Stress Resistance, Adhesion Rate to Porcine Intestinal Epithelial Cells, and Antimicrobial Activity of Probiotic Escherichia coli Nissle 1917 (Postprint)

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Abstract

The present study was designed to investigate the stress resistance, adhesion rate to porcine intestinal epithelial cells, and antibacterial effect of probiotic *Escherichia coli* Nissle 1917 (EcN). In vitro methods were employed to construct the growth curve of EcN and determine its acid tolerance, bile salt tolerance, and heat tolerance. Using porcine intestinal epithelial IPEC-J2 cells as an in vitro cell model, the adhesion rate of EcN to these cells and its adhesion inhibition rate against pathogenic *Escherichia coli* K88 were examined; simultaneously, Western blotting was used to detect the effect of EcN on the levels of β-defensin-2 and Toll-like receptor 4 in IPEC-J2 cells. The results showed that: 1) EcN exhibited certain tolerance to high-acid, high-bile-salt, and high-temperature environments. 2) The adhesion of EcN to IPEC-J2 cells was optimal in the logarithmic phase, with an adhesion rate of 33.96%, which was significantly higher than those in the lag, stationary, and decline phases ($P<0.05$). 3) EcN exhibited good inhibitory effects against pathogenic *Escherichia coli* K88, with an adhesion inhibition rate of 87.84%. 4) EcN could also upregulate the levels of β-defensin-2 and Toll-like receptor 4 in IPEC-J2 cells. These results suggest that probiotic EcN possesses good stress resistance, can effectively adhere to porcine intestinal epithelial cells, and exhibits good inhibitory effects against pathogenic *Escherichia coli* K88.

Full Text

Probiotic *Escherichia coli* Nissle 1917: Stress Resistance, Adhesion to Porcine Intestinal Epithelial Cells, and Antimicrobial Effects

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Abstract

This study aimed to investigate the stress resistance, adhesion rate to porcine intestinal epithelial cells, and antimicrobial effects of probiotic *Escherichia coli* Nissle 1917 (EcN). In vitro methods were employed to determine the growth curve and assess acid, bile salt, and heat tolerance. Using porcine intestinal epithelial IPEC-J2 cells as an in vitro model, we examined EcN adhesion to these cells and its inhibition of pathogenic *E. coli* K88 adhesion. Additionally, Western blotting was used to detect the effects of EcN on β-defensin-2 and Toll-like receptor 4 levels in IPEC-J2 cells. The results showed that: 1) EcN exhibited tolerance to high acidity, high bile salt concentrations, and elevated temperatures. 2) EcN adhesion to IPEC-J2 cells was optimal during the logarithmic phase, achieving an adhesion rate of 33.96%, which was significantly higher than that observed in the lag, stationary, and decline phases ($P<0.05$). 3) EcN demonstrated strong inhibitory effects against pathogenic *E. coli* K88, with an adhesion inhibition rate of 87.84%. 4) EcN upregulated β-defensin-2 and Toll-like receptor 4 levels in IPEC-J2 cells. These findings indicate that probiotic EcN possesses favorable stress resistance, can effectively adhere to porcine intestinal epithelial cells, and exerts significant inhibitory effects against pathogenic *E. coli* K88.

Keywords: *Escherichia coli* Nissle 1917; porcine intestinal epithelial cell; IPEC-J2 cell; adhesion property; stress resistance; heat tolerance; antimicrobial

