“ ROLE OF LACTOSMART AS A NOVEL THERAPEUTIC AGENT IN ANTIMICROBIAL DEFENSE ”

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE
OF

MASTER OF SCIENCE
IN

BIOTECHNOLOGY

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CERTIFICATE

I hereby certify that the Project dissertation titled " Lactoferrin as a Therapeutic Agent in Oral Diseases '' which is submitted by Km Ambika ,2K19/MSCBIO/35, Department of Biotechnology , Delhi Technological University , Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science ,is a record for the project work carried out by the student under my supervision . To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this university or elsewhere. I further certify that the Publication and indexing information given by the student is correct .

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CANDIDATE'S DECLARATION

I hereby certify that the work which I presented in the Major Project-II entitles " Lactoferrin as a Therapeutic Agent in Oral Diseases '' in fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own, carried out during a period from_____, under the supervision of Pro Jai Gopal Sharma .

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University. The work has been communicated in Scopus indexed journal with the following details:

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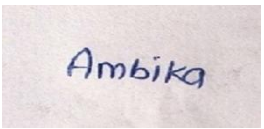

2K19/MSCBIO/35, Km Ambika

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"Only those who risk going too far can possibly find out how far they can go...!''
~ Dr. Walter Bishop

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Km Ambika

Still i rise

Abbreviations

A	Absorbance
A_{260}	Absorbance at 260
AD	Activation domain
ADA	Adenosine deaminase
APS	Ammonium persulfate
ATCC	American type culture collection
Bp	Base pair
bLF	Bovine lactoferrin
CNBr	Cyanogen bromide
CM	Carboxy methyl
cpm	Counts per minute
cm	Centimeter
CBB	Coomassie brilliant blue
cccDNA	Covalently closed circular DNA
$Cacl_2$	Calcium chloride
^{o}C	Degree celsius
DTT	Dithiothrietol

DEAE	Diethylaminoethyl
DBD	DNA binding domain
DMS	Dimethyl sulfate
Da	Dalton
EtBr	Ethidium bromide
ER	Electromagnetic radiation
EB	Elution buffer
g	Gram
GP	Growth pattern
h	Hour
His	Histidine
Hcl	Hydrochloric acid
H2O2	Hydrogen peroxide
IEF	Isoelectric focusing
KD	Kilo dalton
Kcl	Potassium chloride
LB	Luria Bertani medium
LF	Lactoferrin
LPS	Lipopolysaccharide
M	Molar
m	Meter

mm	Milimeter
mg	Miligram
ml	Mililiter
mins	Minutes
mM	Milli molar
MDR	Multi drug resistance
MD	Dynamic stimulations
MIC	Minimum inhibitory concentration
MHB	Muller hinton broth
MBIC	Minimum biofilm inhibitory concentration
Nacl	Sodium chloride
ng	Nano gram
nm	Nanometer
O.D	Optical density
PAGE	Polyacrrylamide gel electrophoresis
PDB	Protein data bank
PAMP	Pathogen associated molecular pattern
rpm	Revolution per minute
RT	Root temperature
r.m.s.f	Root mean square fluctuation
r.m.s.d	Root mean square deviation
SPR	Surface plasmon resonance

SDS	Sodium dodecyl sulfate
TrisCl	Tris (hydroxymethyl) hydrochloride
Tm	Melting temperature
TEMED	N,N,N’,N,’ - tetramethylethylenediamine
v/v	Volume by volume
V	Volt
UV	Ultraviolet
XLD	Xylose lysine deoxycholate agar
YEPD	Yeast extract peptone dextrose
ZOI	Zone of inhibition

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CHAPTER 1

INTRODUCTION

INTRODUCTION

The emergence of multi – drug resistance (MDR) in microorganisms against antibiotics has become a global problem [1,2,3]. Various conventional drugs with promised efficacy and specificity are unable to withstand the threat of antibiotic drug resistance [4,5,6].

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 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 bactericidal and bacteriostatic activities [7,8,9,10,11,12,13] . In the past , several studies have demonstrated the antibacterial and antifungal effects of LF and its derivative peptides, for instance, lactoferricin B [14,15,16,17,18,19] and lactoferrampin [20,21].

Structurally, LF consists of two iron bound lobes, N -lobe (1-333) and C -lobe (345-692) [22,23,24,25]. Amongst the two lobes , the highly cationic properties of N- lobe are responsible for membrane disruption by interacting with anionic components present on bacterial surface [26,27]. It has been established that the lipid A component of the LPS is a known drug target for antimicrobial therapeutics [28,29]. One of the mechanisms by which Lf acts as an antimicrobial agent is through binding to pathogen associated molecular patterns (PAMP) such as Lipopolysaccharide (LPS), thereby disrupting the bacterial membrane integrity and activating the chemical signaling pathway[30-32]. This leads to the secretion of pro- inflammatory responses which

down regulates the release of cytokine production [33,34]. 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 [35-37] .

In the present study , we have performed the partial digestion of LF with trypsin which generates a potent antimicrobial molecule of the size of about 21kDa (85-281). 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 [41]. Our primary focus was on the comparison of LPS binding properties of lactosmart with native LF using surface plasmon resonance technique . The docking and molecular dynamics simulations (MD) 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 which are situated on the N- lobe.

CHAPTER 2

REVIEW OF LITERATURE

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The emergence of multi – drug resistance (MDR) in microorganisms against antibiotics has become a global problem . Various conventional drugs with promised efficacy and specificity are unable to withstand the threat of antibiotic drug resistance .

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RATIONALE

Lactoferrin (LF) is a known antimicrobial protein present in all body secretions. In this study, LF was digested by trypsin, among the hydrolysates, a 21 kDa basic fragment of lactoferrin (termed as Lactosmart) showed promise as a new potent antimicrobial agent. The antimicrobial studies were performed on various microorganisms including -- *Shigella flexineri, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli* as well as fungal pathogens like *Candida albicans, Candida tropicalis*, and *Candida glabrata* . In addition, the lipopolysaccharide (LPS) binding properties of lactosmart were also studied using surface plasmon resonance technique in vitro , along with docking of LPS and molecular dynamics simulation studies.

AIM AND OBJECTIVES

Lactosmart : A novel therapeutic molecule for antimicrobial defense

Objectives:

- ***Lactosmart is a product of tryptic digestion of Lactoferrin.***
- ***Lactosmart can bind to LPS with higher affinity than Lactoferrin.***
- ***It is potent antimicrobial agent against bacteria and fungi.***
- ***Lactosmart can also be used as antibiofilm agent.***

CHAPTER 3

Materials
&
Methods

MATERIALS AND METHODS

Preparation and Purification of Lactosmart

- Bovine lactoferrin was provided from Morinaga Milk Industry Co., Ltd (Japan).
- The powdered lactoferrin was solubilized in 50 mM Tris-HCl pH 7.8 with 0.5M CaCl 2 .
- The trypsin hydrolysis was carried out at a protein: enzyme molar ratio of 50:1 for 30 mins at 37 ℃
- Then the hydrolysis was stopped by adding 50 mM Tris-HCl and the hydrolyzed sample were stored at -20℃ till further use.
- The hydrolysate was subjected to ion exchange chromatography using DEAE in same buffer.
- The unbound fractions were collected as 3 ml fractions in the test tubes and bound protein was eluted with salt gradient of 0 - 0.5 M NaCl.
- Then unbound fractions were concentrated using centricons and subjected to SDS-PAGE analysis.
- The bound fractions were further subjected to gel filtration chromatography using SEPHADEX G-75 column.
- We got three peaks after gel filtration (Fig. 1A).
- All the three peaks were again analyzed by SDS-PAGE.
- The peak corresponding to the molecular weight of 21kDa was desalted and lyophillized for further use (Fig. 1B).

