



## Potential of cocoa powder and dark chocolate in protecting Gram-negative probiotic *Escherichia coli* Nissle 1917

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### Abstract

Probiotic viability is affected by many factors, including the carrier food, processing, storage, and harsh gastrointestinal conditions. Chocolate has shown some potential to protect a wide range of Gram-positive probiotics under such conditions. In this study, *Escherichia coli* Nissle 1917, a typical Gram-negative probiotic strain, was encapsulated using various cocoa powder formulations and incorporated into the chocolate. The encapsulation efficiency, heat resistance, viability during storage, *in vitro* gastrointestinal digestion, and colonic fermentation were evaluated. Gram-positive strain *Lactobacillus rhamnosus* GG was used for comparison. Encapsulation in the combination of cocoa powder and sucrose presented the highest and satisfactory efficiency for both strains, respectively. As a result, good storage stabilities of both encapsulated strains were achieved when they were added to dark chocolate (70% cocoa) and stored at 4 °C or room temperature for 30 days. However, during the *in vitro* gastrointestinal digestion and colonic fermentation of chocolates enriched with these probiotics, the Gram-positive probiotic *L. rhamnosus* GG had a better performance than the Gram-negative probiotic *E. coli* Nissle 1917. Nevertheless, this study demonstrated a satisfactory maintenance of the encapsulated Gram-negative probiotic *E. coli* Nissle 1917 in chocolate up to 48 h of colonic fermentation.

**Keywords:** *Escherichia coli* Nissle 1917; gram-negative probiotics; *Lactobacillus*; probiotic chocolate; *in vitro* digestion; colonic fermentation.

**Practical Application:** The functionality of cocoa powder as an encapsulating agent is extended to next-generation probiotics for potential industrial applications.

## 1 INTRODUCTION

Probiotics have been defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” by the World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) (Hill et al., 2014). In recent years, the beneficial effects of probiotics on human health, such as maintaining healthy gut microbiota, have been widely reported (Ajlouni et al., 2021). However, for a long time, studies on probiotics have mainly focused on the common Gram-positive species, such as *Lactobacillus* and *Bifidobacterium*, although some of the next-generation probiotics discovered in recent years are Gram-negative bacteria (Kandasamy et al., 2017), while the investigation on Gram-negative probiotics is limited.

*Escherichia coli* strain Nissle 1917 is a popular Gram-negative probiotic (Kandasamy et al., 2017). Different from other *E. coli* strains, this strain does not have immunotoxic property (Rietschel et al., 1996). Instead, *E. coli* Nissle 1917 has the potential to inhibit the colonization of other pathogenic *E. coli* strains and stimulate the production of defensin by epithelial cells, leading to the reduction of pathogen invasions (Maltby et al., 2013; Schlee et al., 2007). Furthermore, it can regulate the

levels of pro-inflammatory and anti-inflammatory cytokines to achieve the immunomodulation within hosts (Helwig et al., 2006). Based on these benefits, *E. coli* Nissle 1917 has been widely used to treat gastrointestinal disorders such as diarrhea and inflammatory bowel disease (Henker et al., 2008; Schultz, 2008).

Incorporating probiotics into popular food products is becoming a new trend. However, harsh environments during food processing and storage and in the gastrointestinal transit result in a decrease in probiotic numbers below the recommended minimum ( $10^6$  colony-forming unit [CFU]  $g^{-1}$  or  $mL^{-1}$ ) (Yao et al., 2020). Techniques such as microencapsulation have been widely applied as a strategy to maintain the probiotic viability in food. Apart from traditional biopolymers, edible materials such as cocoa powder, milk fat, fruit peel, and sourdough powders are becoming more popular to protect probiotics in recent years (Hossain, Ranadheera, Fang, & Ajlouni, 2021; Kalandarmahdavi et al., 2021; Muhammad et al., 2021; Silva et al., 2022). Furthermore, choosing the right food product as the vehicle for probiotics is crucial for maintaining sufficient viability levels of probiotics. Some studies have demonstrated that chocolate has the potential to be a suitable carrier for probiotic delivery. However, most of them have focused on the common

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Gram-positive probiotic bacteria (Didar, 2020; Hossain, Ranadheera, Fang, & Ajlouni, 2021; Kaltza et al., 2021; Lasta et al., 2021). Studies on Gram-negative probiotics in chocolate are rare, and the protective ability of chocolate toward *E. coli* Nissle 1917 has not been fully studied.

This study aims to assess the potential of dark chocolate as a food carrier to protect the Gram-negative probiotic strain *E. coli* Nissle 1917 during food processing and storage and *in vitro* digestion and colonic fermentation. Since the investigations on Gram-negative probiotic species are limited, this study could be helpful in developing novel and suitable probiotic food products using Gram-negative probiotics. The traditional Gram-positive lactic acid bacteria *Lactobacillus rhamnosus* GG was used in this study for comparison purposes.

### 1.1 Relevance of the work

The relevance of this study lies in exploring cocoa powder as an encapsulating agent to protect the Gram-negative probiotic strain *Escherichia coli* Nissle 1917 in chocolate. This work extends the functionality of cocoa powder beyond traditional Gram-positive probiotics, supporting the development of innovative functional food products. The findings may broaden the industrial applications of cocoa-based ingredients and provide valuable insights for future research on probiotic delivery systems.

## 2. MATERIAL AND METHODS

### 2.1 Bacterial strains

*L. rhamnosus* GG was obtained from stock strain collections in the Food Chemistry & Microbiology Laboratory at the University of Melbourne. *E. coli* Nissle 1917 was purchased from a commercial preparation capsule of Mutaflor® (Ardeypharm, Herdecke, North Rhine-Westphalia, Germany).

