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A Theoretical Framework for a Non-Antibiotic Approach to Combat *Vibrio cholerae* Outbreaks in Syrian Refugee Camps in Lebanon

Rana Ahmed, PhD Student [1]*, Paula Pineda, BSc Student [2],
Ghosoun Alomari, BSc Student [2]

[1] Department of Microbiology, Faculty of Sciences, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2
[2] Department of Genetics, Faculty of Sciences, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

*Corresponding Author: ahmedr10@myumanitoba.ca

Abstract
Cholera, caused by *Vibrio cholerae*, remains a significant public health challenge, particularly in vulnerable regions such as Lebanon, where a state of emergency was declared in response to outbreaks in Syrian refugee camps. The lack of access to clean water in these camps has made refugees highly susceptible to *V. cholerae* infections, which are characterized by gastrointestinal symptoms, including diarrhea and vomiting. In severe cases, infection can lead to rapid dehydration, and if untreated, may result in organ failure and death. Antibiotics, including tetracyclines, fluoroquinolones, and macrolides, are commonly used for severe cholera cases, particularly when resistance is confirmed. However, *V. cholerae* has developed resistance to these antibiotics through various mechanisms, complicating treatment and increasing the urgency for alternative therapeutic approaches. This theoretical study investigates a cost-effective, non-antibiotic strategy to combat cholera using engineered *Lactococcus lactis* as a probiotic delivery system. We hypothesize that combining conjugated linoleic acid (CLA) with genetically modified *L. lactis* could offer an innovative treatment. CLA, a polyunsaturated fatty acid with well-established antimicrobial properties, inhibits *V. cholerae* growth and toxin production. Additionally, *L. lactis* is genetically engineered to express the chimeric monoclonal antibody ZAC-3, which has been shown to inhibit *V. cholerae* motility, preventing it from colonizing the small intestine and causing infection. ZAC-3 is hypothesized to be displayed on the surface of *L. lactis* using recombinant DNA technology, with a proposed surface-anchoring sequence (AcmA3b) to enable direct interaction with the pathogen. This approach offers a novel, sustainable alternative to antibiotics, with linoleic acid supplementation further enhancing its therapeutic efficacy. Our cost analysis demonstrates that the engineered *L. lactis* strain, when used as a dietary supplement, is affordable and suitable for integration into food donation programs. Its probiotic nature ensures safety without triggering immune responses. In conclusion, while experimental validation is essential, this theoretical research proposes a dual-action approach using CLA and ZAC-3-engineered *L. lactis* to address the growing issue of antibiotic resistance in cholera and other bacterial infections, providing a viable solution without the drawbacks associated with traditional antibiotics.

Keywords: cholera; antibiotic resistance; engineered probiotic; *Lactococcus lactis*; virulence; immunity; refugee; outbreaks

Introduction

On 10 October 2022, Lebanon has declared an emergency state in Syrian refugee camps due to cholera outbreaks in this region and beyond [1,2]. Cholera is an infectious disease caused by gram-negative and toxin-producing bacterium *Vibrio Cholerae*. It is a major cause of dehydrating diarrhea, particularly in developing countries with poor hygienic conditions, and is exacerbated by humanitarian crises [3]. The mechanism of infection of *V. cholerae* is targeting the small intestine for dehydration. The bacteria enter the body and pass through the stomach, where the acidic environment typically acts as a barrier to infection. However, when the infective dose is sufficiently high (typically $>10^8$ pathogens), a portion of the bacteria survive this acidic environment and reach the small intestine, where they can colonize and cause infection [4].The pathogen will move to the small intestine, where it will adhere to the epithelial tissue, multiply further and initiate the release of cholera toxins [3]. It is important to note that *V. cholerae* as a bacterium is not virulent unless it is infected by the bacteriophage CTX which inserts its genome, leading to vibrio toxin production. The toxin leads to the activation of cyclic adenosine monophosphate (cAMP) which inhibits sodium and chloride intake from microvilli, increasing the secretion of chloride and water. The ion imbalance causes fluid to be lost through osmosis,

resulting in acute diarrhea.[3]. Symptoms of the infection start with stomach cramps and vomiting followed by diarrhea, and after the infection progresses, rice water stool characteristic of *V. cholerae* is produced [5]. Severe dehydrationaffecting approximately 5 to 10% of individuals, occurs due to frequent, profuse diarrhea and excessive vomiting, which rapidly depletes body fluids. If untreated, fluid loss can lead to kidney failure, shock, sepsis, and potentially death within hours [6]. The transmission of *V. cholerae* occurs through contact with cholera-contaminated feces. Cross-contamination can happen when water and food are exposed to these contaminants.

