruginosa*, 0.128 mg for *S. aureus*, and 0.032 mg for *E. coli*. These MIC values of lactosmart were much lower than those of LF (**Table 1**). The growth inhibition studies indicate that lactosmart can inhibit *P. aeruginosa* and *S. flexneri* up to 65% at its MIC, while it can inhibit *E. coli* and *S. aureus* up to 70% at its MIC. On the other hand, lactosmart at double the concentration of its MIC value inhibits each strain up to 90%, while LF can only inhibit up to 60% at the same concentration (**Figure 2**). As seen from **Supplementary Figure 1**, the disc diffusion results also indicate that the lactosmart molecule is a more potent antibacterial agent than the native LF. The diameters of the ZOI in each strain were found to be higher in the case of lactosmart.

Antibiofilm Activity

A widely used crystal violet assay to study the inhibition of the biofilm formation by *P. aeruginosa* (9027) was used. The

TABLE 1 | Values of minimum inhibitory concentration (MIC) and zone of inhibition (ZOI) of lactosmart and intact lactoferrin against *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and *Pseudomonas aeruginosa*.

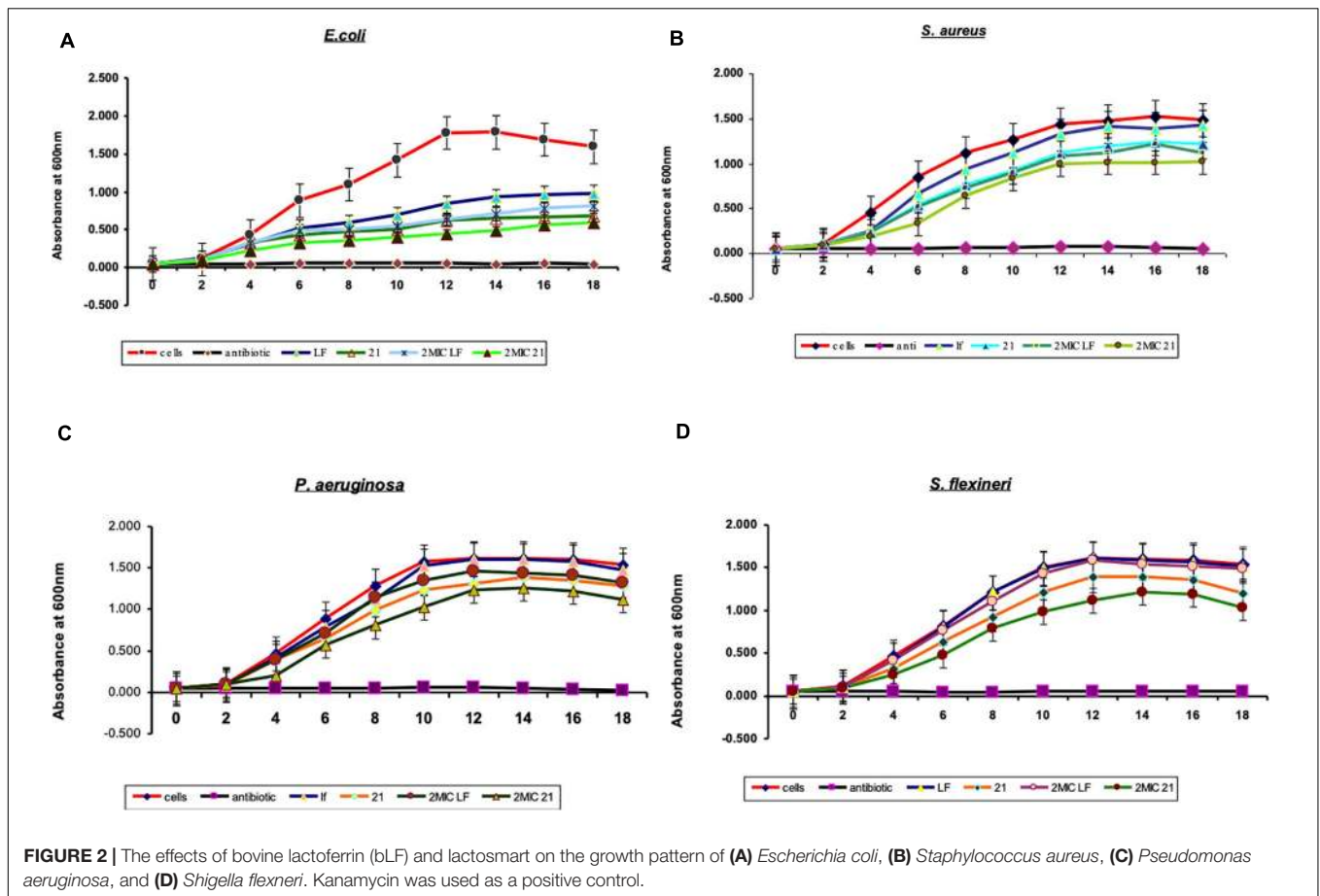| Strains	ATCC	MIC (mg/ml)		ZOI diameter (mm)		
		LF	lactosmart		LF	Lactosmart
E. coli	MG1655	0.5	0.032	MIC	17	18
				2MIC	18	20
S. aureus	6538P	0.8	0.128	MIC	9	12
				2MIC	11	13
S. flexneri	12022	1.5	0.5	MIC	8	10
				2MIC	9	12
P. aeruginosa	9027	1	1	MIC	7	8.5
				2MIC	8	11