Binding of LPS with Lactosmart

- The binding analysis of LPS with lactosmart was carried out in real time using surface plasmon resonance spectroscopy with Biacore- 3000 (Biacore AB, Uppsala, Sweden).
- For SPR 100 ng of protein was immobilized on CM-5 chip using amine coupling kit provided by the manufacturer.
- All the binding studies were carried out in 10 mM PBS pH7.4 buffer. Various concentrations of the LPS were flown at a flow rate of 30 µl/min.
- Various concentrations of the LPS were flown 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 sensogram 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 dissociation (kd) using the formula kd = kd/ka.
- All the results were subjected fitting .

Antibacterial Studies

Medium, Antimicrobial Agent and Bacterial Strains

- The bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA).
- A differential medium xylose -lysine deoxycholate agar (XLD Agar) was used for the cultivation

of Shigella flexineri Luria Bertani (LB Agar) medium was used for cultivation of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa.*

- Muller 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). And all the chemicals were of molecular biology grade.

Measurement of Minimum Inhibitory Concentration (MIC)

- Minimum inhibitory concentration (MIC) was determined using a standard serial dilution broth method as per the guidelines of CLSI reference document M07-A10 [38] for lactosmart fragment and bovine lactoferrin (bLF) against *S. flexneri, E. coli, S. aureus, P.aeruginosa.*

- These bacterial strains were cultured for 24 hrs.

- After preparation of 0.5 McFarland bacterial suspension in Muller Hinton Broth (MHB) medium, highest to lowest concentration of lactosmart and bovine lactoferrin (bLF) was added in each well of a 96-well microtitre plate.

- The protein concentration in the wells ranged from 2 mg to 0.003 mg.

- After incubation at 37℃ 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. flexineri, E.coli, S. aureus, P. aeruginosa,* was examined.

- Bacteria were cultured at 37˚C, with agitation 200 rpm.

- The culture was grown in Muller Hinton broth (MH broth) to an 0.1 at O. D 600 , and then equally distributed into 96 well microtitre plates (200 µl/well) .
- Then bLF and lactosmart were added to their final MIC concentrations. 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 600nm using microplate spectrophotometer (Epoch, Biotek, USA) at 1 hr interval [39].

Disc Diffusion Method

- The method of Bauer et al [40] was used to check the antimicrobial activity of lactosmart fragment and bLF.
- Bacterial culture of *S. aureus, E. coli, P. aeruginosa and S. Flexineri* were grown at their optimum temperature (37˚C) overnight in a Muller Hinton broth medium.
- Thereafter, the bacteria were diluted to about 10 5 colony forming units (cfu/mL).
- The protein minimum inhibitory concentration (MIC) was loaded onto sterile papers (4mm diameter) and placed on the MHA (Muller Hinton agar) surface. Kanamycin (30 µg/ml) was used as a positive control.
- Plates were incubated at the optimal temperature for each strain for 18-24 hrs.
- The diameter of the bacterial inhibition zone indicated the antibacterial activity.

Inhibition of Biofilm Formation

- The antibiofilm activity of lactoferrin and its fragment lactosmart (85-281) was assessed using the broth microdilution method with slight modification [41].
- The bacterial suspension P. aeruginosa (9027) were grown in MH broth (Muller Hinton broth) supplemented with 1% glucose adjusted to 0.5 Mcfarland standard at 37℃ for 24 hr.
- 100 µl of cells were inoculated in 96-well microtitre plate in the presence of lactosmart and lactoferrin at decreasing concentration from 2 to 0.003 mg/ml.
- The wells containing sterile MH broth supplemented with 1% glucose were considered as the negative control and used as a blank.
- After 24 hr of incubation, bacterial cells were discarded and plate was washed thrice with phosphate buffer saline pH 7.4.
- Fixation of the biofilm was done by adding 150 µl of methanol to the well for 20 min.
- Then it was kept at room temperature for 1 hr so that it gets dried up.
- Then 125 µl of 0.1% solution of crystal violet was added to each well and incubated at room temperature for 30mins.
- Excess dye was removed by submerging the plate thrice in PBS buffer and then left overnight for drying.
- 125 µl of 30% acetic acid was added in each well and plate was read at 550nm .

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, 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

- MIC of the lactosmart against Candida strains was determined by broth dilution method as per the guidelines of CLSI reference document M27-A3 [42] and was defined as the lowest concentration that causes 90% decrease in absorbance in comparison to that of control (withotheut protein).

Growth Pattern

- Candida cells were inoculated into fresh YEPD media.
- Varying concentrations of lactosmart was added to the culture and incubated at 37°C with agitation (200 rpm).

- Aliquots were removed after every two hours and growth was recorded in terms of absorbance at nm using Labomed Inc.
- spectrophotometer (USA) for each concentration and plotted against time in hours.

Agar Disc-Diffusion Assay

- 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 [43].
- For higher concentrations, wells were prepared with the help of a sterile syringe.
- The average diameter of zones of inhibition was measured after 48 h.
- Fluconazole (10 μg/disc) was used as a positive control.

Docking of LPS with Lactosmart

- Docking studies were performed using Schrödinger software.
- The target protein lactosmart was selected and prepared for docking by removing waters and adding hydrogens 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, so blind docking method was used to dock the LPS into lactosmart .