### 2.2 Activation of *E. coli* Nissle 1917

The content of one *E. coli* Nissle 1917 capsule (each capsule contains more than  $2.5 \times 10^8$  CFU *E. coli* strain Nissle 1917) was cultured in 400mL Luria-Bertani (LB) broth (Thermo Fisher Scientific Pty Ltd., Melbourne, Victoria, Australia) at 37 °C for 20 h, then inoculated on MacConkey agar (Thermo Fisher Scientific Pty Ltd.), and incubated at 37 °C for 20 h. After incubation, one to two loops of well-isolated colonies were picked and suspended in cryopreservative in the Microbank™ vial. The vial was inverted and stored at -20 °C until use. The activated culture was verified using Gram staining.

### 2.3 Microencapsulation

The stock culture of frozen *E. coli* Nissle 1917 strain (one bead) was inoculated in 400mL LB broth and incubated aerobically at 37 °C for 20 h using a BB-16 incubator (Heraeus Instruments, Hanau, Hesse, Germany). Similarly, *L. rhamnosus* GG was inoculated in 400 mL DeMan, Rogosa, and Sharpe (MRS) broth (Thermo Fisher Scientific Pty Ltd.)

and incubated anaerobically at 37 °C for 48 h. Cells of both probiotics were harvested by centrifugation at  $4000 \times g$ , 4 °C for 15 min in an Allegra X-12R refrigerated centrifuge (Beckman Coulter, Mount Waverley, Victoria, Australia). After centrifugation, the pellet was washed once with 0.85% NaCl solution (saline) and resuspended in cryoprotectant solution (10% sucrose) to achieve a final volume of 5 mL for both strains.

The selected ingredients for microencapsulation included 5g of cocoa powder (PANA Organic, Richmond, Victoria, Australia) and 0.5g of Na-alginate (Sigma Aldrich, Castle Hill, New South Wales, Australia). The microencapsulation mixture was prepared following the method of Hossain, Ranadheera, Fang, and Ajlouni (2021) with some modifications. First, cocoa powder and Na-alginate were dissolved in 100 mL cryoprotectant solution (sucrose 10%), followed by homogenization at 8000 rpm for 15 min using a homogenizer (IKA T25 digital ULTRA-TURRAX®, Staufen, Baden-Württemberg, Germany) to produce the final encapsulant formulation. Second, the mixture was left at room temperature for 2 h, followed by pasteurization at 75 °C for 30 min. Third, the mixture was cooled down and distributed into 30mL sterile Falcon tubes. Fourth, 1mL of suspended cells was added to 30mL of encapsulant formulation, and the mixture was left at room temperature for 1 h. After the full interaction between the probiotics and encapsulants, the mixture was frozen at -20 °C overnight. Subsequently, the frozen mixtures were freeze-dried for 48 h at -60 °C using the benchtop freeze dryer (Dynavac Engineering FD3, Sydney, New South Wales, Australia). The freeze-dried microcapsule powders were designated as “cocoa powder treatment” and stored at 4 °C until use.

Additionally, to evaluate the protective ability of cocoa powder toward probiotics, two control treatments were prepared following the same procedures. The first control was prepared by mixing 1mL of suspended cells with 30mL of cryoprotectant solution only (10% sucrose) and designated as “cryoprotectant treatment,” while the second control was made of 1mL of suspended cells in saline and designated as “saline treatment.”

### 2.4 Encapsulation efficiency

The encapsulation efficiency was tested before and after the encapsulation process. Before the encapsulation, 1mL of suspended cells was directly diluted in saline and serially diluted to proper dilutions; after the encapsulation, 100mg of encapsulated probiotic powders was aseptically dissolved in 10mL of saline and serially diluted using the same diluent. Proper dilutions were plated in duplicate on MRS agar for *L. rhamnosus* GG or LB agar for *E. coli* Nissle 1917 using a pour plate technique (Chen et al., 2017; Fávoro-Trindade & Grosso, 2002). The LBA plates were incubated aerobically at 37 °C for 24 h and the MRS anaerobically at 37 °C for 48 h. The spread plate technique was used to assess the viable counts of the probiotics, and the results were recorded as total viable cells (log CFU). Encapsulation efficiency was calculated based on the viability of probiotics before and after encapsulation. The results were recorded as total viable cells (log CFU).

## 2.5 Chocolate preparation and thermal tolerance of encapsulated probiotics

Preparation of chocolate was conducted following a previous method (Hossain et al., 2022b). Accordingly, 70% dark chocolate was prepared with the following formulation: 70g of cocoa mass (Belgian Delights, Molendinar, Queensland, Australia), 7g of cocoa butter (Belgian Delights), 22.7g of sugar, and 0.3g of soy lecithin (Glentham Life Sciences Ltd., Corsham, Wiltshire, United Kingdom) per 100g. Approximately 45% chocolate contained: 45g of cocoa mass, 7g of cocoa butter, 27g of sugar, 20.7g of milk solids (Woolworths Group Ltd., Melbourne, Victoria, Australia), and 0.3g of soy lecithin per 100g.

In order to study the heat resistance of encapsulated probiotics, cocoa mass and cocoa butter were melted at 60 °C, and other materials were added following the formulations and mixed, and then the temperature was adjusted to 40 °C, 50 °C, and 60 °C, respectively. After reaching the final temperature, freeze-dried microencapsulated powder of each probiotic strain was separately incorporated into chocolate at 2%, followed by gentle stirring until fully mixed (Kemsawasd et al., 2016; Silva et al., 2017). Freeze-dried powders from cryoprotectant treatment and saline treatment were conducted following the same procedures. Initial viability numbers per gram of chocolate for all treatments were kept at the same levels.