FIGURE 2 | The effects of bovine lactoferrin (bLF) and lactosmart on the growth pattern of **(A)** *Escherichia coli*, **(B)** *Staphylococcus aureus*, **(C)** *Pseudomonas aeruginosa*, and **(D)** *Shigella flexneri*. Kanamycin was used as a positive control.

data for the comparison of the minimum biofilm inhibitory concentration (MBIC) of lactosmart and LF are shown in **Table 2**. The MBIC values for lactosmart and LF were 0.25 and 0.50 mg, respectively. These data also indicate the better antibiofilm activity of lactosmart compared to LF (**Figure 3**).

Antifungal Susceptibility and Growth Pattern

The antifungal efficacy of the protein lactosmart against three *Candida* species was studied in terms of MIC and disc diffusion (**Table 3**). All the three *Candida* species gave an MIC of 10 μg/ml for fluconazole, indicating that the strains used in the present study were not resistant to this conventional

antifungal drug. Lactosmart gave an MIC of 1 mg/ml for *C. albicans* and 5 mg/ml for both *C. glabrata* and *C. tropicalis*. Significantly large dose-dependent ZOIs were observed in the presence of lactosmart. At the MIC, the ZOI diameter was 14 mm in *C. albicans*, which increased to 16 mm when the concentration was doubled to 2MIC. At sub-MIC, the ZOI was 12 mm. Non-*albicans* strains gave a higher MIC value of 5 mg/ml. Also, the ZOIs observed at MIC and 2MIC in non-*albicans* species were not very prominent. For all the three *Candida* species, fluconazole gave a ZOI of 25 mm at 10 μg/disc (**Supplementary Figure 2**). The ZOIs formed in the presence of lactosmart were clear and distinct, while those formed in the presence of fluconazole were hazy. Fluconazole is fungistatic (Sharma et al., 2020), and hence, hazy zones were expected. *Candida* growth patterns studied in the presence of lactosmart showed a concentration-dependent decrease. All the three *Candida* species showed a normal growth pattern in control cells (cells only), while the positive control (fluconazole 10 μg/ml) showed complete inhibition of cell growth. The sub-inhibitory concentration of test protein (MIC/2) showed only a slight decrease in growth of all the three cases. At MIC, growth inhibition in *Candida* cells was not comparable to that in the presence of fluconazole but was significant. Growth inhibition due to the test protein was most prominent in case of *C. albicans* (**Figure 4**).

TABLE 2 | The values of minimum biofilm inhibitory concentration (MBIC) of lactoferrin and lactosmart against *P. aeruginosa*.

Microorganism *Pseudomonas aeruginosa*	Biofilm inhibition at MIC (mg/ml)	Adhesion strength of organism
Lactoferrin	0.50	Moderately adherent
Lactosmart	0.25	Moderately adherent

