Engineering probiotic bacteria to express *tcd*B antigen as an oral vaccine carrier against *Clostridium difficile* infection

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Background
Clostridium (now known as *Clostridioides*) *difficile* (*C. difficile*) is a spore-forming, gram-positive organism that can pose serious public health complications. The elderly are especially vulnerable to *C. difficile* infections, which can be fatal. *C. difficile* strains cause symptomatic diseases via the release of two toxins; *tcd*A and *tcd*B, that induce inflammation and tissue damage. Vaccines targeting any of these toxins may offer an effective strategy against *C. difficile* infections.
Objective
This study aimed to use live probiotics as oral vaccines to express the *C. difficile* toxin B gene. Oral vaccination has many advantages over intramuscular injection, as it has higher compliance, feasibility, and simpler administration. In addition, oral vaccines can stimulate both mucosal and systemic immunity against the target antigen. Probiotic bacteria were chosen as they present ideal candidates for this goal in terms of safety and health promotion.
Materials and methods
We chose two probiotic strains: *Lactobacillus gasseri* ATCC 33323 in addition to *Enterococcus faecium* NM1015, which had previously been identified in our lab and is capable of suppressing *C. difficile* colonization. The C-terminal of the *tcd*B gene was amplified by polymerase chain reaction (PCR) from *C. difficile*, cloned, and transformed into an *E. coli* EZ strain. The *tcd*B fragment was digested with *Bam*HI and *Xho*I (NEB, UK) and subcloned into the bile salt-inducible expression plasmid pLB210 (obtained from INRA, France). The transformation and electroporation procedures were employed to insert cloning and expression plasmids into the target bacteria. Colony PCR was used to confirm the engineering strains. Reverse transcription PCR was used to confirm the expression of the *C. difficile tcd*B fragment.
Results and conclusion
The expression vector 'p210-tcdB' was constructed, then introduced into the selected probiotic strains by electroporation and confirmed by colony PCR and plasmid extraction. The reverse transcription (RT)-PCR confirmed the expression of the gene by the engineered strains. No significant difference in the survival rate was observed between the engineered strains and their parental types at pH 2.00 and 1% oxygen. Moreover, the strains showed satisfactory plasmid stability for 210 generations. Future work will involve the *in vivo* evaluation of the engineered probiotic strains as oral vaccines against *C. difficile* using an animal model.

Keywords:
bacterial transformation, bile inducible plasmid, Clostridium *difficile*, cloning *tcd*B, *Enterococcus faecium*, *Lactobacillus gasseri*, oral vaccine carrier, probiotics

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Introduction

Clostridium (now known as *Clostridioides*) *difficile* (*C. difficile*) is a Gram-positive, anaerobic, spore-forming, bacterium that is the leading cause of infectious hospital-acquired diarrhea and public heath complications. Toxins production is considered the main pathogenic factor for disease development. The Centers for Disease Control (CDC) has classified this pathogenic microorganism as one of the three most urgent threats that require fast and crucial action [1]. *C. difficile* infections (CDI) pose a high health risk that can lead to death, especially in the elderly [2]. *C. difficile* strains can cause symptomatic diseases via the release of two proteins known as toxins A and B encoded by (*tcd*A and *tcd*B) genes, respectively [3].

The recommended treatment for the primary episode of CDI includes oral antibiotics; vancomycin, metronidazole, or fidaxomicin [4] these antibiotics are effective in 64% to 82% of the cases [5].

However, the oral antibiotic treatment is costly [6], may lead to the emergence of antibiotic-resistant strains and disruption of the gut microbiota [7]. Moreover, the recurrence of CDI is a major concern with lack of effective standard treatment. Previous studies showed recurrent CDI in 25–30% of the patients after the primary episode [8–10] and in 60% of the patients following the third episode [11,12]. In recent years, fecal or intestinal microbiota transplantation has been used extensively in clinical practice for the treatment of the recurrence of CDI. It is the most effective treatment with a success rate of 60–90%. However, there are significant disadvantages with fecal or intestinal microbiota transplantation such as high cost and infection risks [13,14].