Soon after the solidification, 1 g from each of the chocolate treatments enriched with probiotics was aseptically ground into a powder and homogenized with 9mL saline, followed by proper dilutions, inoculation, and incubation. The spread plate technique was used to assess the viable counts of the encapsulated probiotics in per gram of chocolate. The results were expressed as log CFU g<sup>-1</sup> chocolate.

Since the saline treatment did not offer good protective activity to the tested probiotics, enriching chocolate with cocoa powder and cryoprotectant treatments were used in the following experiments. These two encapsulated probiotics were added to the chocolates and mixed at a predetermined temperature (45 °C) in a water bath (Erdem et al., 2014).

## 2.6 Viability during chocolate storage

To evaluate the viability of probiotics in chocolate during storage, approximately 5g of chocolate enriched with probiotics was poured into a sterilized mold until solidification. Chocolates were then stored at 25 °C and 4 °C under aseptic conditions. Probiotic counts were measured after 1, 15, and 30 days of storage using the same method as described in Section 2.5.

## 2.7 *In vitro* gastric digestion

Since most microorganisms cannot survive the harsh gastric conditions, it is essential to evaluate the protective ability of chocolates toward probiotics during gastric passage (Martinsen et al., 2005). For each probiotic strain, six different types of samples were used in this test to compare the protective abilities against the *in vitro* gastric digestion: (i) 70% chocolate enriched with probiotics from cocoa powder treatments (recorded as L1 and E1); (ii) 70% chocolate enriched with probiotics from

cryoprotectant treatments (recorded as L2 and E2); (iii) 45% chocolate enriched with probiotics from cocoa powder treatments (recorded as L3 and E3); (iv) 45% chocolate enriched with probiotics from cryoprotectant treatments (recorded as L4 and E4); (v) cocoa powder treatments (recorded as LP1 and EP1); and (vi) cryoprotectant treatments (recorded as LP2 and EP2).

The procedures of *in vitro* gastric digestion were conducted as described by Minekus et al. (2014) with some modifications. In the mouth mastication phase, 2.5g of chocolate samples were added to the mixture of simulated salivary fluid (SSF), CaCl<sub>2</sub>, and salivary  $\alpha$ -amylase solution (Sigma Aldrich). The pH was adjusted to 7.0, and Milli-Q water was used to make up to 5mL. The mixture was then vortexed at room temperature for 2 min. Likewise, suitable amounts of powders were added to achieve approximately the same initial probiotic cell numbers.

In the gastric digestion phase, 5mL mouth-masticated samples were added to the mixture of simulated gastric fluid (SGF) with 2000 U mL<sup>-1</sup> porcine pepsin (Sigma Aldrich). The pH was adjusted between 2.0 and 2.3 using 4 M HCl (Darwish et al., 2022), and Milli-Q water was used to make up to 10 mL. The mixture was then aerobically incubated for 120 min in a shaking incubator (37 °C, 120 rpm).

To compare the protective abilities of chocolates as an encapsulant among all treatments during the harsh gastric digestion, 1mL of each gastric digested sample was collected at 30, 60, 90, and 120 min after the start of digestion, and mixed with 9mL saline. Probiotic counts were assessed, and the results were expressed as total viable counts in the SGF suspension at different times of digestion.

## 2.8 *In vitro* intestinal digestion

After the *in vitro* gastric digestion, 10mL of the digested samples was added to the mixture of simulated intestinal fluid (SIF), which contained porcine pancreatin, fresh bile, and CaCl<sub>2</sub>, following the method of Minekus et al. (2014). The pH was adjusted to 7 using 4 M NaOH, and Milli-Q water was used to make up to 20mL. The mixture was anaerobically incubated for 120 min in a shaking incubator (37 °C, 120 rpm). The viability of probiotics was measured after digestion as per the method described in Section 2.5.

After the *in vitro* intestinal digestion, samples were centrifuged at 4000 rpm, 4 °C for 10 min in a refrigerated centrifuge. Residues were collected for the colonic fermentation.

## 2.9 Colonic fermentation

Colonic fermentation was conducted using human feces collected from a 24-year-old healthy male donor, who has not ingested antibiotics for the last 3 months (university human ethics approval ID: 2022-14257-40286). The basal medium and fecal slurry were prepared following the method previously described by Hossain, Ranadheera, Fang, and Ajlouni (2021) with some modifications. Fecal samples were diluted 1:10 (w/v) with 0.1 M anaerobic phosphate buffer and 30% (v/v) glycerol solution and stored at -20 °C until use (Carvalho et al., 2021).

In the colonic fermentation, 5 mL of basal medium and 5 mL of fecal slurry were added to the collected sample residues from *in vitro* intestinal digestion, and the mixture was anaerobically incubated (37 °C, 120 rpm) for up to 72 h. Pour plate count was conducted at 12, 24, 48, and 72 h. The probiotic strain *L. rhamnosus* GG viability was measured following the method described previously in Section 2.5. For the probiotic strain *E. coli* Nissle 1917, proper dilutions were plated on the Violet Red Bile Agar (Thermo Fisher Scientific Pty Ltd.) and incubated aerobically at 37 °C for 24 h (Yu et al., 2018).

