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Efficacy of *Lacticaseibacillus rhamnosus* probiotic strains in treating chromate induced dermatitis

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Chromate induced dermatitis is a significant occupational health concern. Chromate (Cr) resistant *Lacticaseibacillus rhamnosus* strains were isolated from commercial probiotic sachets PREPRO and HiFLORA. Among 13 Cr-resistant bacterial isolates, six were selected based on high chromate resistance at 500 ug/ml. Selected isolates were subjected to biochemical and molecular characterization and in vivo analyses. DPC assay was conducted under controlled conditions in order to determine the reduction potential of the isolated bacteria. The selected isolates were identified as *L. rhamnosus*-L1 (PP493917), *L. rhamnosus*-L2 (PP493918), *L. rhamnosus*-L3 (PP493921) *L. rhamnosus*-L4 (PP493920) *L. rhamnosus*-L8 (PP493922) and *L. rhamnosus*-L12 (PP493923). *Lacticaseibacillus rhamnosus* L1showed highest resistance against Cr (VI) with reduction potential 56%. In vivo experiments were performed to assess the healing effects of isolated bacterial strains on mice skin, with Hematoxylin and Eosin (H&E) staining used to identify severe dermatitis in skin tissue and evaluate therapeutic effects of probiotic strains. The structural determination of flavin reductase protein of *L. rhamnosus* was carried out using bioinformatics tools. These tools predicted the structural-based functional homology of flavin reductase protein in bacterial Cr (VI)-detoxification system. *Lacticaseibacillus rhamnosus* can be effectively used against chromate-induced dermatitis due to its high chromate resistance and reduction potential.

Keywords Eradication treatment, Chromate-associated inflammation, *Lacticaseibacillus rhamnosus*, Flavin reductase, Dermatitis animal studies, Molecular docking

Abbreviations

16S rRNA	16S ribosomal ribonucleic acid
Lacticaseibacillus rhamnosus	*Lacticaseibacillus rhamnosus*
REF	Reference protein
OD	Optical density
PCR	Polymerase chain reaction
chr A	Chromate reductase A
Flavin reductase (F) (R)	Flavin reductase forward and reverse primers

Chromate-induced dermatitis poses a significant occupational and environmental health concern for individuals exposed to chromate compounds in various industrial settings. This condition, varying from mild irritation to severe dermatitis that can hinder quality of life and pose occupational hazards, underscores the importance of gaining a deeper understanding of the underlying mechanisms[1]. To advance preventive and therapeutic strategies, it is crucial to unravel the intricate pathways driving chromate-induced dermatitis. Chromate compounds, commonly found in industrial processes like metal plating, pigment production, and leather tanning, exert cellular toxicity by generating excessive reactive oxidative species and interacting directly with cellular components. It can breach skin barriers to incite multiple inflammatory responses, leading to dermatitis symptoms[2,3]. Probiotics have emerged as a promising experimental remedy for various skin conditions. These beneficial live microorganisms, when administered in sufficient quantities, have the potential to enhance the skin barrier that is compromised by dermatitis. Probiotics have positive effect on human health they can alter the

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function of commensal; microbes, modifying the function of epithelial immune function of host. Dermatitis can occur due to various reasons i.e., food allergen, heavy metals, weak immune system or contact with vulnerable microbe. Probiotics play significant role in treating allergic contact dermatitis and helps in the treatment of anti-allergic therapies[4,5]. Modifying host immune system and skin barrier leads to inhibition of opportunistic pathogens which colonize skin and cause disease[6].

Contact dermatitis triggers when skin comes in contact with a toxic substance causing skin related problems. It can spread rapidly and is prevailing throughout the world because of environmental deterioration. There are two types of contact dermatitis named as irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD). A single exposure of toxic irritant i.e., chromate is enough to trigger cytotoxic effect which develops contact dermatitis. Symptoms include skin burning, irritation, rash, painful fissures and necrosis. The symptoms are observed after few seconds of exposure due to adverse inflammatory reaction. The contact dermatitis is a type IV hypersensitivity reaction. It occurs upon the exposure of low molecular weight chemicals and metal ions such as chromate [Cr] + 6, Nickel [Ni] + 2, Mercury [Hg] + 2 and few others. During allergic reaction both innate and adaptive immune responses occur. During first phase of induction skin sensitization occur when metal allergen i.e., Cr (VI) comes in contact with the skin and penetrates inside the epithelial barrier thus activating effector T cells[7]. In the second phase clinic manifestation and skin damage is observed. Allergens are low molecular weight chemicals which are too small to create immune response, in response to this haptenation process occur. Hapten protein binds with a cutaneous protein through the lipophilic residues which crosses epithelial barrier and form a complex which generates immune response[8]. Innate immunity is then activated in which priming of T cells occur and acquired immunity is stimulated through the production of reactive oxygen species (ROS) and damage associated molecular patterns (DAMPS). Pathogen recognition receptors (PPRs) recognizes DAMPS that resides on antigen presenting dendritic cell surface (DCs) and Langerhans cells (LCs) as Toll like receptors (TLRs)[9,10]. Migrating from epithelial cell layer to lymph nodes where these are processed by dendritic cells and subsequently presented on grooves of MHC (major histocompatiblity complex) which after binding, activated T-cells, CD4 + or CD8-respective antigenic protein determinants[11]. An increase in cytokine and chemokine levels including IL-1ß, IL-8 or even other pro-inflammatory markers such as high levels of type 17 innate lymphoid cells[12,13]. After regulation phase starts different mechanisms occur i.e., antigen clearance that is hapten, for this purpose blood flow increases, metabolization of hapten occurs through regulatory T cells and CD4 + cells. Treg cells help in expansion and priming of specific T cells[14,15].

Patients showing contact dermatitis to chromate and similar toxic chemicals get treated with *Lacticaseibacillus rhamnosus*. *Lacticaseibacillus rhamnosus*stands out for its exceptional ability to diminish chromate levels through a gene, chromate reductase which is present inside the chr operon[16]. Administration of *Lacticaseibacillus rhamnosus* leads to reduction of severe skin reaction. Studies on mice model indicates the probiotic strain *Lacticaseibacillus rhamnosus* reduced levels of immune cells such as interleukins, eosinophils, chemokines which arises in response to allergic reaction[17].

Chromate (Cr (VI)) detoxification in *Lactobacillus* species requires the *chrA* chromate transporter and flavin reductase. Chromate (Cr (VI)) is taken up into the bacterial cell generally by non-specific anion transporters. Once in the cell, Cr (VI) is an extremely potent and mutagenic cancer metal, which generates reactive oxygen species (ROS), results of these reaction might eventually lead to oxidative stress and damage of the cellular system. A flavin reductase is an enzyme that reduces flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) using NAD(P)H as an electron donor. This reduction results in the formation of reduced flavins (FADH2 or FMNH2)[18]. Flavin reductase generates reduced flavins that can directly transfer electrons to Cr (VI), reducing it into the less toxic, trivalent state of chromium (Cr (III)). In *Lacticaseibacillus rhamnosus* two mechanisms are observed; chromate efflux and chromate reduction. Chr A is involved in efflux of Cr (VI) ions out of the cell, reducing its intracellular concentration and providing resistance to bacteria in chromate rich environments[19]. Flavin reductase, however, reduces Cr (VI) to Cr (III) within the cell, decreasing chromate's toxicity[20]. In this study, our aim is to determine the therapeutic potential of *Lacticaseibacillus rhamnosus* strains against Cr induced dermatitis. Flavin reductase directly links with the detoxification of epithelial cells caused by dermatitis. Flavin reductase presence makes *Lacticaseibacillus rhamnosus* a promising probiotic strain to mitigate chromate's harmful effects and focus on eradicating chromate induced dermatitis.

Results

Biochemical and physiological characterization

Biochemical characterization was carried out for the isolated bacterial strains L1, L2, L3, L4, L8 and L12. The strains were tested positive for gram staining and chalk agar test while they were tested negative for catalase test, oxidase test, indole test, citrate test, urease test, H_2S production and pigment production as shown in Table 1. By finding out the ideal growth temperature (Fig. 1), pH (Fig. 2) and sodium chloride concentrations (Fig. 3), physiological characterisation was carried out.