C. difficile can be in a vegetative active form that is oxygen sensitive and disease-causing or as an inactive spore form that is resistant to harsh environmental conditions [15]. The inactive spores when ingested germinate into their vegetative forms and cause disease to their hosts [16]. This spore germination is responsible for the life cycle of *C. difficile* in the host [17]. The disruption of this propagation stage can halt the clostridial infection and release of toxins A and B [18]. Vaccines that target these toxins and/or proteins specific to the spores are a promising strategy to fight CDI. Novel routes of vaccination have been developed in recent years to avoid the drawbacks of injectable vaccines [19]. Many disease-causing microorganisms gain access to the body through mucous membranes. Administering antigens by mouth mimics the natural route of infection; hence it would offer better protection to the host [20]. Oral vaccination is relatively cheap and often triggers both local and systemic immune reactions [21]. By boosting the gut immune response and restoring the balance of the gut microbiota, probiotics can potentiate the treatment of CDI [22]. Probiotics are living microorganisms that have a positive effect on the host's health when they are consumed in sufficient amounts [23], thus they represent attractive candidates for oral vaccination. By producing substances such as bacteriocins, hydrogen peroxide, and short-chain fatty acids, some probiotics can prevent *C. difficile* spores from germination [24,25]. They can also boost the function of the cells in the intestinal mucosa that secrete antibodies and enhance the immune responses to *C. difficile* [26]. In our previous study, three novel probiotic strains capable of hindering *C. difficile* in vitro and in vivo were isolated, characterized and identified as *Enterococcus faecalis* NM815, *Enterococcus faecalis* NM915, and *E. faecium* NM1015 [27].

In this work, we aimed to construct genetically modified probiotic bacteria capable of expressing toxin B antigen and use them as an oral vaccine delivery vehicle against CDI. The probiotics used in this study were *L. gasseri* ATCC 33323 and *E. faecium* NM1015. This study offers an alternative strategy for the prevention and treatment of CDI.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *C. difficile* ATCC 43255 (toxin A+ and toxin B+, RB087) was purchased from American type culture collection (ATCC, USA). *E. coli* (EZ) was purchased from Qiagen, Germany and used for cloning the toxin B encoded by *tcd*B gene and as a host for the antigen expression vector. The human isolated *L. gasseri* ATCC 33323 was obtained from the ATCC, USA. *E. faecium* NM1015 was obtained from our culture collection at the NRC. Plasmid pLB210 was provided by Dr. Luis Bermedze, INRA-France.

C. difficile was grown anaerobically in an anaerobic jar using a gas generation kit (Oxoid LTD, UK) at 37°C in reinforced clostridial agar (Oxoid LTD, U.K). *E. coli*

Table 1 List of strains and plasmids used in this work

Strains or Plasmids	Relevant genotype or phenotype	Sources
C. difficile ATCC 43255	Toxin A+ and Toxin B+, RB 087	ATCC, USA
E. coli (EZ)	EZ Competent Cells	Qiagen, Germany
L. gasseri ATCC 33323	A neotype strain of human origin	ATCC, USA
E. faecium NM1015	Isolated from human stool with the ability to inhibit *C. difficile* 43255	Previous work[27]
E. coli NM0123	*E. coli* (EZ) containing plasmid pMN01, Amp^R	This work
E. coli NM0223	*E. coli* (EZ) containing plasmid pMN02, Cm^R	This work
L. gasseri NM0323	*L. gasseri* ATCC 33323 containing plasmid pMN02, Cm^R	This work
E. faecium NM0423	*E. faecium* NM1015 containing plasmid pMN02, Cm^R	This work
pDrive plasmid	PCR Cloning vector, Amp^R	Qiagen, Germany
pMN01 plasmid	pDrive with insert *tcd*B C-terminal fragment (~1.5 Kb), Amp^R, Kan^R	This work
pLB210 plasmid	Bile salt inducible expression plasmid Cm^R	INRA, France[28]
pMN02 plasmid	pLB210 carrying the *tcd*B C-terminal fragment (~1.5 Kb), Cm^R	This work

was grown at 37°C in Luria-Bertani (LB) broth with shaking at 200 rpm then plated on LB agar plates. *L. gasseri* and *E. faecium* strains were grown at 37°C in DeMan-Rogosa-Sharpe (MRS) broth or agar. Recombinant strains were grown in MRS medium supplemented with 0.3% bile salts (Sigma, USA). Antibiotics were added for the growth of antibiotic resistant strains at the following concentrations: chloramphenicol (Cm) 15 μg/ml for *E. coli* and 17 μg/ml for *L. gasseri,* and *E. faecium* NM1015 strains, ampicillin 100 μg/ml for *E. coli* strains, and X-gal 40 μg/ml for *E. coli* strains.