## 2.10 Statistical analysis

Assessment of the cell viability in all experiments was performed in triplicate, while the counts of each replicate were plated twice. One-way ANOVA was applied to assess the significant difference in cell viabilities among different treatments. Tukey's honest significant difference test was used to separate the means at a 95% confidence level. All results were expressed as means  $\pm$  standard deviation.

## 3 RESULTS AND DISCUSSION

### 3.1 Viability of probiotics after the encapsulation and chocolate-making processes

The initial counts of activated cultures before encapsulation were  $10.8 \pm 0.05$  log CFU and  $10.9 \pm 0.03$  log CFU per mL in *L. rhamnosus* GG and *E. coli* Nissle 1917, respectively. After the encapsulation process, the viabilities of both strains in the cocoa powder treatment were significantly higher ( $p < .05$ ) than in the cryoprotectant treatment and saline treatment (Table 1\_Supplementary data), suggesting that cocoa powder and Na-alginate can be used as a promising encapsulation material combination in the following chocolate-making process.

As the tempering temperatures of different types of chocolates may vary (Afoakwa et al., 2007, 2008), it is necessary to evaluate the viability of probiotics at different temperatures to ensure adequate probiotic survival throughout chocolate processing. Previous studies conducted in our lab by Hossain,

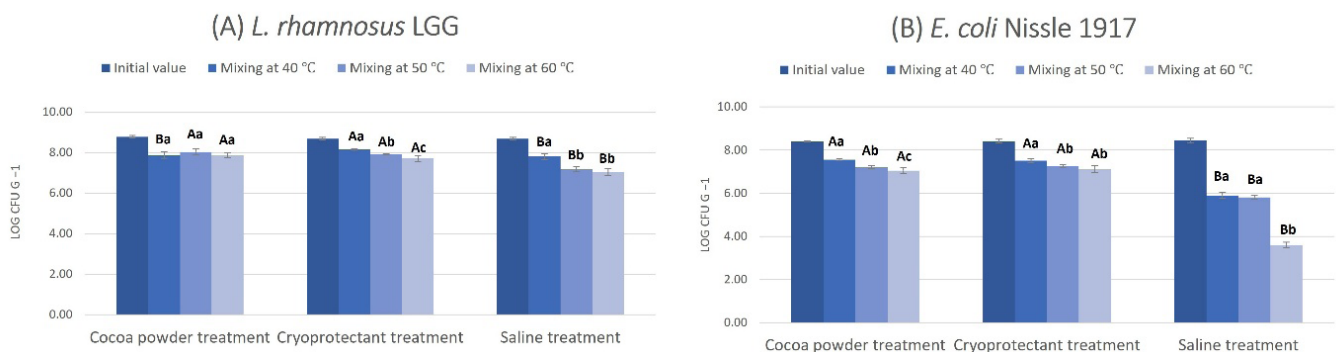
Ranadheera, Fang, and Ajlouni (2021) revealed that the protective ability of 70 and 45% cocoa for chocolate during the chocolate-making process was identical. Hence, in this study, only 70% cocoa chocolate was used in the heat resistance test.

As shown in Figure 1, both probiotics in the cocoa powder treatment presented a decrease of approximately 1 log CFU  $g^{-1}$  when mixing with chocolate at 40 °C, in comparison with the initial counts (numbers of probiotics in the chocolate mixture when added at 2% (w/w) before heat treatment). However, when the saline treatment was added to chocolate, the *E. coli* Nissle 1917 count declined by  $3.59 \pm 0.14$  log CFU  $g^{-1}$ . These results suggested that both the cocoa powders and sucrose were effective in preventing *E. coli* Nissle 1917 from death during chocolate processing.

At the temperatures of 50 °C and 60 °C, the cocoa powder treatment showed a good protection of *L. rhamnosus* GG with no significant difference ( $p > .05$ ) when compared to 40 °C. Both the cocoa powder treatments of *E. coli* Nissle 1917 and cryoprotectant treatment of *L. rhamnosus* GG presented significant reductions ( $p < .05$ ) in viability levels when the mixing temperature increased from 40 °C to 60 °C, while the cryoprotectant treatment of *E. coli* Nissle 1917 showed a significant decrease ( $p < .05$ ) only when the temperature increased from 40 °C to 50 °C. These results indicated that the combination of cocoa powder and sucrose, as well as sucrose alone, can assist probiotics in tolerating heat stress during the chocolate-making process.

### 3.2 Viability of probiotics in chocolate during storage at different temperatures

As shown in Figure 2A, *L. rhamnosus* GG counts from the cryoprotectant treatments added to 45% chocolate (L4) and 70% chocolate (L2) revealed significant loss ( $p < .05$ ) of  $3.58 \pm 0.42$  log CFU  $g^{-1}$  and of  $3.39 \pm 0.33$  log CFU  $g^{-1}$ , respectively, at the end of 30 days' storage at room temperature. In comparison, *L. rhamnosus* GG in the cocoa powder treatments only presented a viability loss of less than 1 log CFU  $g^{-1}$  under the same storage conditions. However, Figure 2B exhibits that *L. rhamnosus* GG in all treatments demonstrated a satisfactory viability retention and no significant decrease ( $p > .05$ ) in viability levels during



Results are expressed as mean  $\pm$  standard deviation of three independent experiments. Cocoa powder treatment: microcapsules consisting of probiotic suspension, cocoa powder, and sucrose. Cryoprotectant treatment: microcapsules consisting of probiotic suspension and sucrose. Saline treatment: microcapsules consisting of probiotic suspension and NaCl. Initial counts represent the number of probiotics in the chocolate mixture when added at 2% (w/w) before heat treatment. Columns with different lowercase superscripts within each treatment represent significant differences ( $p < .05$ ) between the values at different temperatures. Columns with different uppercase superscripts represent significant differences ( $p < .05$ ) between the values for different treatments at the same temperature.

