



Oral vaccination with novel *Lactococcus lactis* mucosal live vector-secreting *Brucella* lumazine synthase (BLS) protein induces humoral and cellular immune protection against *Brucella abortus*

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Abstract

This work aimed to provide recombinant *Lactococcus lactis* as a potential live vector for the manufacture of recombinant *Brucella abortus* (rBLS-Usp45). The sequences of the genes were collected from the GenBank database. Using Vaxijen and ccSOL, the proteins' immunogenicity and solubility were evaluated. Mice were given oral vaccinations with recombinant *L. lactis*. Anti-BLS-specific IgG antibodies were measured by ELISA assay. Cytokine reactions were examined using real-time PCR and the ELISA technique. The BLS protein was chosen for immunogenicity based on the vaccinology screening findings since it had maximum solubility and antigenic values of 99% and 0.75, respectively. The BLS gene, digested at 477 bp, was electrophoretically isolated to demonstrate that the recombinant plasmid was successfully produced. Protein-level antigen expression showed that the target group produced the 18 kDa-sized BLS protein, whereas the control group did not express any proteins. In the sera of mice given the *L. lactis*-pNZ8148-BLS-Usp45 vaccine 14 days after priming, there was a significant level of BLS-specific IgG1, IgG2a ($P < 0.001$) compared to the PBS control group. Vaccinated mice showed higher levels of IFN- γ , TNF α , IL-4, and IL-10 in samples obtained on days 14 and 28, after receiving the *L. lactis*-pNZ8148-BLS-Usp45 and IRBA vaccines ($P < 0.001$). The inflammatory reaction caused less severe spleen injuries, alveolar edema, lymphocyte infiltration, and morphological damage in the target group's spleen sections. Based on our findings, an oral or subunit-based vaccine against brucellosis might be developed using *L. lactis*-pNZ8148-BLS-Usp45 as a novel, promising, and safe alternative to the live attenuated vaccines now available.

Keywords *Brucella* · *Lactococcus lactis* · BLS protein · Mucosal live vector

Introduction

The brucellosis infection is brought on by tiny, aerotolerant, Gram-negative infectious pathogens belonging to the *Brucella* family. The 12 species in the *Brucella* family each have unique host preferences, levels of pathogenicity, and

morphological characteristics (Munson et al. 2022; Dahouk et al. 2017). *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae* are classic *Brucella* species. The zoonotic endemic illness brucellosis is one of the most prevalent in several countries. It generally spreads through contact with infected animal tissues and fluids. It is recognized as an economically valuable livestock sickness that is mainly spread to humans through raw dairy foods and cheese made from unpasteurized milk (Pal et al. 2017). Due to the high prevalence of brucellosis in the Middle East, vaccination of animals is the most effective way to prevent illness and reduce the risk of human infection.

All currently available live attenuated vaccines have drawbacks that make them unsuitable for usage (Klafack et al. 2022), for example, including interference with diagnostics, abortions in given pregnant animals, and others (Klafack et al. 2022). As an alternative to the present brucellosis vaccinations, a new generation of safer, more specific, and more

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cost-effective mucosal-delivered vaccines may be needed to stimulate the immune reaction at the location of clinical disease (Jeyanthan et al. 2022). Today, the use of mucosal administration and probiotic-based systems is encouraged by the vital capacity of *Lactococcus lactis* to express viral and bacterial proteins as a live vector (Wyszyska et al. 2015). Brucellosis has different vaccines, including the S-19 vaccine, used only once in 3–6 months old female calves. The RDS19 vaccine is intended for cows over six months and should not be used in calves under six months. Rev-I vaccine is used to prevent brucellosis in sheep and goats. This vaccine is injected into lambs and goats at four months and is repeated one month before they reach reproductive age. Rev I vaccine should not be used in adult sheep and goats. According to the above content, each of the above vaccines has limitations. Therefore, there is a great need to develop a vaccine that does not have these limitations (Wyszyska et al. 2015; Munson et al. 2022; Dahouk Al et al. 2017).

New methods of treating illnesses brought on by this bacterium are thus needed. Scientific proof demonstrates that different pathogen-fighting vaccinations have eliminated or rendered inactive, such germs. Anti-bacterial vaccines can successfully activate the immune response (Chehelgerdi and Doosti 2020). Determining antigenic components, protein interactions, expression under various circumstances, immunogenicity, and ultimately constructing an appropriate vaccine may all be accomplished through bacterial proteome research. Recently, using proteomics, iron-regulating bacteria molecules were found to exhibit antigenic and immunogenic characteristics (Ansari et al. 2019). Five Outer membrane proteins (OMPs) with significant potential for vaccine development against *S. flexneri* infection were discovered due to the reverse vaccination strategy used to identify *Shigella flexneri* outer membrane antigens as cell-dependent vaccine and antibody candidates (Kazi et al. 2020). The *Brucella* lumazine synthase (BLS) is a viable surface-exposed alternative because of its practical expression, simple subsequent purification, and excellent molecular mass for distribution (Badmasti et al. 2021).

The highly immunogenic protein BLS, which possesses adjuvant qualities, has been proposed as a helpful antigen transporter for vaccine development. This protein is essential for triggering the immune response. One of the low-molecular-weight proteins, the *B. abortus* BLS antigen, has been regarded as an immunodominant antigen (Rezaei et al. 2019). The BLS was chosen for this study primarily because it outperformed other intracellular and periplasmic proteins of *Brucella* previously studied, such as L7/L12 (Ribeiro et al. 2002), GroEL heat-shock protein (Miyoshi et al. 2006), and Cu, Zn superoxide dismutase (Sáez et al. 2012). It also has higher antigenicity and accessibility. It is also a strong contender for utilization as an excellent option for good expression and a simple subsequent purification

procedure. Comprehensive computational approaches predicted the immunogenicity of the BLS as an appropriate candidate for the advancement of the subunit-based vaccine (Rezaei et al. 2019). Therefore, rather than using *Escherichia coli*, we decided to consider *L. lactis* as a suitable host cell for generating the rBLS-Usp45 protein because of the benefits mentioned above.

This study combined the nisin-inducible promoter (PnisA) and the Usp45 signal sequence in a high copy number pNZ8148 expression vector. The protein can be expressed in *L. lactis*. After orally administering *L. lactis*/pNZ8148 and recombinant *L. lactis*/pNZ8148-rBLS-Usp45 to mice, we employed recombinant *L. lactis* inducible expression pNZ8148 vector to produce a *Brucella* BLS antigen and examined the IgG immunological response. In a different group of mice that received an intraperitoneal injection of purified rBLS-Usp45 protein, the anti-rBLS-Usp45 IgG response was also measured.

Materials and methods

Bioinformatics analyses: screening of vaccine candidate proteins

The following procedures were used to find and screen potential vaccinations while adhering to the concepts of reverse vaccinology. The sequences of the genes were typically collected from the GenBank database. PSORTb was used to determine where the protein was located inside the membrane. Using Vaxijen and ccSOL, the proteins' immunogenicity and solubility were assessed. Finally, IEDB (Bepipred Linear Epitope Prediction 2.0) databases were used to evaluate each antigen's epitopes, and the antigens with the right antigenicity were selected (Piri-Gharaghie et al. 2022a).

Identification of potential vaccine antigens

According to the vaxign2 website, six proteins—including surface and non-surface proteins—were chosen to find several potential vaccines. Each protein's DNA sequences that code for its proteins were obtained from the GenBank database (Beiranvand et al. 2021).

B. abortus sequence alignment

A total of six antigens were chosen for BLASTn. The protein sequences were aligned via BLASTn. With a 98% homology cut-off for each molecule, *B. abortus* was chosen as the alignment species. As *B. abortus* strain sequences, the sequences with the most significant homology percentage

were chosen (Beiranvand et al. 2021; Piri-Gharaghie et al. 2022a).

Modeling of antigen localization in bacteria

Initially, UniProtKB (<http://www.uniprot.org/>) was used to obtain the sequence data. Then these antigens were analyzed using PSORTb to predict the subcellular localization. This software effectively determined protein localization by using a cut-off level over 7.5. With “bacteria” and “Gram-negative” selected as the “organism” and “Gram stain” attributes in this program (PSORTb), the functional site and localization of the ligands were identified (Piri-Gharaghie et al. 2022a).

Estimation of the proteins' antigenicity

The www.ddg-pharmfac.net website hosted by Vaxijen is made for categorizing the antigenic characteristics of proteins. Their antigenicity was determined using the chosen

protein sequences, and the results are displayed (Beiranvand et al. 2021).

Projection of protein solubility

The finest and most effective database for determining protein solubility is called ccSOI omics (http://service.tartagliab.com/grant_submission/ccsol_omics). Figure 1 displays the solubility of the molecules as determined by this database along with a graph of the outcomes (Piri-Gharaghie et al. 2022a).