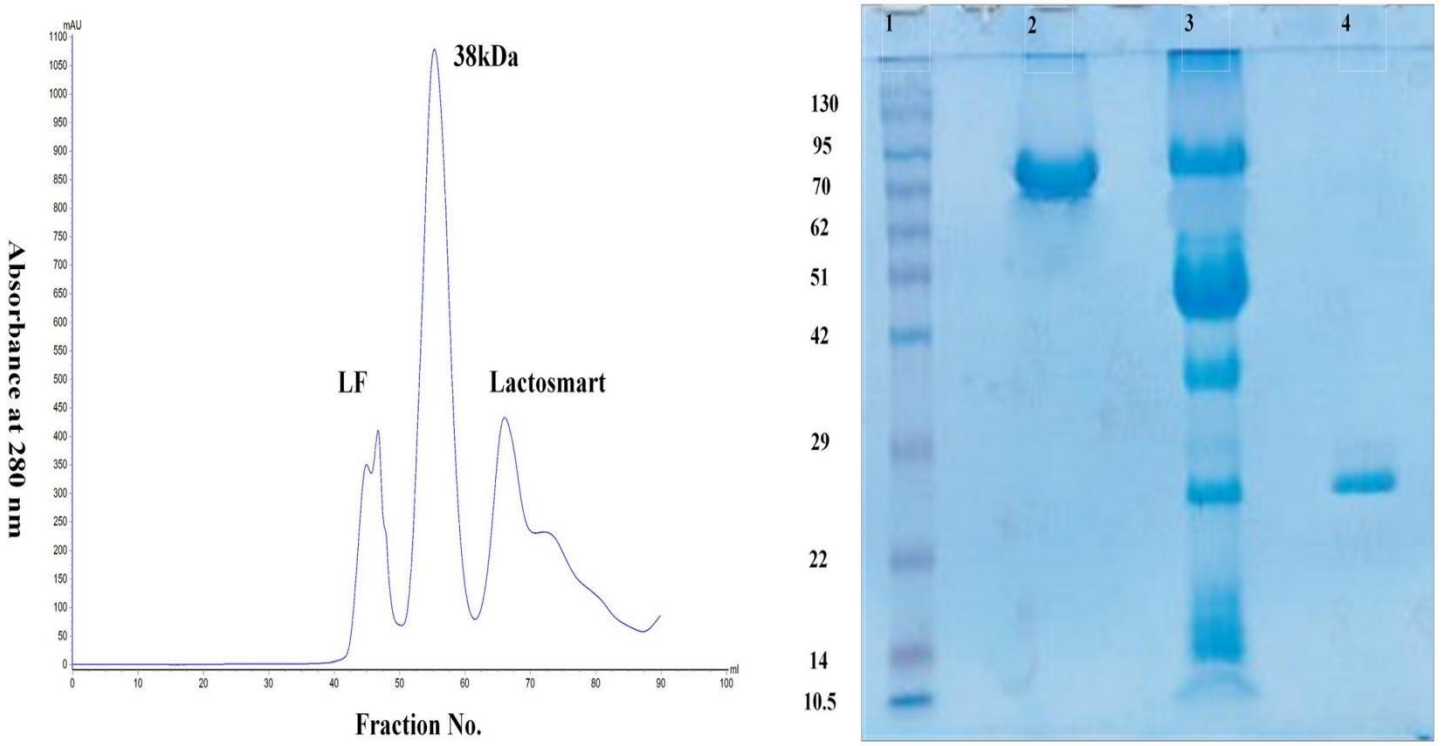
Molecular Dynamics Simulations

- The molecular dynamics simulations of protein-ligand complexes were performed using the AMBER software suite [44].
- The LPS parameters were adopted from the latest version of AMBER compatible GLYCAM_06 force field, which has been thoroughly developed for lipopolysaccharide and related systems [45].
- The protein parameters were taken from the modified ff99SB library [46] 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 ions parameters were taken from the literature [47].
- We used Particle Mesh Ewald (for computing the long-range electrostatics) [48] and periodic boundary conditions along with the SHAKE algorithm (to constraints hydrogens).
- 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 10000 conjugate gradient and 10000 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 molecular dynamics simulations were performed on the systems under consideration.

- We utilized the CPPTRAJ tool [49] in AMBER to process the MD simulation trajectories (to monitor the fluctuations in RMSD, 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 (50).

Figure 1A. Gel filtration profile of trypsin hydrolysate of lactoferrin. Peak 1 indicate the undigested LF, Peak 2 indicate 38kDa fragment and Peak 3 indicate lactosmart.



(A) (B)

Figure 1

Figure 1B. 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.

CHAPTER 4

Results
&
Discussion

RESULTS

Antibacterial Activity and Growth Pattern

The antibacterial activity of the lactosmart was studied and compared with the intact lactoferrin. Minimum inhibitory concentration (MIC) was calculated against four bacterial species namely *S. flexineri, E. coli, P. aeruginosa, and S. aureus*. We found that MIC value of lactosmart against *S. flexineri* 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 the lactoferrin (Table 1). The growth inhibition studies indicate that lactosmart can inhibit *P. aeruginosa* and *S. flexineri* upto 65% at its MIC concentration, while it can inhibit *E.coli* and *S.aureus* upto 70% at their MIC concentration. On the other hand, lactosmart at its double the concentration of MIC value inhibits each strain upto 90% while lactoferrin can only inhibit upto 60% at the same concentration (Fig. 2). As seen from, disc diffusion method is also indicating lactosmart as the better antibacterial agent than the native LF. The diameters of the zones of inhibition in each strain was found to be higher in case of lactosmart (Fig. S1).

Antibiofilm Activity

A widely used crystal violet assay to study the inhibition of the biofilm formation by *P. aeruginosa (9027)* was used. The data for the comparison of the minimum biofilm inhibitory concentration (MBIC) of the lactosmart and LF are shown in (Table 2). The MBIC values for lactosmart and lactoferrin were 0.25 mg and 0.50 mg respectively. This data also indicates the better antibiofilm activity of lactosmart as compared to LF (Fig. 3).

Table 1 : Values of minimum inhibitory concentration (MIC) and zone of inhibition (ZOI) of lactosmart and intact lactoferrin against *E. coli, S. aureus, S. flexineri, and P. aeruginosa.*

Strains	**ATCC**	**MIC (mg/ml)** **LF** **lactosmart**	**ZOI diameter (mm)** **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. flexineri	120222	1.5 0.5	MIC 8 10 2MIC 9 12
P.aeruginosa	9027	1 1	MIC 7 8.5 2MIC 8 11

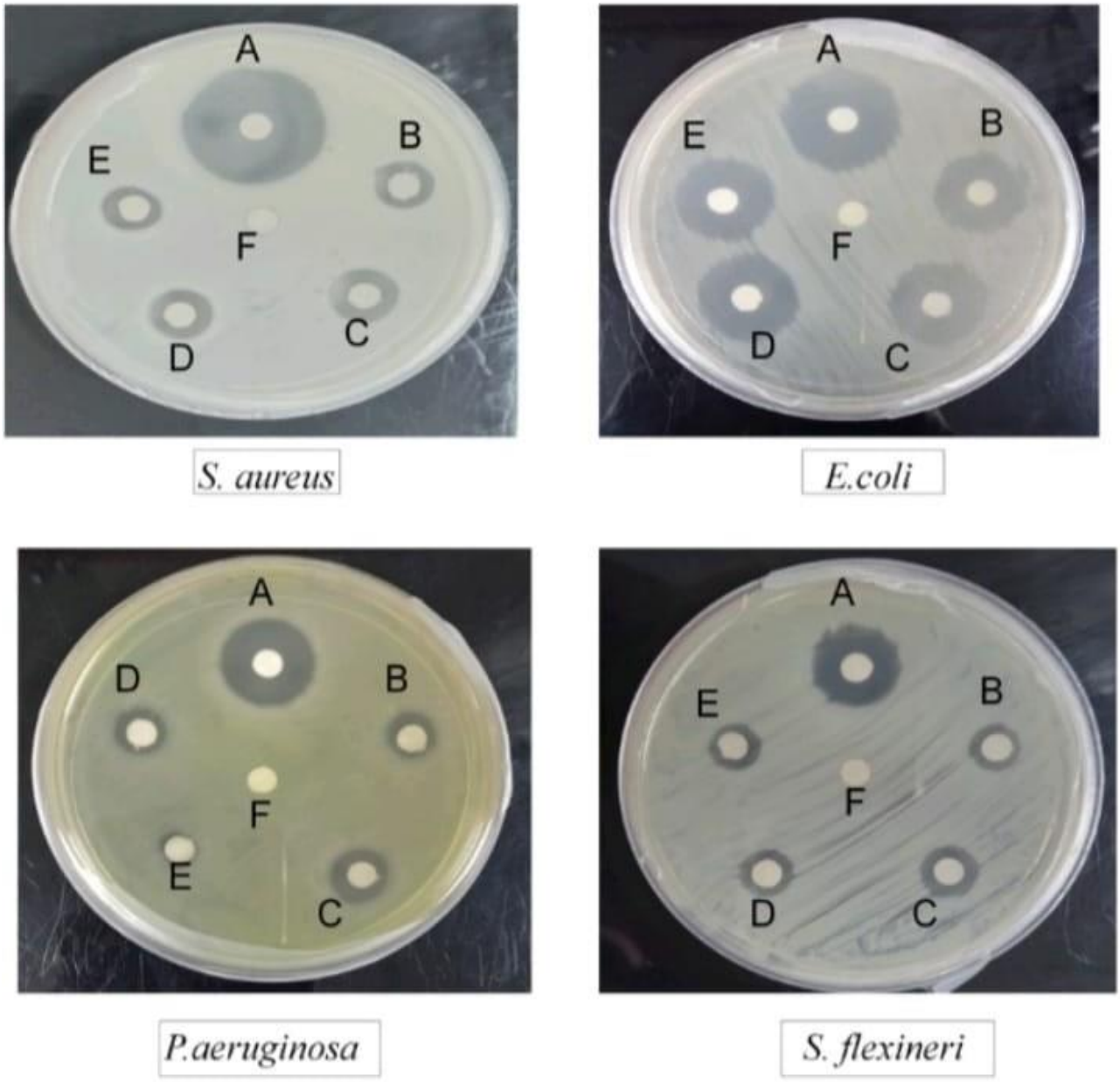


Figure S 1 . Agar disc diffusion assay for *S.aureus* ATCC – 6538P , *E.coli* , *P.Aeruginosa* ATCC-9027 and *S.flexneri* ATCC – 12022 in the presence of test protein at MIC . (A) 30 ug / disc kanamycin were included as positive control , respectively (F) negative control without any agent .

