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RESEARCH ARTICLE

# Formulation of recombinant *Lactococcus lactis* as an oral vaccine candidate for COVID-19

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## Abstract

**Background:** *Lactococcus lactis* is a promising oral vaccine carrier. However, to improve the stability of the bacteria, the freeze-drying method and formulation need to be optimised. **Objective:** To develop a formulation of oral recombinant food-grade *Lactococcus lactis* as a candidate oral vaccine for coronavirus disease 2019 (COVID-19). **Method:** To preserve bacteria during storage, a combination of skim milk, trehalose, and sucrose concentrations was adjusted. The impacts of the freeze-drying procedure were investigated using a bacterial viability test, and bacterial morphology was assessed using scanning electron microscopy. Bacteria produced from the freeze-drying technique were combined with excipients to create granules, tablets, and capsules. After one month of storage, the bacterial viability of each product was assessed after one-month storage at 4°C and 25°C, and physicochemical testing was conducted on each product. **Results:** The cryoprotectant formula containing 8% of skim milk and 7.5% of sucrose protected the bacteria the most from the freeze-drying process. The tablets and capsules complied with current specifications, including disintegration test, tablet hardness, capsule weight uniformity, and bacteria viability. **Conclusion:** Among the products, tablets stored for one month at 4°C had the best bacterial viability. This study demonstrated the potential to develop and administer an easy-to-use oral COVID-19 vaccine candidate using *L. lactis*.

## Introduction

Since the end of 2019, the coronavirus disease 2019 (COVID-19) pandemic has had a massive impact, especially in the health sector worldwide. This disease is caused by SARS-CoV-2, a new virus that originated from mutations in a member of the Coronaviridae family (Chen *et al*., 2022). The World Health Organisation designated COVID-19 a global pandemic because of its rapid spread globally (Chen *et al*., 2022). Transmission of the virus occurs faster through direct patient contact (Rothan & Byrareddy, 2020). All coronaviruses cause general clinical symptoms, including fever, cough, acute respiratory distress, fatigue, and occasional gastrointestinal symptoms such as diarrhoea (Rothan & Byrareddy, 2020). Elderly patients, children, pregnant women, and patients with comorbidities such as diabetes, heart, kidney, and liver diseases exhibit higher mortality rates after contracting COVID-19 (Rothan & Byrareddy, 2020; Tsang *et al*., 2021). COVID-19 limited all social activities outside the home and caused economic losses in various government sectors. The COVID-19 pandemic is a global health concern, and various studies have been conducted to obtain an effective vaccine against it (Ahmed *et al*., 2020; Dhama *et al*., 2020).

Vaccination represents an effective strategy to prevent viral infections. Currently, many injectable vaccines have been developed. Although the injection method is most often used, injectable vaccines have several drawbacks; for example, the immune response is limited to a systemic immune response, and a weak mucosal immune response is produced (Vishweshwaraiah & Dokholyan, 2022). Injectable vaccines are invasive and require specific skills to administer (Vishweshwaraiah & Dokholyan, 2022). According to Santos and colleagues (2021), the Food and Drug Administration (FDA)-approved COVID-19 injectable vaccine requires repeated booster

administration. An oral booster vaccine is more attractive and easier to administer than an injectable vaccine. In addition, the FDA-approved COVID-19 injectable vaccine requires low-temperature storage (−70°C–2°C) to maintain stability. This limitation results in high costs in vaccine distribution and storage in cold chain systems (Uddin & Roni, 2021).

Based on a previous study, *L. lactis*, as an oral vaccine, induced mucosal and systemic immune response in mice. The vaccine induced anti-spike protein IgG and IgA antibodies after mucosal vaccination of recombinant *L. lactis* expressing spike protein (Yurina *et al*., 2023). The spike protein, including the S2 subunit, has been extensively studied as a SARS-CoV-2 antigen (Ng *et al*., 2021; Hu *et al*., 2022). The spike protein, which is found on the surface of the coronavirus, is vital in receptor binding and enables virus entrance into host cells. This protein has been a significant target in COVID-19 vaccine design (Ahmed *et al*., 2020; Smith *et al*., 2020).