Identification of CrR resistance strains

In order to assess the isolated probiotic bacteria resistance to potassium dichromate ($K_2Cr_2O_7$), their minimum inhibitory concentrations (MICs) were calculated shown in Table 2. Furthermore, the diphenylcarbazide (DPC) assay was performed to evaluate isolated probiotic strains ability to reduce chromate. Reduction potential (%) of the isolated strains was measured mentioned in Table 3.

Antibiotic resistance

Antibiotic resistance in *Lacticaseibacillus rhamnosus* strains L1, L2, L3, L4, L8, and L12 determined as shown in the Table 4. Ampicillin resistance (R) was present in all strains. The susceptibility to clindamycin

Sr No	Tests	L1	L2	L3	L4	L8	L12
1	Gram-staining	+	+	+	+	+	+
2	Catalase test	–	–	–	–	–	–
3	Oxidase test	–	–	–	–	–	–
4	Indole test	–	–	–	–	–	–
5	Citrate test	–	–	–	–	–	–
6	Urease	–	–	–	–	–	–
7	Chalk agar test	+	+	+	+	+	+
8	H2S Production	–	–	–	–	–	–
9	Pigment	–	–	–	–	–	–

Table 1. Biochemical characterization of isolated bacterial strains.

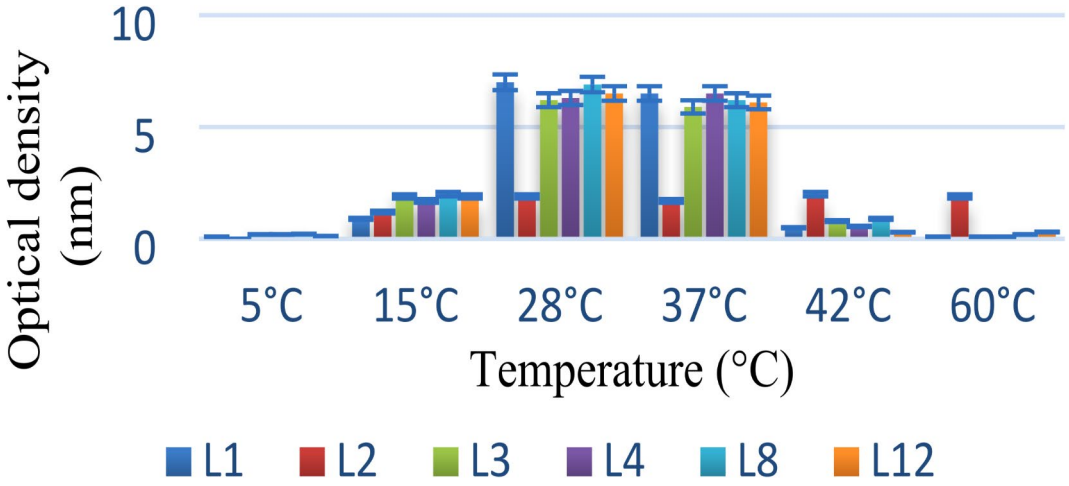

Fig. 1. Determination of optimal growth temperature (°C) of strains L1, L2, L3, L4. L8 and L12.

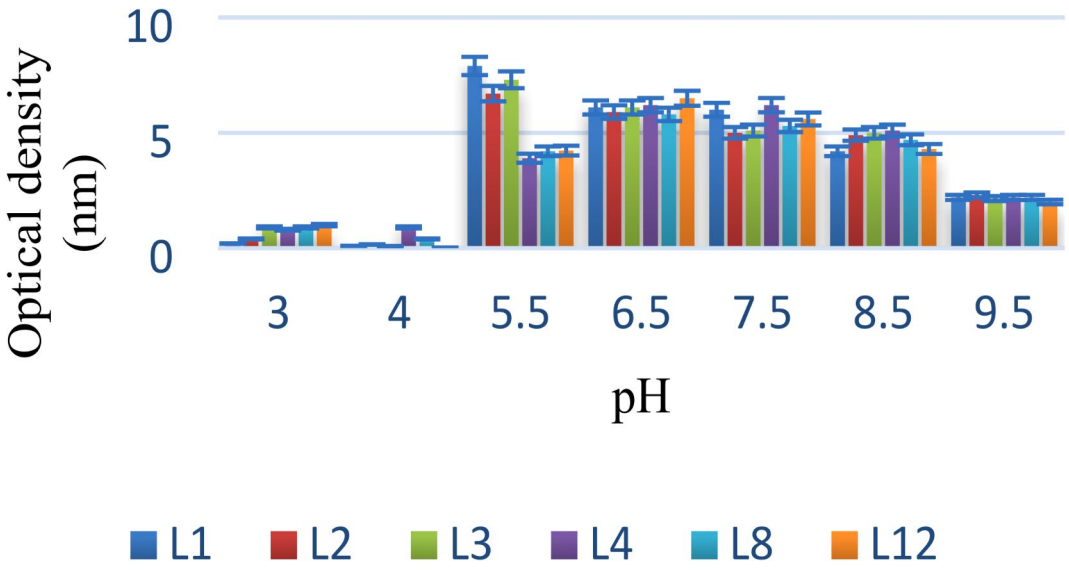

Fig. 2. Resistance to pH variation of strains L1, L2, L3, L4, L8 and L12.

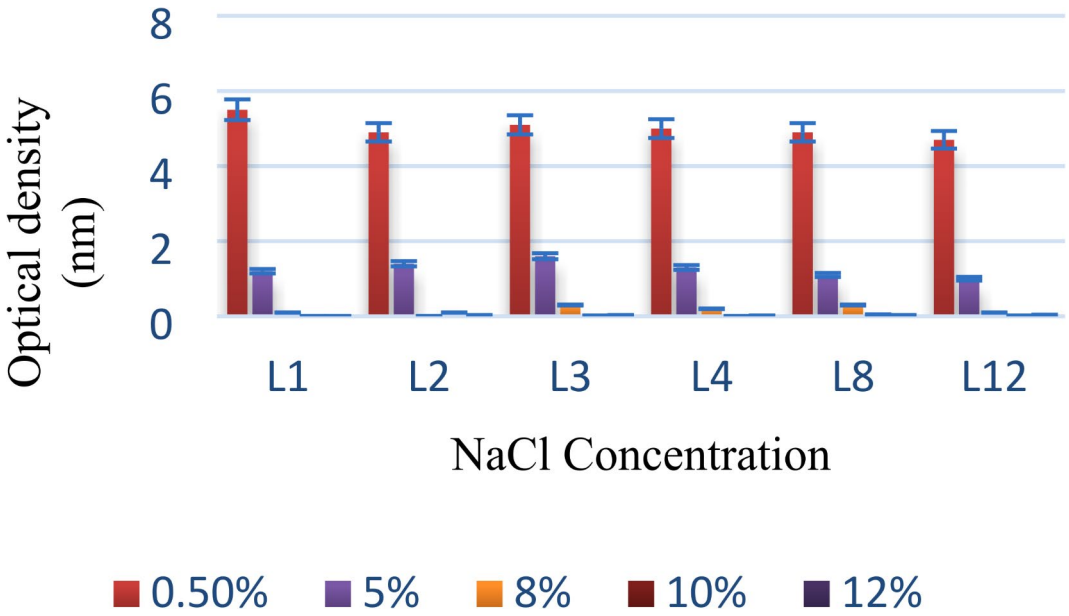

Fig. 3. Examination of optimal growth rate at different NaCl concentrations (%) of strains L1, L2, L3, L4, L8 and L12.

Strain	Concentration of potassium dichromate (ug/ml)			
	500	250	125	62.5
L1	++	++	++	+++
L2	+	++	++	+++
L3	+	+	++	++
L4	+	+	+	+++
L8	W	+	+	+
L9	–	–	–	–
L10	–	–	–	–
L12	W	+	+	+++

Table 2. Minimum inhibitory concentration of cr against probiotic strains. (++) Highly resistant strain, (+) resistant strain, (W) weakly resistant strain showing growth after 48–72 h.

Cr(VI) mg/ml	Reduction potential (%)					
	L1	L2	L3	L4	L8	L12
0.1	56	56.5	48	43	52.1	48
0.3	53	49.3	45	32.4	45	45
0.5	46	46	37.7	29.4	42	37.7
0.7	35.2	39.2	35.2	27	19	31.1

Table 3. Chromate reduction potential (%).

and erythromycin was highest in strains L1 and L12, while several strains exhibited resistance to gentamicin, streptomycin, and tetracycline.