DNA and RNA extraction and manipulation

Genomic DNA for PCR amplifications was extracted using Qiagen extraction kit (Qiagen, Germany). The amplified DNA was excised from the gel and purified using Qiagen Gel extraction kit (Qiagen, Germany). The PCR product was cloned using Qiagen cloning kit (Qiagen, Germany). The plasmid extraction from *E. coli* was performed by Qiagen mini plasmid extraction kit (Qiagen, Germany). Plasmids were digested using restriction enzymes; *Bam*HI and *Xho*I (NEB, UK). Bacterial RNA extraction was performed using RNeasy Mini kit (Qiagen, Germany). All steps were carried out according to the manufacturer's instructions.

Polymerase chain reaction (PCR) and oligonucleotide synthesis

Genomic DNA or colony PCR was performed in 25 μl reactions using Master mix Dream Taq green (Thermoscientific, Lithuania), The primers (Table 2) purchased from Gene-Tech company (Cairo, Egypt) were added in the PCR reaction at a final concentration of 20 pmol. The PCR was performed in a DNA Thermal Cycler (Mj Mini PTC 1148, Bio Rad, USA). PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel supplemented with ethidium bromide (Biotech, Canada). The molecular weight markers: 100 bp and 1 kb (Thermoscientific, Lithuania) were used to estimate the size of the PCR amplicons.

Competent cells and transformation protocols

E. coli competent cells were transformed using electroporation for electro-transformation of ligation products and the calcium chloride heat shock transformation to increase the yields of plasmids [30]. Transformation of recombinant plasmid to *L. gasseri* was carried out by electroporation with some modifications to the method of Mandel and Higa (1970) by increasing the total amount of DNA to be transformed [31]. Electro-transformation of the ligation mixture or plasmid was performed by Micropulser Electroporator (Model 411BR, Biorad, USA) according to the manufacturer's instructions.

DNA sequencing and analysis

The purified PCR products and plasmids were sequenced at Macrogen, (South Korea). DNA similarity investigation was carried out using the basic local alignment search tool on the website of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Testing for plasmid stability

The engineered strains of *L. gasseri* and *E. faecium* carrying the toxin B expression plasmid were examined for plasmid stability. Chloramphenicol was added to MRS broth, and the strains were grown overnight. Then 0.1% of the overnight culture was transferred to MRS broth without antibiotic and grown until they reached the stationary phase (about 10 generations). This process was repeated 21 times (~210 generations). The number of colonies on MRS agar plates with and without antibiotics was counted after 70, 140 and 210 generations. The ratios of colonies on plates without antibiotic to those on plates with antibiotic were calculated as a measure of how stable the cells were without the presence of the antibiotic.

Evaluation of the probiotic properties of the engineered strains

Determination of acid tolerance

Overnight cultures were inoculated as 1% in MRS broth adjusted to pH 2.00 with hydrochloric acid (3.0 M). The samples were kept for 3 h at 37°C. Cells were then diluted in phosphate buffer (0.1 M, pH 6.5) to make the acidity neutral, plated on MRS agar and incubated at 37°C for 24 h. The survival rate was calculated by comparing the number of colonies to the initial number.

Table 2 The sequences and references of the primers used in this work

Primer name	Target gene	Primer sequence 5' –3'	Reference
Primers used for amplification *tcd* Bgenes of *C. difficile*			
TcdB-F TcdB-R	C-terminal –*tcd*B	5'CATGCCATGGGAGAAATTTTATATTAATAACTTTGG3' 5'CGCGGATCCTATTCACTAATCACTAATTGAGC3'	[29]
pDrive cloning vector primers			
M13-F M13-R	Cloning vector binding site	5' GTTTTCCCAGTCACGAC 3' 5' AACAGCTATGACCATG 3'	QIAGEN PCR Cloning Kit

Determination of bile tolerance

The bile tolerance of the engineered strains was determined by inoculating 1% of an overnight culture in MRS broth supplemented with oxgall bile (1% w/v) (Sigma Chemical Co., USA). The cultures were incubated for 3 h at 37°C then plated on MRS agar plates and incubated for 24 h at 37°C. The survival rate was calculated as the number of colonies (cfu/ml) compared with the control without oxgall bile addition.

Evaluation of antigen expression from the constructed probiotic strains

Reverse transcription PCR (RT-PCR)

For reverse transcription, the total RNA was incubated for 5 min at 70°C then, at 4°C for 5 min using a thermal cycler. The reaction was performed in 25 µl of GoScript 5× Reaction Buffer; MgCl2 (1.5–5 mM); PCR Nucleotide Mix; Recombinant RNasin Ribonuclease Inhibitor; GoScript reverse transcriptase; and Nuclease-Free water to reach the total volume. The reaction was performed in a thermal cycler with the following conditions: primer annealing at 25 °C, for 5 min; extension at 42°C, for 1 h; reverse transcriptase inactivation at 70°C, for 15 min. The RT-PCR products were checked by gel electrophoresis to confirm the expression of the *tcd* B gene.