Populations with poor sewage systems with no access to clean water or mechanisms to maintain food free from contaminant are more prone to acquire and spread cholera [7]. As per the Infectious Disease Society of America (IDSA), the primary treatment for cholera is oral rehydration solution (ORS), a powder that can be dissolved in boiled or treated water to restore lost fluids and electrolytes [8]. Antibiotics are also a part of the treatment against cholera and are key to reduce the bacterial load. Antibiotics such as tetracycline, fluoroquinolones, and azithromycin have been used to treat *V. cholerae*, with the goal of reducing pathogen growth by inhibiting essential functions. However, *V. cholerae* has developed resistance to these antibiotics, which has become widespread within the species, complicating treatment efforts. [9]. Other antibiotics have been considered for treatment and, even though they are effective, multidrug-resistance (MDR) is developed eventually. Antimicrobial resistance in *V. cholerae* is driven by a combination of intricate strategies facilitated by specific resistance genes. These mechanisms include reducing antibiotic efficacy, altering drug targets, and directly neutralizing antimicrobial agents. One major mechanism involves limiting antibiotic penetration or actively expelling the drugs through efflux pumps. Efflux pumps in *V. cholerae* are categorized based on their energy sources ATP hydrolysis or the proton-motive force (PMF) generated by transmembrane H+ or Na+ gradients. The PMF-dependent families include MATE (multidrug and toxic compound extrusion), MFS (major facilitator superfamily), RND (resistance–nodulation–cell division), and SMR (small multidrug resistance) systems, which significantly contribute to MDR [10,11]. Another critical mechanism is the modification of antibiotic targets. Mutations in chromosomal genes, such as *gyrA* and *parC*, which encode subunits of DNA gyrase and topoisomerase IV, respectively, reduce the affinity of these enzymes for antibiotics, thereby conferring resistance [12]. Resistance can also arise through the enzymatic breakdown or chemical modification of antibiotics. For instance, extended-spectrum β-lactamases (ESBLs) degrade beta-lactam antibiotics, rendering them ineffective [12]. *V. cholerae* strains display considerable diversity in their antibiotic resistance profiles, with the majority (97%) identified as multidrug-resistant (MDR). Alarmingly, certain strains exhibit extreme drug resistance (XDR), showing insensitivity to a broad spectrum of antibiotics, including advanced agents like fourth-generation cephalosporins (cefepime) and aztreonam. These XDR strains are frequently associated with resistance-related genes, such as those encoding extended-spectrum β-lactamases (ESBLs), highlighting the critical need for enhanced surveillance and targeted interventions to curb the proliferation of antibiotic resistance in *V. cholerae*.[13]. Antibiotic resistance in *V. cholerae* is largely driven by the misuse of antibiotics, particularly tetracycline and fluoroquinolones, by patients or healthcare providers. This misuse, often due to inadequate understanding of the drugs' mechanisms or improper dosing practices, creates selective pressure that allows bacterial populations to adapt and develop resistance, thereby diminishing the effectiveness of these treatments [14].

When bacteria develop antibiotic resistance, patients often require stronger, last-resort treatments, such as carbapenems, which can lose their effectiveness over time [15]. These resistant infections are not only harder and more expensive to treat, but they also lead to longer hospital stays, more frequent doctor visits, and higher medical costs. As a result, patients are at an increased risk of severe illness or even death, highlighting the urgent need to address the growing threat of antibiotic resistance. For health systems that have the resources to provide more complex antibiotics and treatments to counteract the secondary effects of harsh antibiotics, cholera patients may recover successfully. Moreover, fluid replenishment and monitoring of other side symptoms may be controlled in health systems from developed countries whose political and economic status allows stability in the health sector [16]. In developing countries where issues such as corruption, internal political conflicts, and environmental challenges often take precedence over healthcare, people are unable to access complex treatments. In addition to basic necessities like proper nutrition and clean water, these individuals face significant barriers to obtain adequate medical care due to the strain on healthcare systems. . This is the context of Syrian Refugee camps, where Syrian refugees, who flee their homes because of the war that has been going on for almost 12 years, struggle to access clean water and have inadequate sewage systems. On the other hand, in Lebanon, factors such as a fragile water infrastructure, increasing poverty, electricity shortage, and rising fuel prices are contributing to the spread [2,18].

