Screening of Lactobacillus antagonizing Enterotoxigenic Escherichia coli K88 based on Caenorhabditis elegans

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Abstract

The diarrhea caused by enterotoxin-producing Escherichia coli (ETEC) has seriously affected the development of human health and livestock and poultry farming. Studies have shown that Lactobacillus can inhibit the growth of ETEC K88. At present, the Lactobacillus antagonizing ETEC K88 are usually screened by in vitro experiments, but the growth properties of Lactobacillus in vitro and in vivo are different. The selection of high-efficiency and low-cost experimental animal models is an urgent problem to be solved in the breeding process of ETEC K88 Lactobacillus. To this end, we established a Lactobacillus screening model based on the antagonistic K88 of C.elegans. Methods: Firstly, the relationship between OD600 and the number of colonies was obtained by measuring the growth curve of ETEC K88. Secondly, the effects of different concentrations of ETEC K88 on the survival rate of C.elegans were compared to determine the optimal concentration of ETEC K88. Finally, based on the established ETEC Screening of Lactobacillus by C.elegans challenged by ETEC K88. Results and Conclusion: The results indicated that 2×109 cfu/mL was the optimum attacking concentration of ETEC K88 CVCC225. Basis of this model, we successfully obtained a strain L7 capable of antagonizing K88-infected nematode and the survival rate of nematode increased by 24 % and the DT50 time was prolonged 3.11 days, on the 12th day, the protection effect is extremely significant.

Keywords

Lactobacillus, Enterotoxigenic Escherichia coli K88, Caenorhabditis elegans killing model.

1. Introduction

Enterotoxin-producing Escherichia coli (ETEC) is a major pathogen causing diarrhea in humans and livestock. Once infected, its morbidity are very high, causing huge losses to human health and livestock farming. In animal husbandry, the problem of infection is mainly solved by antibiotics, but antibiotics cause problems such as bacterial resistance, and people are beginning to seek effective antibiotic substitutes. In recent years, natural plant extracts, Chinese herbal medicines, enzyme preparations, etc. have been used to replace antibiotics [1-3], but their high raw material cost and functional uncertainty restrict their use in the aquaculture industry. Lactobacillus can synthesize antibacterial peptides, secrete organic acids and improve the immunity, thus Lactobacillus has attracted much attention [4-7]. However, how to select suitable Lactobacillus from a wide range of environmental microorganisms is a major problem that researchers need to solve.

At present, most of the studies use the in vitro screening indicators such as acid production capacity, acid resistance, choline resistance and inhibition zone size to screen the anti-K88 strain, but the complex internal environment required after the intestinal tract of the animal is only used in vitro. The method of screening can not reflect the true state o in vivo, and the direct use of animals to evaluate

the bacteriostatic effect of the strain is too high. Therefore, an efficient and inexpensive in vivo evaluation model is needed to screen Lactobacillus antagonizing K88. C.elegans is an in vivo model accepted and recognized by many scholars. It has a complete digestive system and intestinal tissue and is suitable for the study of intestinal microbes. Moreover, its genetic background is clear and its life cycle is short, which is suitable for screening Lactobacillus that antagonize K88 infection. This study based on C.elegans to construct a Lactobacillus screening model that antagonizes ETEC K88 infection, and provides a basis for the screening and application of Lactobacillus in the future.

2. Materials and Methods

2.1 Materials

ETEC K88 (CVCC 225) is preserved in this laboratory and purchased from the China Veterinary Microorganisms Preservation Center, containing the heat-resistant enterotoxin gene (*estB*) and the heat-labile enterotoxin gene (*elt*). The *estB* and *elt* knockout ETEC K88 was constructed in our laboratory, OP50 and *C.elegans* SS104, purchased from the *Caenorhabditis* Genetic Center (University of Minnesota, Minnepolis), genotype *glp-4(bn2)*, which has temperature-sensitive defects in germline development during larval development, and does not multiply when the ambient temperature is higher than 25 ºC.

