CLINICAL MICROBIOLOGY - RESEARCH PAPER

Streptococcus mutans glutamate binding protein (GlnH) as antigen target for a mucosal anti-caries vaccine

Gisela de Souza Pereira[1] · Milene Tavares Batista[1] · Nayara Fernanda Barros dos Santos[1] · Hélic Moreira Passos[1] · Dalva Adelina da Silva[1] · Ewerton Lucena Ferreira[1] · Luís Carlos de Souza Ferreira[1] · Rita de Cássia Café Ferreira[1]

Abstract

Background In recent years, several studies have demonstrated that bacterial ABC transporters present relevant antigen targets for the development of vaccines against bacteria such as *Streptococcus pneumoniae* and *Enterococcus faecalis*. In *Streptococcus mutans*, the glutamate transporter operon (*glnH*), encoding an ABC transporter, is associated with acid tolerance and represents an important virulence-associated factor for the development of dental caries.

Results In this study, we generated a recombinant form of the *S. mutans* GlnH protein (rGlnH) in *Bacillus subtilis*. Mice immunized with this protein antigen elicited strong antigen-specific antibody responses after sublingual administration of a vaccine formulation containing a mucosal adjuvant, a non-toxic derivative of the heat-labile toxin (LTK63) originally produced by enterotoxigenic *Escherichia coli* (ETEC) strains. Serum anti-rGlnH antibodies reduced adhesion of *S. mutans* to the oral cavity of naïve mice. Moreover, mice actively immunized with rGlnH were partially protected from oral colonization after exposure to the *S. mutans* NG8 strain.

Conclusions Our results indicate that *S. mutans* rGlnH is a potential target antigen capable of inducing specific and protective antibody responses after immunization. Overall, these observations raise the prospect of the development of mucosal anti-caries vaccines.

Keywords *Streptococcus mutans* · GlnH · ABC transporters · Anti-caries vaccines · Mucosal adjuvant · LTK63 · Dental caries

Background

Dental caries is one of the most common infectious diseases affecting humans [1] and has been classified by the World Health Organization (WHO) as a major public health issue [2] affecting almost all adults worldwide [3]. Treatment of dental caries is expensive and disease may cause tooth loss, particularly in developing countries [2].

This disease is the result of a localized tooth dissolution process due to the production of acids by bacteria thriving in the dental biofilm, leading to an imbalance between the processes of demineralization and remineralization [4]. Among all oral biofilm-associated bacteria, *S. mutans* is considered the most prevailing cariogenic species due to its capacity to adhere tightly to the dental surface as well as its acidogenic property [5]. Due to the prevalence of caries, prophylactic strategies are particularly essential as they may mitigate the problem by reducing or eliminating the etiological agent. One of the potential prophylactic approaches is the development of an anti-dental caries vaccine.

The concept of a dental caries vaccine was established shortly after the determination of the role played by *S. mutans* in the disease [5, 6]. However, despite enormous efforts in the last four decades to develop and test different vaccine formulations against human dental caries, no vaccine has been made available for commercial use yet. In part, such failure may be attributed to the difficulties encountered in identifying promising antigens and limitations in their ability to induce immune responses capable of effectively preventing oral colonization by *S.*

Responsible Editor: Luiz Henrique Rosa

✉ Rita de Cássia Café Ferreira
  ritacafe@usp.br

[1] Department of Microbiology, Biomedical Science Institute, University of São Paulo, São Paulo, SP 137405508-900s, Brazil

mutans. While most previous studies focused on bacterial adhesins, particularly the P1 protein, as a vaccine target, recent studies demonstrate the vaccine potential of other less prominent surface proteins. In this context, bacterial carriers of the ABC family (ATP-Binding Cassette) have been characterized as potential targets for neutralizing antibodies due to their important role in the physiology and virulence of different bacterial species [7].

Transport systems like ABC transporters are essential for the passage of substances, such as the nutrients needed for cell growth, through the bacterial envelope. The ABC transporter system consists of three components, one functional and two structural [8, 9]. Two of the components are associated with the cytoplasmic membrane and are responsible for generating energy from the hydrolysis of ATP and promoting nutrient uptake through membrane pores. The third component is a protein involved in binding of the substrate. These proteins are bound to the cytoplasmic membrane and are exposed on the surface of gram-positive bacterial strains [10].