This study aimed to develop an oral formulation based on recombinant *L. lactis* as an oral vaccine candidate for COVID-19. The ability of recombinant *L. lactis* to carry antigens as a vaccine base has been demonstrated (Bermúdez-Humarán *et al*., 2013; Mancha-Agresti *et al*., 2017; Quintana *et al*., 2018). *L. lactis* is resistant to gastric acid and intestinal bases; thus, it can protect antigens from damage caused by digestive juices. The antigen can subsequently be recognised by the mucosal and systemic immune systems, leading to their activation against pathogens (Yurina, 2018; Ma *et al*., 2020; Saleena *et al*., 2022). This bacterium is an effective vaccination delivery system in pre-clinical (Namai *et al*., 2020; Guo *et al*., 2022; Zhai *et al*., 2023) and clinical studies (Mohseni *et al*., 2020).

Because viral infection occurs via the mucosal route, induction of the mucosal immune system is critical in the development of coronavirus vaccines. (Taghinezhad-S *et al*., 2021). Oral vaccines based on *L. lactis*, including tablets, granules, and capsules, are easily consumed and distributed, thereby increasing the convenience of vaccine administration.

Our study specifically aimed to analyse the viability of the bacteria during freeze-drying and storage. To the best of our knowledge, this is the first study to develop an oral formulation of a COVID-19 vaccine candidate using a food-grade recombinant bacterium.

## Methods

### *Bacterial strains and growth*

The main subject of this research was a *Lactococcus lactis* strain NZ3900 (MoBiTec GmbH, Göttingen, Germany) carrying the recombinant plasmid pNZ8149-HCR (*L. lactis* HCR), which has been constructed in previous research (Yurina *et al*., 2023). *L. lactis* was grown in M17 medium (HiMedia, Mumbai, India) supplemented with 0.5% lactose via incubation at 30℃ for 18–24°h. No antibiotics were used for selection. The overnight culture was transferred to a fresh M17 broth medium. After $OD_{600}$ reached 0.8, 40 ng/mL nisin (MoBiTec GmbH) was added to the overnight culture as the inducer, followed by incubation for 18–24 h at 30℃. The cells were harvested using centrifugation at 17,000 × g for 20 min at 4℃, and the pelleted cells were formulated.

### *Cryoprotectant formulation and freeze-drying*

The concentrated cells were resuspended in cryoprotectant (Table I) at a ratio of 1:2 (1 g of bacterial cell concentrate in 2 g of cryoprotectant) before freeze-drying. Before use, each cryoprotectant solution was sterilised at 121℃ for 20 min. One millilitre of the bacterial cell suspension in the cryoprotectant was placed in a 5-mL vial and then freeze-dried at -80°C (freezing rate was 3°C/min). After the freeze-drying process, the powder was stored at 4℃ and 25°C (room temperature) for one month to observe its stability.

**Table I: Formulation of the cryoprotectants**

| Ingredients | Concentration (%) | | | |
|---|---|---|---|---|
| | Formula 1 | Formula 2 | Formula 3 | Formula 4 |
| Trehalose | - | 1 | - | 1 |
| Sucrose | - | - | 7.5 | 7.5 |
| Skim milk | - | 8 | 8 | 8 |

The freeze-dried yield (dry powder yield) was determined as the difference between the weight of the sample before and after drying and calculated as a percentage (n = 3). Values were recorded as the mean ± SD. Following lyophilisation, the products were then tested for total bacterial counts and morphology. The formula with the highest viability was then evaluated for flow properties and moisture content.

***Evaluation of the lyophilised powder***

*Total bacterial counts*

The total *L. lactis* count was determined using the total plate count method after freeze-drying. The procedure was replicated three times at each dilution level. The medium was incubated at 30℃ for 24 h, and the number of microbial units (CFU/mL) was calculated.

*Morphology*

The structure of the lyophilised particles was observed using scanning electron microscopy (SEM), for which the images were systematically observed at 20 kV. Before SEM, the samples were dried. Crushed freeze-dried samples were carefully mounted on a double-sided carbon tape on an aluminium stub. The samples were placed in the chamber, coated with an extremely thin layer of metal gold, and the bacterial morphology was examined via SEM performed at ×5000 and ×20,000 magnification.