**Figure 1.** Viability (log CFU  $g^{-1}$ ) of encapsulated probiotic strain *L. rhamnosus* GG and *E. coli* Nissle 1917 after mixing with 70% dark chocolate at 40 °C, 50 °C, and 60 °C.

storage at 4 °C for 30 days. These results suggest that cocoa powder as an encapsulant, rather than cocoa contents in the chocolate, plays an important role in protecting *L. rhamnosus* GG during storage at room temperature.

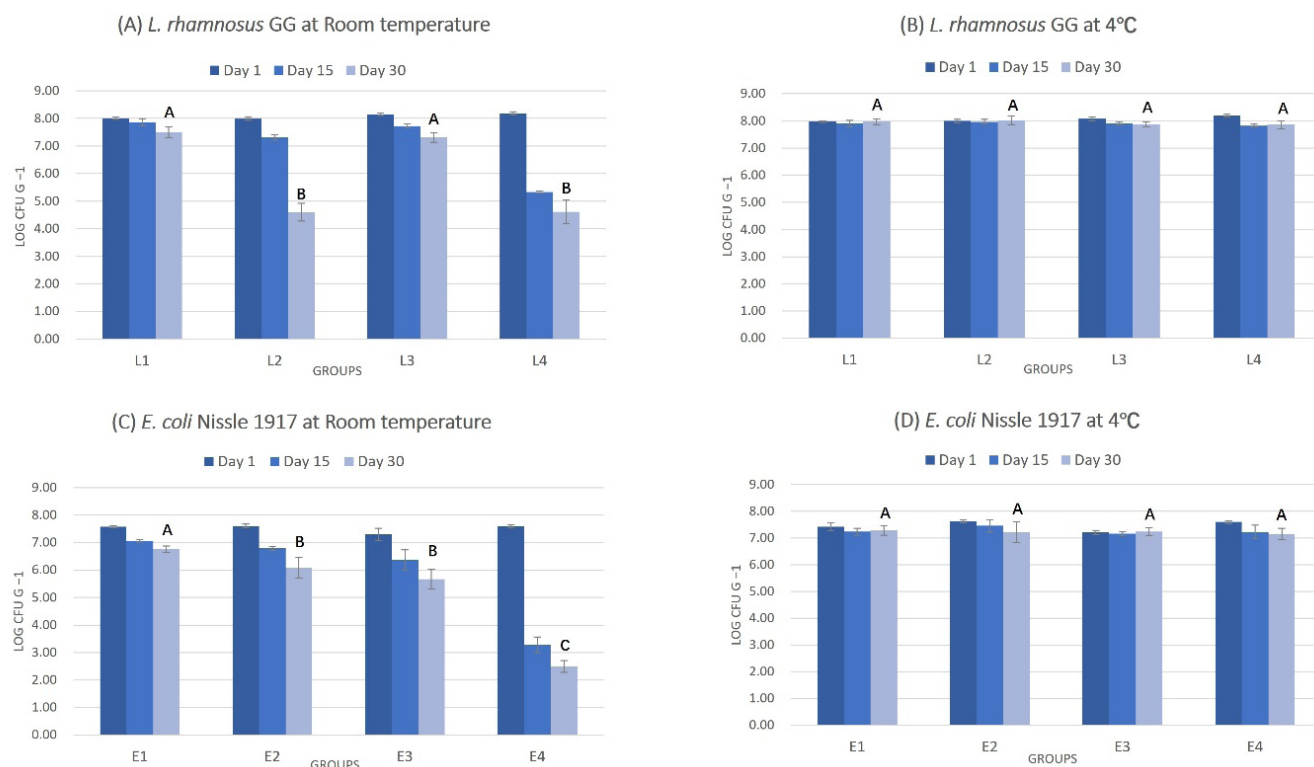
Figure 2C presents a viability loss of  $5.11 \pm 0.22$  log CFU  $g^{-1}$  in E4 after 30 days of storage at room temperature. This decrease was significantly greater ( $p < .05$ ) than the loss in E2 and E3 treatments, which exhibited non-significant loss ( $p > .05$ ) of only  $1.52 \pm 0.37$  log CFU  $g^{-1}$  and  $1.63 \pm 0.36$  log CFU  $g^{-1}$ , respectively, under the same storage conditions. These observations suggest that both the encapsulation material and the chocolate components play a similar role in protecting *E. coli* Nissle 1917 during storage at room temperature. E1 presented a viability loss of only  $0.81 \pm 0.11$  log CFU  $g^{-1}$  at day 30, further demonstrating that both factors are essential to the viability of *E. coli* Nissle 1917 when storing at room temperature. Marcial-Coba et al. (2019) also observed that encapsulated *Akkermansia muciniphila*, another Gram-negative probiotic strain, remained stable in 70% chocolate after storing at 15 °C for 30 days. Additionally, as exhibited in Figure 2D, after storing at 4 °C for up to 30 days, all four treatments presented no significant differences ( $p > .05$ ) in cell numbers, with only  $< 0.5$  log CFU  $g^{-1}$  loss for all treatments.