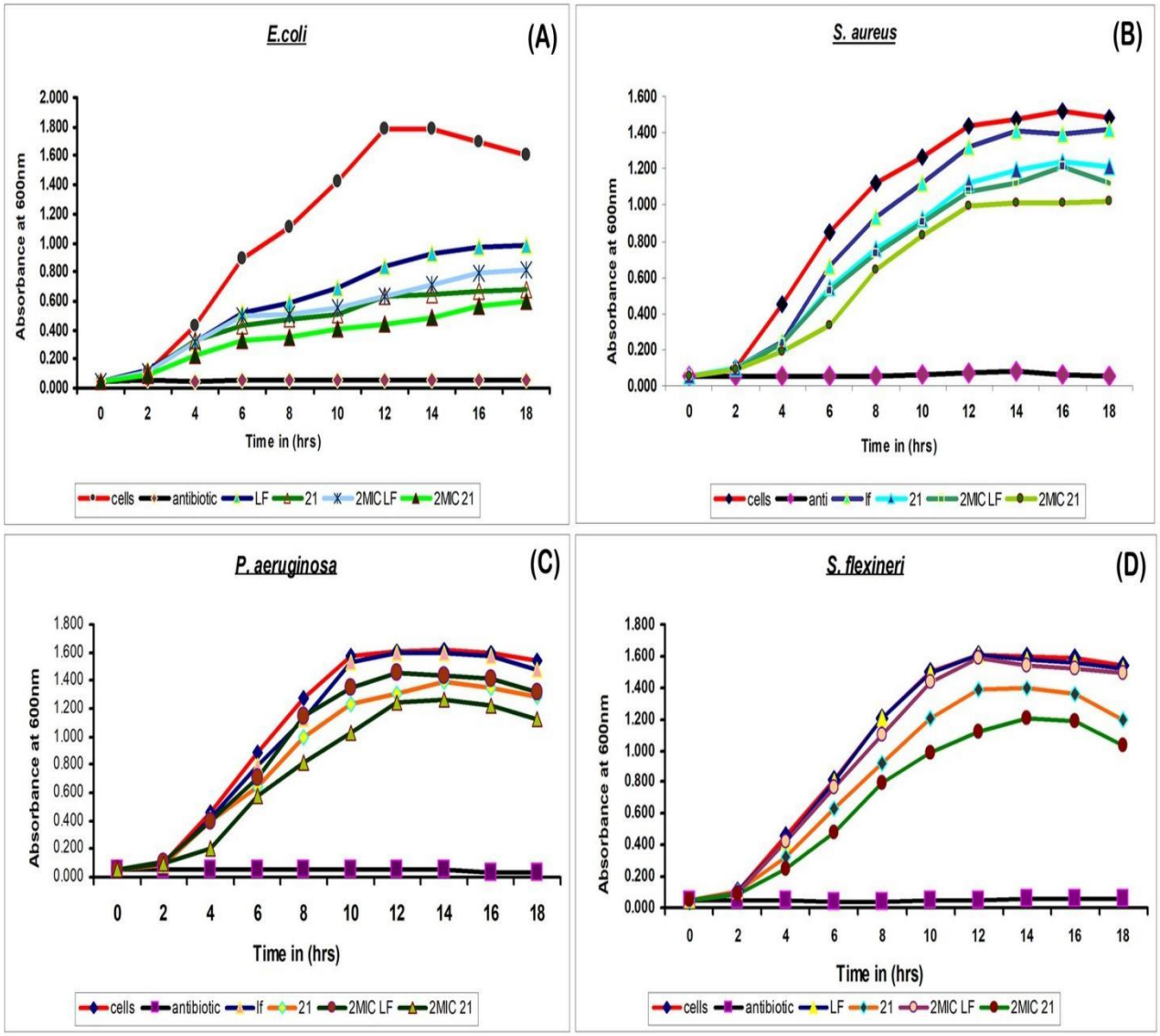


Figure 2

Figure 2. The effects of bLF and lactosmart on the growth pattern of (A) *E. coli* (B) *S. aureus* (C) *P. aeruginosa* and (D) *S. flexineri.* kanamycin was used as a positive control.

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

Table 2: The values of minimum biofilm inhibitory concentration (MBIC) of Lactoferrin and Lactosmart against *P. Aeruginosa.*

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

		2MIC	10
C. tropicalis ATCC 750	5 mg/ml	MIC/2 MIC 2MIC	9 9 10

Table 3: The values of the minimum inhibitory concentration (MIC) and zone of inhibition (ZOI) of lactoferrin against *C. albicans, C. glabrata and C. tropicalis.* The MIC of fluconazole (positive control) was 10 µg/ml and gave ZOI of 25 mm in each strain .

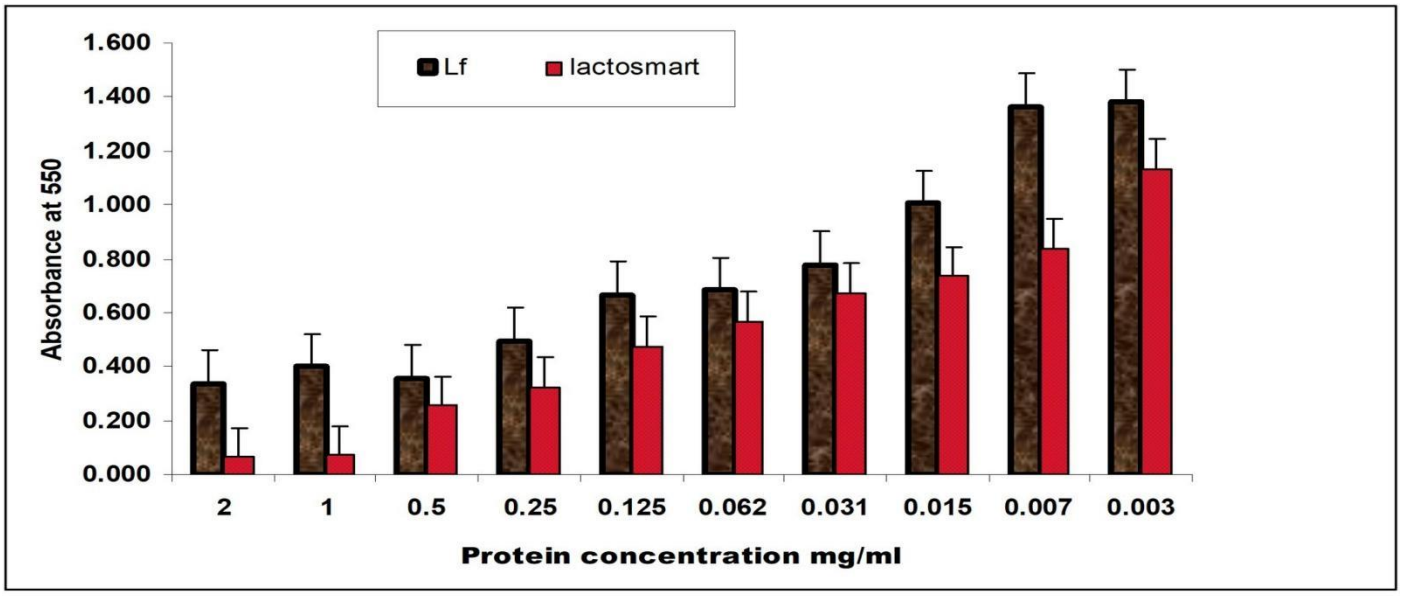


Figure 3

Figure 3. Inhibition of Biofilm formation of *P. aeruginosa* by different concentration of LF and Lactosmart in a decreasing order with 24h incubation at 37℃. Experiments were performed in triplicates and the data were averaged.