Several recent studies have demonstrated the reactivity of sera collected from infected animals or convalescent patients with different elements of bacterial ABC transporters, especially the substrate binding components. This reactivity was demonstrated with *Enterococcus faecium* and *Staphylococcus aureus* [7], and the results point to a possible role of ABC transporters as targets for immunotherapies and/or prophylaxis. Vaccine formulations based on bacterial ABC system components, such as those based on the PstS of *Mycobacterium tuberculosis* and *S. mutans*, which is involved in phosphate transport, PiuA and PiaA from *S. pneumoniae*, which are involved in the uptake of iron, and OppA of *Y. pestis* and *Moraxella catarrhalis*, which is capable of transporting a variety of short peptides, were tested under pre-clinical conditions [11–13]. These studies demonstrate that, in addition to being immunogenic, these proteins may induce protective immunity, at least under experimental conditions.

In *S. mutans*, the *gln* operon is responsible for the transport of glutamate/glutamine, which plays a central role in bacterial metabolism. Deletion of the *gln* operon results in reduction of bacterial tolerance to acidic environments (aciduricity), which is an important virulence factor for the development of dental caries [22]. In this study, we evaluated the performance of a recombinant *S. mutans* GlnH (rGlnH) protein, a substrate-binding component of the ABC transporter, as a vaccine antigen. The purified antigen was combined with a mucosal adjuvant, a non-toxic derivative of the heat-labile toxin (LTK63), and administered to mice via the sublingual route. Our results demonstrate that rGlnH induces the production of antibodies capable of reducing oral colonization by *S. mutans* and is thus a promising antigen candidate for the development of anti-caries vaccines.

Methods

Mice and ethics statement

This study was performed following the guidelines of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). Experimental protocols were approved by the Ethics Committee on the Use of Animals, University of São Paulo (CEUA-ICB/USP). BALB/c mice were obtained from Faculty of Medicine, University of São Paulo (USP). Five animals per cage were housed at the Microbiology Department of the Institute of Biomedical Sciences (USP) with food and water provided ad libitum. At the end of each experiment, mice were sacrificed by carbon dioxide inhalation.

Bacterial strains and growth conditions

E. coli DH5α strain was used for the cloning of *glnH* gene (kanamycin 50 μg mL^{-1}) and *B. subtilis* 1012 strain (chloramphenicol 30 μg mL^{-1}) for protein expression. Both strains were cultivated in Luria–Bertani (LB) broth at 37 °C under aeration. Competent *E.coli* cells were prepared using the CaCl$_2$-mediated transformation protocol (26). The *S. mutans* UA159 strain was cultivated at 37 °C in 5% CO_2 in brain heart infusion broth for genomic DNA extraction, and the NG8 strain was grown in Todd-Hewitt broth supplemented with 0.3% yeast extract for in vivo colonization assays, as previously described [13].

Plasmid construction

The fragment derived from the *glnH* gene was synthesized by GenScript (Piscataway, NJ, USA) based on the original gene sequence of the *S. mutans* UA159 strain and with codon adaptation for optimal expression in *B. subtilis* using the JCAT software (http://www.jcat.de/). In addition, the signal peptide sequence was removed and the GlnH glutamate binding site was modified by replacing the two histidine residues at position 144 and 145 with arginine, as per previous observations [15]. The sequence encoding rGlnH was subcloned into the *B. subtilis* pHT08 expression vector [27]. The recombinant plasmid was extracted and analyzed using 0.8% agarose gel following standard procedure [26]. The constructed expression vector was named pHT08GlnH and subsequently transformed to *B. subtilis* 1012 by electroporation [30].