Our proposal is to use conjugated linoleic acid as well as ZAC-3 antibody in a unified alternative to antibiotics to reduce cholera toxin production and inhibit *V. cholera* motility respectively [19]. Conjugated linoleic acids (CLAs), a class of polyunsaturated fatty acids, hold significant promise in clinical settings due to their wide-ranging physiological benefits. Naturally found in ruminant animal products and available as FDA (U.S. Food and Drug Administration)-approved dietary supplements [20],

CLAs can also be synthesized from linoleic acid-rich oils like safflower, sunflower, corn, and soybeans through an alkaline-catalyzed reaction [21]. Their potent anti-inflammatory properties make them valuable in reducing immune overactivation, which is crucial in managing inflammatory conditions. Additionally, CLAs demonstrate anticarcinogenic, antiobesity, antidiabetic, and antihypertensive effects, underscoring their potential role in treating metabolic syndromes and preventing lifestyle-related diseases [22]. Their versatility and safety profile highlight their importance as a therapeutic agent in modern clinical practice. CLA has shown remarkable potential in enhancing immune function in healthy individuals, positioning it as a superior alternative to antibiotics, which are often associated with immune suppression [23]. Notably, research on pigs demonstrated that those fed CLA-enriched diets had significantly higher antibody titers against *Mycoplasma hyopneumoniae* compared to their counterparts on CLA-free diets [24]. This dual role of CLA—bolstering immune response while exerting antimicrobial effects—highlights its promise as a multifaceted therapeutic agent. Our innovative proposal leverages this dual capability, suggesting CLA as a supplemental therapy for cholera. Specifically, CLA reduces cholera toxin production by inhibiting *V. cholerae* ToxT activity, targeting the pathogen's virulence mechanisms rather than solely focusing on bacterial eradication [19,25]. CLA works on regulator protein, ToxT to inhibit virulence gene expression [19]. This approach not only combats infection but also minimizes the risk of resistance development, offering a sustainable and immune-supportive alternative in cholera management.

In addition, the motility of *V. cholerae* can be inhibited by ZAC-3 antibody that arrests the motility and reduces the ability of *V. cholerae* to colonize the intestinal epithelium by directly interacting with [26]. ZAC-3 is a monoclonal IgA antibody (mAb) that targets the core/lipid A region of the lipopolysaccharide (LPS) of *V. cholerae* [26–28]. Bacterial responses to antibodies are typically more regulated, minimizing the risk of triggering SOS mechanisms that are often associated with toxin release and the dissemination of resistance genes observed with antibiotic use. Monoclonal antibodies (Hu-mAbs) have revolutionized treatments for cancer, autoimmune disorders, and viral infections, yet their application in addressing bacterial infections remains underexplored [29].The ZAC-3 monoclonal antibody exemplifies this untapped potential. Engineered to inhibit the motility of *V. cholerae*, ZAC-3 offers a promising therapeutic option by preventing bacterial colonization and infection. Monoclonal antibodies like ZAC-3 can function both as standalone interventions or in conjunction with antibiotics to enhance treatment outcomes. Moreover, their role extends to prophylactic use, effectively preventing infections before they occur, underscoring their significant promise in transforming bacterial disease management [30].

This article showcases a theoretical approach for developing a potential alternative to antibiotics targeting *V. cholerae O1* strain which has been related to recent cholera pandemics [31]. The research made for this theoretical approach was focused on this strain and its characteristics related to its pathogenicity. However, other cholera strains such as O139 can be susceptible to the treatment as they also show toxin production and motility [32]. Further analysis would be required to adapt this treatment to different strains as they may have different adaptation that confer resistance to antibiotic such as polysaccharide capsule production in O139 strain [32].

Methods

Genetically engineered antibodies designed, and their DNA sequence are incorporated into a vector to be introduced into *Lactococcus lactis* MG1363 using recombinant DNA technology and gene editing techniques. Cloning techniques include CRISPR-Cas9, PCR amplification and ligation into vectors, followed by transformation into *L. lactis* via electroporation [33]. Also, antibody engineering can be achieved with techniques such as *Nicotiana benthamiana*-based rapid antibody-manufacturing platform (RAMP) [34], Additionally, cDNA sequencing and transcriptomics can be used for identifying and optimizing antibody genes. The ZAC-3 antibody gene is expressed by constructing a ZAC-3 plasmid, which is then introduced into the cells. To further enhance expression, linoleic acid is supplemented, as shown in Figures 1 and 2.

ZAC-3 Antibody and Vector Engineering

ZAC-3 monoclonal antibodies can be achieved by designing chimeric mouse-human derivatives of ZAC-3 antibody. A study done by Levinson et al. 2015 applies this methodology [35]. This method from the study assured that the ZAC-3 monoclonal antibody can retain its binding specificity and activities observed in vitro. They initially obtain ZAC-3 B cell hybridoma lines from external resources (Dr. Blaise, CHUV, Switzerland) whose production consist of isolating antibody-producing B lymphocytes from model animals such as mice, sheep or rabbits that have been exposed to the pathogen of interest, *Vibrio cholera* O1 in this case. The isolated B lymphocytes are fused with myeloma cells which are immortal cells that indefinitely proliferate. The product, hybridoma cells, are going to be selected via ELISA where the epitope of the antibody of interest is placed on a plate and the hybridoma cells that react to these epitopes will be recognized and selected [36]. After hybridomas are maintained in fetal bovine serum, the antibody domains (V_L and V_H) are amplified via PCR from cDNA, and codon-optimized to ensure that translation and other mechanisms for its expression have not been affected by the process of cDNA. These corrections are done in the study based on the Kabat and IMGT databases [37]. The V_L and V_H domains from the hybridomas are fused with IgG human antibodies and K

constant regions where the Fab fragments were prepared with IgG Fab preparation kits.