Antifungal Susceptibility and Growth Pattern

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. The lactosmart gave MIC of 1 mg/ ml for *Candida albicans,* and 5mg /ml for both *Candida glabrata* and *Candida tropicalis.* Significantly large dose dependen t zones of inhibition (ZOI) were observed in the presence of the lactosmart. At 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 concentration, the ZOI was 12 mm. Non-albicans strains gave a higher MIC value of 5 mg/ml. Also, the ZOI observed at MIC and 2MIC in non-albicans species were not very prominent. For all the three Candida species, fluconazole gave the ZOI of 25 mm at 10 µg/disc (Fig. S2). The ZOI formed in the presence of lactosmart were clear and distinct while those formed in the presence of fluconazole were hazy. Fluconazole is fungistatic [54] and hence hazy zones were expected. Candida growth patterns studied in the presence of the 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 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* (Fig.4) .

Analysis of LPS Binding

The molecular interaction between lactosmart with LPS were studied in real time using **surface plasmon resonance spectroscopy.** The sensogram for the interaction between LF, lactosmart with LPS were recorded (Fig. 5). The increase in the RU units from the baseline indicate the binding of LPS to the immobilized proteins. The value of the dissociation constant (k D) between LPS and lactosmart was calculated to 4.9 × 10 -11 M, whereas the k D values for LPS and LF was estimated to be 3.2 × 10 -8 M.

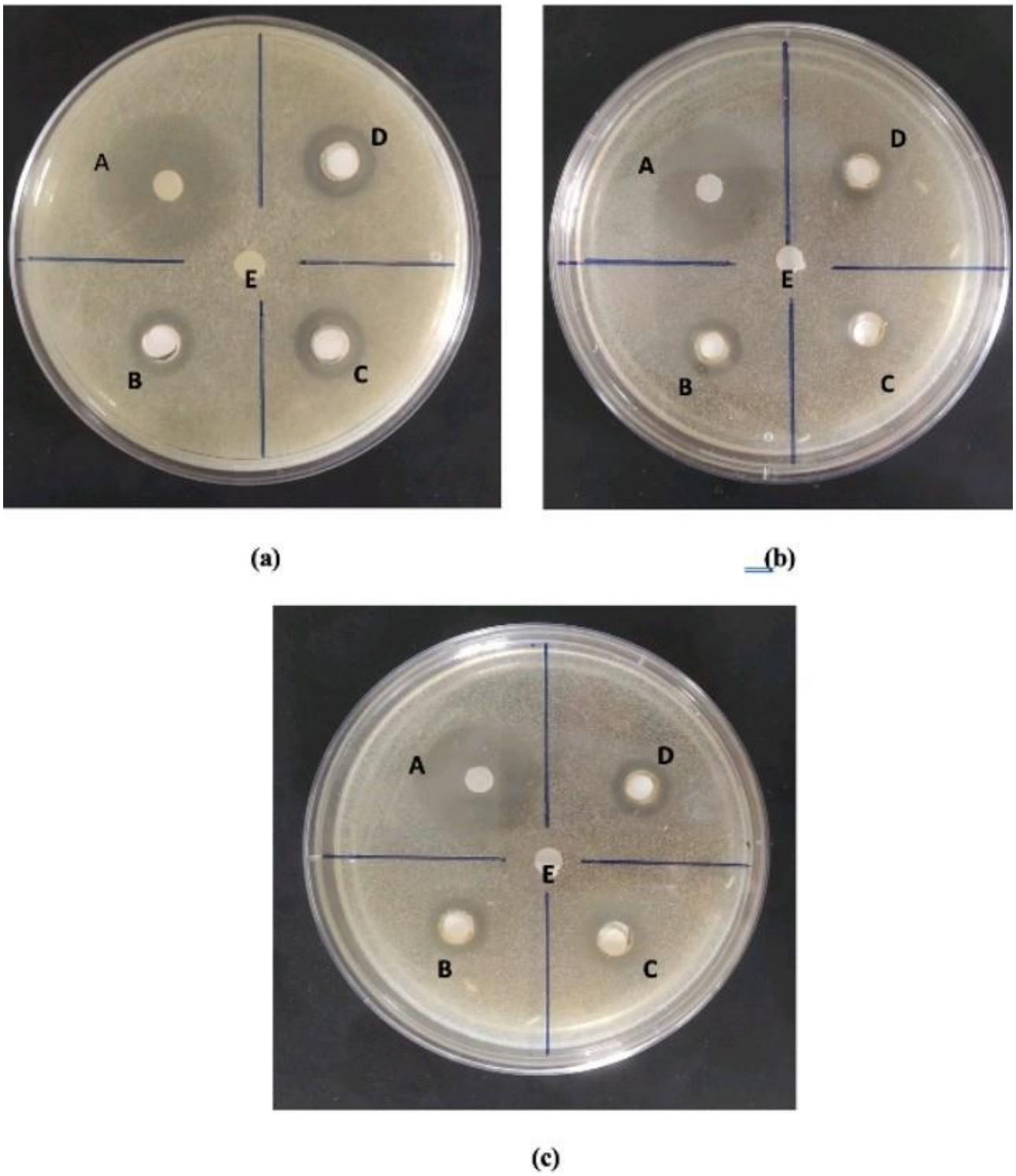


Figure S2 . Agar disc diffusion for (a) *C.albicans* ATCC 5314, (b) *C . glabrata* ATCC 90030 and (C) *C.tropicalis* ATCC 750 in the presence of test protein at MIC/ 2 (B) , MIC (C) , 2MIC (D)ug/disc FLC (A) and only YEPD (E) were included as positive and negative control respectively.