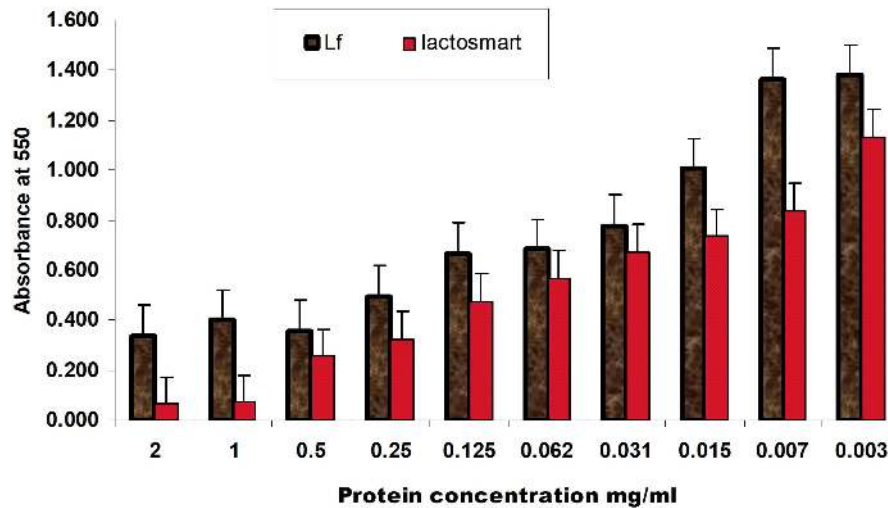*MIC, minimum inhibitory concentration.*

FIGURE 3 | Inhibition of biofilm formation of *P. aeruginosa* by different concentrations of LF and lactosmart in a decreasing order with 24-h incubation at 37°C. Experiments were performed in triplicate, and the data were averaged.

Analysis of Lipopolysaccharide Binding

The molecular interactions between lactosmart with LPS were studied in real time using SPR spectroscopy. The sensogram for the interaction between LF, lactosmart with LPS was recorded (**Figure 5**). The increase in the RU units from the baseline indicates the binding of LPS to the immobilized proteins. Since the rate constants ka and kd are specific for a specific ligand–analyte pair, they do not change upon changing the concentration of either analyte or ligand. The value of the KD, which gives the overall interaction strength between the analyte and ligand complex, was 4.9×10^{-11} M between LPS and lactosmart; it was 3.2×10^{-8} M between LPS and LF.

Docking Analysis

The docking score and glide energy were −6.186 and −64.015 kcal/mol for the site 1 (S1) position in lactosmart. The residues Lys221, Asp225, Glu228, and Asp240 form direct hydrogen bonds with LPS (**Table 4**). LPS also forms hydrophobic interactions with Lys100, Pro219, Glu220, Lys221, Tyr227, Ser235, Arg236, Ala237, Pro238, Asp240, and Glu244 residues (**Figure 6A**). The site 2 (S2) position docked complex has a docking score and glide energy of −6.355 and −59.568 kcal/mol, respectively. LPS in S2 forms hydrogen bonds with Gly120, Arg121, Ser122, Pro251, and Ser252. Tyr92, Gly120, Ile126, Pro188, Phe190, Gly191, Pro251, and His253 residues were involved in the hydrophobic interactions with LPS (**Figure 6B**).

Analysis of Lipopolysaccharide and Lactosmart Complex

All the MD simulation trajectories were first analyzed in terms of RMSD and RMSF fluctuations as a function of run-length. We observed that the RMSD fluctuations (**Figure 7A**) in the protein counterparts were stable throughout the simulations (<2.5 Å) for both the systems considered for the study [called LF_1_P (orange

TABLE 3 | The values of the MIC and ZOI of lactoferrin against *Candida albicans*, *Candida glabrata*, and *Candida tropicalis*.

Candida strains	MIC (mg/ml)	Lactosmart (mg/disc)	Diameter of ZOI (mm)
C. albicans ATCC5314	1 mg/ml	MIC/2	12
		MIC	14
		2MIC	16
C. glabrata ATCC 90030	5 mg/ml	MIC/2	8
		MIC	9
		2MIC	10
C. tropicalis ATCC 750	5 mg/ml	MIC/2	9
		MIC	9
		2MIC	10

The MIC of fluconazole (positive control) was 10 μg/ml and gave a ZOI of 25 mm in each strain.