### 3.3 Viability of probiotics during the *in vitro* gastric digestion

Gastric digestion is an effective process to kill bacteria during food intake (Smith, 2003); thus, in this study, the loss of viability at different time points of the *in vitro* gastric digestion

was evaluated. Although the number of *L. rhamnosus* GG in chocolates (L1, L2, L3, and L4) presented a significant increase ( $p < .05$ ) after 120 min of gastric digestion, its viability in LP1 exhibited a decrease of  $0.1 \pm 0.05$  log CFU after 30 min. This demonstrates that chocolate as a food carrier provides better protective abilities to the encapsulated probiotics than the encapsulant cocoa powder alone. Moreover, the number of *L. rhamnosus* GG in LP2 reduced to zero after exposure to simulated gastric juice for 30 min (Figure 3A), suggesting that cocoa powders have strong protective effects when used as an encapsulation material. This result was in agreement with the study conducted by Doherty et al. (2012), where the free cells of *L. rhamnosus* GG were not able to survive after *in vitro* stomach incubation at a pH less than 3.4 for 30 min. Recently, Hossain, Ranadheera, Fang, Hutchinson, and Ajlouni (2021) also reported that cocoa powders exhibit beneficial impacts on the viability of the *L. rhamnosus* GG during *in vitro* gastric digestion, which is in agreement with our results.

As demonstrated in Figure 3B, encapsulated *E. coli* Nissle 1917 in 45% chocolate (E3 and E4) had a relatively better survivability at the end of gastric digestion than in 70% chocolate (E1 and E2). This could be due to its higher buffering capacity, which comes from the milk solids in 45% chocolate. Previous studies have also demonstrated that higher protein content in milk chocolate contributes to its stronger buffering ability (Mandal et al., 2013; Satapathy et al., 2019). Besides, the viability of *E. coli* Nissle 1917 in EP1 and EP2 decreased rapidly and significantly during the gastric



Results are expressed as mean  $\pm$  standard deviation of three independent experiments. Columns with different uppercase superscripts represent significant differences ( $p < .05$ ) between the values for different treatments at day 30.

**Figure 2.** Viability (log CFU  $g^{-1}$ ) of encapsulated probiotic strain *L. rhamnosus* GG and *E. coli* Nissle 1917 embedded in 70% dark chocolate and stored at 4 °C or room temperature (25 °C) for up to 30 days.

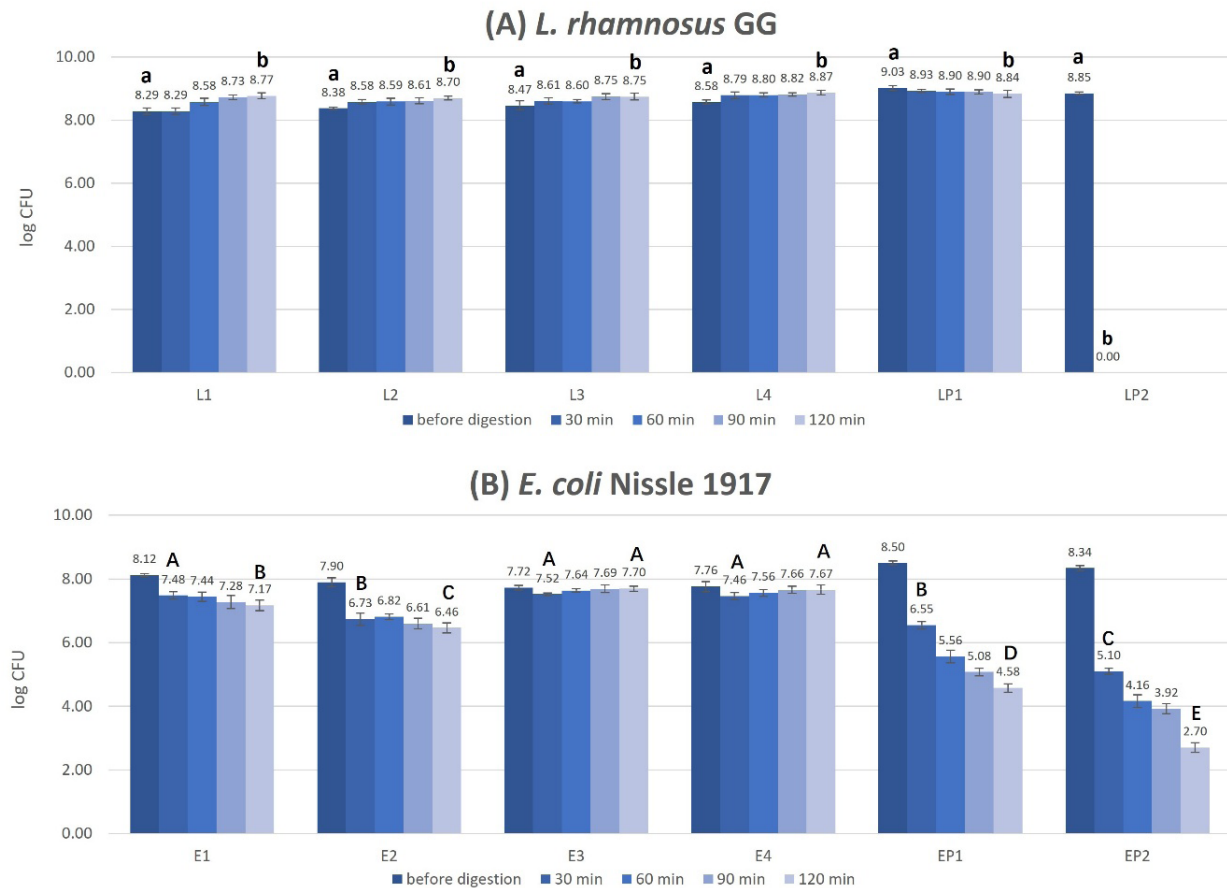
digestion, while incorporating these powders into chocolate as a carrier demonstrated a significantly higher ( $p < .05$ ) viability retention of *E. coli* Nissle 1917 (Figure 3B). This could be due to the multifunctional ability of chocolate in protecting probiotics. On the one hand, chocolate is rich in dietary fibers and phenolic compounds, which increase the resistance to gastric digestion of diverse probiotic strains (He et al., 2021; Hossain et al., 2022a). On the other hand, chocolate has a buffering effect on the pH during gastric digestion, and such an effect has also been observed by Satpathy et al. (2019). Consequently, it could be concluded that chocolate itself can be applied as an ideal food carrier to deliver probiotics.