*Moisture content*

The lyophilised powder was analysed for its moisture content using a moisture balance analyser. Five grams of each sample were tested for humidity, and the results were obtained as the moisture content.

*Flow properties test*

The flow properties of the powder generated via lyophilisation were assessed according to the flow rate and angle of repose (USP, 1174). Flow properties were tested on mixed masses packaged in sachets using a Flowdex Tester. Each sample consisted of 10 g of powder poured into the Flowdex Tester funnel. The time required for the entire sample to fall was calculated and divided by the number of samples. The angle of repose of the falling powder was calculated using the formula: tan ∂ = h (height of the falling powder mass) ÷ r (radius of the falling powder).

***Lactococcus lactis dosage formulation***

The lyophilised *L. lactis* powder was then formulated into an easy-to-use formulation that maintained the stability of the bacterium. In this study, the powder was formulated as tablets and capsules to identify the dosage form with the best stability. The components of the tablet and capsule formulas are presented in Table II. Apart from being formulated into tablets and capsules, the stability of *L. lactis* following lyophilisation was also determined according to the type of packaging used.

**Table II: Formulation of the dosage forms**

| Ingredients | Function | Weight (mg) | | |
|---|---|---|---|---|
| | | Tablet | Capsule | Sachet |
| Dry powder *L. lactis* + cryoprotectant | Active pharmaceutical ingredients | 125 | 130 | 130 |
| Polyvinylpyrrolidone/K30 | Binder | 37.5 | - | - |
| Avicel PH-102 | Disintegrant | 25 | - | - |
| Aerosil 200 | Glidant | 2 | 25 | 25 |
| Magnesium stearate | Lubricant | 2 | 15 | 15 |
| Spray-dried lactose | Filler | 58.5 | 130 | 130 |
| Total weight (mg) | | 250 | 300 | 300 |

*Lactococcus lactis* powder tablets were produced using the direct compression method. All ingredients were weighed, mixed using an IKA Overhead Stirrer (Staufen, Germany), and then compressed using a single-punch tablet press. Each tablet weighed 250 mg. The printed tablets were then tested for physical characteristics and *L. lactis* viability after storage at 4°C and 25°C.

The IKA Overhead Stirrer mixer was used to combine the ingredients before placing them in a hard-shell gelatine capsule. Each capsule weighed 300 mg. The physical characteristics and *L. lactis* viability of the capsules were then examined both before and after storage at 4°C and 25°C. To preserve the viability of *L. lactis*, the powdered samples were then formulated in sachets. To identify which formulation offered the best stability and reliable production feasibility, products currently on the market were used as the basis for the packaging strategy selection. *L. lactis* powder was blended with additional additives before being placed in the sachet to streamline packaging. Similar to tablets and capsules, the physical characteristics and viability of *L. lactis* were evaluated after packaging and storage at 4°C and 25°C.

***Evaluation of L. lactis dosage form formulation***

*Disintegration test*

The disintegration times of tablets and capsules were determined using the Distek Disintegration Tester (USP, 701). Six samples were placed into the chamber, which was heated at 37°C, and the time required for complete disintegration of all samples was determined.

*Tablet hardness test*

Tablet hardness was measured using HC 6.2 Firmware version 01.127. Ten samples were evaluated for hardness. Measurements were performed before and after storage at 4°C and 25°C.

*Capsule weight uniformity test*

Twenty capsules were weighed individually. The capsules were then opened individually, taking care not to lose any of the shells, and the content of each capsule was extracted as fully as possible. Then, the bare shell was weighed. The difference in weight between before and after emptying the capsule was determined as the content mass. No more than two of the individual masses were permitted to deviate from the mean by more than 7.5%.