line) and LF_2_P (purple line)]. For the LPS-bound protein complexes, LF_2_P+L (site 2) (shown in red line) displayed a stable RMSD profile (<2.5 Å). On the other hand, LF_1_P+L (site 1) (blue line) showed some abrupt RMSD fluctuations in the range of 3–5 Å, suggesting that the ligand probably underwent structural/conformational fluctuations that led to an abrupt change in RMSD for the complex (**Figure 7A**). This was anticipated as the ligand comprises several rotatable bonds. The RMSF fluctuations suggested that the fluctuations in the protein residues are reasonably similar for both the systems (**Figure 7B**). We then computed the radius of gyration values, which is known to capture the degree of compactness or expansion as a function of time and plotted in **Figure 7C**. It is evident from the radius of gyration profile that the LF_1_P+L (site 1) is somewhat more open than the LF_2_P+L system (site 2). The slight increment in the radius of gyration values for LF_1_P+L (site 1) is probably due to RMSD fluctuations for the site 1 complex, as seen in the RMSD profile.
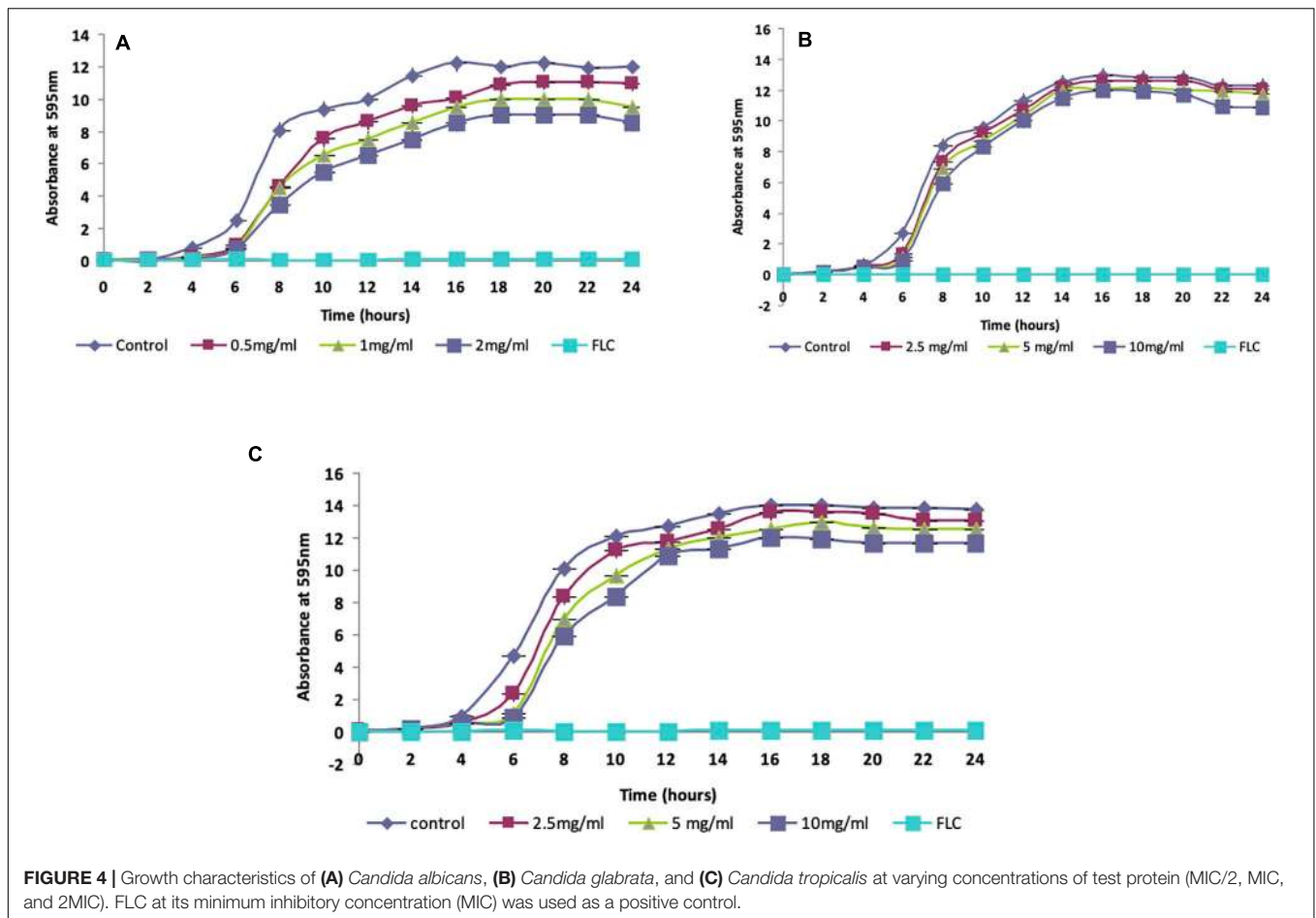
FIGURE 4 | Growth characteristics of **(A)** *Candida albicans*, **(B)** *Candida glabrata*, and **(C)** *Candida tropicalis* at varying concentrations of test protein (MIC/2, MIC, and 2MIC). FLC at its minimum inhibitory concentration (MIC) was used as a positive control.
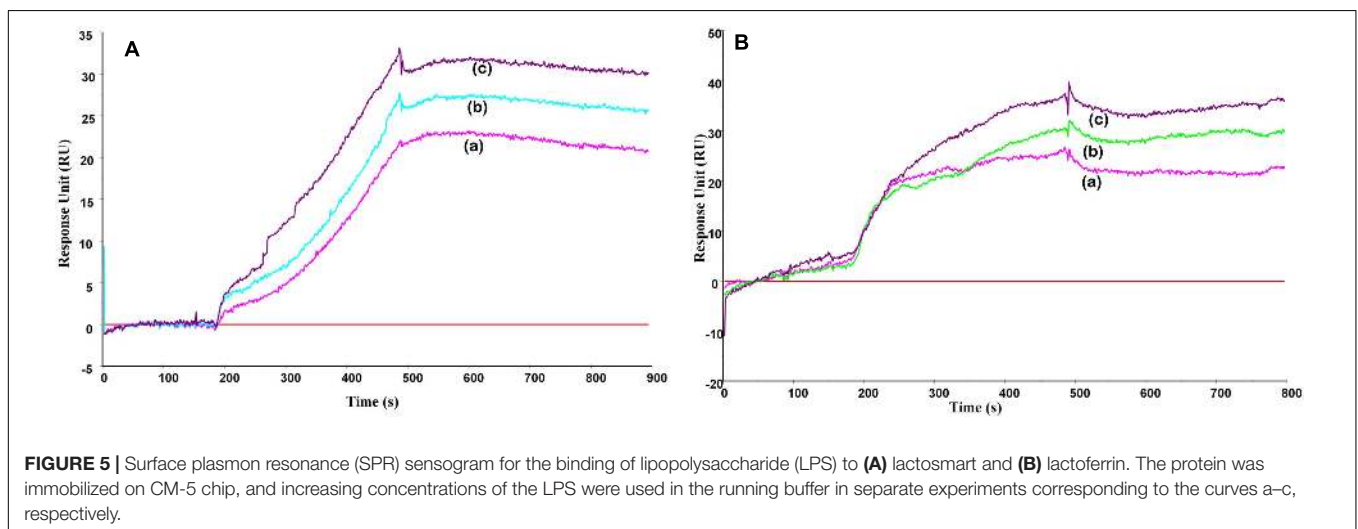