Results and discussion

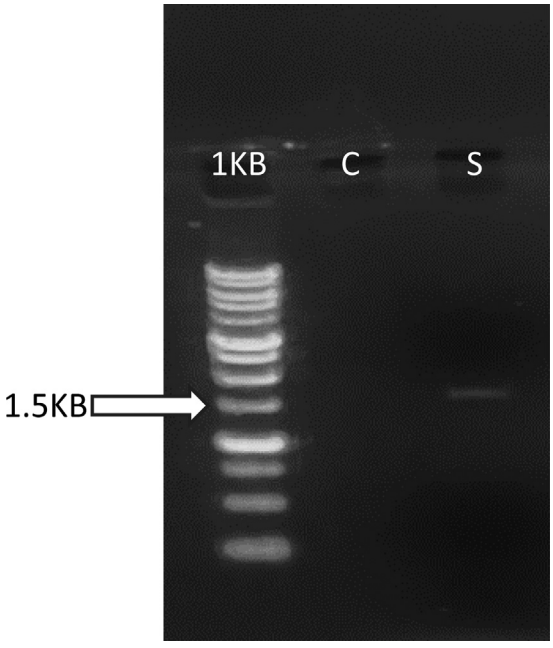
C. difficile, as other pathogens have developed numerous complicated approaches to transport proteins that deactivate vital host factors and disable the host immunity [3]. *C. difficile* secretes two exotoxins, *tcd*A and *tcd*B, which are the key virulence factors responsible for the signs related to CDI [32]. *tcd*A and *tcd*B are homologous toxins sharing 48% sequence identity. Both toxins have high molecular weight, they bind to target cells via receptor-mediated endocytosis and translocate from early endosomes into the cytosol where they are released after auto-proteolytic cleavage. Their subsequent action leads to the breakdown of the intestinal epithelial barrier and inflammation. Several medical strategies for the treatment and prevention of *C. difficile* associated diseases are unsatisfactory; hence, targeting *tcd*A and/or *tcd*B represents a promising tactic for the treatment *C. difficile* infection and associated complications. Specifically, *tcd*B was found to be the main virulence factor of *C. difficile* [33]. Alone, it can cause severe organ damage *in vivo* [34]. In fact, C-terminal-*tcd*B can induce immune responses [35–37]. Therefore, we chose to express the C-terminal-*tcd*B by the probiotic strains.

Previously, *Lactococcus lactis* was used as an oral vaccine using 500 bp of C-terminal of *tcd* gene amplified from *C. difficile* [29]. In this study, we proposed using the *tcd*B fragment from *C. difficile* ATCC 43255, (toxins A+ B+) using *L. gasseri* ATCC 33323 and *E. faecium* NM1015. *E. faecium*, *L. gasseri*, and *L. lactis* are all lactic acid bacteria capable of serving as oral vaccination carriers; but, *E. faecium* and *L. gasseri* have some benefits over *L. lactis*. These advantages include a higher survival rate in the gastrointestinal system, which improves the mucosal immune response and vaccine stability [38,39]. Furthermore, they can colonize the intestinal mucosa and interact with the host immune system, thereby increasing the vaccine's immunogenicity and efficacy [40].

PCR amplification of *C. difficile tcd*B fragment encoding toxin B

To target the *tcd*B gene encoding toxin B and express it using probiotic strains, a set of forward and reverse primers 'TcdB-F and TcdB-R' [29] targeting the C-terminal receptor binding domains of *tcd*B were used to amplify the gene. The amplicon was confirmed by electrophoresis using 1% agarose gel, where a sharp band of 1409 bp was visualized by UV transillumination (Fig. 1). The PCR product was purified and sequenced using the same primers: TcdB-F and TcdB-R. The sequencing analysis using basic local alignment search tool confirmed the identity

Figure 1



Polymerase chain reaction amplification of C-terminal fragment of *tcd*B gene from *C. difficile* ATCC 43255. Lane 1, 1KB DNA Ladder; lane 2, negative control (C); lane 3, polymerase chain reaction product of 1409 bp (S) using primer TcdB-F X TcdB-R.

of the sequence as *tcd*B corresponding fragment from *C. difficile* species with 98% identity to the receptor binding region of the C-terminal region including amino acid residues from 1665 to 2366 [41].