Molecular characterization

"WizPure™ PCR 2X Master Mix" Cat number W1401-5 was used for PCR. The thermal profile for the PCR reaction.

Agarose gel electrophoresis

Gel electrophoresis on a 2% agarose gel was performed as shown in Fig. 4.

Antibiotic	Strains showing inhibition zones					
	L1	L2	L3	L4	L8	L12
Ampicillin	–	–	–	–	–	–
Clindamycin	++	++	–	++	++	++
Erythromycin	++	++	++	++	++	++
Gentamicin	++	–	–	–	–	–
Streptomycin	–	–	++	++	–	–
Tetracycline	++	–	–	–	–	–

Table 4. Antibiotic resistance among different *Lacticaseibacillus rhamnosus* strain. Inhibition zone range ≤ 5 mm: weak, 6–10 mm: Good, 11–20 mm: Very good. Weak (–), good (+) and very good (++)[21].

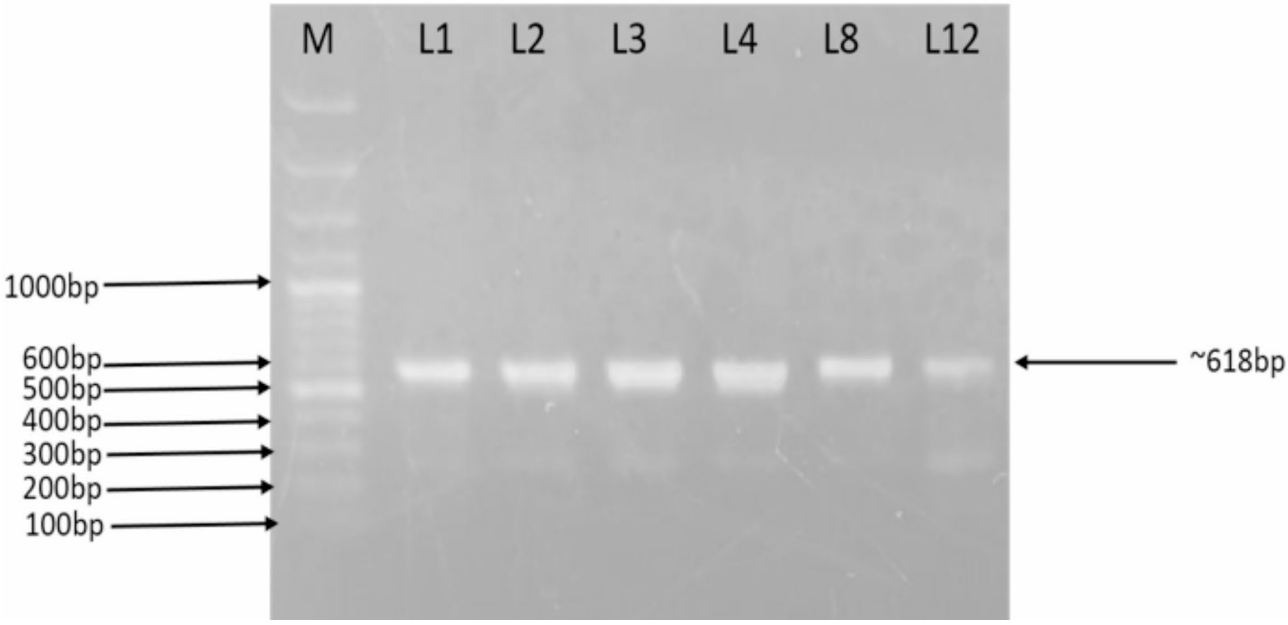

Fig. 4. 2% Agarose gel electrophoresis: Lane M represents Ladder (Thermo Scientific GeneRuler 100 bp Plus DNA Ladder) L1 to L12 represents putative flavin reductase gene.

Bioinformatics analysis

BioEdit

Sequencing chromatogram was obtained from bioedit software (BioEdit 7.2 download (Free) - bioedit.exe (informer.com).

Phylogenetic analysis

Phylogenetic analysis of bacterial species of the *Lactobacillus* on the basis of 16 S rRNA gene.

Phylogenetic analysis revealed that the putative flavin reductase proteinbelongs to the chrR and yieF clade, a specific branch of chromate reductases. Interestingly, this protein shows substantial sequence and structural divergence from other characterized chromate reductases. Furthermore, the amino acid sequence of flavin reductase and REF was paired with other characterized protein sequences of *Bacteroides eggerthii* (PDB:4ICI), *Bacteroides uniformis* (PDB:4J8P), *Bacteroides fragilis* (PDB:3KLB) and *Bacteroides fragilis* (NCBI ID: CAH09789.1). Notable residues (of flavin reductase/REF) 42Ala and 90Leu and 112Asp is highly conserved in all proteins, while some residues like 44Phe, 46Thr and 156Ile exhibit absolute conservation among all proteins.

Protein structure prediction

Physiochemical characterization and domain analysis

Protein family, domain, and motif identification were performed using the InterPro online portal available at (InterPro (ebi.ac.uk)), which combines predictions from multiple signature databases. Amino acid sequence positioned the protein within the FMN-binding split-barrel superfamily and flavin reductase-like domain. This domain organization implies FMN-dependent catalytic activity.

The Expasy ProtParam tool (Expasy - ProtParam) was utilized to determine the protein's physiochemical parameters, including molecular weight (MW), isoelectric point (pI), grand average of hydropathicity (GRAVY),

and instability index as shown in Table 5. These calculations facilitated the prediction of its functional behaviour and stability.

Molecular docking
Structural comparison of flavin reductase and 3hmz was conducted.

Simulation of FMN to flavin reductase and REF protein
Molecular docking simulation of FMN bound to flavin reductase protein, generated using AutoDock Vina. The predicted binding energy is -8.8 kcal/mol, indicating strong affinity (Fig. 9).

Molecular docking simulation of FMN bound to REF protein, generated using AutoDock Vina. The predicted binding energy is -9.1 kcal/mol, indicating strong affinity (Fig. 10).

Animal studies
Histopathology (H&E) results for hexavalent chromium induced allergic contact dermatitis[22].

Histopathological analysis of skin samples shows a progression of chromate heavy metal-induced damage from normal tissue to severe damage. With its intact epidermis, sebaceous glands, and hair follicles, as well as the absence of any granulomas, burns, or infections, the control sample exhibits normal skin architecture. The remodeled skin, on the other hand, shows epidermal growth along with regions of epithelial thinning, destruction, and irregular growth of hair follicles and sebaceous glands. Active remodeling and inflammation are indicated by the dermis's fibrocollagenous tissue, edema, mild chronic inflammatory cell infiltrates, and inflamed granulation tissue.

The chromate-induced samples exhibit the most drastic changes, with the dermis exhibiting severe damage, inflammation, and bleeding, and the epidermis displaying widespread necrosis and desquamation. The results demonstrate how chromate heavy metals harm skin tissue, causing necrosis, inflammation, and serious structural damage[23].

A common occupational hazard for construction workers exposed to wet cement is cement allergy, a type of allergic contact dermatitis. The presence of heavy metal dichromates in cement, specifically hexavalent chromium (Cr(VI)), is the main cause of this condition. One well-known environmental contaminant that has serious health effects is Cr(VI) as stated by Kridin et al., 2016[23]. Through anion transporters, Cr(VI) enters host cell membranes and causes a series of detrimental effects inside the cells. Hossini et al., 2022 reported similar toxic effects of chromate these include DNA damage, which plays a major role in the development of acute inflammatory responses, allergic reactions, and carcinoma, similar affects of chromate were reported by[24]. Interestingly, a number of investigations have shown that some bacterial species can convert Cr(VI) to its less hazardous trivalent form (Cr(III)). Plestenjak et al., 2022 studies different bacterial isolates and their bioremediation potential against hexavalent chromium. It has been suggested that this microbial reduction process could be used to lessen the negative effects of Cr(VI), including the way it can cause irritant dermatitis. These results are consistent with the goal of our study, which was to investigate the potential of probiotic bacterial strains to treat chromate-induced dermatitis Because of their resistance qualities, probiotic bacteria present a viable facultative approach to reducing the harmful effects of heavy metals like chromium. By leveraging their endurance potential, these bacteria can facilitate the transformation of chromium from its more toxic hexavalent form (Cr(VI)) to its less toxic trivalent form (Cr(III)), thereby protecting against cellular deterioration[25].