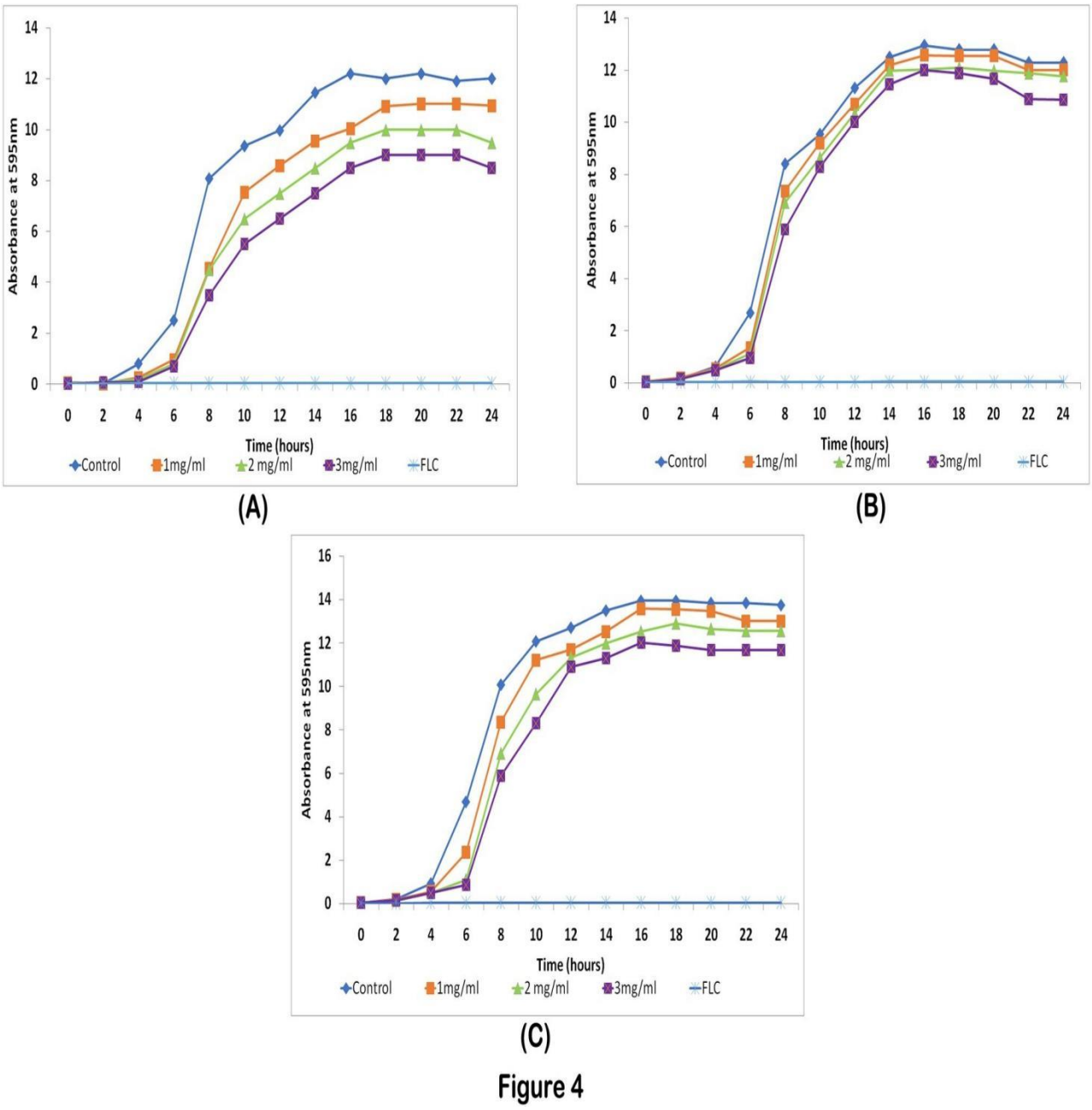


Figure 4. Growth characteristics of (A) *C. albicans* (B) *C. glabrata* and (C) *C. tropicalis* at varying concentrations of test protein (MIC/2, MIC, 2MIC). FLC at its MIC was used as a positive control.

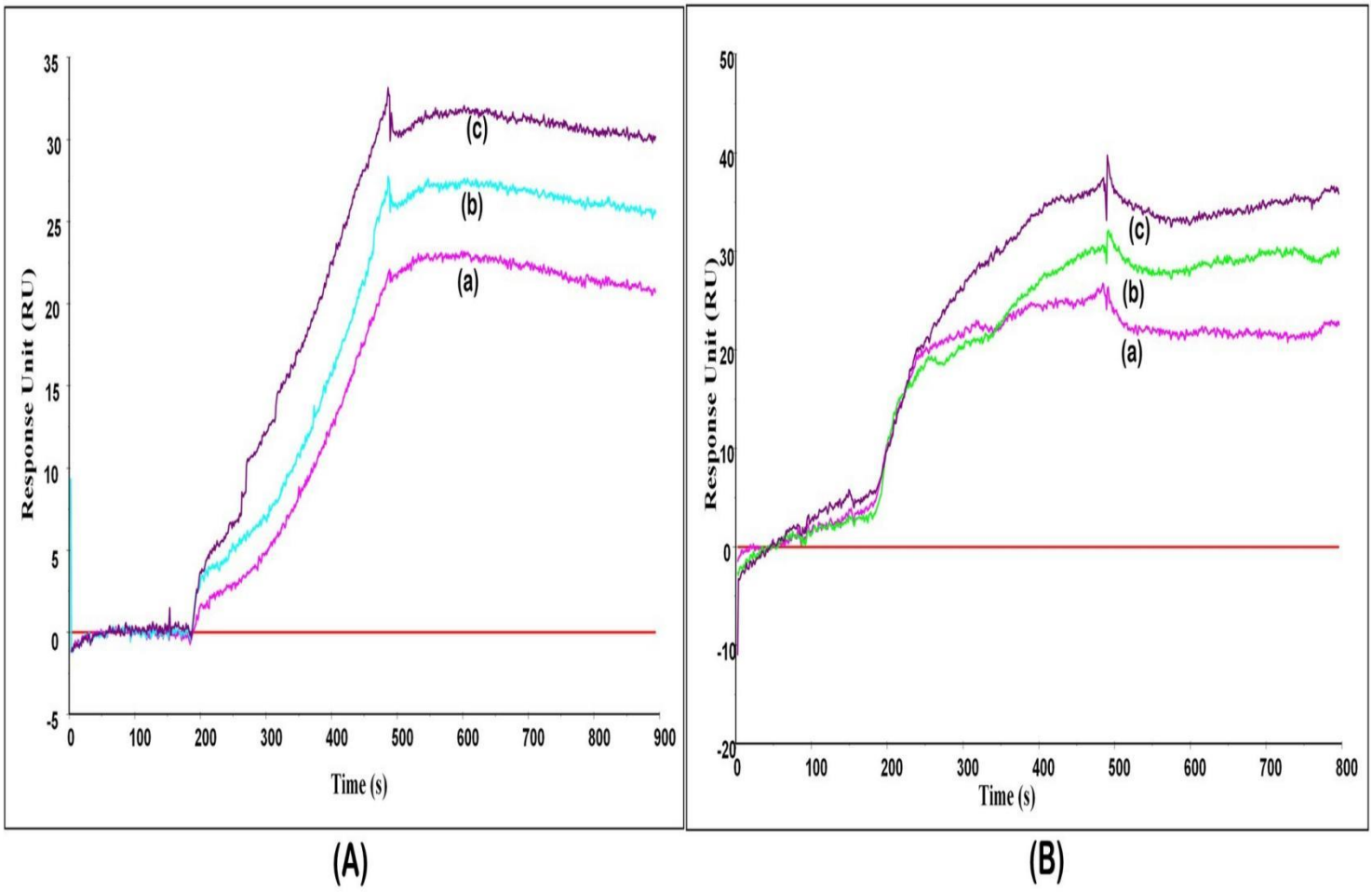


Figure 5

Figure 5. SPR sensogram for the binding of LPS to (A) lactosmart (B) lactoferrin. The protein was immobilized on CM-5 chip and increasing concentrations of the LPS was used in the running buffer in separate experiments corresponding to the curves a, b and c respectively.

Docking Analysis

The docking score and glide energy are -6.292 and -61.069 kcal/mol for the site 1 (S1) position in the Lactosmart. The residues Arg224 act as an acceptor and Asp225, Asp240 act as the donors in the hydrogen interaction (Table 4). It is also forming hydrophobic interactions with the Glu85, Gln87, Arg89, His91, Tyr93, Glu211, Ser212, Phe215, Glu216, Glu221, Pro238, Val239, Lys241, Phe242 and Lys243 residues (Fig. 6A). The site 2 (S2) position docked complex has a docking score and glide energy of -6.061 and -60.459 kcal/mol, respectively. The ligand was interacting with the Tyr92, Thr122 acting as an acceptor and Thr122, Tyr192, Pro251, Ser252 and His253 residues acting as the donor in the hydrogen bond interaction. The Leu119, Arg120, Arg121, Ala123, Gly160, Phe183, Ser191, Ser193, Arg210, Ser212, Thr213, Glu216, Asp217 and Val250 residues were involved in the hydrophobic interactions with the LPS (Fig. 6B).

Analysis of LPS and Lactosmart Complex

All the molecular dynamics simulation trajectories were first analyzed in terms of RMSD and RMSF fluctuations as a function of run-length. We observed that the RMSD fluctuations (Fig. 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 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 Å to 5 Å, suggesting that the ligand probably underwent structural/conformational fluctuations that led to an abrupt change in RMSD for the complex (Fig. 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 (Fig. 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 (Fig. 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