Expression and purification of rGlnH

Expression and purification of the rGlnH protein by *B. subtilis* cells were carried out according to previously described methods [28, 29]. Transformants were cultured overnight in LB, inoculated (1:100) in 50 mL of LB medium, and grown until an OD_{600} of 0.6–0.8 was achieved. Production of the recombinant protein was induced by addition of 1 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside) and incubation for 16 h at 37 °C with constant shaking at 200 rpm. Culture samples before and after addition of IPTG were collected at different time points (2, 4, and 16 h) and analyzed using SDS-PAGE. The induced cultures were harvested by centrifugation at 10,000 rpm for 5 min at room temperature and suspended in 5 mL of Tris–HCl buffer (100 mM Tris–HCl, 500 mM NaCl, pH 7.5) and lysozyme (800 μg/ml). Thereafter, they were kept on ice for 30 min. Then, 0.01% SDS and 0.01 mM PMSF (phenyl methane sulfonyl fluoride) were added followed by sonication using 40% pulsed cycle of 30 s burst and 1 min pause for a total duration of 2.5 min, 3 times. After centrifugation, the soluble supernatants were loaded into 5 mL HisTrap HP columns (GE Healthcare, IL, USA) for purification by affinity chromatography using an AKTA FPLC device (Amersham Biosciences). The bound proteins were eluted from the column with an imidazole gradient of 0.02–1 M in Tris–HCL buffer. The eluted fractions containing purified protein were analyzed using SDS-PAGE, quantified by Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, MA, USA), and stored at − 20 °C.

Vaccine regimen and sample collection

Groups of five 6–8 week old BALB/c female mice were immunized sublingually in a three-dose regimen with 2-week intervals, by topical application with a micropipette under the tongue after anesthetizing with ketamine (80 mg/kg of body weight) and xylazine hydrochloride (8 mg/Kg of body weight). Each animal received 5 μg of rGlnH alone or the protein added with 5 μg of LTK63, a non-toxic derivative of the ETEC LT [31] as a mucosal adjuvant. The humoral response of the immunized animals was measured in blood samples collected one day before each dose and two weeks after the last dose by submandibular puncture. The samples were incubated for 30 min at room temperature and another 30 min at 4 °C, followed by centrifugation at 5,000 rpm for 30 min at 4 °C. The serum was collected from the supernatant and stored at − 20 °C for further analysis.

Detection of rGlnH-specific antibody response

Anti-GlnH IgG responses were analyzed by ELISA using rGlnH as solid-phase bound antigen. Serum samples of each immunized mouse were pooled and diluted serially twofold in PBS-Tween (0.05%). After incubation with the tested sera, the wells were incubated with peroxidase-conjugated goat anti-mouse IgG or anti-mouse IgG1 and IgG2a (Sigma or Southern Biotech) as secondary antibodies. The absorbance values measured in sham-treated mice were lesser than those measured in serum samples of vaccinated mice. Concentrations of antigen-specific antibodies were determined with a standard curve prepared with known concentrations of mouse IgG, IgG1, and IgG2a antibodies (SouthernBiotech, AL, USA). Immunodetection of the native GlnH expressed on the surface of bacterial cells was performed with *S. mutans* NG8 cells as the solid-phase bound antigen in ELISA plates, as previously described [31].

Protective role of anti-rGlnH antibodies to oral colonization challenge with *S. mutans*

The effect of anti-rGlnH antibodies on in vivo tooth colonization by *S. mutans* was tested under previously described experimental conditions using Balb/c mice [13, 32, 33]. Cells from *S. mutans* NG8 strain were pre-incubated with pooled serum samples (two weeks after the last dose of the immunization regimen) and diluted 1:100. The controls were prepared with bacterial cells incubated with PBS or serum pool collected from sham-treated mice. Before challenge, *naïve* BALB/c female mice were fed an ad libitum diet and with water containing 1% sucrose for one day. Before inoculation, mice were anesthetized, and the oral cavities were cleaned with chlorhexidine (0.12%) and treated with clarified human saliva. *S. mutans* NG8 cells were applied on the surface of the tooth with a micropipette. After six hours, the oral cavities were sampled with swabs, plated on Mitis-Salivarius (MS) agar plates, and incubated at 37 °C for 48 h in anaerobic conditions. Colony-forming units (CFU) were counted to determine the number of *S. mutans* recovered from the mice oral cavity. Protective immunity against *S. mutans* was also assessed by carrying out oral colonization in mice immunized with rGlnH, according to previously described conditions [13]. Mice subjected to the complete vaccine regimen were inoculated two weeks after the third dose, following the same procedure described above, with *S. mutans* NG8 cells applied directly to their teeth. Sample collection and processing for determination of the number of *S. mutans* cells followed the same procedure described above.