Cloning of the *C. difficile tcd*B fragment

Cloning using the Qiagen PCR cloning kit was used to obtain the *tcd*B gene in satisfactory yield with stability. The cloning process facilitates further subcloning through digestion by wide range of the restriction enzymes located in the cloning vector pDrive. *E. coli* strain was transformed and incubated on ampicillin LB agar plates at 30°C with shaking overnight. Colonies resistant to ampicillin (Amp^R) were picked and checked by colony PCR using primers M13-F and M13-R from the pDrive to confirm the presence and the orientation of the *tcd*B gene within the cloning vector (Fig. 2). The band with the correct predicted size confirmed the presence of the target gene (Fig. 2). The plasmid pDrive with the *tcd*B insert was then extracted and sequenced; the sequence results confirmed the presence of the *tcd*B gene sequence orientation. Thus, the *E. coli* strain and the plasmid were named 'NM0123' and 'pMN01', respectively.

Construction of *C. difficile* C-terminal *tcd*B – expression plasmid

The expression vector pLB210 was used for the construction of the expression *C. difficile tcd*B antigen (Fig. 2). The *tcd*B fragment was isolated from the cloning vector pDrive-tcdB (pMN01) by restriction enzymes *Bam*HI and *Xho*I. The vector pLB210 was digested by the same two restriction enzymes and checked on 1% agarose gel (Fig. 3). The ~1.5 kb *tcd*B fragment and the large fragment (~3 kb) from the pLB210 vector were excised from the gel and purified before ligated together using ligase 4 enzyme. The ligation reaction was examined on 1% agarose gel where plasmid pMN02 was formed by ligation (Fig. 4). The ligated product was introduced by electroporation into *E. coli* (Qiagen EZ) competent cells and was plated on LB agar containing 15 µg/ml