FIGURE 5 | Surface plasmon resonance (SPR) sensogram for the binding of lipopolysaccharide (LPS) to **(A)** lactosmart and **(B)** lactoferrin. The protein was immobilized on CM-5 chip, and increasing concentrations of the LPS were used in the running buffer in separate experiments corresponding to the curves a–c, respectively.
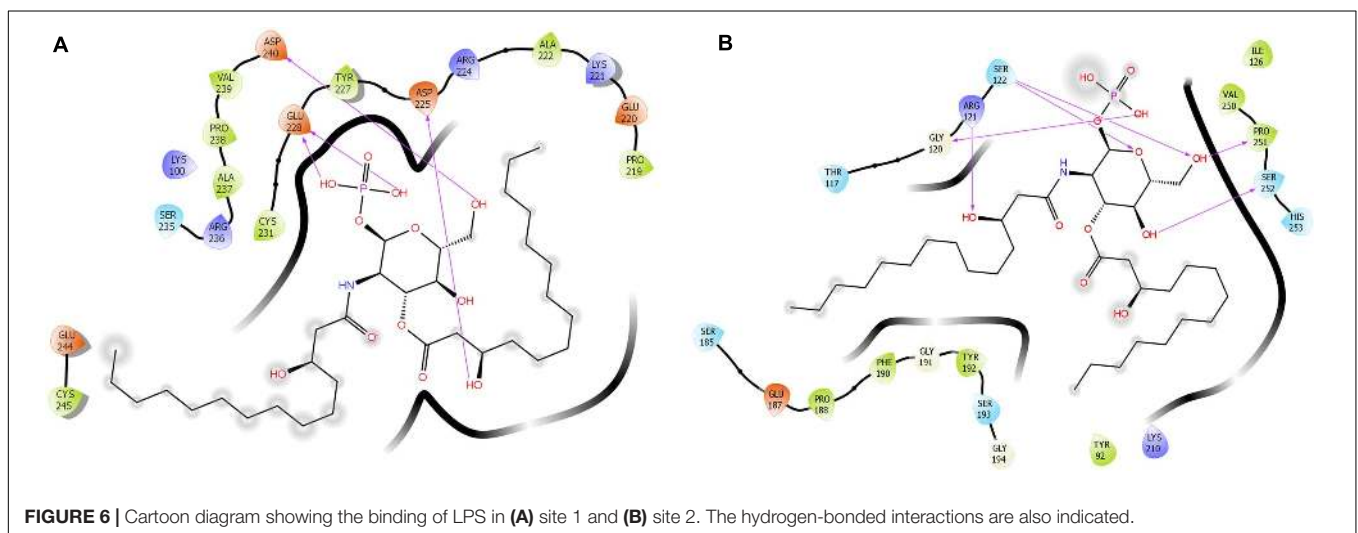
Furthermore, to understand the binding of the ligand in the two possible binding sites offered by the protein, we monitored the change in the number of H-bonds with respect to the simulation time (shown in **Figure 7D**). After some initial fluctuations in both the systems considered (probably due to the changes in the ligand conformation, as noticed in RMSD fluctuations), there are two hydrogen bonds (average) between the ligand and the protein. The protein showed a few dominating movements in the loop region, which were anticipated in such secondary structural elements.