effects

Escherichia coli Nissle 1917 (EcN) (serotype O6:K5:H1) is a non-pathogenic Gram-negative probiotic strain discovered and isolated by German physician Alfred Nissle during World War I [1]. The probiotic functions of EcN are associated with its modulation of intestinal microbiota and induction of immune responses through cytokines [2]. EcN plays an important immunological role in the intestine by regulating antimicrobial peptide expression, increasing immunoglobulin A (IgA) and mucin secretion, and promoting anti-inflammatory immune responses [3]. Additionally, EcN enhances tight junctions in porcine intestinal epithelial cells and improves mucosal barrier function by upregulating the expression of zonula occludens tight junction proteins and promoting their redistribution [4-5]. Oral administration of EcN significantly reduces diarrhea rates in weaned piglets and competitively inhibits *Salmonella* invasion [6], while also effectively preventing enterotoxigenic *E. coli* infections [7]. These findings demonstrate that EcN plays a crucial role in enhancing intestinal immune function and protecting the intestinal barrier in animals. The survival and adhesion of probiotics to intestinal epithelial cell surfaces are prerequisites and fundamental for exerting their beneficial effects. Therefore, this study aimed to elucidate the growth characteristics, acid, bile salt, and heat tolerance, adhesion properties to porcine intestinal epithelial cells, and antimicrobial effects against pathogenic bacteria, thereby providing a reference for the application of EcN in animal production.

Materials and Methods

1.1 EcN Growth Curve Determination and Bacterial Preparation

The EcN strain was purchased from the German Collection of Microorganisms and Cell Cultures (DSM 6601). The *E. coli* K88 strain was obtained from the China Institute of Veterinary Drug Control. The growth curves of EcN and *E. coli* K88 were constructed using turbidimetry, with culture time (0–30 h) as the abscissa and corresponding absorbance values at 600 nm (OD600) as the ordinate. EcN and *E. coli* K88 cultures were grown in LB medium, and bacterial suspensions were adjusted to approximately 1.0$×$10^8 CFU/mL using sterile phosphate-buffered saline (PBS) for subsequent in vitro stress resistance, adhesion to porcine intestinal epithelial cells, and antimicrobial effect assays.

1.2 IPEC-J2 Cell Culture

Porcine jejunal epithelial IPEC-J2 cells were obtained from frozen stocks maintained in the laboratory of the College of Animal Science and Technology at Southwest University. Resuscitated cells were cultured in flasks containing 5 mL of complete DMEM cell culture medium (supplemented with 5% fetal bovine serum and 1% antibiotics) at 37°C in a 5% CO2 incubator. Upon reaching 85%

confluence, cells were trypsinized and adjusted to a concentration of approximately 2.0$×$10^5 cells/mL, then seeded into six-well plates and cultured until forming a monolayer for subsequent experiments.

1.3 Stress Resistance Tests

1.3.1 Acid and Bile Salt Tolerance Equal volumes of EcN suspension were exposed to simulated gastric fluid at pH 1.5, 2.0, and 2.5, with pH 7.0 serving as the control. Additionally, equal volumes of EcN suspension were placed in LB liquid medium containing porcine bile salt concentrations of 0, 0.30%, 0.60%, and 0.90%, with three replicates per group. The acid and bile salt treatment groups were incubated at 37°C with shaking at 200 r/min for 3 h and 24 h, respectively. The survival rate of EcN was calculated as the percentage ratio of viable bacterial counts after treatment to those before treatment.

1.3.2 Heat Tolerance Four tubes containing 9 mL of sterile physiological saline were preheated to 80°C, and 1 mL of bacterial suspension was added to each. After heat exposure for 30, 40, 50, and 60 seconds, the tubes were rapidly cooled. Bacterial suspensions from each time point were serially diluted tenfold, and 100 L of the optimal dilution was spread onto LB agar plates. Following incubation at 37°C for 24 h, colonies were counted (two replicates per group). The experiment was repeated at 70°C and 65°C, and EcN survival rates were calculated using the method described in Section 1.3.1.

1.4 IPEC-J2 Cell Adhesion and Antimicrobial Tests

For the adhesion assay, 2 mL of incomplete DMEM cell culture medium (without antibiotics) and 1 mL of EcN suspension from different growth phases (lag, logarithmic, stationary, and decline) were added to IPEC-J2 cell monolayers. An equal volume of sterile PBS replaced the EcN suspension in control groups, with three replicate wells per group. After incubation at 37°C with 5% CO2 for 2 h, cells were washed five times with PBS, air-dried, and fixed with methanol for 20 min, followed by Gram staining. Under oil immersion microscopy, 25 fields were randomly selected for photography and enumeration of EcN bacteria adhering to 100 cells. The adhesion rate was calculated as the percentage ratio of adherent bacteria to the total bacteria added.