Another study shows a similar method applied for the strain Vibrio cholerae (O395) to produce polyclonal as in the previous paper. This shows the effectiveness of the protocol and its reproducibility [38].

Both papers can be referenced to engineer the monoclonal ZAC-3 antibody and obtain their DNA sequence to engineer the vector for *Lactococcus lactis* transformation. Once the chimeric antibody has been reverse-sequenced, the fragment can be inserted in a vector. A mechanism to insert the ZAC-3 antibody gene is CRISPR-Cas9 by homology-directed repair (HDR) [39]. The design of CRISPR-Cas9 experiment initiates by synthesizing the single-guide RNA (sgRNA) which will be complementary to the region of insertion and breakage at the vector by Cas9. Cloning will occur in *Escherichia coli* bacteria where a vector with antibiotic-resistance gene, promoter, replication region and marker gene will be present and will be the one going through the insertion of the ZAC-3 (Fig 1). The Cas9-sgRNA complex can be incorporated in E. coli in the form of ribonucleoprotein particles (RNP) via electroporation, viral vector or microinjections [40]. Along with Cas9-sgRNA, a donor DNA fragment is incorporated in the complex. Donor DNA will contain ZAC-3 gene and regions complementary to the hanging ends produced from Cas9 double-stranded break. The Cas9-sgRNA recognizes the protospacer adjacent motif (PAM) where the double-stranded break is going to occur [41]. Once the vector has been restricted, cross over between the vector and donor DNA occurs, inserting ZAC-3 to the vector. To select for the cloned bacteria, they go through two screening processes: selecting for vector presence by exposing to antibiotic resistance and selecting for ZAC-3 insertion by blue-white screening [42]. First, cloned culture is going to be exposed to ampicillin and the bacteria that have the vector will survive the medium as it contains ampicillin resistant gene. Second, screening will be blue-white screening which consists of initially having the vector with a lacZ gene which produced beta-galactosidase protein which hydrolyzes X-gal and produced a blue pigment. When lacZ is disrupted, beta-galactosidase is not produced and thus X-gal is not hydrolyzing, and no pigment is produced [42]. Therefore, in the case of ZAC-3 insertion, when *E. coli* culture is exposed to X-gal, we expect to see no pigment as a sign that *E. coli* was cloned and thus, we have the vector required for transformation of *L. lactis*.

Note that the Cas9-sgRNA design can be done by external companies where there is a diverse catalog with a variety of Cas9 proteins that depend on interests such as design and delivery method, and screening and validation. For example, ThermoFisher provides personalized CRISPR genome editing resource guides depending on the interest of each project and their limitations [43].

Lactococcus lactis Transformation

After ZAC-3 vector engineering, the vector will be extracted from the cloned *E. coli* culture through plasmid DNA extraction. A variety of kits are sold to perform these tasks where all are based on cell lysis, purification and isolation of plasmid DNA such as the plasmid DNA isolation kit from QIAGEN which effectively isolates plasmid without its degradation for sequencing or cloning which indicates that it is high yield [44].