2.2 Strains culture

Lactobacillus were cultured in MRS medium, and ETEC K88 and OP50 were cultured in LB medium. The strain stored at - 80 °C was inoculated into a solid medium, and single colonies were picked to liquid medium, and MRS was filled up in a culture vessel to ensure anoxic state. The inoculated liquid medium was cultured overnight at 37 °C, 160 rpm to a log phase in a constant temperature shaker.

2.3 Growth curve of ETEC K88

The ETEC K88 in log phase after activation was inoculated to 100 mL of LB liquid medium in a 1:100 inoculum and cultured in 37 °C shaker for 16-20 h.Different cultivated time bacteria were removed the supernatant, resuspend the cells with the same volume of S-Medium, and measure with a microplate reader. Bacterial absorbance OD600, and diluted to the appropriate multiple. The Number of bacteria was count with LB plate.

2.4 C.elegans maintenance

The cultivation of *C.elegans* SS104 is based on the method of Stiernagle et al[8].The culture medium was centrifuged to remove the medium, and the cells were resuspended in M9 buffer, and the suspension was added to the center of the NGM medium, uniformly coated, and the edge of the medium was kept at about 1 cm. The inside of the table was air-dried and cultured in an incubator at 37 °C for about 16 h. The thicker lawn was grown on the surface of the medium and stored in a 4 ºC refrigerator for later use.

2.5 Nematode killing assay

The method of *nematode* synchronization is based on the method of Schreiber et al[9]. Approximately 18 ~ 25 *C.elegans* were transferred from a lawn into a 24-well culture plate containing the pathogen, ETEC K88 and OP50 in a liquid medium. ETEC K88 was diluted to 1×10^7 ~ 2×10^9 cfu/mL using S-Medium. 2×10^9 cfu/mL of $K88^-$ (*estB* $^-$, *elt* $^-$) or OP50 was added to the control group. The culture was incubated for 20 days at 25 ºC, and the number of worms deaths was recorded every day.

2.6 Screening of antagonistic K88 Lactobacillus

The *Lactobacillus* L1-L7 were screened by in the laboratory exhibited different inhibitory K88 activities *in vitro*. About 1×10^8 cfu/mL *Lactobacillus* were added into 24-well plate and co-cultured with *nematode* for 20 hours. Then removed the bacterial solution and washed the surface bacteria with

S-Medium by 3 times. 2×10^9 cfu/mL ETEC K88 was used to kill *nematode*, and record the number of worms deaths every day. The culture condition was same as *nematode* challenging assay.

3. Results

3.1 Effect of different killing concentration K88 on nematode survival rate

At first, in order to obtain accurate treatment concentration, the standard curve of absorbance value OD600 and bacterial concentration (cfu/mL) was determined (Fig. 1) . According to the calculation, the function relation between absorbance OD600 and bacterial concentration is: $Y = 3\times10^9x-4\times10^8$, $R^2>0.95$.