Abdel-Megeed et al., 2021 stated about probiotics as promising agent for heavy metal detoxification. Among these, *Lactobacillus* strains are widely recognized for their safe application in the dairy and food industries. They are especially effective because of their strong oxidative stress-reducing properties and high capacity to detox heavy metals like chromium. By blocking its absorption and actively keeping it out of cells via particular operons, these strains can regulate the accumulation of chromium. In our study, we selected six strains of *Lacticaseibacillus rhamnosus* to evaluate their efficacy in treating heavy metal-induced dermatitis. These strains were picked because of their proven safety record and ability to reduce oxidative stress[26].

In vivo tests using mice with chromium-induced dermatitis were conducted to assess the ability of these probiotic strains to mitigate allergic reactions and reduce chromium levels. The results underscore the therapeutic potential of *L. rhamnosus* strains in managing chromium-induced cellular damage and allergic conditions, highlighting their role as a safe and effective intervention[27].

Characteristics	REF	Flavin reductase
No of Amino Acids	205	205
Molecular weight	22581.2	22625.32
Theoretical PI	6.51	6.98
No of + ve charges residues	16	17
No of -ve charges residues	17	17
Instability Index	37.26	40.07
Aliphatic Index	113.85	115.87
Gravy	0.145	0.172

Table 5. Physiochemical characteristics of REF and flavin reductase.

This study focused on the potential of probiotics—live microorganisms that provide health benefits when given in sufficient amounts—to reduce chromium toxicity. Quadrant streaking was used to find chromium-resistant strains, and the colonies were then purified and compared. Only six of the eight tested *Lactobacillus* strains—designated L1, L2, L3, L4, L8, and L12—showed resistance to media supplemented with hexavalent chromium as potassium dichromate[28]. These six strains were then chosen for in-depth examination. Biochemical characterization of the isolates was performed using standard test procedures. All strains were confirmed as gram-positive based on gram staining, with no gram-negative strains detected. This observation aligns with prior findings that probiotics are predominantly gram-positive bacteria. Additional biochemical tests, including catalase and oxidase tests, were conducted to further characterize the isolates, confirming their identity as lactic acid bacteria[29]. The capacity of these isolates to produce lactic acid—a characteristic that distinguishes *Lactobacillus* species—was demonstrated by particular tests, such as the use of chalk agar as reported by Salem et al., 2023 where the study discussed about the production of lactic acid on chalk agar confirming that the bacterial isolate under study is a lactic acid bacteria[30]. These strains biochemical traits and resistance to Cr (VI) highlight their potential use in treating illnesses brought on by heavy metal exposure. These results support the hypothesis that *Lactobacillus* strains, particularly those resistant to Cr(VI), are robust candidates for therapeutic interventions aimed at reducing chromium toxicity and its associated health impacts[31]. The presence of clear zones surrounding the growth of the isolated colonies indicated that all six strains tested positive in the chalk agar test. This result validates the strains capacity to generate lactic acid, which is a crucial trait of lactic acid bacteria. Significantly, comparable patterns of activity were noted in earlier assessments, confirming strains dependability and consistency in displaying this characteristic. These results highlight the chosen strains functional potential in applications needing strong lactic acid production.

The chromium resistance of the six chosen probiotic strains against potassium dichromate at concentrations of 500, 250, 125, and 62.5 µg/ml was assessed using the minimum inhibitory concentration (MIC) test. The strains differing degrees of resistance were shown by the results. Across all tested concentrations, strains L1 and L2 demonstrated moderate to good resistance. At 125 µg/ml and 62.5 µg/ml, strain L3 showed good resistance; at higher concentrations (250 µg/ml and 500 µg/ml), moderate resistance was seen. Greater sensitivity to higher chromium concentrations was indicated by strain L4-low inhibition at 125 µg/ml and high inhibition at 62.5 µg/ml. Whereas strain L12 showed a pattern of weak to high resistance as the chromium concentration dropped, strain L8 showed weak to moderate resistance, especially at higher concentrations[32]. Chromium resistance was further examined using a diphenyl carbazide (DPC) assay stated by Lace et al., 2019. It made use of the DPC reagent's capacity to bind unreduced hexavalent chromium (Cr (VI)) and generate a purple hue, which enabled the measurement of Cr (VI) levels. To make the analysis easier, a standard curve was created that connected chromium concentrations with optical density at 540 nm. These results highlight the probiotic strains potential for use in reducing chromium toxicity and offer important insights into their capacity for chromium resistance[33,34].

Significant new information about the genetic and structural characteristics of the flavin reductase protein in relation to the reference (REF) gene from *Lacticaseibacillus rhamnosus* was revealed by the bioinformatics analysis in order to understand the structural homology of protein under study as stated by Amin et al., 2022. The six samples alignment and sequencing showed significant nucleotide changes from the reference sequence, but no distinct mutations were found. Despite multiple mutations, structural analysis revealed that the protein's secondary and tertiary structures only slightly differed, indicating that the protein's global fold and overall integrity were largely maintained. The FMN-binding motif was discovered through functional domain analysis, highlighting its catalytic function in chromium reduction. As seen in Fig. 8, conserved areas inside the binding pockets further emphasized their functional significance. Strong interactions between flavin mononucleotide (FMN) and the reference and variant proteins were found by molecular docking simulations. The REF protein showed slightly higher stability, suggesting its strong catalytic potential, even though both proteins showed high binding affinity. Together, these results highlight the flavin reductase protein's functional robustness and crucial function in chromium detoxification procedures[35].

Even though our study yielded important results, there is room for more research to improve comprehension and application of these findings. The identification and analysis of six chromium-resistant strains of *Lacticaseibacillus rhamnosus* provided a solid basis for future research examining the molecular mechanisms underlying their capacity for chromium detoxification. Furthermore, although in vivo assessments employing mouse models provided insightful information, additional research utilizing human models may be able to more accurately depict the intricacies of chromium-induced dermatitis in clinical settings. A more thorough grasp of these probiotic strains therapeutic potential may also be obtained by investigating their stability and long-term effectiveness in a range of physiological and environmental settings. Lastly, the bioinformatics analyses paved the way for experimental validation to completely clarify the catalytic activity and interaction dynamics of the flavin reductase protein, providing crucial insights into its function in chromium reduction. These directions for further study have the potential to improve the application of probiotics in the treatment of heavy metal toxicity and related illnesses.

Conclusion

*Lacticaseibacillus rhamnosus*strains were used as therapeutic agents for chromate-induced dermatitis. The strains showed resistance to Cr (VI), a high chromate reduction potential, and positive physiological properties, making them promising candidates for treatment of heavy metal induced dermatitis. The research concludes that *Lacticaseibacillus rhamnosus* contains flavin reductase gene making the strain resistant to chromate[36]. Through in vivo studies conducted on mice model it was determined that administration of *Lacticaseibacillus rhamnosus* helped reducing sensitivity and allergic reaction caused by chromate induced contact dermatitis[37]. Our molecular docking investigation concludes by showing that the flavin reductase protein (ΔG = -8.8 kcal/

mol) still displays strong ligand interactions, despite the reference protein (REF) showing somewhat higher stability (ΔG = -9.1 kcal/mol). The flavin reductase protein's binding sites have been shown to be mutated, suggesting increased adaptability and functionality that may have special benefits for its biological functions"[38].

Methods

Isolation of *Lactobacillus rhamnosus*

Commercial probiotic sachets PREPRO (GG) manufactured by Matrix Pharma (PVT.) LTD and Hiflora manufactured by Unicare Pharmaceuticals (PVT.) LTD., containing *Lacticaseibacillus rhamnosus* strains were used. Subcultures were prepared from each isolate after sampling following the serial dilution method. The samples were diluted in 0.9% normal saline in 10-fold serial dilution from 10^{-1} to 10^{-8}. After dilution, 1 mL samples from each dilution were plated onto MRS agar plates and spread over the surface using a sterile glass spreader. The plates were incubated at 37 °C for 24 h in anaerobiosis. Isolated colonies were further purified on MRS agar plates.