Statistical analysis

The results were analyzed using the GraphPad Prism 5 software and expressed as the mean $\pm$ SD. Statistically significant differences ($p < 0.05$) were determined using Student's t-test and Mann–Whitney post-test.

Results

Production of recombinant *S. mutans* GlnH (rGlnH) by *B. subtilis*

The *S. mutans* UA159 *glnH* gene (744 bp) was subcloned into *B. subtilis* expression vector pHT08 (8 kb), forming the pHT08GlnH vector (approximately 8.8 kb) (Fig. 1(a), data not shown). Chemically competent *B. subtilis* 1012 cells were transformed with pHT08GlnH, and rGlnH was highly expressed as a soluble protein with an approximate weight of 70 kDa, which is different from the predicted molecular weight of approximately 29 kDa. The abnormal SDS-PAGE migration observed was not due to polymerization since treatment with reducing agents (e.g., urea and guanidine) did not affect protein migration (data not shown). However, this is a highly acidic protein (composed of around 7.5% of glutamate and aspartate), a property that is reportedly associated with slower migration in SDS-PAGE [23–25]. The expressed rGlnH was detected with a His tag-specific antibody in immunoblots (Fig. 1b and c). The protein was highly expressed at different experimental conditions (Fig. 1(d)) and purified from the soluble fraction of the cell extracts by affinity chromatography (Fig. 1(e)).

Sublingual immunization with rGlnH induces specific IgG serum response in mice

Groups of female BALB/c mice were immunized with rGlnH via the sublingual route. An increase in the serum rGlnH-specific IgG response was observed in animals immunized with the protein, with or without the combination of a non-toxic mucosal adjuvant derived from the LT produced by ETEC (LTK63) (Fig. 2a). Co-administration of LTK63 further increased the anti-rGlnH serum IgG titer levels, even with the administration of a single dose (Fig. 2a). Immunization with rGlnH either alone or adjuvanted with LTK63 stimulated high levels of the IgG1 (Fig. 2b). In contrast, no significant changes in IgA levels were detected in mice immunized with rGlnH even with co-administration of LTK63. These results indicate that rGlnH is highly immunogenic after sublingual administration, resulting in the induction of specific serum IgG responses.

Induction of protective immunity by rGlnH

We initially tested the ability of immune sera to recognize native *S. mutans* GlnH protein. Sera obtained from mice immunized with rGlnH recognized native GlnH expressed on the pathogen surface (Fig. 3a). This result demonstrates that rGlnH preserves relevant epitopes found on the native protein expressed in *S. mutans*. In order to evaluate the protective role of the GlnH-specific antibody responses, we applied two previously established challenge protocols based on a mouse oral colonization model [13]. The first protocol measured the neutralization effectiveness of anti-rGlnH antibodies. *S. mutans* NG8 strain was incubated with serum pools collected from animals immunized with three doses of the tested vaccine formulations. Mice orally inoculated with bacteria previously incubated with anti-rGlnH sera, either alone or co-administered with LTK63, showed significant reduction in oral colonization by the NG8 strain compared to mice inoculated with bacteria treated with sera from non-immunized mice (Fig. 3b). The second protocol evaluated the protective immunity induced in animals immunized with rGlnH by orally inoculating mice previously immunized with rGlnH with *S. mutans* NG8. As shown in Fig. 3c, mice immunized with rGlnH, either alone or co-administered with LTK63, showed a reduction in oral colonization compared to sham-treated mice. These results indicate that mucosal immunization with rGlnH induces protective immunity against oral colonization by *S. mutans* in the tested experimental conditions.

Discussion

Dental caries is a highly widespread disease globally [14]. Several studies have demonstrated that *S. mutans* is the most epidemiologically relevant pathogen associated with dental caries [4]. In the last 40 years, several groups have pursued the ideal of an effective anti-*S. mutans* vaccine capable of preventing tooth decay [4, 6]. In this regard, the identification of potential antigen targets is an essential step toward the generation of antibodies capable of reducing or preventing oral colonization by the pathogen. Although previous studies dealing with anti-caries vaccines mainly focused on the P1 protein (antigen I/II) as the vaccine target [4, 5, 21], more recent evidence indicates that ABC transporter components play an essential role in the infection process and may contribute to the establishment of protective immunity against the disease [7, 13]. Therefore, our study evaluated the use of a recombinant form of the *S. mutans* glutamine/glutamate binding protein (rGlnH), a component of the glutamate transporter system and a virulence-associated factor, as a potential antigen target for vaccine formulations against dental caries.