Prediction of B-cell epitope

The most antigenic molecules were chosen based on the peptides' “maximum antigenic scores.” Immunogenicity was estimated using the Kolaskar and Tongaonkar antigenic scales from the IEDB dataset. The maximal, median and minimal B-cell epitope immunogenic scores were obtained using this dataset. The threshold for antigenic scoring was

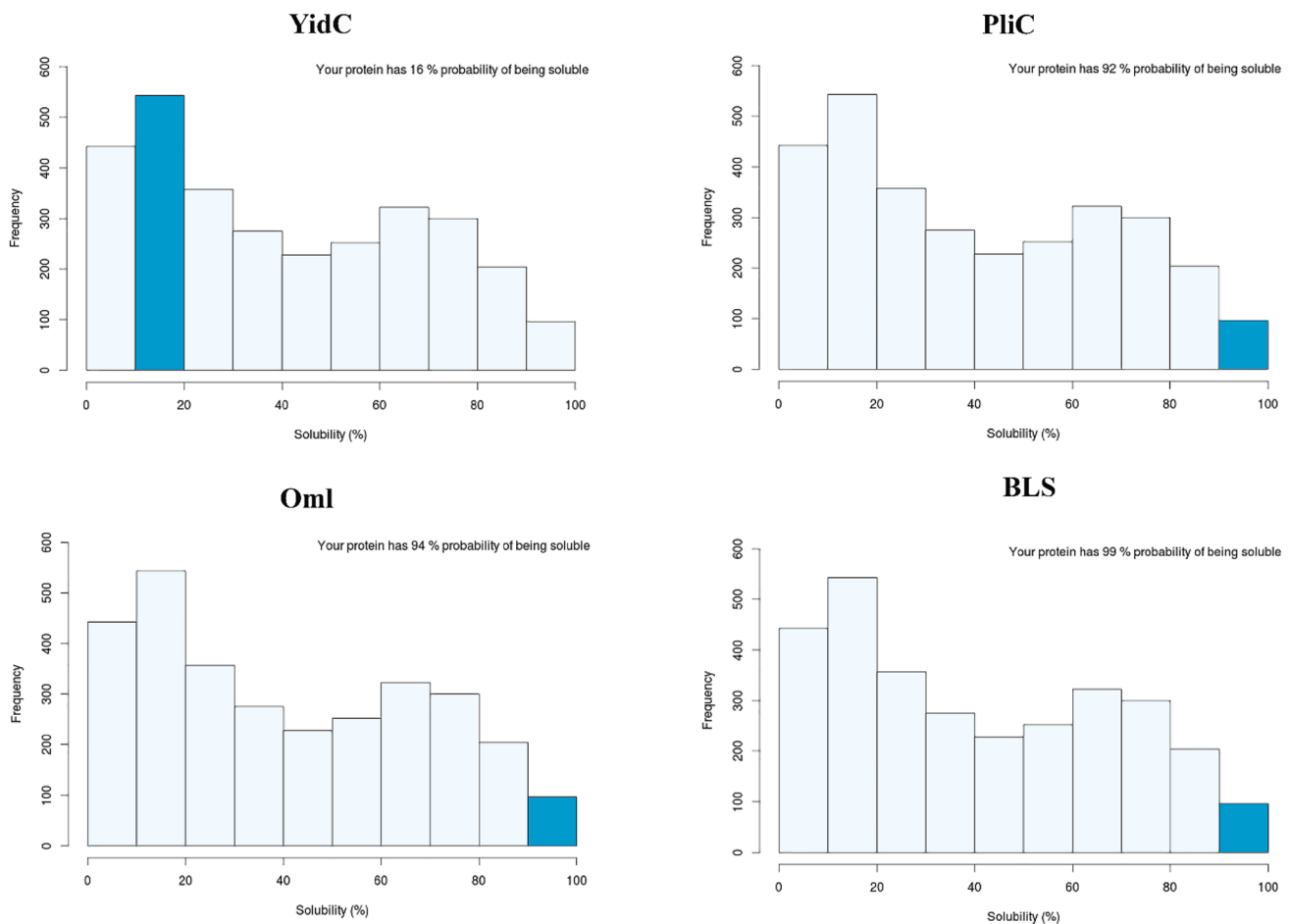


Fig. 1 Protein solubility diagram. According to the results, BLS, Oml and plcC proteins showed more solubility than 70%. However, the YidC proteins solubility was less than 20%

(1000). In this database, total proteins are generally categorized as immunogenic if their score is more than 1000 (Beiranvand et al. 2021; Piri-Gharaghie et al. 2023).

The analysis of the protein surface sequence

PRED-TMBB is a database for detecting and recognizing protein sequence data and membrane localization was employed since vaccine design must anticipate protein epitope clustering and their exterior placement surface. Only multiple alignments are required for this database to make predictions; amino acid patterns and other data are not required. The ability of this website to find exterior membrane water-soluble molecules in a sizable dataset makes it a powerful tool for discriminating research. This tool provides a reliable and robust way of screening whole genomes. The functions of this web server include locating membrane strands, determining ring topology, and applying discriminating procedures to any amino acid sequence (Piri-Gharaghie et al. 2022a; Piri-Gharaghie et al. 2023; Ishwarlall et al. 2022).

In-vitro analysis

Bacterial strains and culture media

Escherichia coli Top 10F and *Lactococcus lactis* PTCC1336 bacteria were produced by the Iranian Biological Resource Center for this investigation. The *Lactococcus lactis* bacteria were grown in M17 broth (DM565) produced by Quelab, Canada. Using a shaker incubator, *Escherichia coli* species Top 10F was grown in LB broth (Merck company, Germany) at 37 °C (200 rpm). Microbiological agar medium made by Merck was introduced to each of the media at a ratio of 1.5% to create agar media in the plates.

Construction of *L. lactis*/pNZ8148 -rBLS-Usp45

The Addgene website was used to gather details on the nisin-based expression vector pNZ8148, and Gene Runner was used to choose the restriction enzyme cleavage sites. An origin of replication (ORI), a gene for chloramphenicol resistance, two genes for the replication proteins repA and repC, a nisin-inducible promoter (P nisA), and a transcription terminator are all included in the expression vector pNZ8148 (T). This vector is made to allow cloned sequences to be expressed in gram-positive bacteria, particularly in species of lactic acid bacteria. The base pairs in this expression vector total 3167. The final organism was created using the 477 bp BLS gene fragment with accession number Q2YKV1.1 and a 27 aa signal peptide sequence known as Usp45 with accession code ABY84357. Finally, the gene along with the signal peptide (Usp45-BLS) was synthetically

cloned in the nisin-based vector pNZ8148 between KpnI and XbaI cutting sites by Generay, China.

Confirmation of BLS gene cloning in pNZ8148 vector

To validate the correctness of the cloning, polymerase chain reaction (PCR), enzyme digestion, and sequencing methods were performed. Using specified primers, the BLS gene's PCR was carried out, and the amplicon was electrophoresed on 1% agarose gel. With the help of the restriction enzymes KpnI and XbaI from GENERay products, the recombinant expression vector was digested. Nucleotide sequencing of the recombinant vector was performed by GENERay.

Transformation

By using the electroporation technique, the recombinant plasmid pNZ8148-rBLS-Usp45 was successfully transformed into the *L. lactis* strain. Briefly, utilizing an electroporator (Xcell BIO-RAD Gene Pulser; with 2500 V, 25 microfarads, and 200 ohms' resistance), 400 µL of *Lactococcus lactis* PTCC1336 host cells were transfected with 6 µL (0.2 µg/µL) of the recombinant plasmid pNZ8148-Usp45 carrying the BLS gene as well as 6 µL (0.2 µg/µL) of the pNZ8148 vector without the BLS gene. A suspension of *Lactococcus lactis* bacteria was cultured on an M17Agar medium containing the antibiotic chloramphenicol (25 µg/mL). It was heated for 72 h at 30 °C as a positive and a suspension of *Lactococcus lactis* bacteria containing recombinant vector as a negative control. The chloramphenicol-resistant gene in pNZ8148 was used to select the positive transformed *L. lactis*/pNZ814-8rBLS-Usp45, which was then identified by colony PCR using the specified primers.

Forward primer: 5'-ATGAATCAAAGCTGTCCAAATAAAAC-3'.

Reverse primer: 5''TTAAACAAGAGCAGCAATACGTGAAC'3'.

The PCR procedure was carried out under optimal conditions, which included preheating at 94 °C for 5 min, 35 stages of denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min, and elongation at 72 °C for 1 min followed by the final elongation at 72 °C for 10 min.