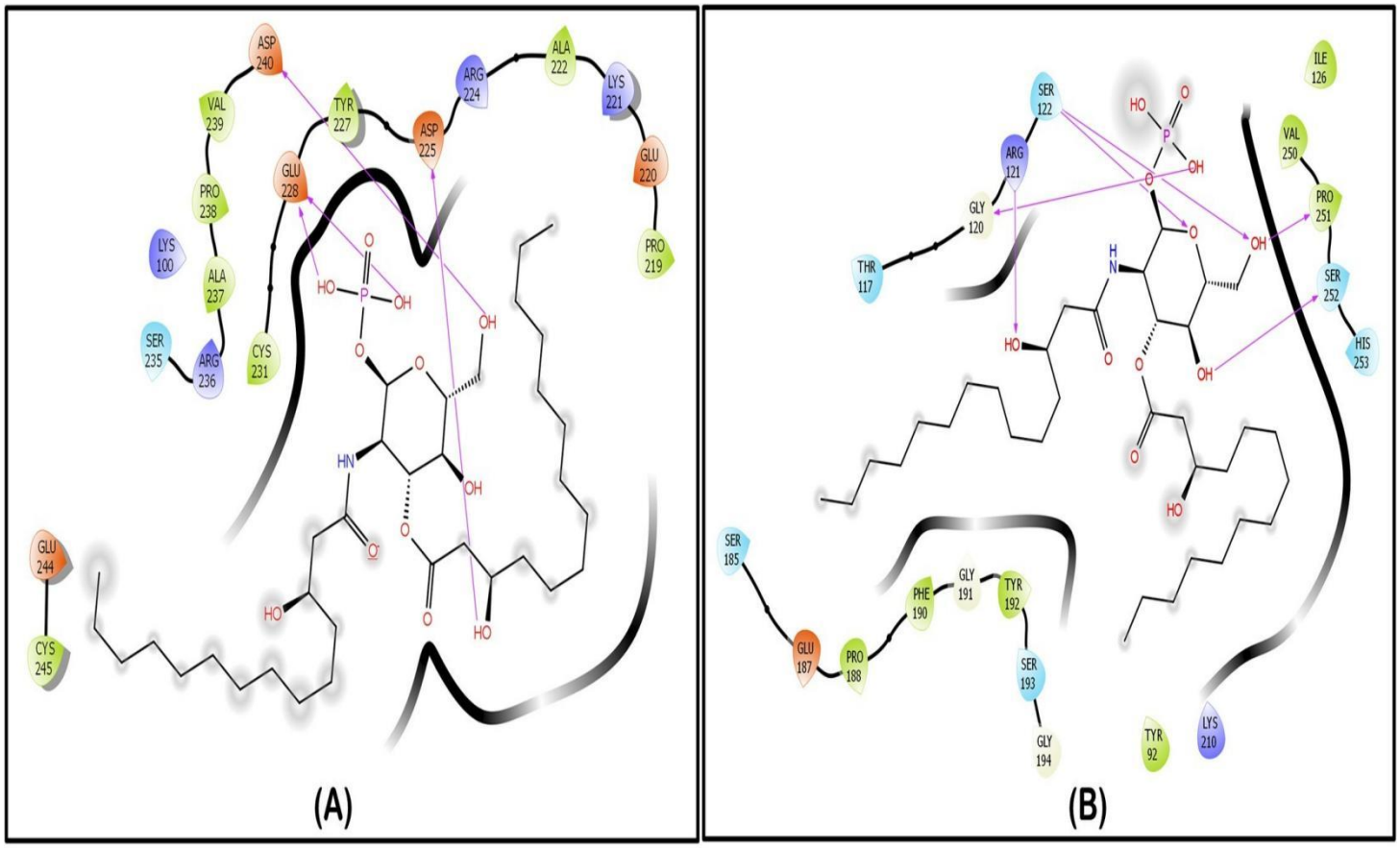


Figure 6

Figure 6. Cartoon diagram showing the binding of LPS in (A) site 1, (B) site 2. The hydrogen bonded interactions are also indicated.

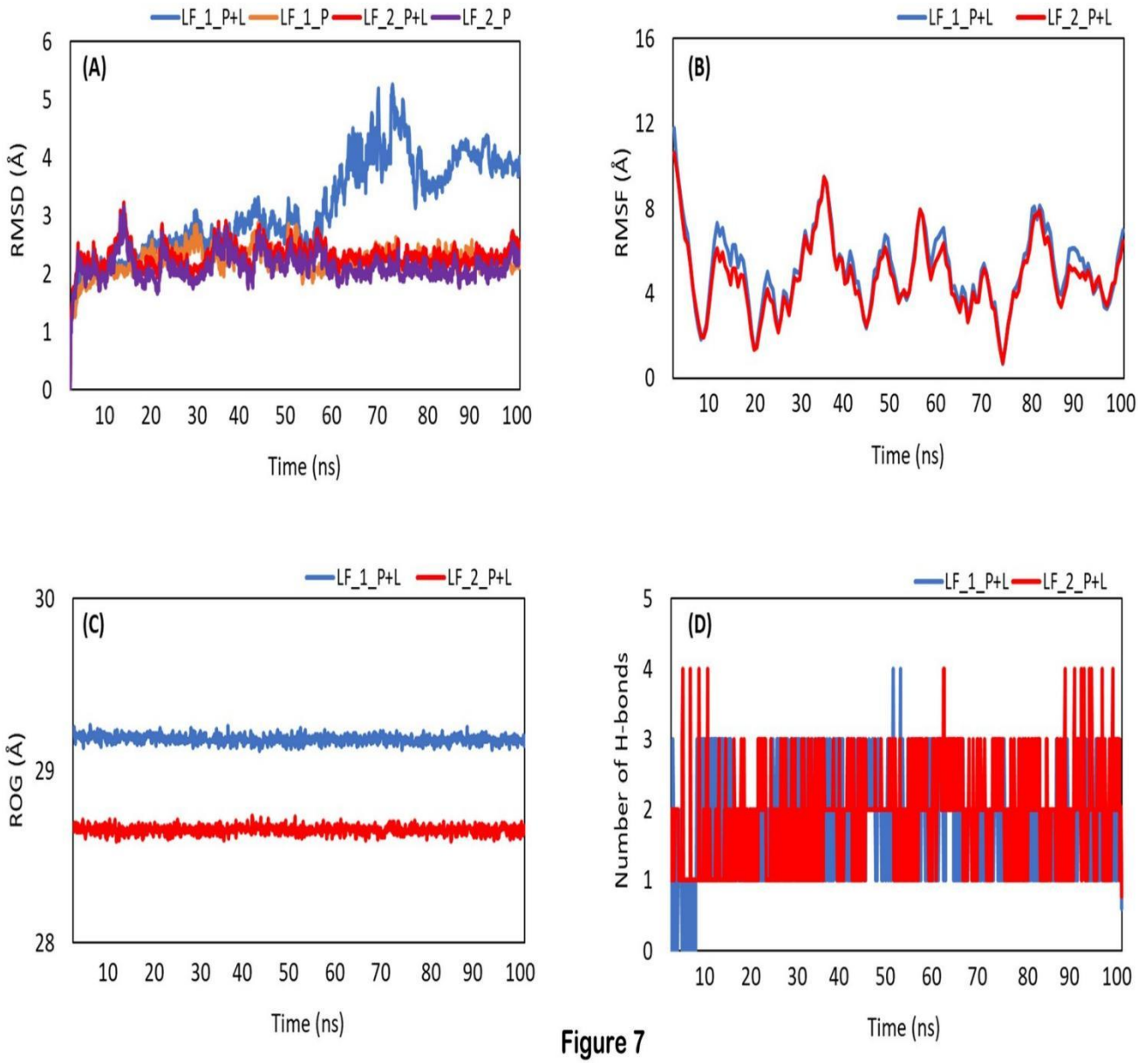


Figure 7. Plots showing the (A) RMSD (B) RMSF (C) Radius of gyration (Rg) and (D) the number of stable hydrogen bond with the function of time. Blue colour indicates the complex of LPS and lactosmart at site 1. Red colour indicates the complex of LPS and lactosmart at site 2.

for LF_1_P+L (site 1) is probably due to RMSD fluctuations for the site 1 complex, as seen in the RMSD profile.

Further, 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 (Fig. 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 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 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 binding of -8.97 ± 0.56 kcal/mol (Fig. 8).

Upon examining the structure of the native bovine lactoferrin, it is clear that the lactosmart molecule is generated by two clean cleavages of the native lactoferrin molecule at the two beta strands e (residues 90-100) and j (residues 247 to 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 e and j respectively.

The lactosmart molecule has two binding sites for LPS, termed as S1 and S2 respectively. The two LPS binding sites are located at the opposite sides of lactosmart molecule Figs. 2. 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, The site S2 can be accessible only after the hydrolysis of LF takes place using trypsin, generating the lactosmart molecule.