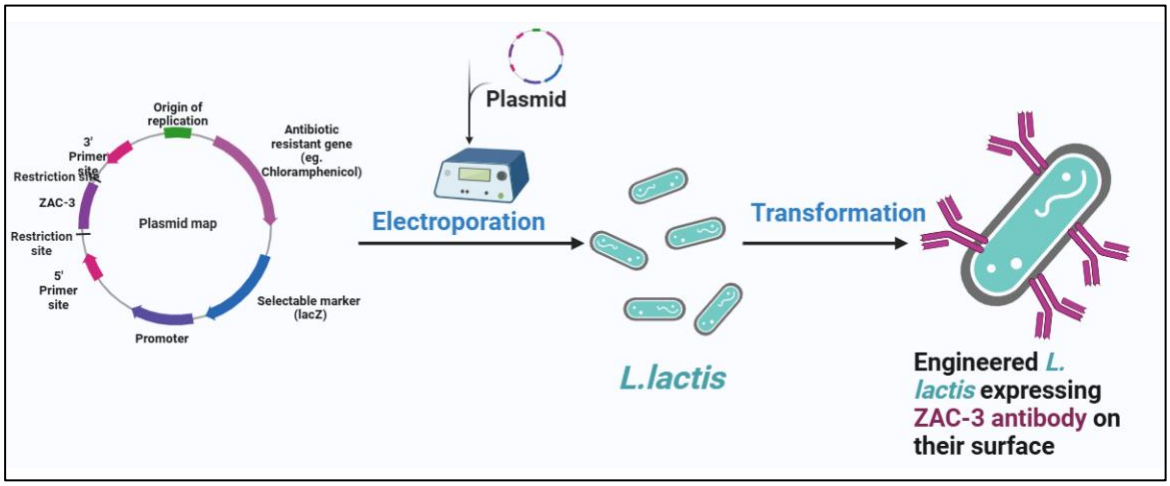

Figure 1. Engineering Process of Lactococcus lactis Transformation. The engineering process involves cloning target components into plasmids and transforming them into Lactococcus lactis cells to express ZAC-3 for our probiotic application. This stepwise approach allows for the modification and manipulation of bacterial genetics, enabling the expression of the desired antibody tailored specifically for our probiotic use**.** Image created with BioRender.

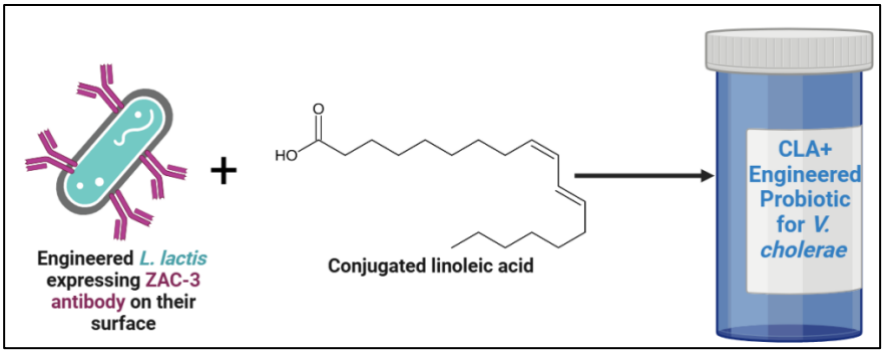

Figure 2. Final Product Incorporating Conjugated Linoleic Acid (CLA) and the Engineered Probiotic. This figure illustrates the final product comprising Conjugated Linoleic Acid (CLA) and the engineered probiotic. This combined formulation represents the culmination of our efforts, merging the health benefits of CLA supplementation with the therapeutic potential of the genetically engineered probiotic. Image created with BioRender.

After ZAC-3 isolation, transformation process of *Lactococcus lactis* can start. To prepare *Lactococcus lactis* competent cells, bacterial cultures are grown to the mid-exponential phase (OD600 0.5–0.6) where most of the cells should be metabolically active harvested by centrifugation, and washed multiple times with cold, sterile, low-salt solution to remove any residual growth media and increase cell permeability. This process enhances the cells' ability to take up plasmid DNA during electroporation, making them electrocompetent. The washed cells are then resuspended in a cold, sterile solution of 10% glycerol to maintain their competency. The constructed plasmid containing the ZAC-3 sequence is introduced into the electrocompetent. *L. lactis* cells via electroporation. For electroporation, competent cells are mixed with plasmid DNA in a chilled electroporation cuvette. The cells are subjected to a high-voltage pulse (e.g., 1.8 kV, 25 µF, 200 Ω) using an electroporator [45–47]. Following the electroporation, the cells are immediately transferred to a suitable recovery medium, such as M17 broth supplemented with glucose or other nutrients and incubated at 30°C for 1–2 hours with gentle shaking to allow for the recovery and expression of the plasmid [48]. After the recovery period, the cells are plated on selective agar plates containing the appropriate antibiotic (e.g., chloramphenicol, depending on the plasmid marker) to select for successful transformants [47].

The recombinant *L. lactis* strain is cultured in selective media made of ampicillin and blue-white X-gal to ensure *L. lactis* have been effectively transformed. The same selection principles used in *E. coli* transformation are applied. The final plasmid construct is designed for optimal expression of the ZAC-3 antibody on the bacterial surface. Colonies are then screened for the presence of the ZAC-3 construct using colony PCR or restriction digestion analysis.

In Vitro Culture

Transformed *Lactococcus lactis* cells should be cultured in GM17 medium supplemented with 0.1% Tween 80 to optimize the expression and secretion of the ZAC-3 antibody. Tween 80 aids in enhancing membrane stability and promoting the secretion of recombinant proteins [49]. The cultures then incubated at 30°C under microaerophilic to anaerobic conditions (with approximately 5% CO2 and <1% O2) to mimic the natural growth conditions of *L. lactis* and promote efficient protein expression [50,51]. The pH of the culture medium maintained between 6.5 and 7.0 using pH buffers to ensure optimal growth and expression conditions. Antibody expression was induced at the mid-log phase (OD600 0.4–0.6) by adding nisin, a natural inducer that activates the *nisin* promoter within the plasmid to initiate protein expression [47]. Antibiotics, such as chloramphenicol, were included in the medium to maintain plasmid selection pressure and prevent the loss of the plasmid from the transformed *L. lactis* cells. The cultures were grown with gentle agitation (150–200 rpm) to ensure proper aeration and nutrient distribution. The secreted ZAC-3 antibody is anchored to the surface of the *L. lactis* cells through the AcmA3b surface-anchoring sequence. This sequence facilitates the stable attachment of the antibody to the bacterial cell wall, ensuring the antibody is properly displayed for potential immunological applications [52,53]. After cultivation, the cells are harvested by centrifugation, and the supernatant is collected for analysis of the secreted protein, while the bacterial cell pellet is analyzed for surface expression.