Fig. 1 Expression and purification of the *S. mutans* rGlnH in recombinant *B. subtilis* strain. **A** Restriction analysis of the plasmid pHT08GlnH. **B** Expression of the rGlnH by a recombinant *B. subtilis* strain. Polyacrylamide gel results of whole extracts of *B. subtilis* carrying the pHT08GlnH vector before (T_0) and after different induction times (T_2, T_4, T_{16}). **C** Western blot of the whole extracts of *B. subtilis*-pHT08GlnH. For immunodetection, the anti-His tag polyclonal antibody was used. **D** Evaluation of the influence of growth conditions on the expression of GlnH protein. Expression of rGlnH by the *B. subtilis* strain induced at different temperatures, with different aeration conditions and aliquots, before and after induction, was analyzed by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250. **E** SDS-PAGE of the eluted fractions obtained from the immobilized metal affinity chromatography of rGlnH protein. Flow: flow through; 5 to 200 mM: eluted fractions from imidazole gradient
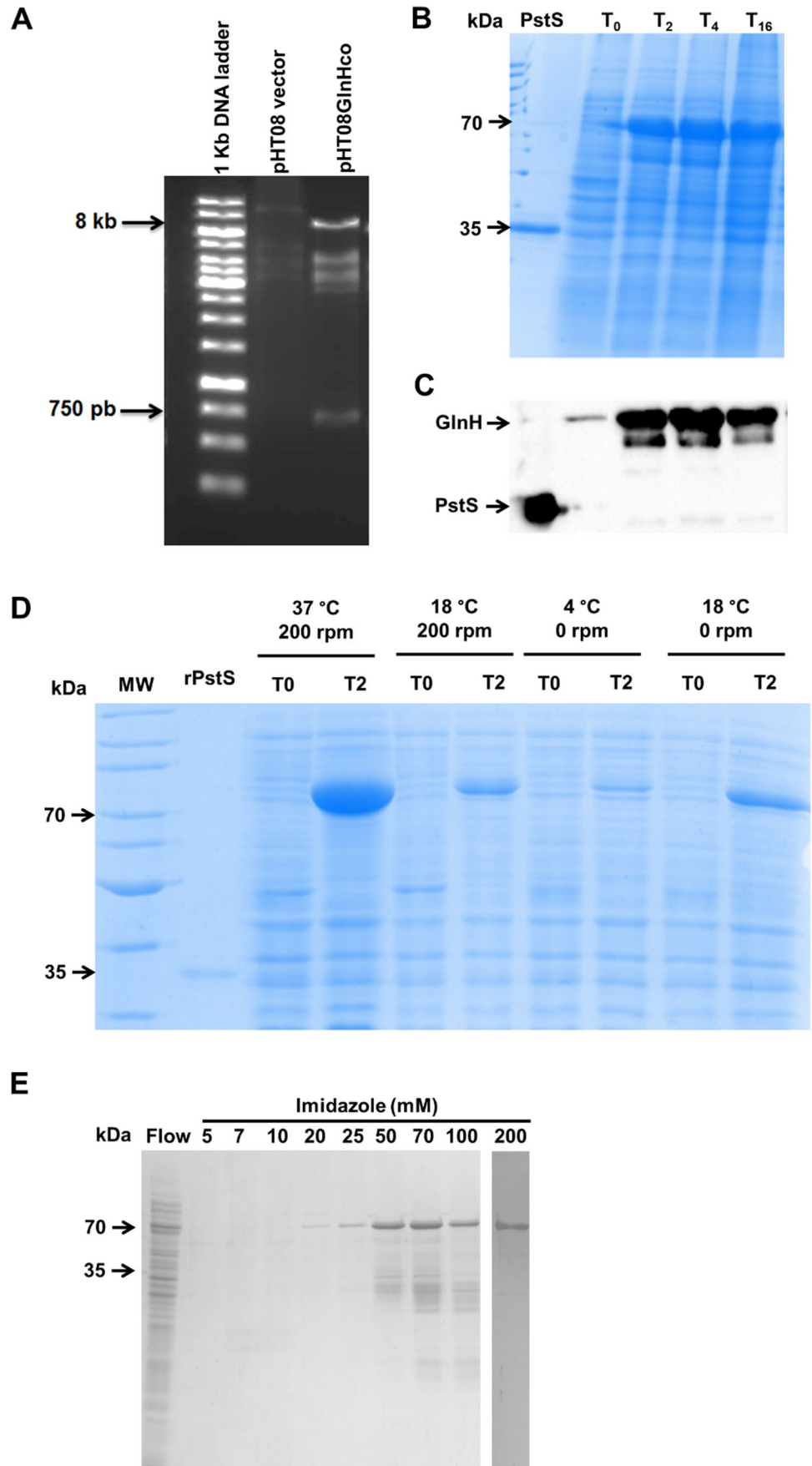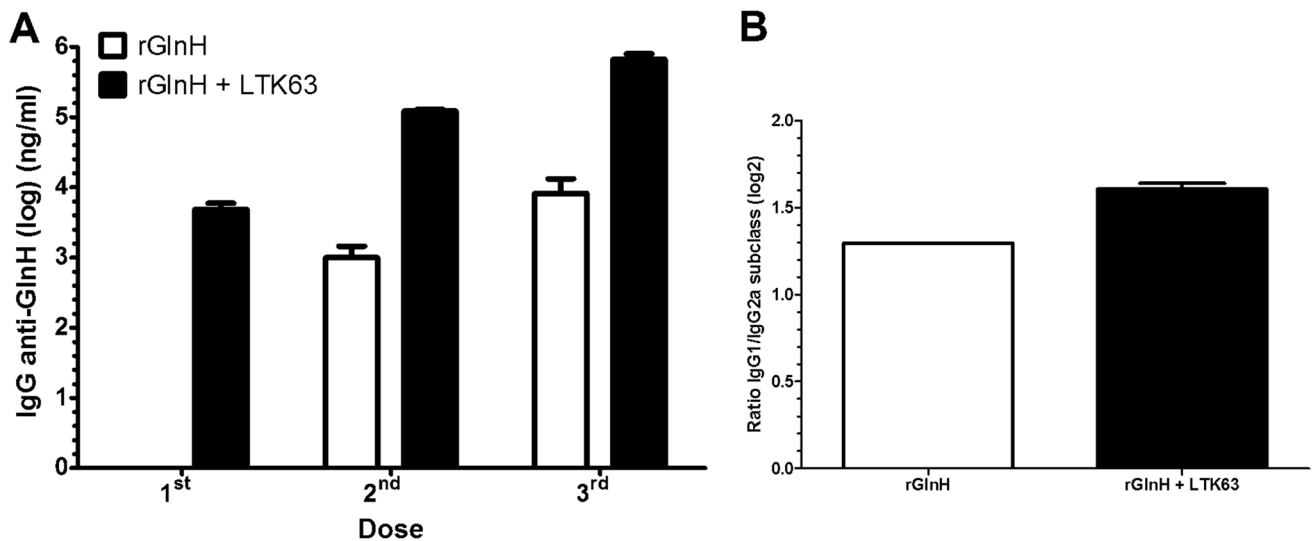
Fig. 2 GlnH-specific antibody responses after sublingual immunization. **A** Serum anti-GlnH IgG responses. Sera from BALB/c mice immunized sublingually with 5 μg of rGlnH alone (white bars) or in combination with 5 μg of LTK63 as an adjuvant (black bars) were collected two weeks after each dose. **B** Serum anti-GlnH IgG subclass responses. ELISA was performed with pooled sera samples ($n = 10$ or 11) using purified rGlnH as the immobilized antigen. Data is based on two independently performed immunization experiments and expressed as means $\pm$ SEM