The S1 consists of residues of Helix 8a, i strand and the loop between the two beta strands i and j. Its phosphate group is found to be anchored deep inside a spherical, charged groove which consists of three aspartic acids and one glutamic acid

residues Figs. 3. The oxygen atoms of the phosphate group make two hydrogen bonds with Arg224. The LPS molecule further makes tight hydrogen bonds with Asp240,Asp225 and Asp224. Several other hydrophobic interactions further strengthen the LPS binding and are provided by residues Lys100, Pro219 Glu220, Lys221, Ala222, Tyr227, Cys231, Ser235, Arg236, Ala237, Pro238, Val239, Glu244 and Cys245.

The second LPS binding site, S2 consists of three charged residues from the j strand and the loop between the beta strand f and alpha helix 5. Two conformationally significant residues, Pro251 and Gly120 are situated at the ends of the S2, making hydrogen bonds with the LPS molecules. The charged residues which interact with the pho sphate group of the LPS molecule are two serines, Ser122 and Ser252 and one arginine residue, Arg121, which is involved in the iron binding. The hydrophobic arms of the LPS molecule are anchored by hydrophobic interactions provided by Tyr92, Thr117, Ile126, Ser185, Glu187, Pro188, Phe190, Gly191, Tyr192, Ser193, Gly194, Lys210 and Val250.

DISCUSSION

Antibiotic resistance has been identified as a global crisis which is expected to cause a medical catastrophe in the future. We need to urgently address this problem and discover new antimicrobial agents which can be used to fight against this menace.

Lactoferrin is an abundant iron-binding protein which is the part of our innate immune system. In the past, there have been many reports which have established lactoferrin as a potent antimicrobial agent. In this study, a novel hydrolytic molecule from the N-lobe of lactoferrin, lactosmart, has been generated using trypsin.

Lactosmart has been tested against different strains of bacteria namely *E. coli, S. aureus S. flexineri* and *P. aeruginosa.* The MIC values against these strains were 0.03, 0.12, 0.50, 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, 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 the lactosmart against fungal pathogens.

To explore another function of lactosmart, the binding affinity of LPS was studied using docking and molecular dynamics simulations studies in-silico and binding studies using surface plasmon resonance 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 -6.292 and -61.069 kcal/mol respectively for site 1 while for the site 2, the values of docking score and glide energy were -6.061 and -60.459 kcal/mol respectively. There were no major changes in the r.m.s.d and r.m.s.f values during the molecular dynamics simulations run. The binding affinities were calculated using MM-PBSA approach. S1 showed the binding affinity of -7.22 kcal/mol while S2 showed binding affinity of -9.38 kcal/mol. The binding affinity using SPR showed very high value of dissociation constant as 4.9x10 -11 M. The two binding sites have been structurally characterized. These studies showed the wide spectrum role of lactosmart in the antimicrobial defense.

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

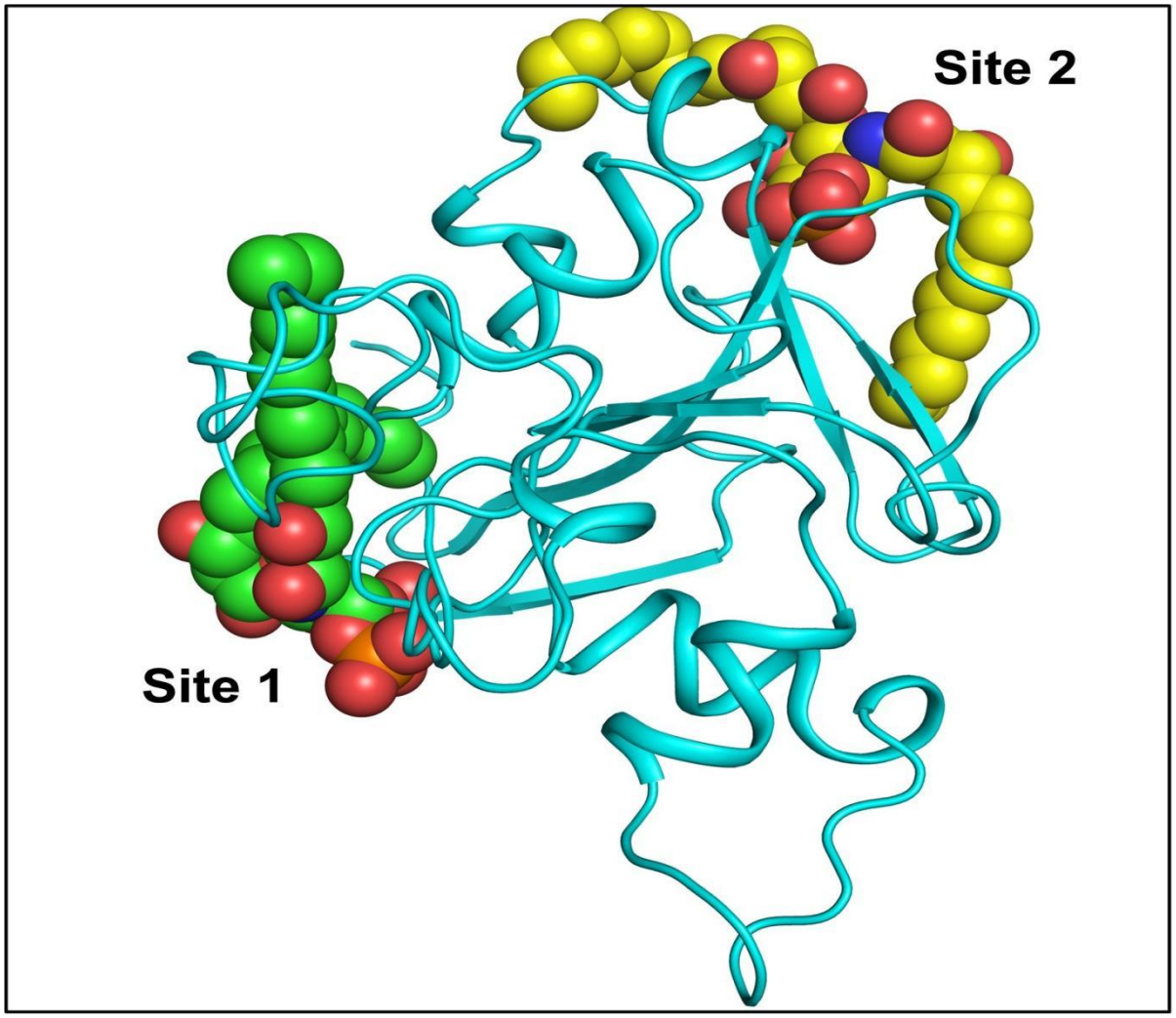


Figure 8

Figure 8 : Cartoon diagram showing the presence of LPS at both the sites.

CHAPTER 5

CONCLUSION

CONCLUSION

The problem of antibiotic resistance has prompted researchers around the globe to search for new antimicrobial agents. Antimicrobial proteins and peptides 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, among the hydrolysates, a 21 kDa basic fragment of lactoferrin (termed as Lactosmart) showed promise as a new potent antimicrobial agent. The antimicrobial studies were performed on various microorganisms including *Shigella flexineri, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* as well as fungal pathogens like *Candida albicans, Candida tropicalis,* and *Candida glabrata* . In addition, the lipopolysaccharide (LPS) binding properties of lactosmart were also studied using surface plasmon resonance technique in vitro, along with docking of LPS and molecular dynamics simulation studies. The results showed that lactosmart had better inhibitory effects against pathogenic microorganisms as compared to LF. The results of docking and MD simulation studies further validated the tighter binding of LPS to lactosmart as compared to LF. Since LPS is an essential and conserved part of the bacterial cell wall, the proinflammatory 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 the antimicrobial defense. **We propose that lactosmart can be further developed as antibacterial, antifungal and antibiofilm agent**.

CHAPTER 6

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