In-vivo analysis

Preparation of *Lactococcus lactis* cells for immunization

In M17 medium supplemented with 1% glucose and 5 µg of chloramphenicol (Sigma-Aldrich) per mL at 30 °C without shaking, the *L. lactis*/pNZ8148 (Harboring the empty vector) as control and pNZ8148-BLS-Usp45 strains are grown to an optical density at 600 nm of 1.0. Following a second centrifugation (4000g at 4 °C) wash in sterile, ice-cold PBS, the

collected cells are suspended in vaccination buffer (0.2 M sodium bicarbonate, 5% casein hydrolysate, and 0.5% wt/vol glucose) at a dosage of 5×10^{10} CFU/mL (Bermúdez-Humarán et al. 2003).

Animals and feeding procedure

One hundred twenty female BALB/c mice weighing 15–20 g and 6 to 8 weeks old were used in this study. Through oral vaccination with 100 µg of pDNA, pNZ8148-BLS-Usp45 has been administered to animals. 100 mice were divided into five groups of 20, and PBS was administered to an extra 20 mice used as negative controls ($n=20$). Table 1 displays the number of mice utilized in each group. Nisin was used to culture and stimulate the control and recombinant strains of *L. lactis*. A group of 20 mice was given 10^8 colony-forming units (CFU) of *L. lactis* pNZ8148-BLS-Usp45 three times by oral pipette. The three treatment days were spaced 15 days apart (days 0–2, 14–16, and 28–30). 10^8 *L. lactis* or PBS vaccines were given to the animals in the standard control. The second set of mice underwent a subcutaneous injection immunization with the IRIBA vaccine containing 2×10^8 CFU (Razi, Iran). As a positive control, 200 µl of PBS were vacuum-cleaned at zero time.

Indirect ELISA for detection of anti-rBLS-Usp45 IgG responses

Two days before each vaccine and 15 days after the final immunization, the serum IgG (IgG1, IgG2a) of each sample was measured using indirect ELISAs. The purified rBLS-Usp45 was employed to coat the wells of a polycarbonate plate with 100 µL of a 5 µg/mL solution in carbonate buffer (pH 9.6). To prevent non-specific binding, the plates were inhibited with 5% skim milk in PBS containing 0.5% Tween 20 for an additional overnight incubation at 4°C after being rinsed with PBS containing 0.05% (w/v) Tween 20. Plates were incubated with 100 µL of diluted serum samples diluted 1:100 in preventing buffer for 2 h at room temperature while being rocked after the blocking buffer was removed. Goat Anti-Mouse IgG (Bio-Rad Cat no:

170-6516) labeled with HRP was incorporated into the wells at a dilution of 1:2000 in PBS-Tween and cultured at room temperature for one hour after three further PBS-Tween washes were conducted between incubations. Following a final washing procedure, the administration of 50 µL/well of the enzyme–substrate TMB for 30 min at 37 C was used to measure specific reactivity. When 2 M H₂SO₄ was added to each well, the reaction was halted. After 10 min, the ideal density (OD 492) at 492 nm was determined. Every test was run in triplicate.

Detection of cellular immune response

ELISA assay The levels of IFN-γ, TNF-α, IL-10, and IL-4 were evaluated to assess the cellular immunological response brought on by oral vaccination. According to the user's guide, karmania pars gene ELISA kits were used to measure the levels of proinflammatory cytokines in the serum sample (KPG, IRAN). A 96-well plate was coated with the coating antibody overnight at 4 °C (0.2 µg/well). The plate was then blocked using PBS containing 0.5% BSA and 0.1% Tween 20. The material was then divided into two duplicate wells with a volume of 100 µL, and 50 µL of a detecting antibody at a concentration of 1 µg/mL was added. At room temperature, the plate was covered and shaken for two hours (700 rpm). Streptavidin-HRP (1:1024) was incorporated and incubated for 30 min at room temperature on a shaker following washing with PBS, including 0.1% Tween 20 (700 rpm).

Real-time PCR assay Following the anesthesia of the mice, the spleen was removed under aseptic conditions and kept in liquid nitrogen at – 198 °C. Then, spleen tissue was processed using the YTA kits' instructions for RNA extraction and cDNA synthesis (Yekta Tajhiz, Iran). The GAPDH gene was utilized as an internal control during real-time PCR using the YTA SYBR Green master mix (Yekta Tajhiz, Iran). For real Time-PCR, a reaction volume of 15 µL, composed of 0.5 µL cDNA, 0.5 µL forward primer, 0.5 µL reverse primer, 10 µL master mix, and 3.5 µL of double sterile distillation water, was employed. Along

Table 1 The number of mice used in this experiment

Group number	Injection composition	Number of mice	Average weight of mice (gr)	Type of injection	Time of injection	Injection day
1	<i>L. lactis</i> /pNZ8148-BLS-Usp45	20	18.7 ± 0.2	oral	Every 8 h for 3 days	0–2, 14–16, 28–30
2	<i>L. lactis</i> /pNZ8148	20	18.5 ± 0.6	oral	Every 8 h for 3 days	0–2, 14–16, 28–30
3	<i>L. lactis</i>	20	18.6 ± 0.4	oral	Every 8 h for 3 days	0–2, 14–16, 28–30
4	IRIBA Vac	20	18.3 ± 0.8	Subcutaneous injection	Every 8 h for 3 days	1, 14, 28
5	pNZ8148	20	18.6 ± 0.7	oral	Every 8 h for 3 days	0–2, 14–16, 28–30
6	PBS	20	18.7 ± 0.9	oral	Every 8 h for 3 days	0–2, 14–16, 28–30

with initial denaturation for 10 min, the temperature cycle program also included 40 cycles at 95 °C for 20 s, annealing at 53 °C for 1 min, and elongation at 72 °C for 1 min, followed by the final elongation at 72 °C for 10 min. The relative gene expression of IFN- γ , TNF- α , IL-10, and IL-4 was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH levels in each sample (Sunita et al. 2020).

Survival rate of mice

Following Hosseinezhad-Lazarjani et al. (2023), the protection tests were carried out (Hosseinezhad-Lazarjani et al. 2023). Two weeks following the initial treatment, ten mice from each group received intraperitoneal (i.p.) exposure to 10^4 CFU of *B. abortus*. Each group's body weight, clinical rating, and survival rate were noted daily during the seven-day observation of the mice. The overall clinical sign for each mouse was graded on a sliding scale from 0 to - 5. Clinical evaluations of each animal were given a score between 0 (standard, active, healthy), - 1 (slightly sick, slightly ruffled fur, otherwise standard), - 2 (ill, ruffled fur, sluggish movement, hunching), - 3 (extremely sick, ruffled hair, prolonged movement, stooped, eyes shut), - 4 (moribund), and - 5 (dead). The infected mice were slaughtered according to ARRIVE recommendations [PLoS Bio 8(6), e1000412, 2010] (Hosseinezhad-Lazarjani et al. 2023). Two weeks later, and the spleens were removed under aseptic circumstances, homogenized, and plated in Blood Agar (BIO3P, Iran) to count the number of *B. abortus* CFU per organ. The average log₁₀ CFU for the treatment and control group was subtracted from the median log₁₀ CFU for the standard matching control to get the average log₁₀ CFU.

Histopathological examination

In an aseptic procedure, livers were removed and fixed in 10% formalin. Slices were embedded in paraffin, stained with hematoxylin and eosin, and then inspected histopathologically under a microscope (HE). An ImagePro macro was used to evaluate liver damage by dividing the lesion area by the total liver area.

Statistical analyses

Analyzing the data and running statistical tests were done with GraphPad Prism 5.0. The means were compared using a one-way analysis of variance (ANOVA), and then a Tukey–Kramer post hoc test with a 95% confidence interval was performed. The survival rates of vaccinated mice and the control group were compared using

a Chi-square test with Yates' adjustment; when differences were $P < 0.05$ or $P < 0.01$ significant, they were deemed highly significant.

Results and discussion

Results of computer studies based on reverse vaccinology

Protein subcellular localization prediction using PSORTb

With localization values over 7.5, 5 of the six selected proteins were anticipated to be surface proteins. For additional analysis, they were employed along with BLS, Oml, YidC, PliC, BamE. Protein described as periplasmic included BamD. BamD was therefore disqualified from further research. According to the PSORTb analysis results (Table 2), 5 external membrane antigens were chosen and progressed to the next study stage.

Protein antigenicity

The antigenicity was assessed using the VaxiJen in-silico tool and the chosen protein sequences, with a cut-off value of 0.6. The antigens BLS, Oml, YidC, PliC, were chosen as having suitable antigenicity (≥ 0.6), as shown in Table 2's results. BamE, one of the five proteins mentioned above, was eliminated due to its limited antigenicity. BLS, Oml, YidC, PliC were utilized as suitable proteins to assess solubility and flexibility.