For the inhibition assay, 500 L of EcN suspension was pre-incubated with IPEC-J2 cells for 2 h, followed by the addition of 500 L of *E. coli* K88 suspension and 1 mL of incomplete DMEM medium. The control group received *E. coli* K88 suspension without EcN pre-treatment. After further incubation at 37°C with 5% CO2 for 2 h, cells were washed five times with PBS, lysed, and the lysate was serially diluted tenfold before plating on violet red bile agar (VRBA) selective medium for colony counting (four replicate wells per group, averaged). The adhesion inhibition rate was calculated using the formula: Inhibition rate (%) = 100 × (control group − treatment group) / control group.

For the antibacterial zone test, 100 L of *E. coli* K88 suspension was spread evenly on agar plates and air-dried at room temperature in a sterile laminar flow hood. Wells were created using Oxford cups (8 mm diameter), and 200 L of EcN suspension was placed into each well. Following incubation at 37°C for 24 h, the diameter of the inhibition zone was measured (three replicates, averaged) to evaluate antimicrobial efficacy.

1.5 Detection of β-defensin-2 and Toll-like Receptor 4 Levels

The experiment consisted of four groups: EcN group (1 mL EcN), *E. coli* K88 group (1 mL *E. coli* K88), EcN + *E. coli* K88 group (1 mL of a 1:1 mixture), and a PBS control group (equal volume). After adding 2 mL of incomplete DMEM medium and incubating for 3 h at 37°C with 5% CO2, cells were washed five times with PBS, trypsinized, and transferred to 1.5 mL centrifuge tubes. Following centrifugation at 12,000 r/min for 5 min at 4°C, the supernatant was discarded. Cells were washed again, centrifuged, and the supernatant removed before adding 1 mL of protein lysis buffer. After centrifugation for 10 min, the supernatant was collected, and β-defensin-2 and Toll-like receptor 4 levels were detected by Western blotting.

1.6 Statistical Methods

Data were organized using Excel 2007 software, and one-way ANOVA was performed using SAS 9.1.3 software. Results are expressed as means ± standard error of the mean (SEM).

Results

2.1 EcN Growth Curve

The EcN growth curve is shown in [Figure 1: see original paper]. A rapid increase was observed 4 h post-inoculation, indicating entry into the logarithmic growth phase. After 10 h of culture, the culture gradually entered the stationary phase, followed by a slow decline after 18 h. This declining trend became more pronounced after 24 h, marking the transition into the death phase.

Figure 1. The growth curve of EcN

2.2 EcN Stress Resistance In Vitro

The results of the acid tolerance test are presented in . EcN survival rate decreased significantly with decreasing pH of the simulated gastric fluid ($P<0.05$). At pH 2.5, the survival rate was 30.26%, representing a 51.58% reduction compared to the control group. At pH 2.0 and 1.5, survival rates were 17.69% and 10.44%, respectively, demonstrating that strongly acidic environments inhibited EcN growth.

Table 1. Test results of acid tolerance of EcN

The bile salt tolerance test results are shown in . The control group exhibited a survival rate of 84.95%, which decreased significantly with increasing bile salt concentration ($P<0.05$). At 0.30% bile salt concentration, survival rate dropped sharply to 27.34%. As bile salt concentration increased from 0.30% to 0.90%, the decline in survival rate was more gradual, with survival rates of 21.03% and 15.21% at 0.60% and 0.90% concentrations, respectively. High bile salt environments inhibited EcN growth.

Table 2. Test results of bile salt tolerance of EcN

The temperature tolerance test results are depicted in [Figure 2: see original paper]. EcN survival rate gradually decreased with increasing water bath temperature and exposure duration. At 80°C, survival rates after 30, 40, 50, and 60 s were 15.86%, 11.01%, 5.29%, and 2.20%, respectively, indicating low survival. At 70°C, corresponding survival rates were 59.03%, 49.78%, 43.61%, and 30.40%, while at 65°C, survival rates were substantially higher at 80.18%, 73.57%, 60.79%, and 49.34%.