***Total bacterial counts in oral products***

Total *L. lactis* counts were determined using the total plate count method on days zero (pre-storage) and 30. A crushed tablet/capsule was diluted in 5 mL of sterile PBS. Three hundred milligrams of powder from a sachet were diluted in 5 mL of PBS. Before being applied to M17 agar, the capsule contents and granules were also diluted in sterile PBS. The procedure was performed three times. The agar was incubated for 24 h at 30°C. The following equation was used to calculate the number of viable *L. lactis* cells:

$$\text{CFU/ml} = \frac{Number\ of\ colonies\ formed \times dilution\ factor\ of\ sample}{1\ mL\ of\ sample}.$$

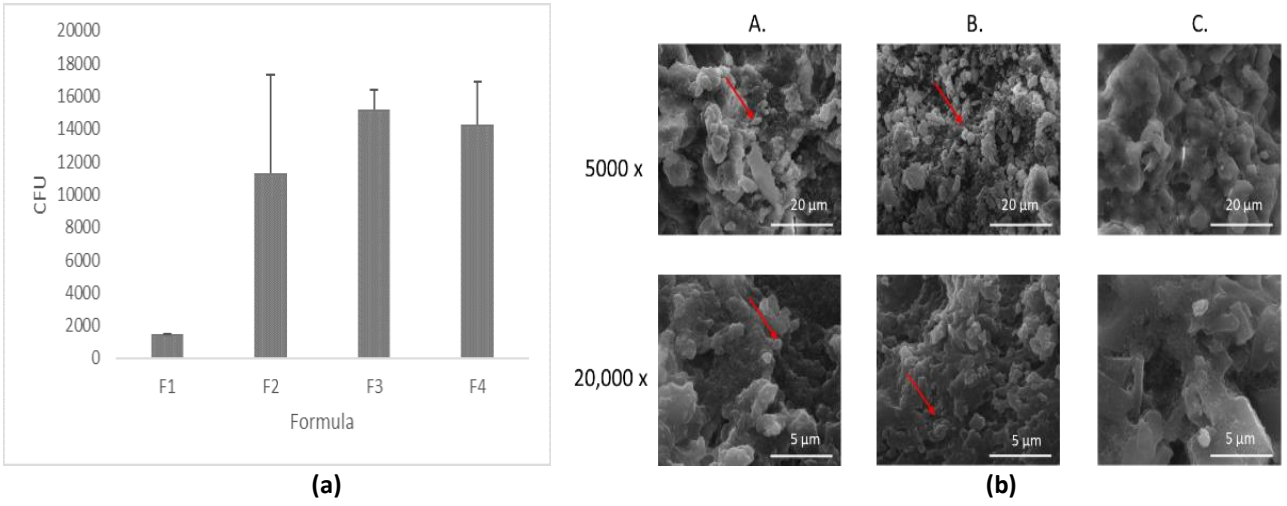
## Results

***Evaluation of the lyophilised powder***

In our experiment, 50 mL of culture were lyophilised at −55°C and vacuum pressure of 60 mmHg for 62 h, yielding a powder product (Table III). The result of the viability test is presented in Figure 1 (a). Based on the viability test, the optimal formula was formula 3 (F3), which contained 7.5% trehalose and 8% skim milk.

**Table III: The yield of *L. lactis* powder using different cryoprotectants**

| Formula | Initial weight (g) | Final weight (g) | Yield (%) |
|---|---|---|---|
| Formula 1 (control) | 26.026 | 0.656 | 2.521 |
| Formula 2 | 28.348 | 2.083 | 7.348 |
| Formula 3 | 29.261 | 4.715 | 16.112 |
| Formula 4 | 30.561 | 4.484 | 14.671 |




**(a)** **(b)**

**Figure 1: (a) Viability of *L. lactis* in the presence of different cryoprotectants (mean ± SD); (b) Scanning electron micrographs of freeze-dried recombinant *L. lactis* in the presence of (A) 1% trehalose and 8% skim milk, (B) 7.5% sucrose and 8% skim milk, and (C) 1% trehalose, 7.5% sucrose, and 8% skim milk. Red arrows denote encapsulated bacteria cells. Encapsulated cells were magnified 5000× and 20,000×.**

In addition, the effects of freeze-drying were examined using SEM (Her *et al*., 2015), as presented in Figure 1 (b). SEM illustrated that the cells' morphology was best when encapsulated in the presence of 7.5% sucrose and 8% skin milk. As presented in the SEM results, three types of cryoprotectants caused excessive cell wrapping.