Solubility of proteins

Two proteins, BLS, and Oml, showed suitable solubility ranges, as shown in Fig. 1. Solubility was appropriate, as evidenced by proteins BLS, Oml and pliC which had scores of 99% and 94% and 92%, respectively. YidC (16%), protein's poor solubility prevented it from being included in the subsequent study.

Immune epitope database B-cell epitope prediction (IEDB)

The BLS, Oml and pliC proteins have been identified as having the proper immunogenicity, solubility, and antigenicity. The best antigenic option was BLS, with a median antigenic value of 1.043. Following Fig. 2's findings, protein BLS displayed 4 great epitopes, while Oml and pliC proteins displayed 5 and 4 tiny epitopes for B-cells. Also, out of 158 amino acids of BLS protein, 116 amino acids (73.41%) were identified as epitopes and only 42 amino acids (26.58%) were not epitopes. Out of 99 amino acids of pliC, only 46 amino acids (46%) were

Table 2 Preliminary screening and identification of proteins

Protein	Symbol	Accession numbers	PSORTb	Score	Vaxign	Y/N	VaxiJen
			Region localized		Similarity to Host Proteins		
AltName: Full = BLS	BLS	Q2YKV1	OuterMembrane	8.2	Human Mouse Pig	N N N	0.72
Outer membrane lipoprotein	Oml	AAB06277	Periplasmic/OuterMembrane	7.5	Human Mouse Pig	N N N	0.65
Membrane protein insertase yidC	YidC	AZS93164	Periplasmic	7.5	Human Mouse Pig	N N N	0.64
<i>Brucella abortus</i> PliC crystal form	PliC	4MIS_B	Periplasmic/extracellular	8.0	Human Mouse Pig	N N N	0.52
Outer membrane protein assembly factor BamE	BamE	AZS90979	OuterMembrane	10	Human Mouse Pig	N N N	0.31
Outer membrane protein assembly factor BamD	BamD	AZS91551	Cytoplasmic/extracellular	7.5	Human Mouse Pig	N N N	0.28

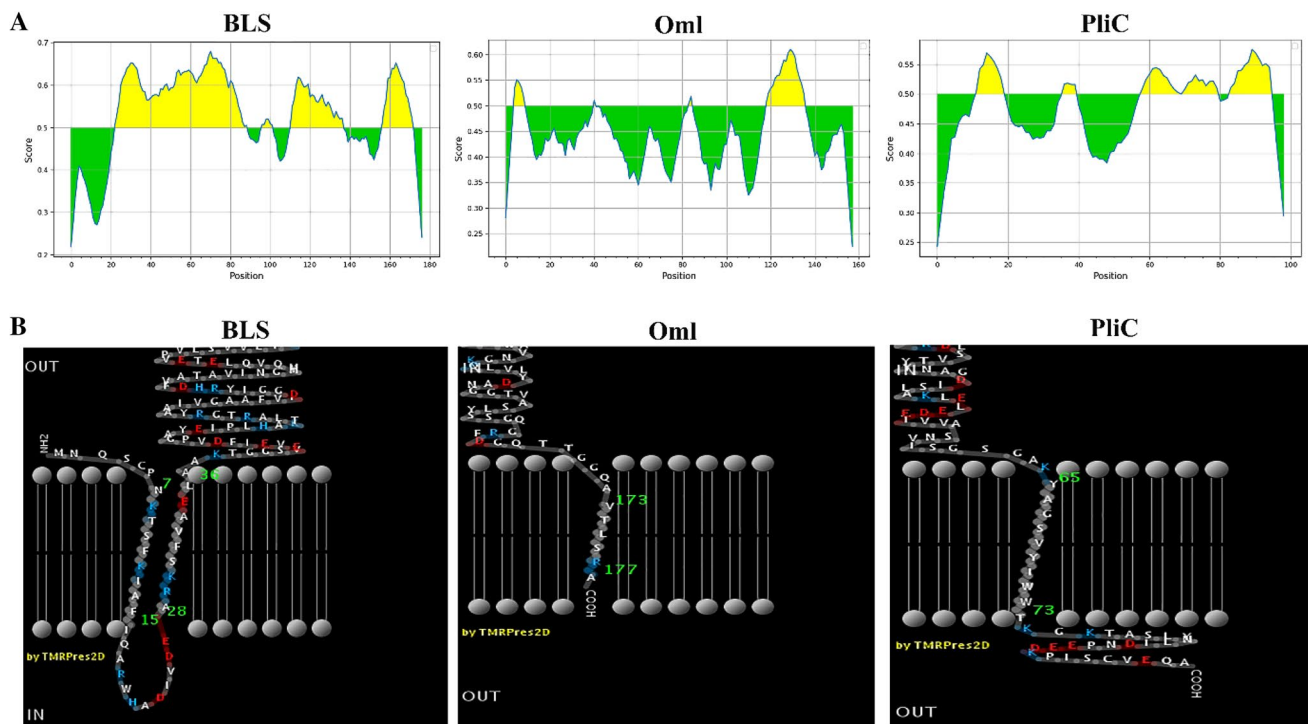


Fig. 2 a IEDB dataset for linear B-cell epitopes. The sequence of the YELLOW gradient protein displays positions above the threshold epitope. As a result, all proteins that passed the screening and had a YELLOW coloration of at least 70% had robust epitope features. b

Localization of proteins epitopes in the bacterial cell membrane the outer membrane, periplasmic space, and inner membrane sequences are shown in the 2D representation (color figure online)

epitopes and 54 amino acids (54%) were not identified as epitopes. But the Oml protein has 177 amino acids, of which only 14.68% (26 amino acids) were identified as epitopes.

Identification of external epitopes and epitope localization

PRED-TMBB prediction was employed to weigh specific protein epitopes in the bacterial surface following the findings of Fig. 2a. Green portions of the proteins were found inside the internal membrane, red portions crossed the membrane, and blue portions were found on the exterior of the outer layer or exterior of the bacterial membrane. According to Fig. 2b, each of the chosen proteins had its specific membrane weight, consistent with the results.

Each protein's outer and inner membrane portions were discovered and later recognized as functional areas by overlaying the sequence onto the PRED-TMBB findings and using the starting and ending amino acid sequences of the epitope. The resulting protein's immunogenicity will be more potent as the more epitopes protrude from the surface, the longer the projecting amino acid sequences. BLS protein has scored much higher than the cutoff criterion based on this study (Table 3). BLS is thus chosen as the most antigenic molecule since it has functional exterior epitopes.

In-vitro analysis

Confirmation of transformed pNZ8148-BLS-Usp45 in *L. lactis*

Using direct colony PCR and double digestion assessment with two restriction enzymes, the recombinant plasmid pNZ8148-BLS-Usp45 was verified. DNA analysis showed that the recombinant plasmid's virulence gene sequence was 100% identical to that of the *B. abortus* bacterium.

The recombinant plasmid was then provided the GenBank nucleotide sequence Accession number MH734194.1 after DNA sequencing. The BLS gene digestion fragments at 477 bp were electrophoretically isolated to ensure that

the recombinant plasmid was successfully produced (Fig. 3a).

Expression of recombinant *L. lactis* /pNZ8148-BLS-Usp45 in mRNA and protein level

Reverse transcriptase-PCR was used to measure the relative levels of the expression pattern of the BLS Gene delivery (RT-PCR). The 477 bp band on the agarose gel indicates that the gene has undergone transcription (Fig. 3b). Rojan Azma's SDS-PAGE and Western blot analyses additionally demonstrated that the particular proteins were produced at the protein level. The target group produced the 18 kDa-sized BLS protein, as evidenced by antigen expression at the SDS-PAGE, but the control group did not (Fig. 3c). In Fig. 3d, samples of *L. lactis* protein from our recombinant *L. lactis*/pNZ8148-BLS-Usp45 organism and the bacteria transformed with an empty vector (pNZ8148) are also subjected to Western blot examination.