Freeze Drying and Supplementation

The engineered *Lactococcus lactis* probiotic is provided as a freeze-dried powder to enhance its stability and facilitate ease of storage and application. Freeze-drying is a widely used preservation method in the pharmaceutical industry, and several studies have explored the impact of various cryoprotectants on the stability of probiotics during the freeze-drying process [54] Freeze-drying (lyophilization) is employed to preserve the probiotic by removing water content while maintaining the viability of the bacterial cells. To further optimize its functionality, the freeze-dried powder is supplemented with conjugated linoleic acid [54].

Regulatory and Safety Considerations

As engineered probiotics are being developed, it is crucial to consider regulatory aspects related to the use of genetically modified organisms (GMOs) in humans. Testing for safety and toxicity must be conducted, including assessing whether the engineered *L. lactis* strains can survive in the human gastrointestinal tract without producing harmful effects. Furthermore, antibiotic resistance associated with plasmids should be carefully monitored to avoid the spread of resistance traits. Additionally, consultation with regulatory bodies, such as the FDA or EFSA (The European Food Safety Authority), is required for approval before human clinical trials or commercialization.

Results

Our proposed study outlines the development of a genetically engineered *Lactococcus lactis* MG1363 strain designed to express the ZAC-3 monoclonal antibody, which has shown promise in inhibiting the motility of *V. cholerae*. ZAC-3, targets a specific virulence factor in *V. cholerae*, and impeding the pathogen's ability to colonize and infect the host.To optimize secretion and surface display of the ZAC-3 antibody on *L. lactis*, the ZAC-3 plasmid is engineered with signal peptides for efficient secretion into the growth medium, alongside the AcmA3b sequence. The AcmA3b sequence is derived from the *Lactococcus* surface protein AcmA and functions as a surface-anchoring motif, to ensure stable integration of the ZAC-3 antibody on the bacterial cell wall. This allows the engineered probiotic to directly interact with *V. cholerae* in the host environment.

We anticipate a transformation efficiency of approximately 85%, indicating efficient integration of the ZAC-3 plasmid into the bacterial genome, resulting in a stable *L. lactis* strain capable of expressing the ZAC-3 antibody. Upon induction with nisin, we expect a significant increase in ZAC-3 antibody production compared to non-induced cells.

The engineered *L. lactis* strain will be freeze-dried to retain high viability over storage periods, ensuring the stability and practicality of this probiotic for future use. Additionally, supplementation with linoleic acid is predicted to enhance the probiotic's therapeutic potential by inhibiting *V. cholerae* virulence. Linoleic acid has been shown to reduce ToxT-mediated gene expression, and we expect a reduction in virulence factor expression, as expected to be confirmed by qPCR analysis.

Future work will focus on in vivo studies to evaluate the probiotic's efficacy in reducing cholera symptoms in animal models, with the ultimate goal of achieving similar inhibitory effects in clinical settings. We hypothesize that this combined strategy—antibody expression and linoleic acid supplementation—could provide a novel, effective approach to cholera prevention and treatment, capitalizing on the stability and functionality of the engineered probiotic.

As shown in Table 1, a detailed comparison is provided between traditional antibiotic treatment and the proposed engineered probiotic approach for managing cholera. The table highlights several potential advantages of the engineered probiotic, including its ability to target *V. cholerae* virulence factors without compromising the immune system, unlike conventional antibiotics, which can suppress immune function.

Table 1. Comparison of Traditional Antibiotic Treatment and Engineered Probiotic Approach for Cholera Management

Aspect	Traditional Antibiotic Treatment	Engineered Probiotic Approach
Efficacy	Effective in reducing bacterial load and symptoms of cholera.	Targeted approach against Vibrio cholerae without disrupting gut microbiota. May require longer treatment duration for symptom relief.
Side Effects	Common side effects include gastrointestinal discomfort, allergic reactions, and development of antibiotic resistance.	Engineered probiotic is generally recognized as safe (GRAS) and well-tolerated by most individuals.
Cost	Costs may vary depending on the type of antibiotic used, healthcare setting, and duration of treatment. Can be expensive, especially in resource-limited settings.	Relatively low production cost of engineered probiotic. Affordable and suitable for large-scale implementation in resource-limited settings.
Sustainability	Antibiotic resistance poses a significant threat to long-term effectiveness. Continued reliance on antibiotics may contribute to the development of multidrug-resistant strains.	Engineered probiotic offers a sustainable alternative to antibiotic treatment, reducing the risk of antibiotic resistance and preserving the efficacy of existing antibiotics. May contribute to long-term public health goals by promoting microbial balance in the gut.