The F3 lyophilised dry powder (freeze-dried) had a moisture content of 0.25%. This value represents the residual moisture in the product, which is influenced by the freeze-drying duration and the cryoprotectant's nature (Hansen *et al*., 2015). Empirical studies are used to determine the ideal value for any product. Nonetheless, according to Chavez and Ledeboer (2007), the moisture content of dried probiotics should be less than 5% to ensure stability. The results of the flow properties test showed that the angle of repose's mean was 23.22±1.0, and the flow rate mean was 1.65±1.0 (g/s). According to the calculations, the powder had extremely good flow properties, with an angle of repose of 23.22 based on the criteria stated in USP General Chapter 1174 Powder Flow.

***Evaluation of capsule preparations***

The weight uniformity and disintegration time of the capsules were determined. All capsules met the specification: no more than two of the individual masses deviated by 7.5% from the mean (Prichard, 1884). The disintegration time was determined immediately after the capsule was formulated (month 0) and after one month of storage in a refrigerator and at room temperature. Table IV shows the results of the stability test. Based on the stability test results, the capsules met the specifications, being stable at 4°C or 25°C for one month. The capsule formulation maintained its physical stability in this study.

**Table IV: Stability test of the dosage form**

| Parameter | Storage condition | Month | |
|---|---|---|---|
| | | 0 (pre-storage) | 1 month |
| **Capsule** | | | |
| Disintegration time (<30 min (Convention, 2012)) | Room temperature | 3.93 min | 2.67 min |
| | Refrigerator (4°C) | 3.93 min | 3.3 min |
| **Tablet** | | | |
| Disintegration time (<30 min (Convention, 2012)) | Room temperature | 28 min | 27 min |
| | Refrigeration (4°C) | 28 min | 25 min |
| **Hardness (N)** | Room temperature | 33.33 ± 5.05 | 85.1 ± 5.12 |
| | Refrigeration (4°C) | 33.33 ± 5.05 | 78.23 ± 8.05 |

***Evaluation of tablet preparations***

Next, the hardness and disintegration time of tablets were evaluated before and after one month of storage (Table IV). The results showed that the tablet disintegration time was shorter than 30 min. The initial hardness of the tablet supported the physical resistance of the tablets, but after one month of storage in a refrigerator and at room temperature, the hardness significantly increased. Fortunately, the increase in the hardness did not worsen the disintegration time.

The finished tablet and capsule products were kept in a room at cold temperatures. According to the results, the bacteria remained viable after one month of storage. The viability test results are depicted in Figure 2. As presented in the graph, the tablets were more viable than the capsules and sachets.

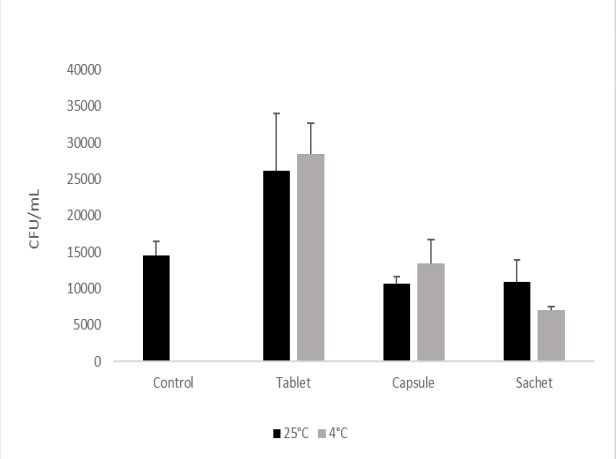



**Figure 2: The viability of *L. lactis* in different formulations after one month of storage at 4°C and 25°C. Tablet formulations displayed the highest viability among the formulations. Note: The control is the product without excipients**

## Discussion

In this study, we demonstrated the viability of recombinant *Lactococcus lactis* formulated as tablets, capsules and granules after freeze-drying. To our knowledge, this is the first study to develop an oral formulation of a COVID-19 vaccine candidate. To select *L. lactis,* a food grade using a lactose-containing medium was used, meaning that no antibiotics needed to be used.

***A combination of cryoprotectants provides better stabilisation of the bacteria***

Lyophilisation is a drying technology that involves freezing water or other solvents and then sublimating the ice under vacuum and low temperatures (Elliott *et al*., 2017). The method involves using cryoprotectants like skimmed milk and different carbohydrates like trehalose, glycerol, and sucrose to protect bacteria from damage during freezing. The chosen cryoprotectants for evaluating survival rate include trehalose, sucrose, skimmed milk, maltodextrin, and a combination of skimmed milk and sucrose. This method maintains viability and purity over time (Yuste *et al*., 2021).