The analysis of the IgG titer

Sera were obtained from several BALB/c mice vaccinated intragastrically with *L. lactis*/pNZ8148, *L. lactis*/pNZ8148-BLS-Usp45, *L. lactis*, pNZ814, and PBS, as well as from a group of mice that had been vaccinated subcutaneously with the IRIBA Vac. The analysis of the IgG response showed a significant increase in comparison to the control (PBS) administered group, and the changes in IgG antibody response in the group immunized with intragastric *L. lactis* / pNZ8148-BLS-Usp45 are reported as significantly different between before and after last vaccination ($P < 0.01$) (Fig. 4a). After 28 days, no significant antibodies were produced in the PBS-administered group ($P = 0.226$). Serum IgG antibody titers were more significant in the *L. lactis*/pNZ8148 group than in the PBS and pNZ814 groups ($P < 0.05$). The serum IgG antibody values in the *L. lactis*/pNZ8148 administration group were slightly greater than those in the *L. lactis* administration group; however, the differences were not statistically significant ($P = 0.312$) and were lower than those in the *L. lactis*/pNZ8148-BLS-Usp45 and IRIBA-vac administration group ($P < 0.001$). When mice were vaccinated intragastrically with *L. lactis*/pNZ8148-BLS-Usp45 and subcutaneously with IRIBA Vac, a substantial increase in the antibody levels was seen between before (day 0) and after the final vaccination (day 28) ($P < 0.001$). Additionally, it demonstrated no discernible changes between mice that had been inoculated via subcutaneous injection and those who had been given *L. lactis*/pNZ8148-BLS-Usp45 intragastrically. Additionally, an indirect ELISA was used to examine BLS-specific IgG1 or IgG2a in BALB/c mice. As shown in Fig. 4b, c, mice immunized with *L. lactis*/pNZ8148-BLS-Usp45 produced significantly more BLS-specific IgG1 and

Table 3 B-cell epitope prediction. Antigenic scores of the analyzed proteins

Protein	Minimum antigenic score	Maximum antigenic score	Average antigenic score	Threshold antigenic score
BLS	0.884	1.195	1.043	1.000
PliC	0.243	0.575	0.477	1.000
Oml	0.225	0.610	0.440	1.000

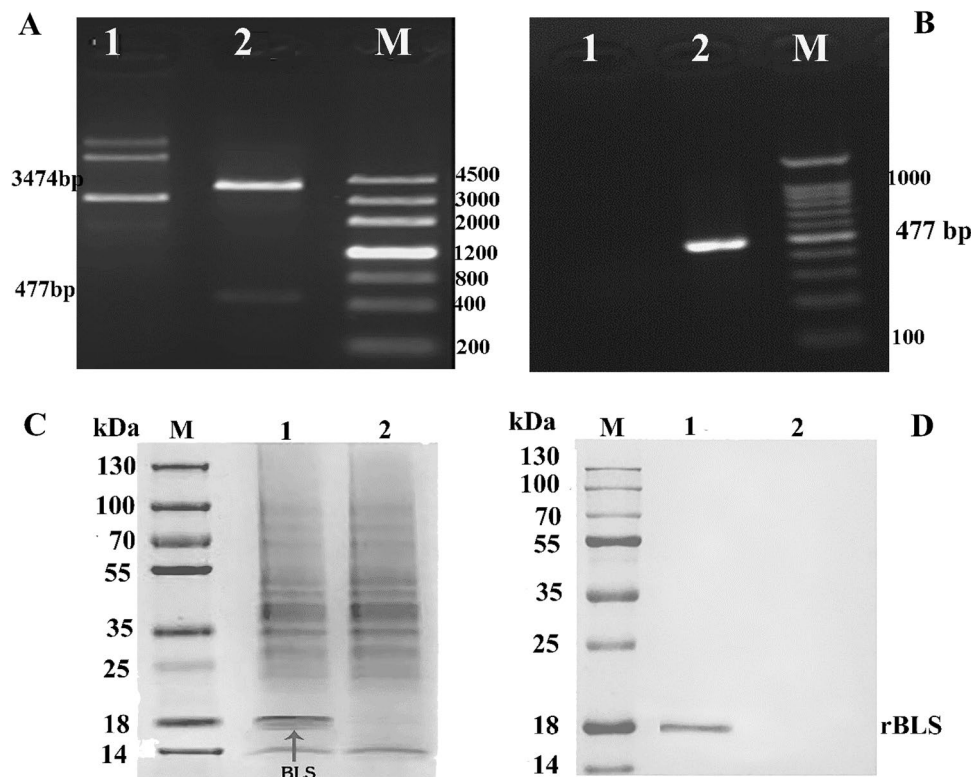


Fig. 3 **a** Lane 1: recombinant plasmid before enzymatic digestion, Lane 2: double enzyme digestion shows the 477 bp band of the gene along with the signal peptide. **b** Line 1: *L. lactis* transformed with a vector lacking the target gene, line 2: expression of BLS gene transcription at the mRNA level and the formation of 477 bp band in *L. lactis* transformed with a recombinant vector. **c** SDS-PAGE electrophoresis of the recombinant *L. lactis* protein mixture. *L. lac-*

tis bacteria were altered in lane 1 using the recombinant BLS vector pNZ8148-Usp45. Lane 2: *L. lactis* bacteria were altered by an empty vector that did not express the desired protein. Lane M: marker. **d** Verify the presence of recombinant protein in transformed *L. lactis* bacteria using a western blot. Line 1: *L. lactis* bacteria transformed with the recombinant vector pNZ8148-BLS-Usp45, M: marker, Line 2: *L. lactis* bacteria transformed with a vector lacking the target gene

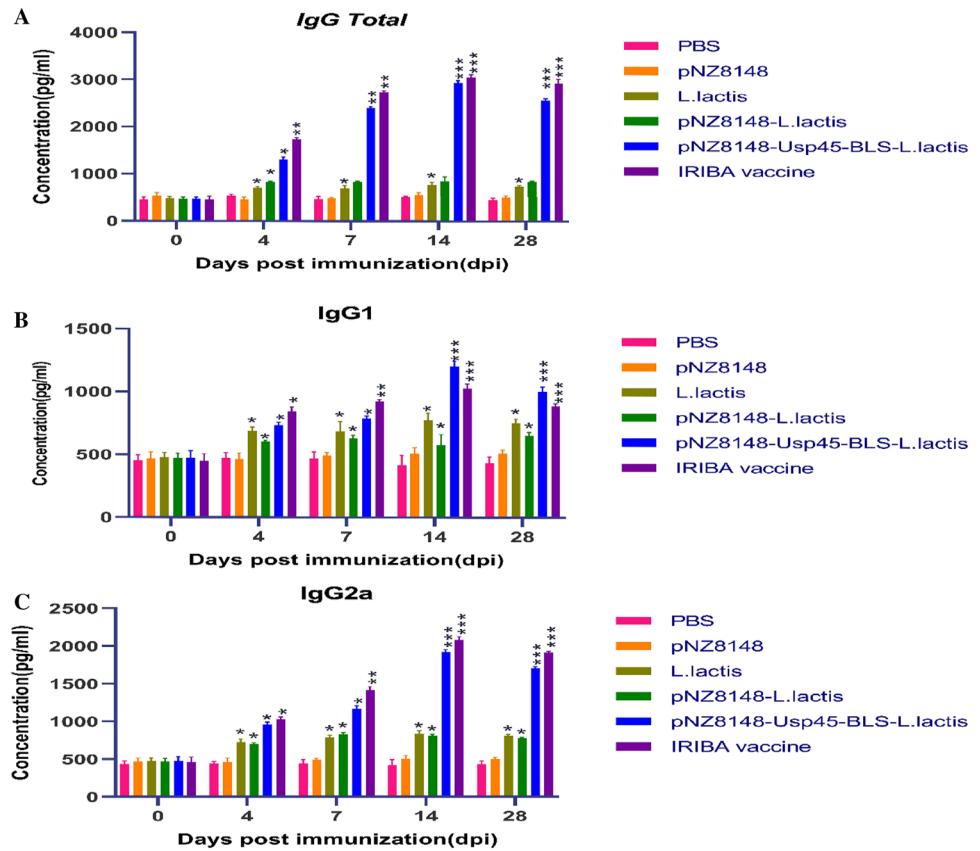
IgG2a in their serum 14 days after stimulation compared to the PBS control group. At day 28, comparable significant values were observed; however, the levels had recovered to baseline compared to the PBS negative control groups. The results showed that levels of BLS-specific IgG1 and IgG2a were considerably ($P < 0.001$) higher 14 days after vaccination in both the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccine (positive control) groups compared to the values from mice in the PBS control group.

Cytokine production

In vitro research was conducted on the cytokine secretion of spleen cells upon re-stimulation. Splenocytes from vaccinated and non-immunized mice were generated in response to a challenge. Animals given PBS, pNZ8148, *L. lactis*, and *L. lactis*-pNZ8148 did not significantly alter the unstimulated state of their cells ($P > 0.05$). Unimmunized (saline inoculation) animal cells, on the other hand, did not significantly increase their IFN- γ and TNF- α production following re-stimulation ($P > 0.05$). After 14 days,

L. lactis/pNZ8148-BLS-Usp45 and IRBA vaccine-infused mouse cells released higher IFN- γ and TNF- α (Fig. 5). On days 14 and 28, samples from mice who received the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccines, as well as the negative control groups, showed higher levels of IFN- γ and TNF- α , respectively ($P < 0.001$) (Fig. 5). The levels of IFN- γ and TNF- α in the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccination groups at day 28 were remarkably equal to those in the control groups ($P < 0.001$). Despite a reduction compared to controls, IFN- γ and TNF- α concentrations were still considerably greater on day 28. The induction of IL-4 and IL-10 in mice that had received vaccinations was also assessed. The findings demonstrated that no mice given the vaccine had produced IL-4 or IL-10 14 days following immunization. Additionally, animals were vaccinated with the *L. lactis*/pNZ8148-BLS-Usp45 vaccine. They administered the IRBA vaccination displayed substantially greater IL-4 and IL-10 titers at 28 days following vaccination ($P < 0.05$) than mice exposed to the control groups of the pathogen.