Additionally, the economic feasibility of the engineered probiotic is emphasized in Table 2. As indicated in the table, the production cost of *Lactococcus lactis* expressing the ZAC-3 antibody is relatively low compared to traditional antibiotic treatments. This cost efficiency further supports the viability of the engineered probiotic as an accessible and economically sustainable alternative for cholera management.

Table 2. Estimated Cost Analysis for Producing 100,000 Capsules of Engineered Probiotics with CLA for Cholera Treatment

Cost Break Down							
Equipment		**Consumables**		**Manufacturing**		**General**	
Molecular Biology Equipment	$6,000	Molecular Biology Reagent	$7,300	Bioreactor Equipment	$9,000	Business and Product Liability Insurance	$5,000
Engineering Equipment	$8,000	Engineering Materials	$9,000	Prototyping	$30,000	Advertising and Marketing	$1,000
Total (75,300 Canadian Dollars)							

Discussion

The development of a genetically engineered *L. lactis* expressing ZAC-3 antibody offers a promising non-antibiotic therapeutic strategy to combat cholera outbreaks in Syrian refugee camps in Lebanon. This approach offers a promising alternative, especially considering recent cholera outbreaks in countries like Niger and Thailand, and the global depletion of oral cholera vaccine stocks. The versatility of this engineered probiotic could prove crucial in managing future outbreaks, especially when traditional vaccine and antibiotic resources are limited. Between January 1 and September 29, 2024, a cumulative total of 439,724 cholera cases and 3,432 deaths were reported globally. While the number of cases has decreased by 16% compared to the previous year, the concerning 126% increase in mortality underscores the challenges of managing the disease in certain regions. Outbreaks in conflict zones, areas affected by severe flooding, and regions with inadequate healthcare infrastructure contribute to these higher death rates [55]. This approach has the potential to significantly reduce cholera's impact in these vulnerable regions. Safety is a paramount concern in the development of any therapeutic intervention, particularly in vulnerable populations such as refugee camps. Our engineered *L. lactis* is a familiar component of the human digestive system, commonly found in many fermented foods, therefore it is "generally recognized as safe" (GRAS) and has been suggested as a potential carrier for delivering therapeutic molecules in the gastrointestinal tract. Certain natural isolates of *L. lactis* have shown anti-inflammatory properties, while an *L. lactis* strain sourced from fermented milk has been noted for its ability to modulate the intestinal microbiota [56]. Moreover, conjugated linoleic acids (CLAs), which are FDA-approved dietary supplements, further enhance their safety profile and therapeutic potential in clinical applications [20]. Many monoclonal antibodies are currently FDA-approved for clinical use [57] however, the ZAC-3 antibody would also need to undergo clinical trials in animal models to ensure its safety and effectiveness before being considered for human application. This familiarity reduces the risk of adverse reactions and supports the probiotic's acceptance as a safe treatment option. Moreover, the engineered probiotic's mode of action, targeting *V. cholerae* with specific antibodies, minimizes the risk of damage to the gut microbiota. Unlike antibiotics, which can disrupt the balance of beneficial bacteria and lead to secondary infections, our probiotic maintains the integrity of the digestive tract, reducing the likelihood of subsequent health issues. Another notable benefit is the simplicity of administration. While antibiotics often require strict dosage regimens and medical supervision, our probiotic, which contains the ZAC-3 monoclonal antibody, can be consumed as a dietary supplement. Monoclonal antibodies, like ZAC-3, are designed to target specific proteins or cells, offering precise treatments with fewer off-target effects. Their long-lasting effects typically result in less frequent dosing, making them suitable for long-term disease management. Unlike antibiotics, which target broad bacterial functions and follow standard dosing regimens, ZAC-3 potentially reduces the risk of resistance development by focusing on specific disease mechanisms. This precision makes ZAC-3 a promising alternative for cholera outbreaks, while antibiotics are less sustainable for long-term use due to their broader action and tendency to promote resistance [58,59]. This flexibility is particularly beneficial in refugee camp settings, where access to healthcare facilities and medical personnel may be limited. Therefore, even though dosage cannot be clearly established from our theoretical alternative, we can assume that this treatment can be consumed as a regular probiotic. This means that 1 to 10 billion colony-forming units (CFU) can be consumed per

day or more often [60]. In the case of individuals with cholera, this dosage may be significantly higher and administered more frequently per day until stabilized. Refugees can incorporate the probiotic into their daily diet without the need for specific medical instructions or frequent visits to health centers. This approach reduces the logistical and practical barriers to treatment, facilitating widespread adoption and compliance.