Figure 2. Test results of temperature tolerance of EcN

2.3 EcN Adhesion to IPEC-J2 Cells

Based on the established EcN growth curve, cultures grown for 2, 8, 16, and 26 h were selected to represent the lag, logarithmic, stationary, and decline phases, respectively, for subsequent experiments.

The adhesion of EcN to IPEC-J2 cells is shown in [Figure 3: see original paper] and . Adhesion rates differed significantly among EcN from different growth phases ($P<0.05$). Logarithmic-phase EcN exhibited the strongest adhesion, with 3,056 bacteria adhering per 100 cells and an adhesion rate of 33.96%. This was followed by stationary-phase EcN, with 2,043 adherent bacteria per 100 cells and a 22.70% adhesion rate. Lag-phase and decline-phase EcN showed the third and fourth highest adhesion performance, with 1,174 and 661 bacteria per 100 cells, corresponding to adhesion rates of 13.04% and 7.34%, respectively.

Figure 3. The adhesion of EcN to IPEC-J2 cells (Gram stain, 1,000×)

Table 3. Adhesion rate of EcN at different growth phases to IPEC-J2 cells

2.4 EcN Inhibition of *E. coli* K88

The adhesion inhibition test results for EcN against *E. coli* K88 are presented in . EcN achieved an adhesion inhibition rate of 87.84% against *E. coli* K88. The antibacterial zone test measured an inhibition zone diameter of 4.73 mm. These results demonstrate that EcN exerts strong inhibitory effects against *E. coli* K88.

Table 4. Adhesion inhibition test results of EcN to *Escherichia coli* K88

2.5 Effects of EcN on β-defensin-2 and TLR-4 Levels

The effects of EcN on β-defensin-2 and Toll-like receptor 4 levels are shown in [Figure 4: see original paper]. The control group exhibited significantly lower β-defensin-2 levels compared to the EcN and EcN + *E. coli* K88 groups ($P<0.05$), but did not differ significantly from the *E. coli* K88 group ($P>0.05$). TLR-4 levels in the control group were significantly lower than in the other three groups ($P<0.05$). β-defensin-2 and TLR-4 levels in the EcN group were lower than in the EcN + *E. coli* K88 group, though the differences were not significant ($P>0.05$). Additionally, the *E. coli* K88 group showed slightly lower β-defensin-2 and TLR-4 levels compared to the EcN and EcN + *E. coli* K88 groups, but these differences were not statistically significant ($P>0.05$).

Figure 4. Effects of EcN on β-defensin-2 and toll-like receptor-4 levels

Discussion

3.1 EcN Stress Resistance In Vitro

Research indicates that EcN possesses six unique iron uptake systems that produce siderophores [enterobactin, salmochelin, yersiniabactin, aerobactin, the ferric dicitrate transport system, and the chu heme transport locus] to acquire ferric iron (Fe3+) from the environment for energy production during metabolism. Neutral to slightly acidic conditions are more favorable for EcN to produce four of these siderophores (enterobactin, salmochelin, aerobactin, and yersiniabactin) [8], suggesting that such environments are more suitable for EcN survival. Although EcN survival rate decreased with declining pH in our simulated gastric fluid experiments, the strain maintained certain viability, demonstrating its tolerance to acidic environments. The pH in the porcine stomach typically ranges from 2.0 to 3.5, though dietary changes can cause fluctuations between 1.5 and 5.0. Our results showing EcN survival at pH 2.5 indicate its potential to transit through the gastric environment and exert probiotic effects in the intestine. In addition to acid tolerance, EcN must withstand the high osmotic pressure created by bile salts in the intestine. Porcine intestinal bile salt concentrations generally fluctuate between 0.03% and 0.50%, yet our experiments demonstrated EcN survival even at high bile salt concentrations (0.90%), confirming its tolerance to intestinal bile salt environments. Furthermore, EcN maintained viability under high temperature conditions, indicating its thermotolerance. Regarding the specific mechanisms underlying probiotic acid, bile salt, and heat tolerance, Hatice et al. [9] found that lactic acid bacteria produce exopolysaccharides that confer tolerance to low pH and high bile salt environments. Turpin et al. [10] identified multiple genes associated with acid, bile salt, and heat tolerance in probiotic *Pediococcus acidilactici* at the molecular level, including heat shock protein (groEL) genes, D-alanine transferase (dltD) genes, clpL (an ATPase) genes, and bile salt hydrolase (bsh) genes. Therefore, whether EcN employs similar or other potential mechanisms to tolerate strongly acidic, high bile salt, and high temperature environments requires further investigation.