Bioremediation potential

In order to assess the resistance of isolated probiotic bacterial strains to potassium dichromate ($K_2Cr_2O_7$), their minimum inhibitory concentrations (MICs) were calculated. Out of the 13 isolated probiotic strains, eight showed growth on selective MRS media used for the growth of lactic acid bacteria. The colonies were further characterized for chromate resisting ability. Out of eight isolates, six strains—designated as L1, L2, L3, L4, L8, and L12—were selected based on their ability to grow in chromium-enriched media. Minimum inhibitory concentrations (MICs) of potassium dichromate were determined for each strain. The strains L1, L2, L3, L4, L8, and L12 showed high to moderate growth at concentrations of potassium dichromate ranging from 62.5 µg/mL to 500 µg/mL. Under comparable circumstances, however, the remaining strains showed no growth. Furthermore, the diphenylcarbazide (DPC) assay was utilized to evaluate chromate reduction potential of the isolated strains. With the use of this test, it was possible to quantify chromate reduction profiles at different $K_2Cr_2O_7$ concentrations and observe the transformation of hexavalent chromium [Cr(VI)] into trivalent chromium [Cr(III)]. After preparing a stock solution of $K_2Cr_2O_7$ (10 mg/mL), 20 ml of MRS broth were inoculated with the bacterial test organisms. The cultures were incubated for 72 h at 37 °C on a shaker set to 150 rpm after being exposed to four different chromate concentrations (100 µg/mL, 300 µg/mL, 500 µg/mL, and 700 µg/mL). After the incubation period, each culture was centrifuged for five minutes at 8,000 rpm to extract 100 µL of the supernatant. Each supernatant sample was combined with 8.5 ml of distilled water, 1 ml of DPC reagent, and a few drops of concentrated H_2SO_4. To allow for color development, the reaction mixtures were incubated for 30 to 40 min at room temperature. Using a spectrophotometer, the absorbance of the reaction products was measured at OD 540 nm and compared to a colorized reference control. This made it possible to quantify the reduction of chromate[34]. *Lacticaseibacillus rhamnosus* ability to reduce chromate was efficiently measured using the DPC test, which also gave important information about the bacterial strain possible use in the bioremediation of hexavalent chromium-contaminated environments.

Reduction was determined by the formula:

$$\text{Chromate reduction } (\%) = [\text{OD of Control of Sample/OD of Control}]$$

Characterization of biochemical and physiological traits

The morphological and biochemical characteristics of Cr resistant bacterial strains L1, L2, L3, L4, L8, and L12 were examined, along with motility tests, to identify them at gene level. These analyses included Gram staining, catalase, oxidase, chalk agar, citrate, and H_2S production assays. By finding out the ideal growth temperature, pH, and sodium chloride concentrations, physiological characterisation was carried out. Following a 24-hour incubation period, the optical density of each strain was determined by measuring at 600 nm. The strains were grown at temperatures of 5 °C, 15 °C, 28 °C, 37 °C, 42 °C, and 60 °C. Furthermore, the strains were incubated in MRS medium at various pH values 3.0, 4.0, 5.5, 6.5, 8.5, and 9.5. Testing basic pH conditions because probiotic lactic acid bacterial strains may come into contact with basic pH conditions in some external environments (such as during industrial fermentation or food processing) or in host systems with different local pH levels. A better understanding of lactic acid bacteria metabolic limits, stress responses, and potential for genetic or functional modification is gained by investigating how they react to basic pH. Lastly, the effects of different sodium chloride concentrations (0.5%, 5.0%, 8.0%, 10.0%, and 12.0%) on growth were evaluated, and the results were recorded.

Molecular characterization and analysis: DNA extraction, primer design, PCR, and gel electrophoresis

After additional analysis it was determined that these strains were resistant to high chromate concentrations. Genomic DNA from the isolates was isolated. Single colonies from the master plate bearing the labels L1, L2, L3, L4, L8, and L12 were selected and subjected to processing with the GeneAll® ExgeneTM kit (cat number 106 – 101) in order to isolate DNA[39]. Primers were designed by MACROGEN and were developed in accordance with the reference nucleotide sequence of *Lacticaseibacillus rhamnosus*, a member of the flavin reductase family protein, which is accessible in the NCBI database under accession number NZ_CP040780.1.

Polymerase chain reaction (PCR) was carried out. The procedure was divided into five steps; denaturation, annealing, extension, cycling and final extension. The double-stranded DNA was denatured into single strands by heating the reaction mixture to 95°C for 30 seconds. To enable the primers to anneal to their complementary sequences on the single-stranded DNA templates, the temperature was lowered to 55°C for 30 seconds. The following primer sequences were used during annealing; flavin reductase (5' ATGATTAACCTATCTGCCAG

3') and flavin reductase (5' TTATTTTGGTCGCCGCAATG 3') Taq DNA polymerase extended the primers (by creating new DNA strands in the 5' to 3' direction while the temperature was raised to 72 °C for one minute. The target DNA was amplified exponentially in 30 cycles. To make sure DNA strand synthesis was finished, a final extension was carried out for five minutes at 72 °C. To preserve the amplified DNA for later use, the reaction was maintained at 4 °C[40]. An electrophoresis on a 2% agarose gel was performed.

Bioinformatics analysis

Sequencing was done for bioinformatics analysis by first creating a 50 µl PCR reaction. The entire sample was put onto an agarose gel and run at 70 V for 45 min after the PCR was finished. After being purified with the WizPrepTM Gel/PCR Purification Mini Kit (Cat# W70150-300), the putative flavin reductase genes were shipped to Macrogen, Korea, for sequencing. FASTA and AB1 files were produced as the sequencing results, and BioEdit software was used to evaluate the AB1 files. FASTA sequences were obtained following analysis, and NCBI BLAST was performed for comparison. Using ClustalW for multiple sequence alignment and MEGA-X software (ver.05) for phylogenetic tree construction, a phylogenetic study based on 16 S rRNA shown in Fig. 5 and flavin reductase genes shown in Fig. 6 was carried out[41]. When compared to the reference sequence (NZ_CP040780.1), mutational analysis revealed many mutations (C121A, C144T, C200T, C264T, A276G, G339A, A366G, C532A, T546C, T552C, C555A, T570C) but no novel mutations in any of the six sequenced samples (L1, L2, L3, L4, L8, L12). The Expasy translate tool was utilized for primary structure prediction, which involved predicting amino acid sequences from the nucleotide sequences of the flavin reductase and REF genes[42]. Clustal Omega was used to align the amino acid sequence, and ColabFold v1.5.5: AlphaFold2 and UCSF Chimera were used to produce the secondary and tertiary structure predictions of flavin reductase and REF gene as shown in Figs. 7 and 8, resulting in pdb files for additional analysis. Domain representation of both the proteins were seen for modeling and organizing biological data to enable effective analysis, interpretation, and computational processing as shown in Fig. 11. Using the InterPro portal, physiochemical characterization and structural comparison for both the proteins under study were carried out as shown in Fig. 9. The protein was classified into the FMN-binding split-barrel superfamily, suggesting that FMN-dependent catalytic activity was present[43].

Additionally, by aligning the sequences of flavin reductase and REF with proteins from Bacteroides species, comparative analysis revealed residues that were both varied and conserved. Using homology-based techniques and structural comparison with the flavin reductase protein (PDB ID: 3HMZ), binding site prediction was carried out. Chimera software was used to identify important motifs and active sites. AutoDock Vina was used to perform molecular docking of flavin mononucleotide and flavin reductase as shown in Figs. 10 and 11. Simulations for the binding of flavin mononucleotide (FMN), which revealed strong ligand interactions for both flavin reductase (ΔG = -8.8 kcal/mol) and REF protein (ΔG = -9.1 kcal/mol) as shown in Figs. 12 and 13 respectively. Due to changes in the binding locations, the flavin reductase protein showed increased flexibility in ligand binding, whereas the reference protein showed slightly higher stability[44].

Animal studies

To determine the healing effects of allergic contact dermatitis, mice model was designed. In vivo analysis was carried on mice owned by Institute of Microbiology and Molecular Genetics, University of the Punjab. Based on weight of the mice the study used combination of xylacine and ketamine, a safe and effective combination which was administrated intraperitoneally. In surgical procedure, ketamine and xylocaine were administered in required amount depending on the weight of mice (grams) as shown in Table 6. Six albino mice (30–33 g) of either sex on Swiss background (Swiss strain) were acclimatized to laboratory conditions in Departmental animal house in the context of chromate induced dermatitis[44]. The anesthetic caused the mice to remain unconscious for half an hour.