An essential aspect of our approach is its capacity to mitigate the emergence of antibiotic resistance. Overuse and misuse of antibiotics have led to a global health crisis, with many bacterial strains developing resistance to multiple drugs. Infections with antibiotic resistance bacteria may cause severe illness, higher mortality rates, increased amount of healthcare costs and an increased risk of complications and admission to hospital [61]. By providing an alternative treatment for cholera that does not rely on antibiotics, we contribute to the broader effort to preserve the efficacy of existing antibiotics. This approach not only addresses the immediate health needs of the refugee population but also supports long-term public health goals.

Regarding the economic impact of this probiotic alternative, cost-effectiveness is crucial for large-scale implementation in resource-limited settings such as refugee camps. Health organizations can include the probiotic in food donations, providing a dual benefit of nutritional support and disease prevention. The affordability of this intervention ensures that it can be sustainably integrated into ongoing humanitarian efforts without imposing significant financial burdens.

To enhance the probiotic's therapeutic potential, we supplemented it with linoleic acid, a fatty acid known for its antimicrobial properties[62].This supplementation aims to boost the overall effectiveness of the treatment, providing an additional layer of defense against *V. cholerae*. Linoleic acid's role in disrupting bacterial membranes complements the antibody-mediated targeting, creating a multi-faceted approach to cholera prevention and treatment.

Conclusion

In conclusion, our study demonstrates the feasibility and potential benefits of using genetically engineered *Lactococcus lactis* as a non-antibiotic treatment for cholera outbreaks in developing countries. This probiotic offers a safe, cost-effective alternative to traditional antibiotics, addressing immediate health concerns while reducing the risk of antibiotic resistance. By facilitating easy administration and ensuring long-term health benefits, this innovative approach has the potential to improve health outcomes for vulnerable populations, particularly in refugee camps, and contribute to global efforts in combating antibiotic-resistant bacteria.

The use of *L. lactis* expressing the ZAC-3 antibody presents a solution that preserves the potency of antibiotics, maintains gut microbiome composition, and targets specific bacterial infections. With its anticipated cost-effectiveness, large-scale implementation of this technology in resource-limited environments is feasible, providing both nutritional support and disease prevention during crises. This aligns with the United Nations Sustainable Development Goals, particularly those focused on Good Health and Well-being and Innovation, Infrastructure, and Industry.

If proven effective, this technology could represent a paradigm shift in the management of infectious diseases. Rather than replacing antibiotics entirely, it offers a complementary strategy that preserves the potency of existing antimicrobials while mitigating the risks of resistance.

This could have far-reaching implications for global health, particularly as the world faces an increasing burden of multi-drug-resistant infections. For instance, diseases such as tuberculosis, which have become resistant to standard treatments, could benefit from this targeted approach. By focusing on specific disease mechanisms rather than broad bacterial functions, this technology offers a promising pathway for combating not only cholera but other challenging infections as well. Further research should explore the long-term effects of continuous consumption of engineered probiotics, their application to other infectious diseases, and optimization of antibody expression for maximum therapeutic efficacy. This approach represents an exciting advancement in infectious disease management, offering sustainable, targeted alternatives to conventional antibiotics and contributing to the fight against antimicrobial resistance globally.

List of Abbreviations Used

cAMP: cyclic adenosine monophosphate
CLA: conjugated linoleic acid
EFSA: The European Food Safety Authority
FDA: U.S. Food and Drug Administration
GRAS: generally recognized as SafeIDSA (Infectious Disease Society of America)
MATE: multidrug and toxic compound extrusion
MDR: multidrug-resistance
MFS: major facilitator superfamily
ORS: oral rehydration solution
PAM: protospacer adjacent motif
PMF: proton-motive force
RAMP: rapid antibody-manufacturing platform
RND: resistance-nodulation–cell division
SMR: small multidrug resistance systems
XDR: extreme drug resistance

Conflicts 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.

Ethics Approval and/or Participant Consent

This study did not require ethics approval or participant consent as it primarily involved the genetic engineering of *Lactococcus lactis* and laboratory-based research. No human

or animal subjects were involved in the experimental procedures, thereby eliminating the need for ethical review and consent. The focus was on developing a probiotic therapeutic strategy at the molecular and microbial levels, ensuring compliance with ethical standards for non-human research.

Authors' Contributions

RA: contributed to the conception and design of the study, writing (review & editing), supervision, drafting and critically revising the manuscript, project administration, and investigation, and gave final approval of the version to be published.
PP: contributed to the conception and design of the study, writing (introduction and methods), drafting the manuscript, and reviewing., and gave final approval of the version to be published.
GA: contributed to the conception and design of the study, writing (results and discussion), drafting the manuscript, and reviewing, and gave final approval of the version to be published.

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