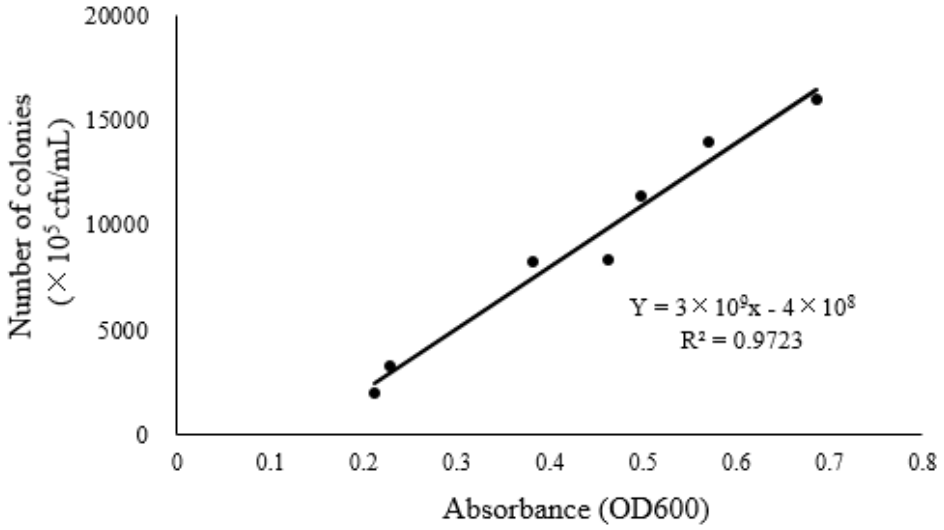

Fig. 1 Growth curve of ETEC K88

Secondly, the effects of different concentrations on the survival rate of *C.elegans* was compared (Fig. 2) . The results showed that the enterotoxin gene *estB* and *elt* knockout strain K88$^-$ (*estB* $^-$, *elt* $^-$) had no significant effect on the *nematode* death rate compared with the OP50 control group ($P > 0.05$), indicating that the cause of death of K88 on *nematode* was related to the toxicity of STb and LT. At the five concentrations set, the K88 lethal effect at low concentrations (1×10^7 cfu/mL) was not significant ($P > 0.05$). As the concentration of ETEC K88 increased, the survival time of *nematode* gradually shortened, and the lethal effect of *nematode* was significant ($P < 0.05$). When the DT_{50} time exceeded 10 days, the whole treatment cycle was longer. According to other literatures, 2×10^9 cfu/mL was selected as ETEC K88 challenge (Table 1) . At this time, all nematodes in this group died after 13 days of infestation, and the DT50 time was 8.25 days. However, if the treatment concentration is continuously increased, the *nematode* activity is not high, which affects the screening effect of the *Lactobacillus*.

Table 1 Statistical analysis of different concentrations of ETEC K88 challenging *C.elegans*

Group	DT_{50} (day) [a]	Survial time (day) [b]	P_1-value [c]	P_2-value [d]
OP50	12.24±0.14	19	-	-
K88$^-$	11.67±0.31	18	0.0871	-
K88 1×10^7	12.9±0.39	19	0.6225	0.034
K88 1×10^8	12.14±0.20	19	0.0406	0.433
K88 5×10^8	11.15±0.09	16	<0.0001	0.234
K88 1×10^9	9.99±0.54	13	<0.0001	0.003
K88 2×10^9	8.25±0.43	13	<0.0001	0.000

Note: a: DT_{50} indicates the time required for *C. elegans* death 50 %; b: the last *C. elegans* death time; c: log-rank test result; d: indicates the significant level of DT_{50} in OP50 vs K88 challenge group.