Our finding is consistent with similar studies reporting that the combined use of multiple cryoprotectants produced better results (Jouki *et al*., 2021; Oluwatosin *et al*., 2022). Previous research illustrated that sucrose was a better cryoprotectant for *Lactobacillus plantarum* than inulin and maltodextrin. Sucrose has multiple effects, including prebiotic, cryoprotectant, and preservative effects (Oluwatosin *et al*., 2022). Another study examined the interactions among cryoprotectants, including milk, sucrose, and trehalose. The researchers observed synergistic effects between milk and sucrose and between sucrose and trehalose on the viability of lactic acid bacteria (Gisela *et al*., 2014).

As presented in the SEM result (Figure 1b), our findings are in line with those reported by Chen and colleagues in 2023, who demonstrated that combinations of cryoprotectants provide better stabilisation of the bacteria during storage. The composite cryoprotectants inhibited the formation of ice crystals during freeze-drying, thereby improving the surface properties of the powder, effectively sustaining the structural integrity of the cell membrane, and maintaining cell membrane permeability, which could improve the stability of the stored powder (Chen *et al*., 2023).

***Tablets and capsules met the standard product requirement***

Probiotic bacteria should retain high levels of viability throughout processing and stay alive during storage and delivery, such as while passing through the GIT. Probiotic survival and dosage levels during storage and administration are critical criteria for probiotic effectiveness. Probiotics are exposed to water, air, heat, strong acids, and bile during storage and oral administration. To address these issues, several dose forms, such as capsules, pills, powders, and liquids, have been optimised (Wang *et al*., 2022). Our study focused on the capsules, powder, and tablet forms.

Delivering viable bacteria cells to the GIT is challenging, especially when the probiotic product is in liquid or powder form. Several studies used capsules which contain lactic acid bacteria in powders or microcapsules, with excipients like diluents, glidants, disintegrants, and fillers added to preserve the bacteria's physiology. The capsule shell protects the bacterial core from the acidic environment (How & Yeo, 2021; Wang *et al*., 2022).

The tablet, a dosage form with a large worldwide market share, has numerous advantages, including physicochemical stability, a simple production method, cheap manufacturing costs, and a high degree of consumer acceptability (Sierra-Vega *et al*., 2019). Tablets containing lactic acid bacteria have been studied extensively and demonstrated high beneficial health effects (Chuang *et al*., 2011; Nishihara *et al*., 2014; Suzuki *et al*., 2017). The standard probiotic tablet formation process involves combining the powder with an excipient after drying and pressing the tablets into shape. However, these procedures can damage various cellular and physiologically active components of probiotics, posing a challenge in the design of probiotic tablets (Byl *et al*., 2019; Vorländer *et al*., 2020).

Based on our results, the capsules and the tablets met the product requirement. The disintegration time limit for capsules and tablets was based on the monograph in US Pharmacopeia Chapter 701-Disintegration (Convention, 2012). Because the product of this research had not been established in any official monograph, a time disintegration limit of 30 min was considered safe. Given that some marketed tablets have a disintegration time limit of up to 60 min, our selected limit was logical.

The increased tablet hardness can be explained by interparticle deformation and water content within the tablet after tablet compaction. The strength of the tablet changes after its ejection from the die, which leads to the interparticle attraction during the post-compaction storage phase. Interparticle attraction in tablets is primarily due to solid material rearrangement at particle surfaces, particle deformation after compaction, and dissolved material crystallisation due to water movement within the tablets, resulting in a compact structure (Shotton & Rees, 1966; Alderborn & Ahlneck, 1991).

## Conclusion

In conclusion, our research found that the combination of 7.5% sucrose and 8% skim milk best protected *L. lactis* during freeze-drying. The powder formulated as a

tablet had the highest viability and met the tablet product requirements. However, our study was limited by the fact that antigen stability within the cells was not determined. Although our previous study demonstrated that bacteria express the spike protein antigen, the antigen expression after formulation should be determined.

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