### 3.4 Viability of probiotics during the *in vitro* intestinal digestion and colonic fermentation

*In vitro* gastrointestinal digestion usually consists of gastric digestion and intestinal digestion. In order to compare the performance of probiotic chocolates during the *in vitro* intestinal digestion and colonic fermentation, chocolates enriched with *L. rhamnosus* GG and *E. coli* Nissle 1917 were subjected to intestinal digestion. Results in Figure 4 demonstrate that chocolate

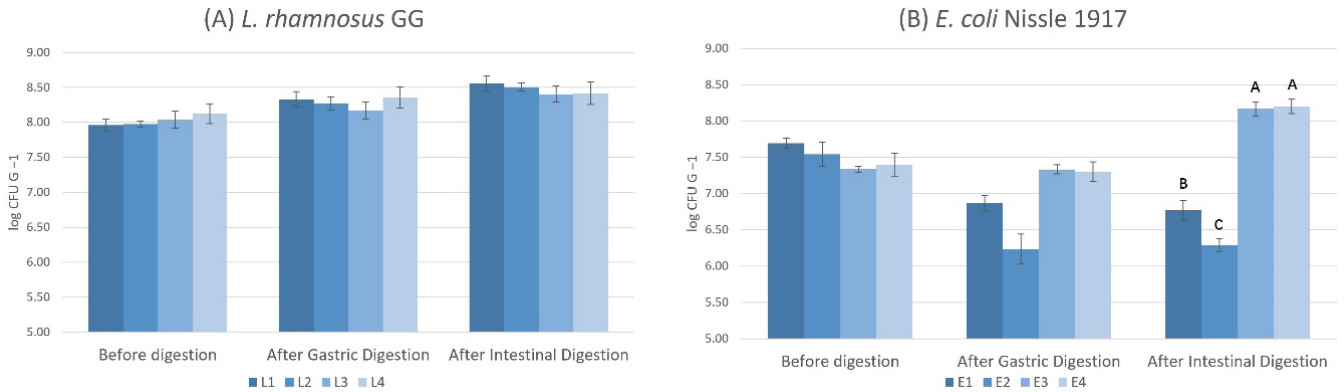
can provide better protection for probiotics during intestinal transit. Viability levels of *L. rhamnosus* GG in all four treatments increased slightly during the whole intestinal digestion process. However, the counts of *E. coli* Nissle 1917 in both 70% chocolates (E1 and E2) did not show any significant change ( $p > .05$ ), but in the 45% chocolates (E3 and E4), the viability levels of *E. coli* Nissle 1917 were increased.

During the *in vitro* colonic fermentation (Figure 5), *L. rhamnosus* GG in all types of chocolates increased by approximately 1 log CFU  $g^{-1}$  after 12 h, then remained at the same level without any significant changes ( $p > .05$ ) in L1, L3, L4, or an increment in L2 after 72 h. Viability levels of *E. coli* Nissle 1917 increased between 9.3 log CFU  $g^{-1}$  and 9.6 log CFU  $g^{-1}$  in all samples within 12 h. This increasing trend of both probiotic strains can be attributed to the favorable growth environment in the colon, which was also recorded by Hossain, Ranadheera, Fang, and Ajlouni (2021). Unlike *L. rhamnosus* GG, viability levels of *E. coli* Nissle 1917 significantly reduced ( $p < .05$ ) after 48 h. However, a significantly higher ( $p < .05$ ) viability of *E. coli* Nissle 1917 in 70% chocolates than in 45% chocolates at 48 h was reported in our study. This indicates that chocolate with more cocoa content helps *E. coli* Nissle 1917 to remain viable