Our initial attempts to express rGlnH in *E. coli* and *B. subtilis* strains failed due to slow bacterial growth and the low expression levels of the protein. We hypothesized that, even at low concentrations, the recombinant protein binds to glutamate/glutamine inside the cell and thus prevents adequate bacterial growth and protein biosynthesis. To overcome this, we designed a new synthetic gene with a major modification, i.e., replacement of the histidine residues at positions 144 and 145 by arginine, as previously reported by Armstrong et al. [17], leading to loss of substrate binding activity. This modification had a significant impact on protein expression, resulting in high yields of rGlnH by a recombinant *B. subtilis* strain.

Mucosal epithelial surfaces that comprise the gastrointestinal, urogenital, and respiratory tracts form a physical and immunological barrier to the entrance of pathogens [16, 17]. Immunization via mucosal routes can stimulate both systemic and mucosal immune responses, leading to the production of antibodies (IgA and IgG) and T cells. Particularly, the sublingual immunization route has proven to be efficient in inducing an immune response against different pathogens [18]. In our study, sublingual immunization with rGlnH induced high concentrations of antigen-specific serum IgG. On the other hand, no significant secretory anti-rGlnH IgG or IgA levels were detected in vaccinated mice. Despite that, the immunization of mice with rGln conferred a significant reduction in oral colonization by the *S. mutans* NG8 strain, which demonstrates the efficacy of the immunization strategy. This protective effect may,

therefore, be ascribed to the passage of local serum IgG antibodies to the dental periodontal region.

Adjuvants play an important role in the efficacy of different vaccine formulations, particularly those based on purified recombinant proteins. In contrast to parenteral vaccines that require adjuvants to promote local inflammatory reactions, mucosal adjuvants usually exert their effects by enhancing mucosal permeability. The heat-labile toxin (LT) produced by some enterotoxigenic *E. coli* (ETEC) strains is one of the most extensively studied and effective mucosal adjuvants under experimental conditions. The non-toxic LT derivative LTK63 was obtained after insertion of a mutation at the A subunit at position 63, leading to a blockage of its toxic effects while preserving its adjuvant effects [19, 20]. The association of rGlnH and LTK63 impacted the serum IgG responses elicited in vaccinated mice. The adjuvant effects were observed after administration of each dose of the vaccine formulation, three in total. Addition of LTK63 also modulated the IgG subclass responses, leading to enhanced production of IgG1 without any significant increase in salivary IgA. Similar results were observed in a previous study based on the phosphate binding protein of *S. mutans* [13]. In both cases, experimental evidence demonstrates that protective effects against oral pathogen do not require the induction of local secretory IgA responses and may be ascribed to other mechanisms involving systemic antibody responses.
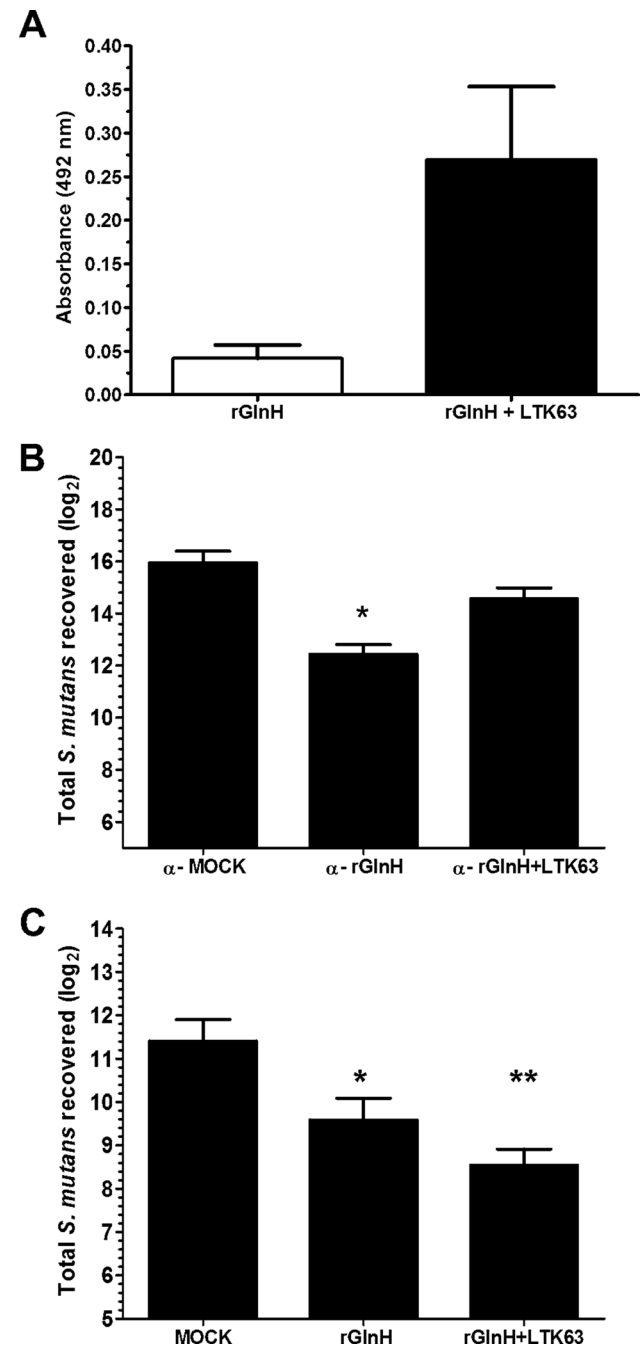
Important evidence of the protective role of the tested vaccine formulations was the observation that anti-rGlnH