3.2 EcN Adhesion and Antimicrobial Properties

This study compared the adhesion capacity of EcN from four different growth phases to IPEC-J2 cells, with logarithmic-phase EcN showing optimal adhesion. Xiong et al. [11] reported that *Lactobacillus reuteri* adhesion to Caco-2 cells increased and then decreased with bacterial age, suggesting that EcN adhesion to IPEC-J2 cell surfaces may be associated with its growth stage. Fimbriae are important structures for bacterial attachment to animal intestinal mucosal epithelial cells. EcN' s genome encodes F1A, F1C, and curli fimbriae that facilitate colonization on IPEC-J2 cells and biofilm formation to resist pathogen invasion. Deficiency in F1C fimbriae and flagella expression reduces adhesion performance and weakens inhibition of *Salmonella* invasion [12]. Literature also reports that EcN regulates cell adhesion and antimicrobial activity through F1C fimbriae. EcN induces β-defensin-2 secretion and Toll-like receptor 4 expression in IPEC-J2 cells, which are important for inhibiting pathogen invasion and alleviating intestinal inflammation. EcN-induced β-defensin-2 secretion may be associated with flagella; deletion of genes encoding F1C fimbriae and flagella reduces adhesion rates by 99.9% and 50.0%, respectively, while also decreasing antimicrobial capacity [13]. Furthermore, EcN forms flagella during adhesion and growth on IPEC-J2 cells, with flagellar length and number increasing with culture time [14]. Thus, F1C fimbriae and flagella play crucial roles in EcN adhesion to cell surfaces and probiotic function. Our investigation of EcN-induced β-defensin-2 and TLR-4 expression and adhesion mechanisms in IPEC-J2 cells was preliminary, and deeper exploration of adhesion performance and antimicrobial mechanisms is needed.

In our experiments, pre-incubation of IPEC-J2 cells with EcN before *E. coli* K88 infection resulted in an adhesion inhibition rate of 87.84%. Boudeau et al. [15] compared two approaches: pre-incubating IPEC-J2 cells with EcN before adding pathogenic *E. coli* versus adding both bacteria simultaneously, finding inhibition rates of 97.2%–99.9% and 78.0%–99.9%, respectively. Kleta et al. [16] and Malchow et al. [17] also reported that pre-incubation of IPEC-J2 cells with EcN reduced infection rates by pathogenic *E. coli* and affected pathogen adhesion and invasion capacity, while simultaneous addition of EcN and *Salmonella* only reduced *Salmonella* invasion by 70% [18]. These findings suggest that EcN adhesion and physiological function may exhibit time-dependent effects. Research indicates that increasing pre-incubation time and EcN dosage enhances pathogen inhibition [12]. Pre-incubation with higher EcN doses also upregulates intestinal mucin mRNA expression [19]. Collectively, these studies suggest that early and high-dose administration of EcN preparations in production practice may optimize probiotic efficacy.

This study provides only a preliminary exploration of EcN mechanisms. Further molecular-level research is needed to elucidate EcN adhesion mechanisms, pathogen adhesion inhibition, and stress resistance properties.

EcN adhesion to porcine intestinal epithelial cells is optimal during the log-

arithmic phase, and it exhibits strong inhibitory effects against pathogenic *E. coli* K88.

EcN demonstrates stress resistance to high acidity and bile salt concentrations, facilitating its physiological function in the gastrointestinal tract.

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Note: Figure translations are in progress. See original paper for figures.