Fig. 4 **a** BLS-specific mucosal total IgG antibodies in mice. Sample obtained from mice after oral immunization with different groups of vaccines. **b** Thirty days following the last vaccination, BALB/c mice exhibit humoral immunological response. 1:200 serum dilutions of rBLS-specific IgG1 from various groups of mice. **c** BLS-specific mucosal IgG2a antibodies. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$



The transcription level of cytokines increased in the spleen and small intestine

The quantity of cytokine transcription in the spleen and small intestine was assessed using a quantitative real-time PCR technique. In the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccination groups, the IFN- γ and TNF- α transcription levels were significantly higher than those in other groups. The small intestine and spleen had the same results. Real-time PCR findings from the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccination groups showed significantly different levels of IFN- γ and TNF- α gene expression from those from the other groups, and these results were also consistent with ELISA. IL-4 and IL-10 transcription levels were considerably higher in the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccination groups than in the other groups. The outcomes in the spleen and small intestine were the same. In comparison to other groups, the *L. lactis*/pNZ8148-BLS-Usp45 and IRBA vaccination groups had significantly greater transcription levels of IL-4 and IL-10 (Fig. 6).

Protective activity of vaccinated mice against *B. abortus*

The mice were exposed to pathogenic *B. abortus* isolates to test any potential protective effects of the different immunization groups. The *B. abortus* isolates were used in

three different replicates of the study. The spleen weights of mice given various control vaccines 15 days after infection considerably reduced compared to those inoculated with the *L. lactis*-pNZ8148-BLS-Usp45 and IRBA vaccine (Table 4). Mice immunized with IRBA, or *L. lactis*-pNZ8148-BLS-Usp45, did not exhibit any statistically significant alterations ($P > 0.05$). The IRBA vaccine groups, *L. lactis*-pNZ8148-BLS-Usp45, and non-challenge groups did not differ from one another substantially ($P > 0.05$). Six *B. abortus*-exposed mice were randomly chosen, and their mortality, weight fluctuations, and general health were tracked every day for 15 days.

All of the mice in the control group perished seven days after the experiment, according to Table 4. The 15-day survival rates of mice models immunized with *L. lactis*-pNZ8148-BLS-Usp45 were 87.5% when challenged with a lethal dosage of *B. abortus* isolates. These values were much more significant than mice given the IRBA vaccine (62.5%). Seven days following the challenge, each group's body mass and clinical symptom scores reached their lowest points. Fifteen days following the trial, the mice's body weight was normal, and the symptoms vanished.

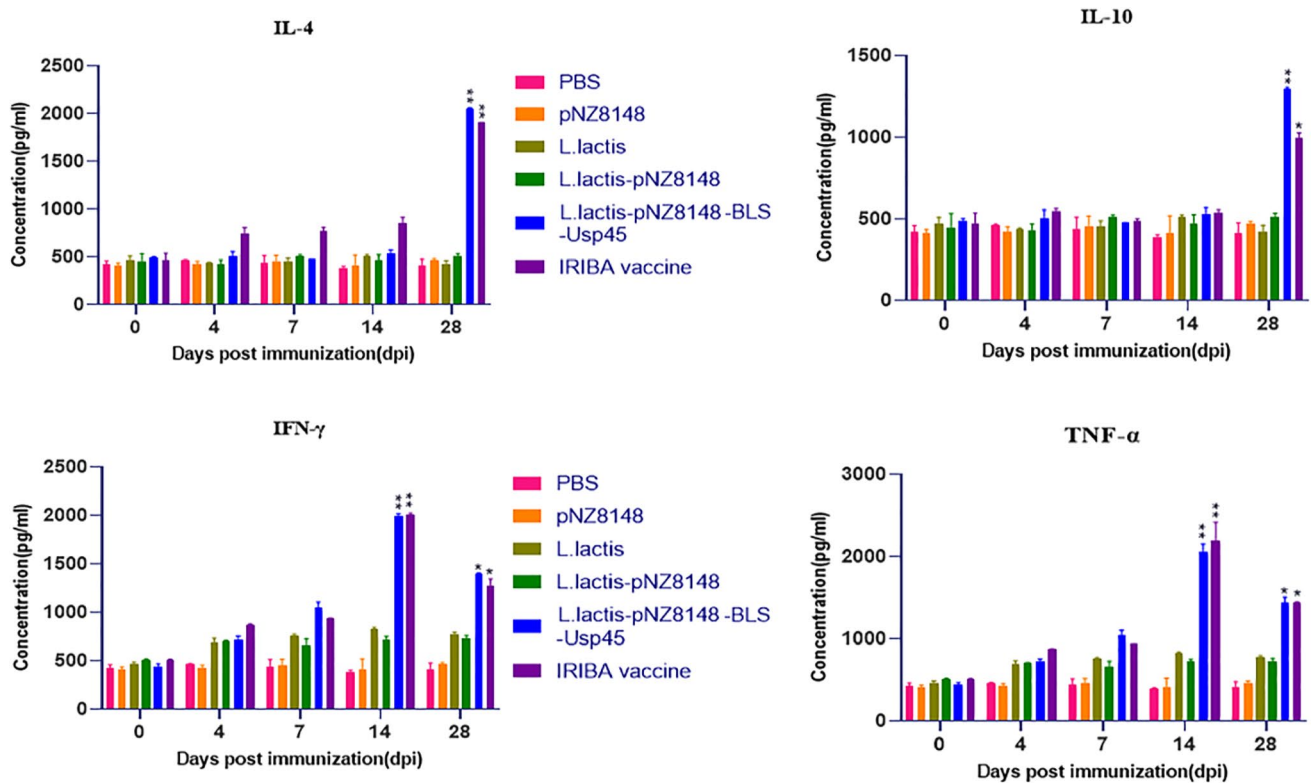


Fig. 5 The splenocyte supernatants of the control and vaccinated groups included cytokine levels. In mice inoculated with various vaccinations, the levels of inhibitory cytokines (IL-4, IL-10) and func-

tional cytokines (IFN- γ , TNF- α) were measured in the spleen. PBS served as a standard. * $P < 0.05$, ** $P < 0.01$

Vaccinated mice showed lower bacterial loads and pathological lesions

We evaluated the efficacy of the *L. lactis*-pNZ8148-BLS-Usp45 vaccine using a challenging experiment in BALB/c mice to assess protection. Three isolates of clinical *B. abortus* were given to each group. Six mice from each category were randomly chosen to determine how many germs were present in the spleen tissue (Fig. S1). Forty-eight hours after the challenge, six mice from each group were removed, serially diluted, and then plated on *Brucella* agar with 5% blood plates. The plates were then incubated at 37 °C overnight. The log₁₀ CFU/mL ratio was computed and compared following the CFU count. Compared to the animals in the other groups, the mice that received the *L. lactis*-pNZ8148-BLS-Usp45 vaccination had reduced spleen bacterial loads (Fig. S1). Following the challenge, the left spleen tissue was aseptically removed, preserved in a 4% formalin solution, stained with hematoxylin–eosin, and examined under a microscope. Figure 7 shows that the *L. lactis*-pNZ8148-BLS-Usp45 and IRIBA-vac group's spleen parts had less severe spleen damage, alveolar edema, lymphocyte infiltration, and topological damage as a result of the inflammatory process than other groups. The *L. lactis*-pNZ8148-BLS-Usp45 vaccination

groups had cleaner alveoli and were more normal than the other groups. Spleen failure in the *L. lactis*-pNZ8148-BLS-Usp45 groups was much less severe than in the other groups after the mice were exposed to the vaccination, indicating that the animals had a less inflammatory response to spleen failure.

The Middle East has a high prevalence of brucellosis, and vaccination is strongly advised to avoid the illness due to the high expenditures associated with the cattle business (Dorneles et al. 2015). Novel vaccination approaches with the goals of maximizing protective immunity, minimizing side effects, safe handling, straightforward administration, and low manufacturing and delivery costs could reduce safety issues and outweigh the drawbacks of the live attenuated strains of *Brucella* currently used to restrict brucellosis (Saez et al. 2012). The appealing vaccination strategies depend on locating and utilizing novel *Brucella* immunogenic proteins to create novel recombinant vaccines (Vishnu et al. 2015). *Escherichia coli* has only been used in limited research to produce the preventative recombinant vaccine against brucellosis (Gupta et al. 2012).