Finally, we computed the binding affinities of the protein–ligand systems by utilizing the last 20 ns of the simulation

TABLE 4 | Docking score and glide energy (in kcal/mol) of lipopolysaccharide (LPS) against Lacto smart (LS) (PDB ID: 1BLF).

Compounds	Docking score (kcal/mol)	Glide energy (kcal/mol)	Hydrogen-binding interactions	Distance (Å)	Hydrophobic interactions
LPS (Site 1)	−6.186	−64.015	Lys221 NZ. ….O6	2.95	Lys100, Pro219 Glu220,
			Asp225 OD2. …..O43	2.70	Lys221, Tyr227, Glu228,
			Glu228 OE2. ….O47	2.53	Ser235, Arg236,
			Glu228 OE2. ….O48	2.82	Ala237, Pro238, Asp240,
			Asp240 OD1. …..O6	2.64	and Glu244
LPS (Site 2)	−6.355	−59.568	Gly120 O. …. …..O47	2.78	Tyr92, Gly120, Ile126, Ser122, Pro188, Phe190, Gly191, Pro251, and His253
			Arg121 NE. …. …..O44	3.21	
			Arg121 NH2. …..O44	2.99	
			Ser122 N. …. …..O47	3.18	
			Ser122 OG. …. …O5	3.03	
			Pro251 O. …..O6	2.83	
			Ser 252 O. …..O4	2.75	
			His253 ND1. …..O6	3.19	