Fig. 3 Protective immunity induced by immunization with rGlnH. **A** Reactivity of anti-GlnH antibodies to native GlnH protein expressed on the surface of *S. mutans* cells. Immunoassays were performed using pretreated *S. mutans* cells (1×10^8 CFU/well) as solid-phase bound antigens in ELISA plates. **B** Serum neutralization of the adherence of *S. mutans* to the oral cavity of naïve mice. Animals ($n = 5$) were pretreated with chlorhexidine and human saliva, then treated with *S. mutans* NG8 strain previously incubated with serum pools collected from vaccinated mice. *S. mutans* without pre-incubation with serum (untreated) and incubated with sera collected from sham-treated mice were used as negative controls. Data represents two independent experiments and are expressed as the mean $\pm$ SEM. *, $p < 0.05$ (Student's *t*-test and Mann–Whitney test as post-test). **C** Inhibition of *S. mutans* oral cavity colonization following sublingual immunization with rGlnH protein. Three weeks after the last dose, the mice ($n = 5$) were pretreated with human saliva and inoculated with the *S. mutans* NG8 strain. Oral cavity swabs were collected 6 h after inoculation and plated on -MS agar containing bacitracin (0.2 U/ml) and sucrose (5%). Data represent two independent experiments and are expressed as the mean $\pm$ SEM. *, $p < 0.05$; **, $p < 0.01$ (Student's *t*-test and Mann–Whitney test as post-test)

antibodies efficiently bound to the protein expressed on the surface of *S. mutans* cells. This was further supported by additional tests where vaccination with rGlnH prevented tooth colonization based on a murine model. These results demonstrate that immunization with rGlnH conferred partial protection to the colonization of teeth by the *S. mutans* NG8 strain. Notably, mice immunized with the adjuvanted formulation showed significant reduction in the colonization of the oral cavity, a feature that may reflect the amount of neutralizing antibodies and epitope specificity of the antigen-specific antibodies. Thus, co-administration of the mucosal adjuvant improved the immune responses elicited in vaccinated mice both quantitatively and qualitatively. A previous study demonstrating the modulation of epitope specificity of the induced antigen-specific antibodies by incorporation of LT [21] may help in understanding the relevance of both the adjuvant and antigen for the development of an effective dental caries vaccine.

Conclusions

In the present study, an experimental mucosal-delivered vaccine formulation containing a recombinant form of rGlnH showed positive results and could potentially be developed further as an effective anti-caries vaccine. Our results indicate that co-administration of rGlnH with a mucosal adjuvant induces antigen-specific serum IgG responses capable of recognizing the native protein expressed in *S. mutans* cells and confers protective immunity. Collectively, these results show promising prospects for the development of antibacterial vaccines based on the ABC transporter components.

Data availability Data supporting the conclusion in this article is included within the article and all of the datasets analyzed in this article are available from the corresponding author.

Declarations

Ethics approval and consent to participate All experiments involving animals were previously approved by the Ethic Committee on Animal Use of the Institute of Biomedical Sciences of São Paulo University (CEUA-ICB/USP), and in accordance with the guidelines from Brazilian National Council for Control of Animal Experimentation (CONCEA).

Competing interests The authors declare no competing interestsss.

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