Hair from abdominal region was depilated before application of 1.5% potassium dichromate in petrolatum. For dose preparation 0.75 g of $K_2Cr_2O_7$ was mixed with 50 g petroleum jelly and heated for about a minute in microwave to form a fine mixture. Immobilization for manipulation required an intraperitoneal injection of 250 µL of anesthesia. Mice weighing 30 g was considered as reference weight and according to this weight aliquots of anesthesia dose were prepared as mentioned in Table 6. Anesthesia led to mice immobilization for about 30 min. The induction of dermatitis consisted in applying mixture of chromates to the skin and bandaging the region. Mice were checked 7 days post exposure to ensure the onset of dermatitis as shown in Fig. 14. According to reported literature, *Lacticaseibacillus rhamnosus* has significant role in treating chromate induced dermatitis[44]. In order to treat chromate induced dermatitis in mice 24 h fresh bacterial culture broth was prepared.173µL from broth was taken and mixed with 40 ml PBS diluent. The probiotic strain was injected in mice. The isolated probiotic strains showed better healing effects on dermatitis within 7 days of administration.

Anesthetic used

Ketamine and Xylocaine.

Rationale

This approach offers a regulated and repeatable model to evaluate *Lacticaseibacillus rhamnosus* effectiveness in treating chromate-induced dermatitis. Standardized dermatitis induction, calibrated probiotic dosage, and well-established anesthesia methods guarantee dependable outcomes while preserving animal safety.

Euthanasia method

Anesthesia was administered to the mice prior to sacrifice, and euthanasia was performed using a dissection kit. Following euthanasia, skin samples were collected and sent for histopathological analysis. Ketamine/Xylocaine mixture was intraperitoneally injected with a sterile syringe. The amount of anesthesia used was determined

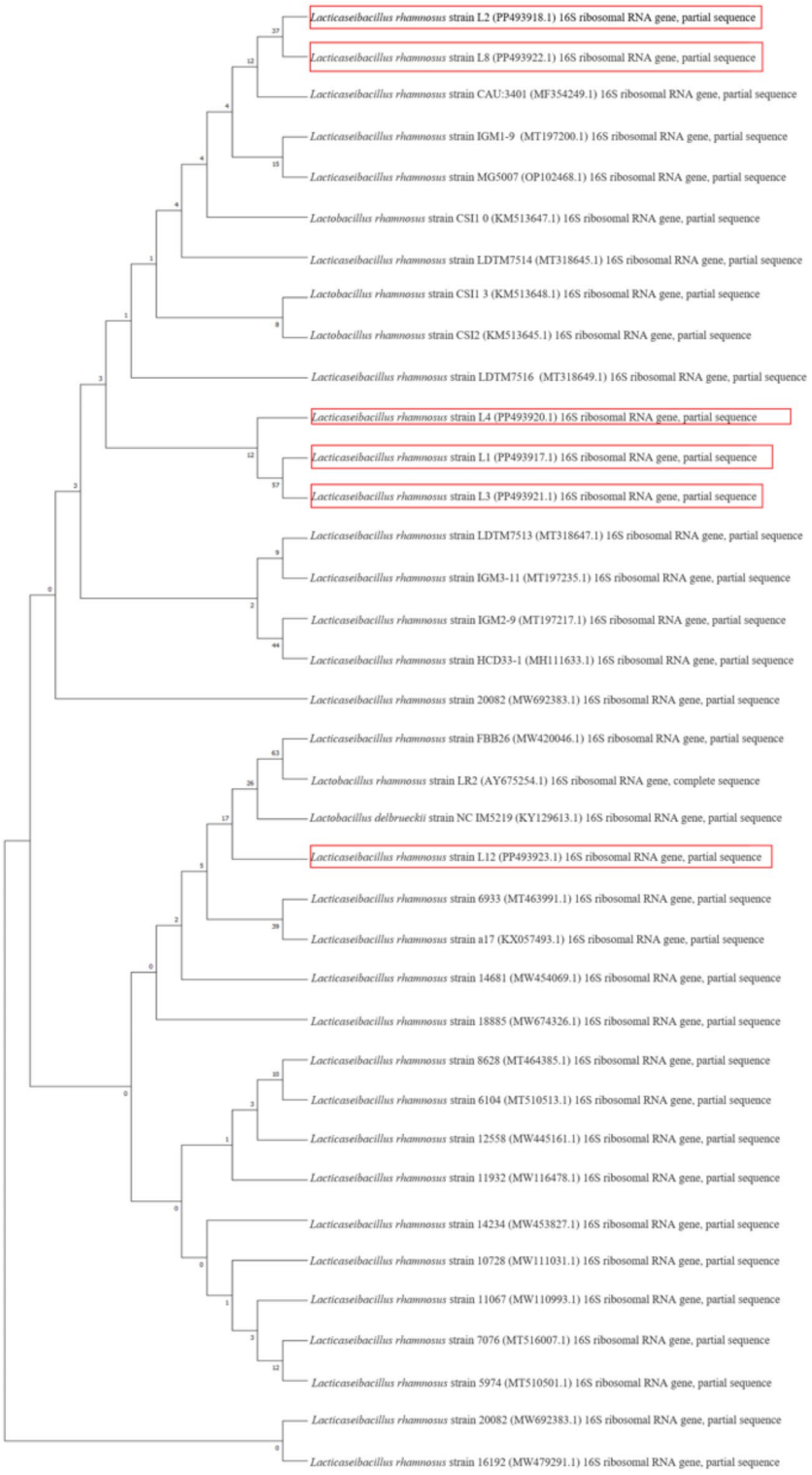

Fig. 5. Phylogenetic tree constructed from MEGA-X through neighbor joining method.

according to the mouse body weight as mentioned in Table 6. Anesthesia was given, it took 5–10 min for the anesthetic to take effect. The absence of a response to toe or tail pinch stimuli confirmed the efficacy of the anesthesia in the mice. Throughout the procedure, the respiratory rate, heart rate, and reflexes of the mice were closely monitored. The mice were carefully assessed to ensure they were fully anesthetized before proceeding with euthanasia.

Using sterile scissors and forceps, an incision was made along the abdominal midline and extended to the desired area of skin for collection. The skin was carefully separated from the underlying tissues using blunt