Most pathogenic microorganisms start their infectious cycles on mucosal surfaces. Therefore, if the colonization and penetration of infectious organisms were to halt at this

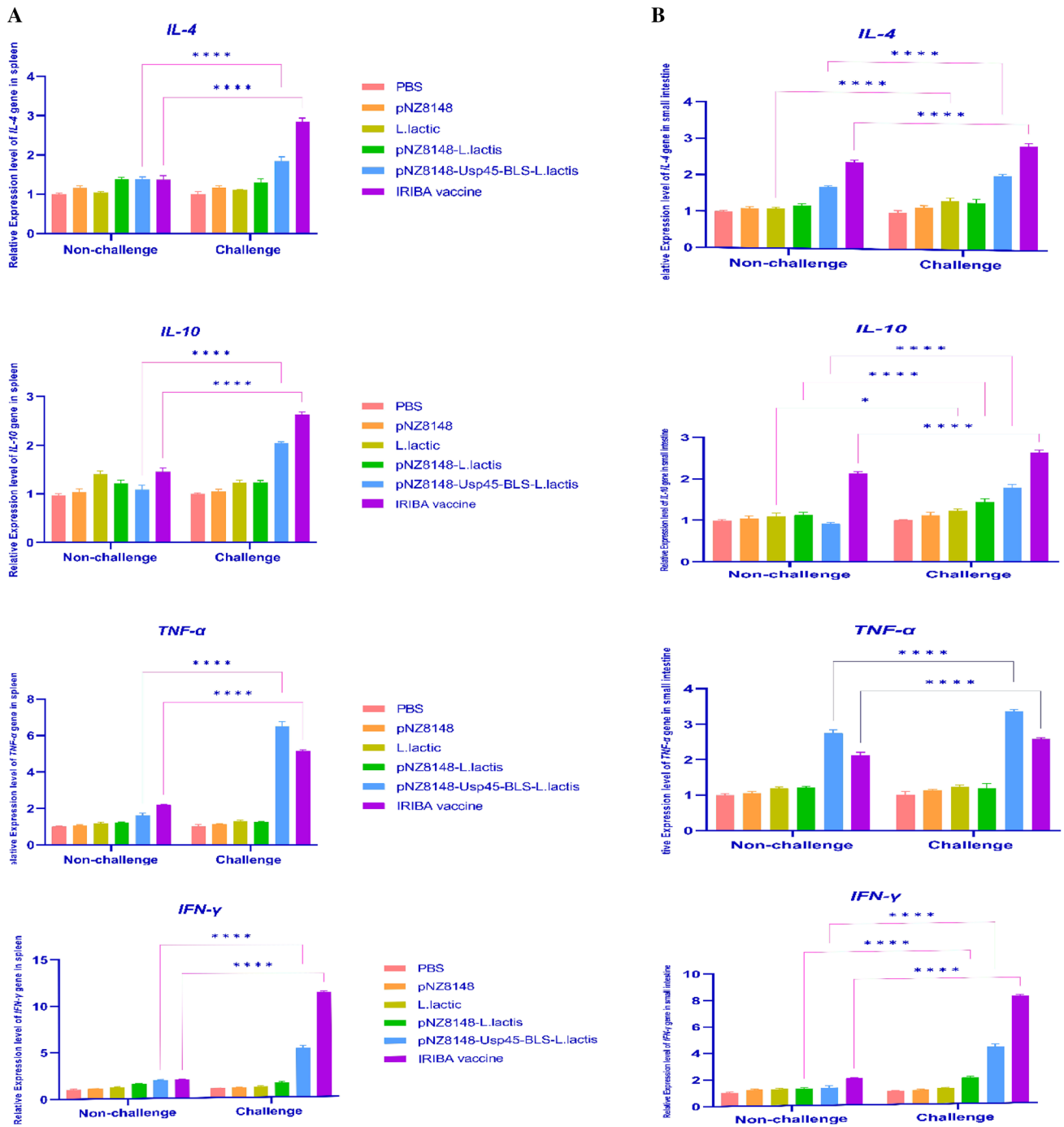


Fig. 6 **a** Cytokine levels were found in the spleen of the control and immunized groups. **b** Cytokine levels were found in the small intestine of the control and immunized groups. **** $P < 0.0001$

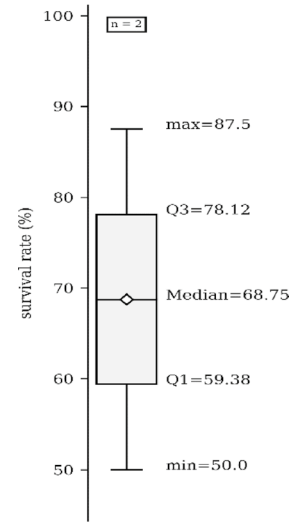
point, infection would not occur. For this reason, a vaccine must be developed to promote cellular and mucosal protection. According to current research, lactic acid bacteria (LAB) are one of the most effective options for creating mucosal vaccines because they may generate mucosal immunity when used as a live delivery vector for antigens. The current work aims to introduce *Lactococcus lactis* as

a beneficial, non-pathogenic mucosal immunotherapy and manufacture the immune-stimulatory rBLS-Usp45 protein as a substitute for the *E. coli* manufacturing system owing to its benefits (Rezaei et al. 2021). We looked into the capacity of *L. lactis* to transfer the protein antigen to the gastrointestinal tract and subsequently immunize the mice by carrying blank vector pNZ8148 and rBLS-Usp45. However,

Table 4 Survival percentage and clinical indicators of each group

Group	Before challenge			After challenge			survival rate (%)
	body weight (g)	clinical score	spleen weights (mg)	body weight (g)	clinical score	spleen weights (mg)	
PBS	18.3	0	409	15.75	-5	786	0
pNZ8148	18.7	0	400	15.67	-4	803	0
<i>L. lactis</i>	18.6	0	398	15.87	-3	794	12.5
pNZ8148- <i>L. lactis</i>	18.3	0	385	16.93	-1	601	30
IRIBA Vac	18.5	0	417	15.83	0	456	62.5
pNZ8148-Usp45-omp25- <i>L. lactis</i>	18.7	0	409	16.94	0	463	87.5

0 (normal, active, healthy), -1 (slightly sick, slightly ruffled fur, otherwise normal), -2 (ill, ruffled fur, sluggish movement, hunching), -3 (extremely sick, ruffled hair, very slow movement, stooped, eyes shut), -4 (moribund), and -5 (dead).



0 (normal, active, healthy), -1 (slightly sick, slightly ruffled fur, otherwise normal), -2 (ill, ruffled fur, sluggish movement, hunching), -3 (extremely sick, ruffled hair, very slow movement, stooped, eyes shut), -4 (moribund), and -5 (dead)