Figure 2

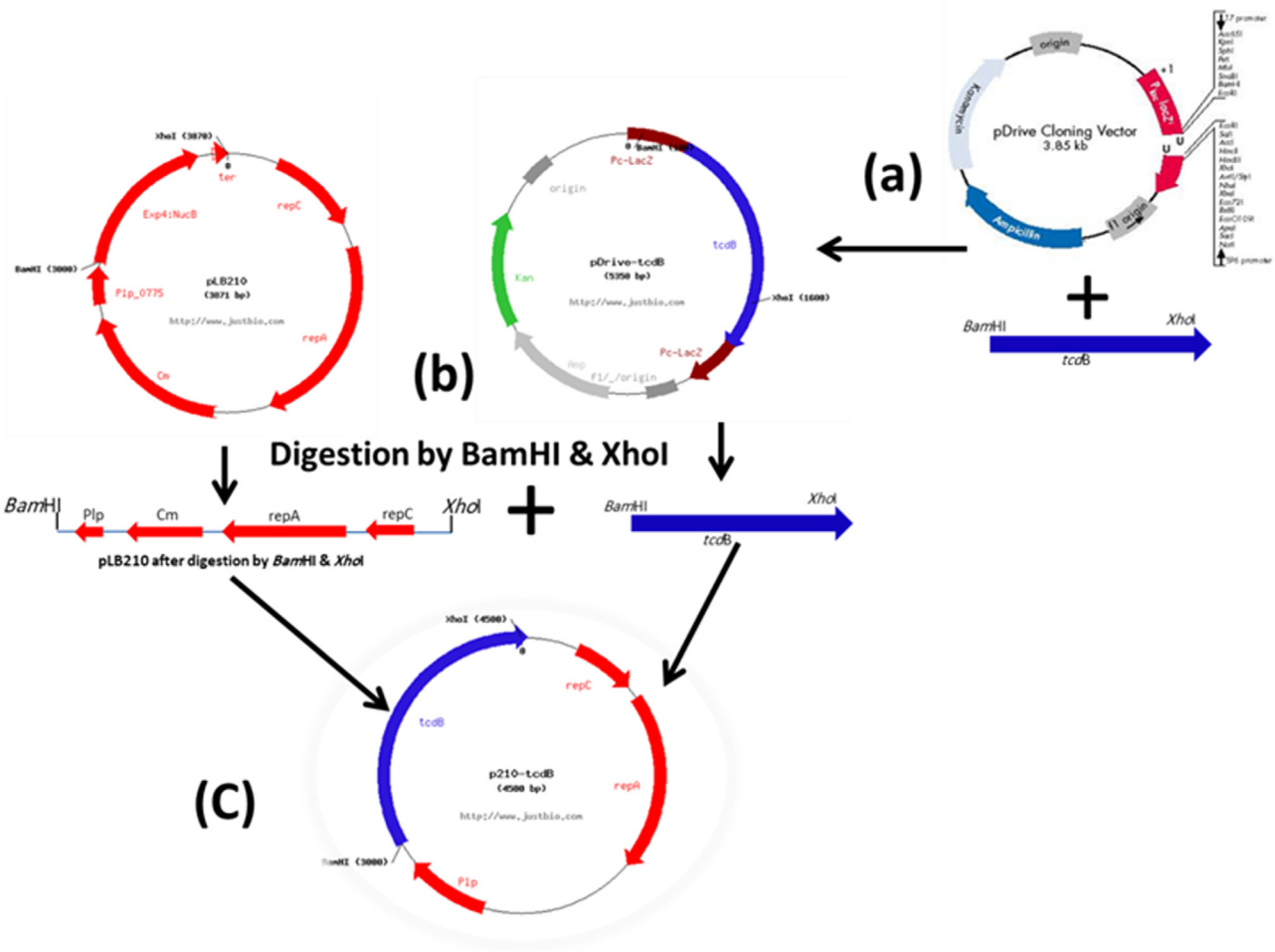
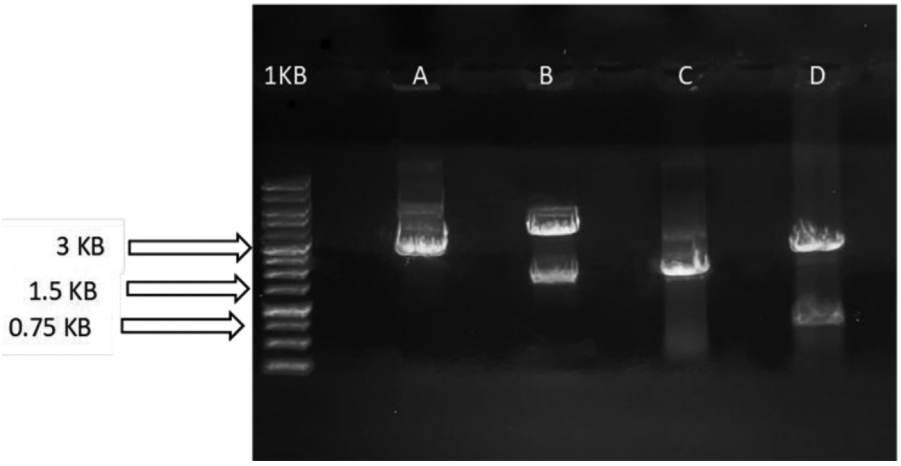


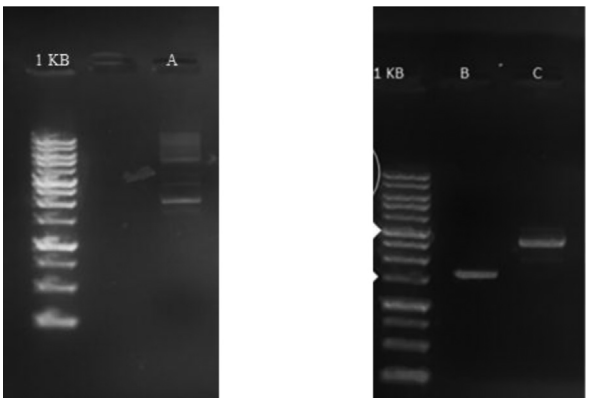
Diagram of the construction of the pMN02 expression vector. (a) Cloning of *C. difficile tcd*B gene encoding Toxin B, (b) Digestion of both plasmids pMN01and pLB210 with *Bam*HI and *Xho*I, (C) Ligation between the two fragments and the construction of the expression vector p210-tcdB (pMN02).

Figure 3



Digestion of cloning vector pMN01 and expression plasmid pLB210 using restriction enzymes *Bam*HI and *Xho*I. Lane 1, 1KB DNA Ladder; A: undigested cloning vector; B: digestion of cloning vector; C: undigested expression plasmid; D: digested expression plasmid.