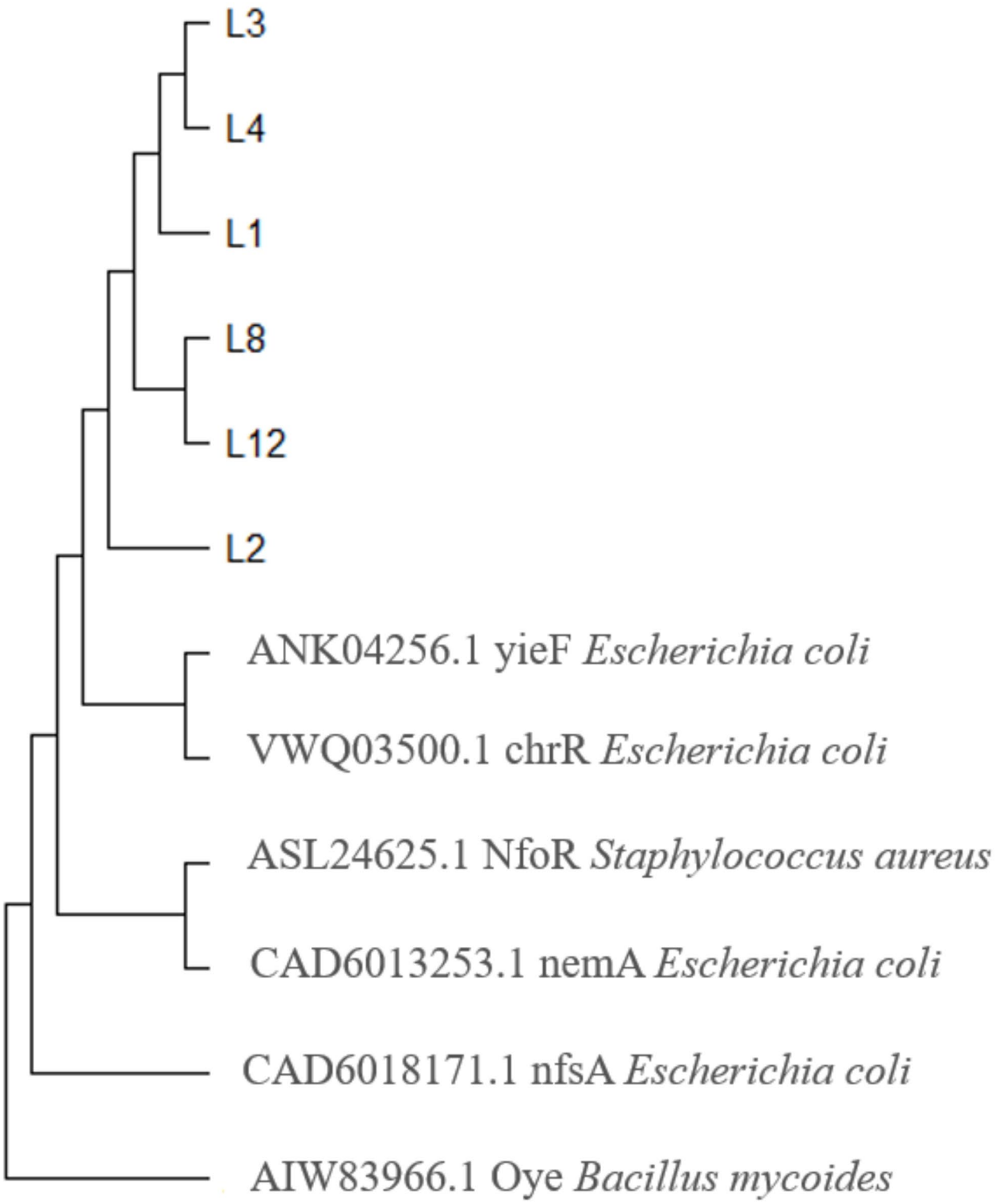

Fig. 6. Phylogenetic tree showing association between flavin reductase and different classes of chromate reductase.

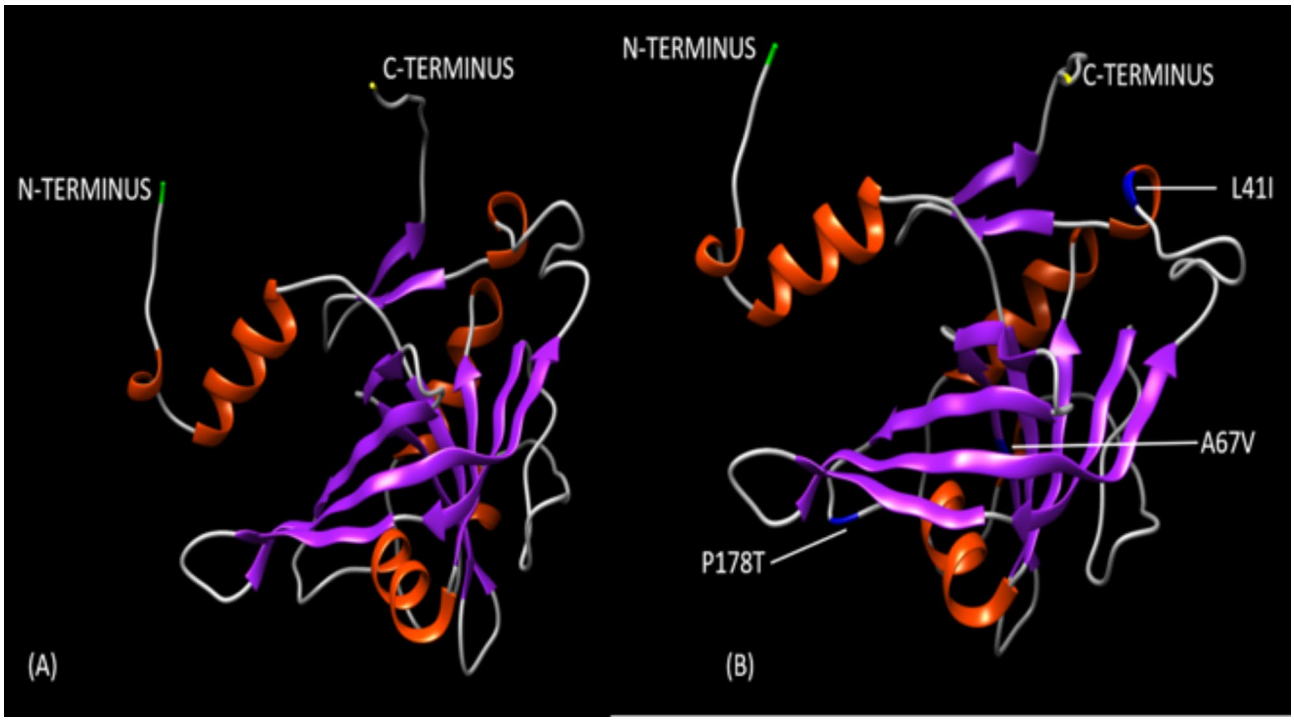

Fig. 7. (**A**) Tertiary structure of REF: (**B**) tertiary structure of flavin reductase (Mutations sites are highlighted in blue).

Fig. 8. Superimposed tertiary structure of "REF" in red colour and "flavin reductase" in blue colour.

dissection. Skin samples, approximately 1 × 1 cm, were collected from the area of interest. The excised skin samples were immediately transferred to labeled sterilized falcon tubes containing 15 ml of 10% formalin for histological examination.

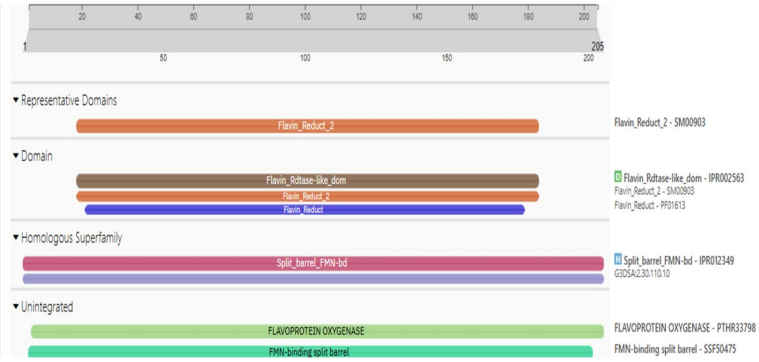

Fig. 9. Domain representation for the targeted protein.

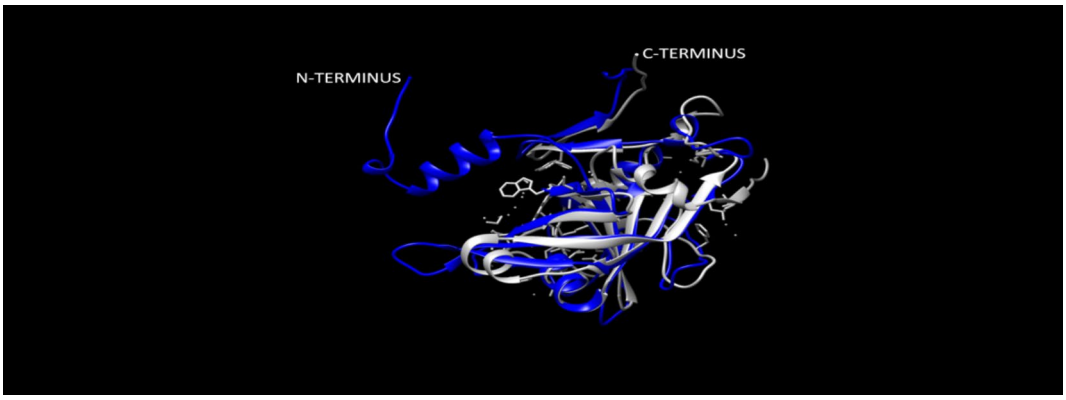

Fig. 10. Structural comparisons of flavin reductase (Blue colour) and 3hmz (Grey Colour) proteins.