FIGURE 6 | Cartoon diagram showing the binding of LPS in **(A)** site 1 and **(B)** site 2. The hydrogen-bonded interactions are also indicated.
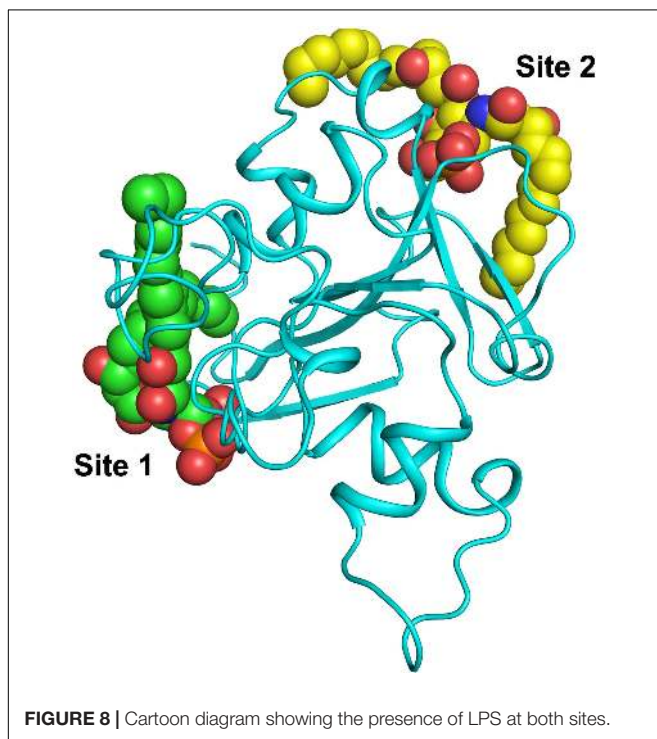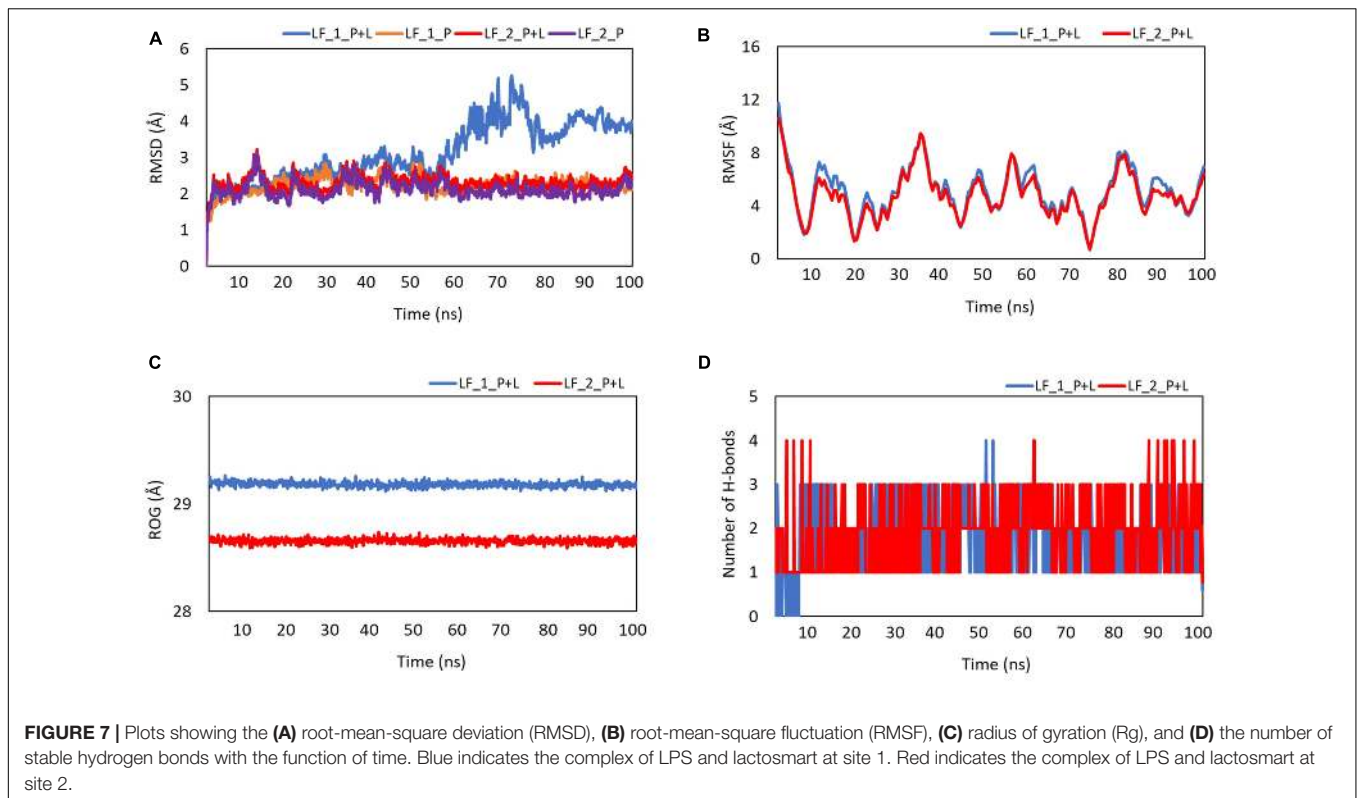
trajectories in both cases with the MM-PBSA approach. The LF_1_P+L system (site 1) showed a predicted binding affinity of −7.89 ± 0.87 kcal/mol, and the LF_2_P+L system (site 2) showed a binding affinity of −8.97 ± 0.56 kcal/mol. Upon examining the structure of the native Blf, it is clear that the lactosmart molecule is generated by two clean cleavages of the native LF molecule at the two beta strands βe (residues 90–100) and βj (residues 247–257). Interestingly, these two beta strands make the supportive floor of the iron binding site. Two major iron-binding residues, Tyr92 and His253, are placed in the βe and βj, respectively.

The lactosmart molecule has two binding sites for LPS, termed S1 and S2, respectively. As seen from **Figure 8**, the two LPS-binding sites are located at the opposite sides of the lactosmart molecule. While S1 is found on the surface of the LF molecule, S2 is found closer to the iron-binding site. It is fair to assume that

while S1 is accessible to LPS binding even in native LF, site S2 can be accessible only after the hydrolysis of LF takes place using trypsin, generating the lactosmart molecule (**Figure 8**).