Figure 4



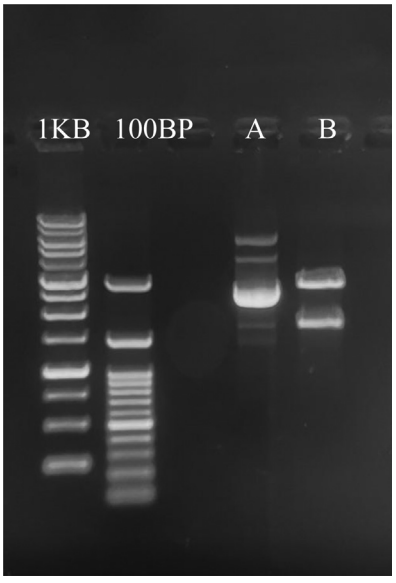
Ligation reaction between *tcd*B fragment and pLB210 fragment after excision from the gel. Lane 1, 1KB DNA Ladder; A: Ligation product of B and C; B: Purified fragment of *tcd*B gene restricted from cloning vector; C: Purified fragment of restricted pLB210 plasmid.1KB 100BP A B

chloramphenicol then incubated at 37°C for 18 h. The colonies obtained were confirmed by colony PCR using primers TcdB-F and TcdB-R where the PCR product of ~1.5 Kb was confirmed by agarose gel electrophoresis (Fig. 6). One positive colony was chosen and named *E. coli* NM0223 and then was deposited at the NRC collection culture. The recombinant vector pMN02 was extracted from *E. coli* NM0223 and digested by the same two restriction enzymes and checked on 1% agarose gel to confirm successful ligation (Fig. 5).

Introducing the *C. difficile* C-terminal *tcd*B – expression plasmid (pMN02) into the *L. gasseri* 33323 and *E. faecium* NM1015

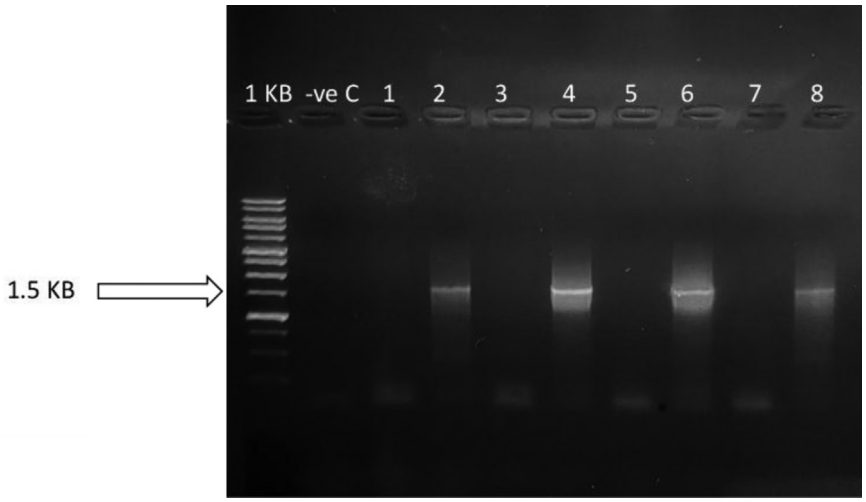
The *C. difficile tcd*B-expression plasmid (pMN02) was introduced into the selected probiotic bacteria *L. gasseri* 33323 and *E. faecium* NM1015 by electroporation. Minor modifications were done for a successful transformation where the probiotic strain was grown to $O.D_{600}$ 0.8 and in the final step the competent cells were divided into aliquots of 150 µl in addition to 15 µl of the *C. difficile tcd*B expression plasmid (pMN02) in the electroporation process. The cells were incubated in MRS broth with 0.5 M sucrose and 10 mM $CaCl_2$ in a shaking incubator at the appropriate temperature of 30°C for 5 hours before plating on chloramphenicol (17 µg/ml) MRS agar plates. The colonies were checked by colony PCR and the colonies with the

Figure 5



Digestion of the recombinant expression vector pMN02 using restriction enzymes *Bam*HI and *Xho*I. Lane 1, 1KB DNA Ladder; A: undigested recombinant expression vector; B: digested recombinant vector.

Figure 6



Colony polymerase chain reaction for the colonies obtained from the transformation and electroporation using primers TcdB-F and TcdB-R. Lane 1, 1KB DNA Ladder; lane 2, negative control; 1. Colony polymerase chain reaction of *E. coli*; 2. *E. coli* containing the cloning vector; (pMN01) 3. *E. coli* with no vector; 4. *E. coli* containing the expression plasmid (pMN02); 5. *L. gasseri* containing empty plasmid; 6. *L. gasseri* NM0323 containing the expression plasmid (pMN02); 7. *E. faecium* containing empty plasmid; 8. *E. faecium* containing the expression plasmid (pMN02).

expression plasmid without insert were used as control then visualized on agarose gel (Fig. 6). The results confirmed the presence of the *tcd*B-expression plasmid (pMN02) by giving ~1.5 kb band within the engineered strains. The engineered *L. gasseri* 33323 and *E. faecium* NM1015 were named NM0323 and NM0423, respectively.