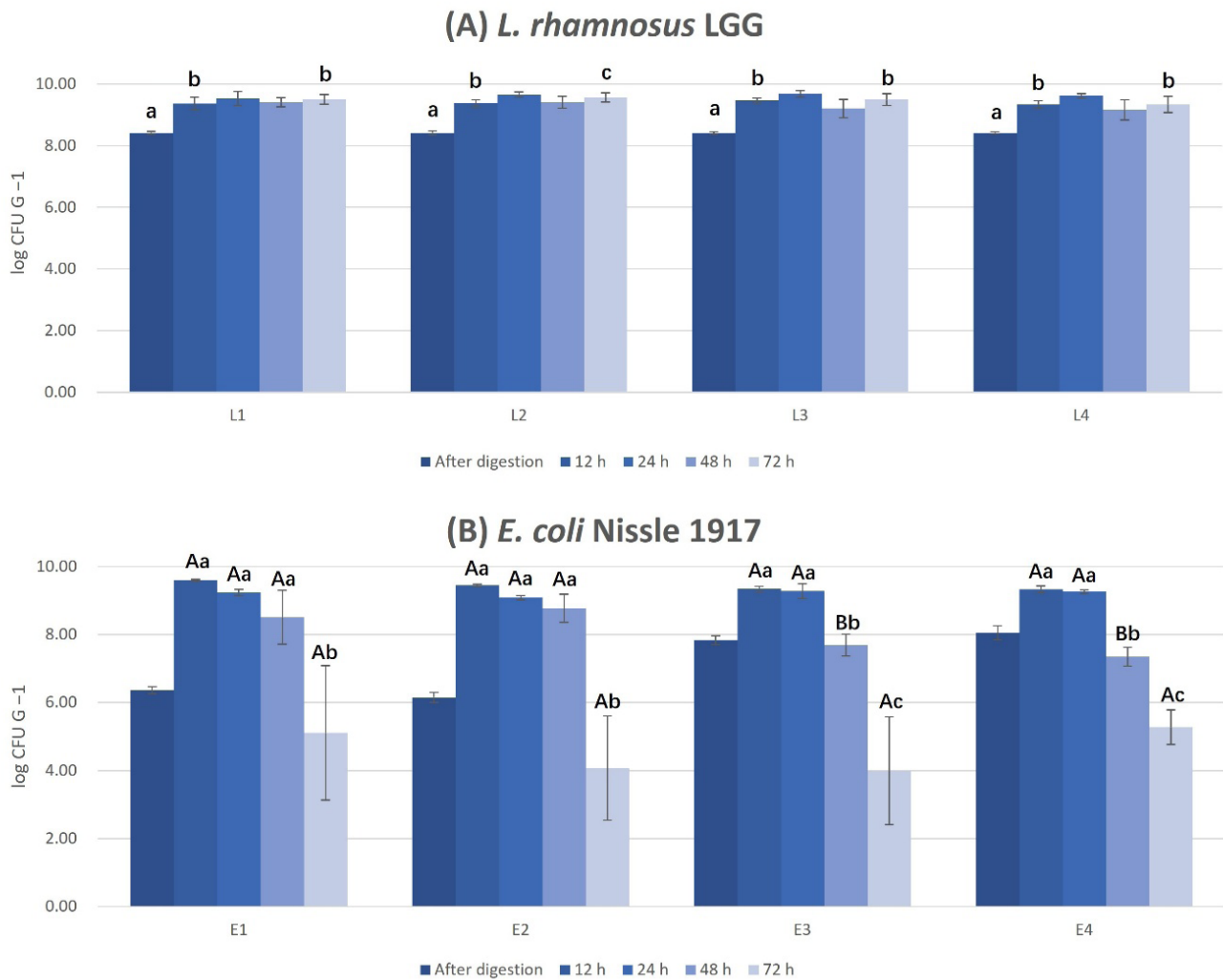
Results are expressed as mean  $\pm$  standard deviation of three independent experiments. Columns with different lowercase superscripts within each treatment represent significant differences ( $p < .05$ ) between the values at different time points. Columns with different uppercase superscripts represent significant differences ( $p < .05$ ) between the values for different treatments at 30 min and 120 min.

**Figure 3.** Viability (log CFU) of *L. rhamnosus* GG and *E. coli* Nissle 1917, either in cocoa powder treatment or cryoprotectant treatment, incorporated into the 45% chocolate and 70% chocolate during the *in vitro* gastric digestion.



Results are expressed as mean  $\pm$  standard deviation of three independent experiments. Columns with different uppercase superscripts represent significant differences ( $p < .05$ ) between the values for different treatments after intestinal digestion.

**Figure 4.** Viability (log CFU g<sup>-1</sup>) of *L. rhamnosus* GG and *E. coli* Nissle 1917 incorporated into the 45% chocolate and 70% chocolate during *in vitro* gastrointestinal digestion.



Results are expressed as mean  $\pm$  standard deviation of three independent experiments. Columns with different lowercase superscripts within each treatment represent significant differences ( $p < .05$ ) between the values at different time points of colonic fermentation. Columns with different uppercase superscripts represent significant differences ( $p < .05$ ) between the values for different treatments after colonic fermentation for 12 h, 24 h, 48 h, and 72 h.

**Figure 5.** Viability (log CFU g<sup>-1</sup>) of *L. rhamnosus* GG and *E. coli* Nissle 1917 incorporated into the 45% chocolate and 70% chocolate after colonic fermentation for 12 h, 24 h, 48 h, and 72 h.

for a longer time in the colon. Nevertheless, in this experiment, after 72 h, significant viability decreases ( $p < .05$ ) were observed in all treatments, suggesting that *E. coli* Nissle 1917 was not able to stay in the colon for 72 h. One possible explanation is that the growth of *E. coli* Nissle 1917 is more readily affected by the lack of nutrients for its growth and survival in the colon than *L. rhamnosus* GG.

#### 4 CONCLUSIONS

This study showed that microencapsulation of Gram-negative probiotic strain *E. coli* Nissle 1917 in the combination of cocoa powder and sucrose exhibited protective effects on the probiotic during chocolate processing. Furthermore, incorporating this encapsulated probiotic into 70% chocolate maintained sufficient probiotic numbers ( $> 10^6$  CFU  $g^{-1}$ ) during storage at either 4 °C or room temperature, as well as during the *in vitro* gastrointestinal transition. This protective effect was also reproduced in the Gram-positive probiotic *L. rhamnosus* GG. During the colonic fermentation of probiotic-enriched chocolates, *E. coli* Nissle 1917 demonstrated highly satisfactory viability levels up to 48 h in all chocolate samples. Given the potential health benefits of *E. coli* Nissle 1917, especially their ability to maintain healthy gut microbiota, this finding is highly encouraging. Future studies should be focused on embedding Gram-negative probiotics into popular food and beverages to harness the full potential of probiotics.

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