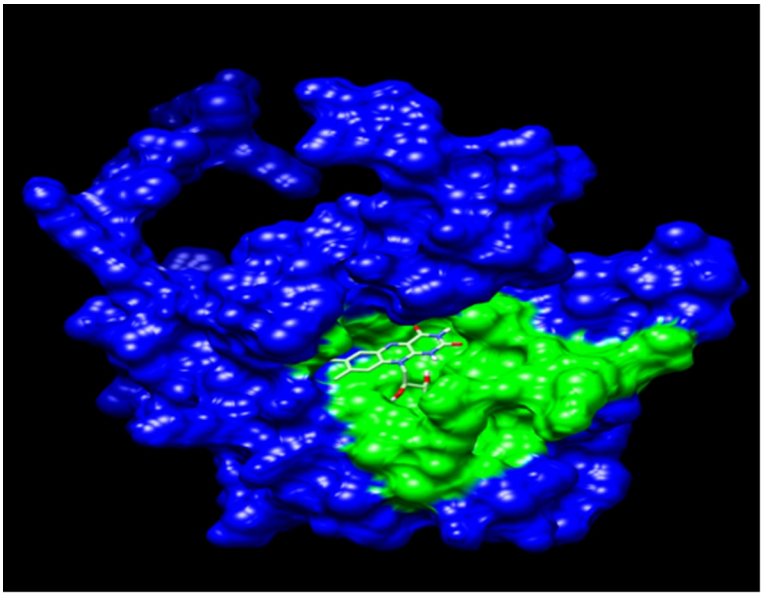

Fig. 11. Docking of flavin mononucleotide into the active site of flavin reductase. The docking pose, generated using AutoDock Vina, reveals key interactions between FMN and flavin reductase. (Green colour indicates the predicted binding pocket)

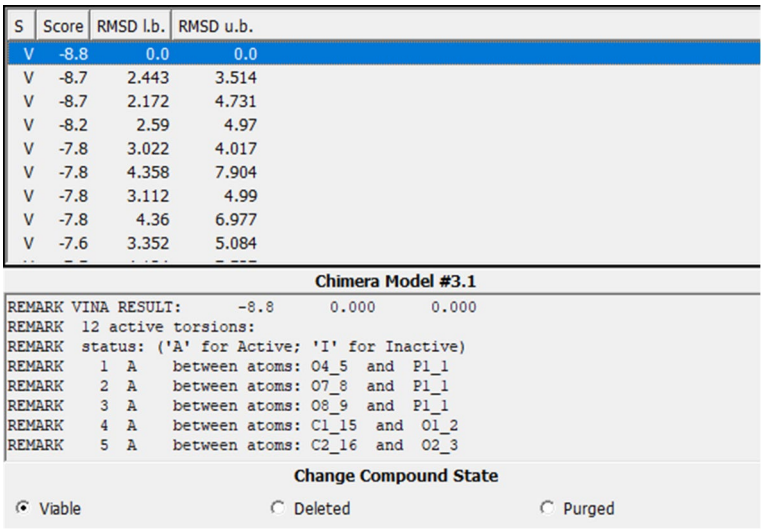

Fig. 12. Predicted binding energy for flavin reductase is -8.8 kcal/mol.

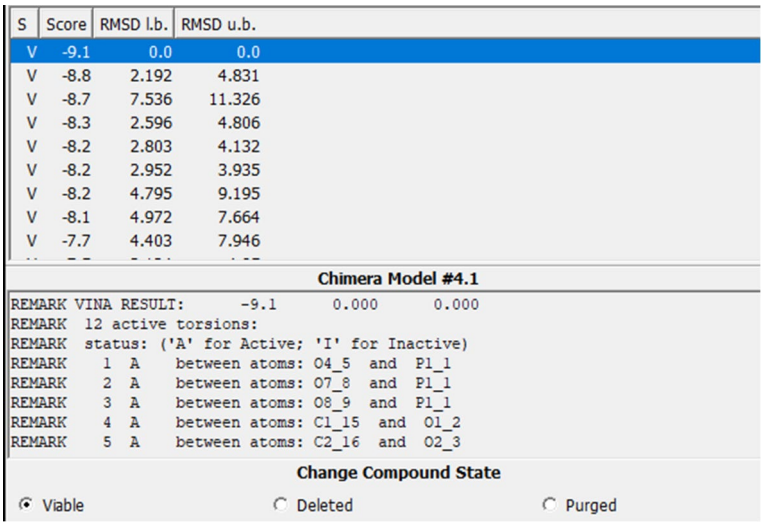

Fig. 13. Predicted binding energy for REF is -9.1 kcal/mol.

Sr no.	Ketamine (µL)	Xylocaine (µL)	Autoclaved water	Weight of mice (g)
1	68	16.6	165.4	33
2	62	15.1	173	30
3	64	15.6	170.4	31
4	66	16.1	167.9	32
5	68	16.6	165.4	33
6	64	15.6	170.4	31

Table 6. Local anesthesia dosage according to different weight of mice in.

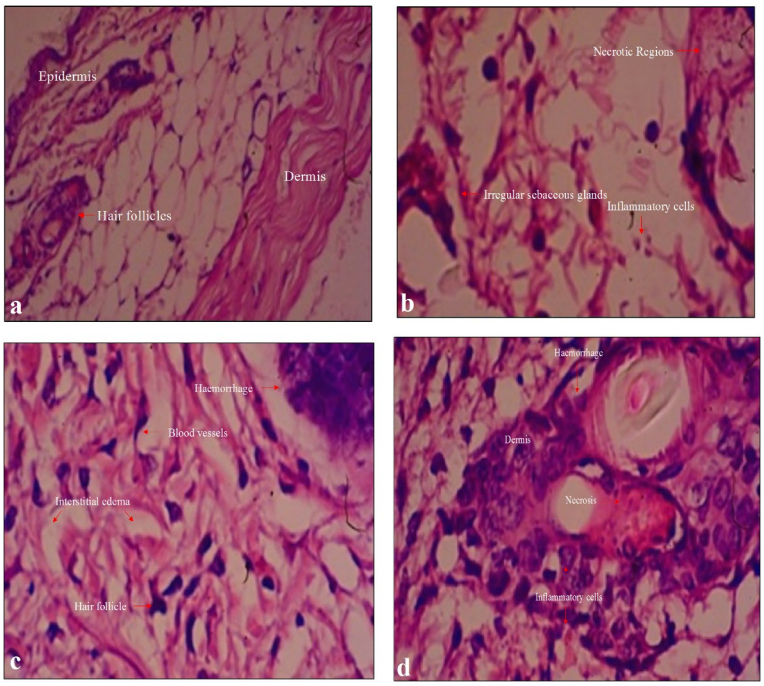

Fig. 14. (**a**) Normal skin tissue: Section shows normal skin with intact epidermal layer, hair follicles and dermis. (**b**) Skin remodeling: Section reveals epidermal growth with few areas of thinned out epithelium and necrotic regions with irregular growth of sebaceous glands and presence of inflammatory cells. (**c**) Dermis reveal hemorrhage, edema and irregular hair follicles. (**d**) Severe damage: Section reveals skin induced by chromate heavy metal. Skin tissue showing moderate to severe necrosis and desquamation in epidermis and inflammation and hemorrhage in dermis.

Data availability

All 16S sequences obtained in this study were deposited in NCBI with the accession numbers PP493917, PP493918, PP493921, PP493920, PP493922 and PP493923.

Received: 7 December 2024; Accepted: 10 March 2025
Published online: 14 March 2025

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Acknowledgements

The authors would like to thank the University of Central Punjab, Lahore, Pakistan and University of the Punjab, Lahore, Pakistan for providing experimental facilities to conduct the experiments.

Statement

This study is performed in accordance with relevant guidelines and regulations. All methods are reported in accordance with ARRIVE guidelines.

Author contributions

Author 1/ Mahnoor Ali: PhD Scholar publishing research work. Performed wet and dry laboratory experimentation, written the research article. Designed research work. Author 2/ Aatif Amin: Formatting the article, guiding Author 1 and helped with chromate bioremediation experimentation.Author 3/ Zaigham Abbas: Providing research guidelines, helped and performed in vivo trials with Author 1.

Funding
This research work was supported by University of Central Punjab and University of the Punjab Lahore, Pakistan.

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval
All animal experiments were conducted in strict compliance with established ethical guidelines and standardized protocols.

Consent to participate
Informed consent was obtained from the owner to use animals in the study.

Consent for publication
All authors are agreed to publish this study.

Human or animal participate
Author using animals in experimental research take over the responsibility for the welfare and wellbeing of their animals.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-93732-9.

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