Reverse transcription PCR (RT-PCR)

Reverse transcription is the use of RNA to produce DNA of the gene of interest by an RNA-dependent DNA polymerase. Thus, the RT-PCR was used to confirm the expression of the *tcd*B C-terminal fragment by the recombinant vector pMN02 introduced into *E. coli* (pMN02), *L. gasseri* (pMN02), and *E. faecium* (pMN02). The strains were grown in the appropriate broth supplemented with chloramphenicol and bile salts for induction of the expression. RNA from each strain was extracted. RT-PCR was carried out with the RNA from the three strains and three negative controls which carry pLB210 without insert. The products were analyzed on 1% agarose gel (Fig. 6). The three strains carrying the (pMN02) expression vector produced bands of the predicted size, while the strains carrying the empty expression vector (without insert) did not produce any bands.

Evaluation of plasmid pMN02 stability

The stability of the constructed plasmid pMN02 in *L. gasseri* NM0323 and *E. faecium* NM0423 was evaluated in the absence of chloramphenicol. Approximately 90% ±2.0 of *L. gasseri* NM0323 and *E. faecium* NM0423 colonies grown in the absence of chloramphenicol remained resistant after 70 and 140 generations. However, after 210 generations, there was a small reduction of the number of colonies of both strains where 85.3%±1.0 of the colonies showed resistance. Random colonies PCR confirmed the presence of the plasmid pMN02 (data not shown).

Evaluation of the probiotic properties of the engineered strains

Probiotic properties such as tolerance to acidic condition of the stomach and bile salts are essential for the bacteria ability to colonize the host's intestines and delivery of the proteins. To determine whether introducing the plasmid pMN02 affected the probiotic properties of *L. gasseri* NM0323 and *E. faecium* NM0423, their survival in pH 2.00 and 1% oxgall bile salts for 3 h was evaluated. No significant difference in the survival rate was observed between the engineered strains and their parental types (Table 3). The survival rates of *L. gasseri* NM0323 and *E. faecium* NM0423 in pH 2.00 were 85.5%±1.0 and 70.2%±2.0, respectively. The survival rates of *L. gasseri* NM0323 and *E. faecium* NM0423 in 1% oxgall

Table 3 Tolerance of the engineered strains to pH 2.00 and 1% oxgall bile salt

Strains	Survival (%) 3 h at pH 2.0	Survival (%) 3 h at 1% Oxgall
L. gasseri NM0323	85.5±1.0	87.2±1.0
L. gasseri ATCC 33323	84.9±1.3	86.4±0.9
*E. faecium*NM0423	70.2±2.0	84.7±0.7
E. faecium NM1015	70.5±1.5	85.0±1.0

bile salt were 87.2%±1.0 and 84.7%±0.7 respectively. These tolerance percentages are acceptable and in agreement with previous results obtained with the parental strain *E. faecium* NM1015 [27] in addition to the results obtained by other researchers [42,43]. The results confirm the stability of the probiotic properties of the engineered strains.

Conclusion

For an alternative strategy to combat CDI, we can use probiotic bacteria as an oral delivery vehicle of *C. difficile* antigen. This strategy has two advantages: first, the use of probiotic strains as antigen carriers is both safe and has health benefits; and second, problems arising from traditional vaccination methods are avoided.

This study used the bile salt-inducible expression vector pLB210 to design probiotic bacteria, specifically *L. gasseri* ATCC 33323 and *E. faecium* NM1015, to express the C-terminal of the *tcd*B gene, which encodes for *C. difficile* toxin B. This effort led to the successful creation of the *C. difficile tcd*B-expression plasmid and its introduction into *L. gasseri* ATCC 33323 and *E. faecium* NM1015. Moreover, the production of genetically modified organisms expressing the *C. difficile tcd*B C-terminal segment was validated. Furthermore, the modified bacteria maintained plasmid stability after 210 generations and exhibited favorable probiotic activities. Future in vivo testing as an oral vaccination against CDI will be conducted in an animal model.

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Conflicts of interest

There are no conflicts of interest.

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