The S1 consists of residues of Helix α8a, βi strand, and the loop between the two beta strands βi and βj. Its phosphate group is found to be anchored deep inside a spherical, charged groove, which consists of two aspartic acids and one glutamic acid residues. The LPS molecule makes tight hydrogen bonds with Asp240, Asp225, and Glu228. Several other hydrophobic interactions further strengthen the LPS binding and are provided by residues Lys100, Pro219, Glu220, Lys221, Tyr227, Ser235, Arg236, Ala237, Pro238, Asp240, and Glu244.

The second LPS binding site, S2, consists of three charged residues from the βj strand and the loop between the beta strand βf and alpha helix α5. Two conformationally significant residues,

FIGURE 7 | Plots showing the **(A)** root-mean-square deviation (RMSD), **(B)** root-mean-square fluctuation (RMSF), **(C)** radius of gyration (Rg), and **(D)** the number of stable hydrogen bonds with the function of time. Blue indicates the complex of LPS and lactosmart at site 1. Red indicates the complex of LPS and lactosmart at site 2.

FIGURE 8 | Cartoon diagram showing the presence of LPS at both sites.

Pro251 and Gly120, are situated at the ends of S2, making hydrogen bonds with the LPS molecules. The charged residues that interact with the phosphate group of the LPS molecule are

two serines, Ser122 and Ser252, and one arginine residue, Arg121, which is involved in iron binding. The hydrophobic arms of the LPS molecule are anchored by hydrophobic interactions provided by Gly120, Arg121, Ser122, Pro251, and Ser252. Tyr92, Gly120, Ile126, Pro188, Phe190, Gly191, Pro251, and His253.

DISCUSSION

Antibiotic resistance has been identified as a global crisis that is expected to cause a medical catastrophe in the future. We need to urgently address this problem and discover new antimicrobial agents that can be used to fight against this menace. LF is an abundant iron-binding protein that is part of our innate immune system. In the past, there have been many reports that have established LF as a potent antimicrobial agent. In this study, a novel hydrolytic molecule from the N-lobe of LF, lactosmart, has been generated using trypsin. Lactosmart has been tested against different strains of bacteria, namely, *E. coli*, *S. aureus*, *S. flexneri*, and *P. aeruginosa*. The MIC values against these strains were 0.03, 0.12, 0.50, and 1.00 mg/ml, respectively. The MBIC value against the biofilm-forming bacteria *P. aeruginosa* was 0.25 mg/ml, while the MIC value against the planktonic cells of *P. aeruginosa* was 1 mg/ml. This shows that lactosmart was more effective against the cells forming biofilm than the planktonic cells of *P. aeruginosa*. The antifungal activity of lactosmart was also tested against *C. albicans*, *C. glabrata*, and *C. tropicalis*, and the MIC values were 1 mg/ml for *C. albicans* and 5 mg/ml for *C. glabrata*

and *C. tropicali* each. The zone of inhibition values along with MIC values clearly indicate the effectiveness of lactosmart against fungal pathogens.

To explore another function of lactosmart, the binding affinity of LPS was studied using docking and MD simulation studies *in silico* and binding studies using SPR technique *in vitro*. LPS was found binding at two different sites, S1 and S2, which are situated at opposite sides of lactosmart. The average docking score and glide energy were −6.186 and −64.015 kcal/mol, respectively, for site 1, while for site 2, the values of docking score and glide energy were −6.355 and −59.568 kcal/mol, respectively. There were no major changes in the RMSD and RMSF values during the MD simulation run. The binding affinities using MM-PBSA approach showed the binding affinity of −7.22 kcal/mol for S1, while S2 showed a binding affinity of −9.38 kcal/mol. The binding affinity using SPR showed a very high value of dissociation constant at 4.9×10^{-11} M. The two binding sites have been structurally characterized. These studies showed the wide-spectrum role of lactosmart in antimicrobial defense.

This is the first study in which the generation and purification of a novel antimicrobial fragment of LF termed lactosmart have been described. It is proposed that this molecule should be further investigated and developed as a future antibiotic to combat antimicrobial resistance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JS, PS, and SS conceived and designed the study and drafted the manuscript. JS, PS, SA, VV, and PP performed the experiments and collected the data. PS, SS, TS, and NM analyzed the data. TS drafted the manuscript. JS, SA, VV, PP, NM, TS, PS, and SS approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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