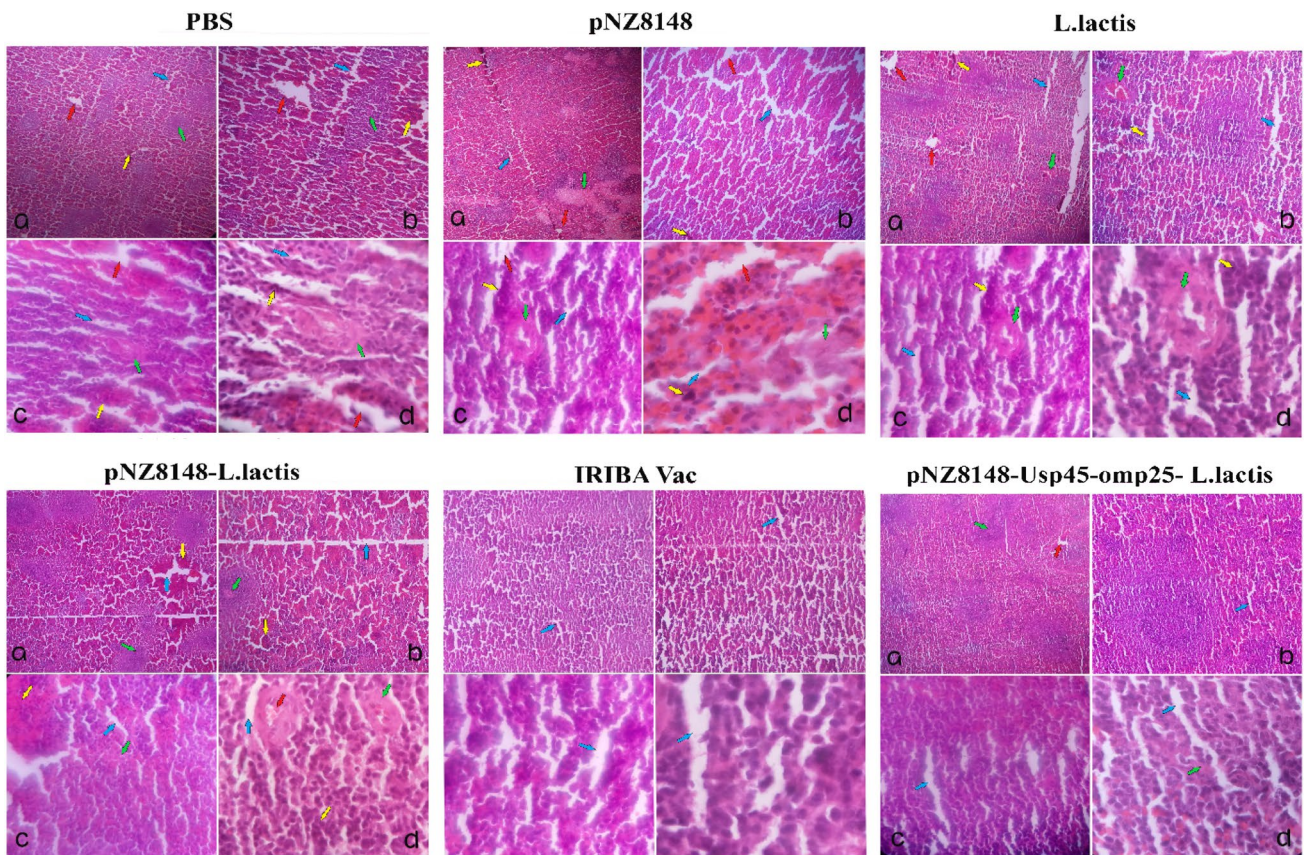


Fig. 7 Hematoxylin–eosin staining of spleen tissue sections. **a–d** Respectively, magnification 4 ×, 10 ×, 40 ×, 100 ×—increased space of sinusoids (blue arrow), spleen tissue necrosis (green arrow), bleed-

ing in the tissue (yellow arrow), abscess in the tissue, brucellosis (red arrow) (color figure online)

the challenge assay in mice against a pathogenic *Brucella* strain is necessary to address the assumption to investigate the effectiveness of oral vaccination. The findings validated earlier studies linking high IgG reactions to the fusion rBLS-Usp45 antigen as an effective immunogen to elicit specific antibodies (Ghasemi et al. 2015).

In contrast to live attenuated strains of *Brucella*, which carry the risk of reverting to their original virulent form, choosing the preferred antigens of bacteria because they only contain the preferred immunogenic epitopes in future subunit-based vaccines rather than the entire bacterium could be the ideal choice toward establishing novel vaccination strategies (Delany et al. 2013). Studies on reverse vaccination are one of the novel sciences that may be used to find potential antigenic options (Beiranvand et al. 2021). A genetic investigation to identify a potential vaccination against *B. abortus* isolates is included in this publication. The *B. abortus* genome is being used uniquely to discover vaccine candidates. An effort to find potential vaccine candidates for *meningococcus serogroup B* (*Neisseria meningitidis*), reported in 2000, was the first traditional instance of practical reverse vaccinology (Kelly and Rappuoli 2005). After some time, a *meningococcus serogroup B* vaccine was discovered in 2006 (Giuliani et al. 2006), and encouraging findings were shown after human clinical tests in 2011 (Toneatto et al. 2011). Pathogen antigen discovery continued until Chiang et al. (2015) used reverse vaccinology to effectively identify three antigens as vaccine candidates (Chiang et al. 2015). Reverse vaccinology allows us to filter the proteome using practical criteria, such as protein localization, non-similarity to the human proteome, and MHC binding affinity, reducing the trial-and-error required to uncover potential vaccine candidates (Piri-Gharaghie et al. 2022a). Reverse vaccinology algorithms may quickly identify a particular antigen or a collection of antigens for vaccine design programs using the genetic and cellular protein data on infections. In addition, it is a cost-effective strategy because most screening algorithms and genetic data are readily available. Reverse vaccination is also effective against various diseases, including parasites, viruses, fungi, and bacteria. Using the BLAST program, this strategy may be used for any microorganism (Piri-Gharaghie et al. 2022b).

In the theory that the live vector mucosal vaccine can increase the efficacy of our constructed vaccine because of its various roles in innate and adaptive immunity by contributing to the generation of antigen-specific immune function and its adjuvant effect on IgG1 and IgG2a antibody production, we conjectured the BLS as an immunogenic surface-exposed antigen with the Usp45 secretion factor could be a rational choice toward achieving this goal. Previously, Steindler had noted that administering immunoregulatory cytokines and antigens improved a vaccine's efficacy (Steindler et al. 1998; Piri-Gharaghie et al. 2023).

Many authors have looked at using lactobacilli as oral vaccine vectors. Since probiotics readily live in the digestive tract without harming it and retain tight contact with the epithelium, this idea has been investigated. Moreover, several writers have investigated their immunomodulatory abilities (Yu et al. 2013; Piri Gharaghie et al. 2023; Pouwels et al. 1998). Because of this last characteristic, they have been used in conjunction with vaccinations for intracellular microbes. For example, *L. rhamnosus* acts as an adjuvant for the polymorphic membrane protein C (N-Pmpc) vaccine and boosts both humoral and specific cell immune function (Inic-Kanada et al. 2016). Better results have been observed when probiotics are used alongside immunizations to prevent brucellosis.

A ribosomal protein called L7/L12 causes a reaction specific to cells. *L. lactis* was altered by Ribeiro et al. (2002) utilizing the gene L7/L12. When given orally to BALB/c mice, the vaccination produced large quantities of IgA versus L7/L12 in the feces. However, no specific antibodies were discovered in the blood, indicating that no systemic response was elicited. A more excellent defense was produced by administering the probiotic intraperitoneally.

According to Bermdez-Humarán et al. (2003), when given to C57BL/6 mice via the nasal route, *L. lactis* converted to generate IL-12 alongside *L. lactis*, and E7 antigen of the human papillomavirus type 16 induced a Th1-type reaction with significant IFN- γ secretion in splenocytes. Based on this research, oral administration of two recombinant isolates of *L. lactis*, one encoding Cu/Zn SOD and the other generating IL-12, was performed on BALB/c mice. In reaction to re-stimulation with SOD or crude *Brucella* protein extract, the animals generated notable amounts of SOD-specific slgA in nasal and bronchoalveolar lavage and T cell proliferation.

Real-time PCR and ELISA techniques were used in this work to assess the transcriptional and expression levels of inhibitory and functional cytokines. According to our findings, significant levels of IFN- γ and TNF- α cytokines were produced on day 14 following the last vaccination and IL-10 and IL-4 cytokines on day 28. Additionally, the transcriptional data and the amount of expression of the immune cells' proteins were both consistent.

An earlier live vaccination based on the beta antigen C (Bac) molecule was created by Gupalova et al. (2018). To immunize mucous membranes, they employed the probiotic bacteria *Enterococcus faecium* L3. The probiotic strain's capacity to grow at the mucosal level increases the amount of protein by the approach, which is advantageous. However, the development of immune tolerance through ongoing antigenic stimulation must be considered. *L. lactis*, in contrast, has a GRAS status of food grade and is a non-invasive, non-pathogenic bacterium (Bermúdez-Humarán et al. 2011). As a result, it may be utilized to create live vaccines without

running the risk of colonization, which lowers the danger of tolerance development. Its peptidoglycan wall also functions as a biological adjuvant (Medina et al. 2010).

In this situation, we have seen a substantial reduction in bacterial load in the mice inoculated with *L. lactis*, suggesting that live vector vaccine can reduce bacterial colonization. Live vector vaccine intrinsic features for triggering a particular immune reaction against antigens of different infections have been previously revealed (Medina et al. 2010).

These findings suggest that *rL. Lactic*-BLS as an expression vector can produce live oral vaccines that are inexpensive, simple to administer, and effective at preventing *B. abortus* infection. Because of their opsonophagocytic activity, serum antibodies play a crucial role in mucosal defense. Additionally, *B. abortus* could not propagate throughout the body due to the unique serum humoral reaction to BLS.

Conclusion

As a result, intraperitoneal and oral administration of routes is appropriate, even though the route has been found to differ in IgG response for immunization mice. However, a mucosal-administered vaccine based on *L. lactis* can live there, withstand stomach acid, and adhere to mucosal surfaces, which is a sensible alternative for creating a brucellosis-controlling vaccine. In conclusion, the orally administered live *Lactococcus*-based vaccine can be viewed as an alternative possible vaccine delivery vector for the pathogenic live *Brucella* vaccines now in use to create a future vaccine that is both effective and safe against brucellosis.

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Data availability Data available at request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval The animal experiments were approved by the Ethical Research Committee in the Islamic azad university of Shahrekord branch (IR.IAU.SHK.REC.1401.004).

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