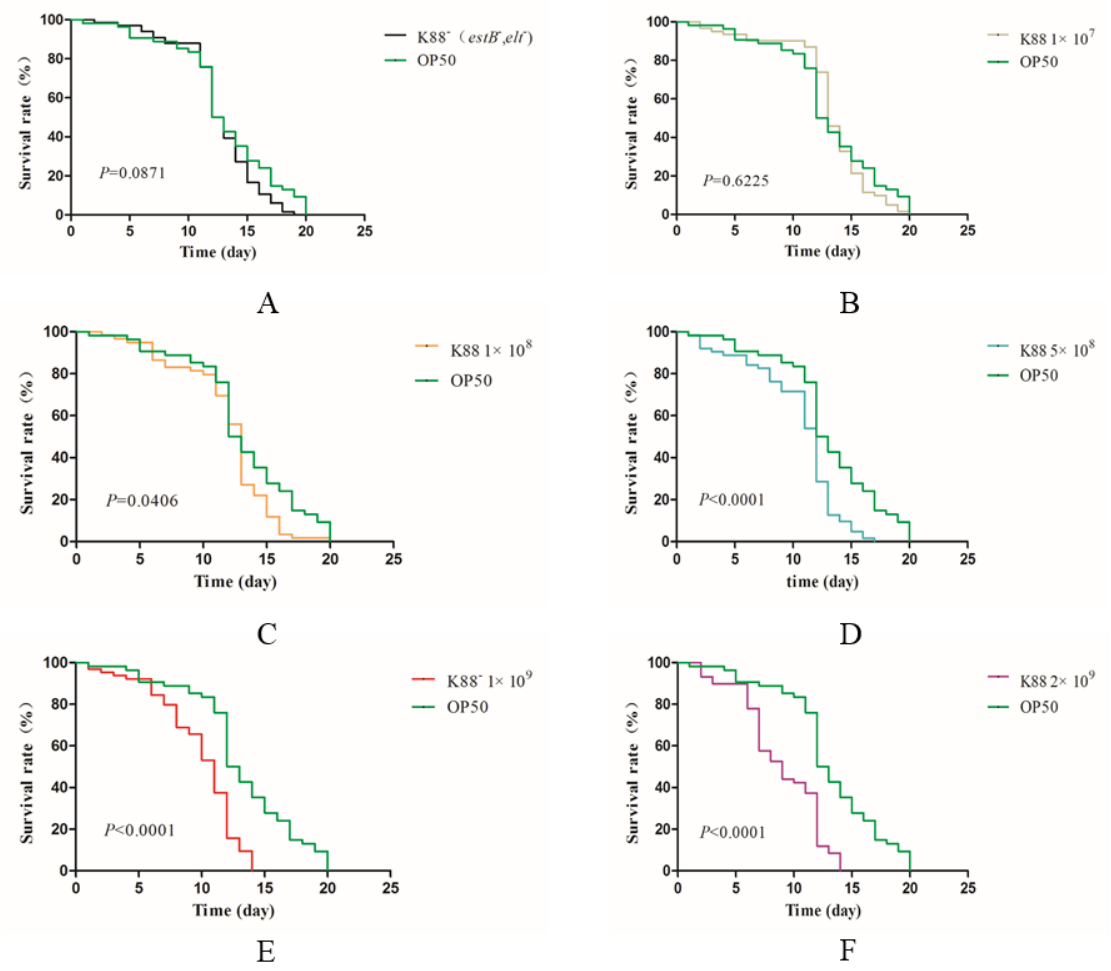

Fig. 2 Survival curves of *C.elegans* after challenging with ETEC K88 of different concentration

3.2 Screening of Lactobacillus for protection of C.elegans

To compare *Lactobacillus* protective effects against *nematode*, seven strains of *Lactobacillus* that inhibited ETEC K88 *in vitro* were selected. The results showed that (Table 2) , the DT50 of the infected group was only 8.25 days, and the survival rate on the 12th day was only 11.86%. But the DT50 of the nematode infected with ETEC K88 after treatment with *Lactobacillus* were significantly longer than that of OP50+K88 group (P<0.001). DT50 time was extended for 2 ~ 3 days. Among them, the *nematode* survival rate after L7 treatment was higher, and the DT50 time was extended by 3.1 days, and the protective effect was remarkable.

In vitro experiments showed that L1-L7 bacteria showed certain antibacterial activity, and the protective effects of these seven strains on *nematode* were also extremely significant in the same line, and the inhibition zone size and DT50 of L1, L3, L4, L5 and L7. The time is relatively large, and the results *in vitro* and *in vivo* are consistent at this point. However, it can be seen that there is no difference in the inhibition zone size of L4 and L7 *in vitro*, but its protective effect on *nematode* is not as good as L7, indicating that the *in vitro* and *in vivo* screening results are different. This may be related to the physiological characteristics of the strain such as acid resistance and bile salt resistance.

Table 2 Statistical analysis of 7 strains of *Lactobacillus* protecting *C.elegans* inhibiting ETEC K88 infection

Group	*Lactobacillus*	Survival rate	DT_{50} (day)	Survival time	*P*-value▪	Zone of Inhibition

OP50+K88	-	11.86	8.25±0.43	13	-	0
OP50+K88$^-$	-	50.00	11.67±0.31	19	0.000	0
OP50	-	46.67	12.24±0.14	20	0.000	0
L1+K88	0.3	33.87	11.43±0.11	16	0.000	23.60±0.56 ^{b}
L2+K88	0.3	32.00	10.53±0.29	18	0.000	20.55±0.14 ^{b}
L3+K88	0.3	26.42	11.35±0.04	13	0.000	21.81±0.26 ^{b}
L4+K88	0.3	22.92	11.18±0.22	14	0.000	23.36±0.30 ^{b}
L5+K88	0.3	28.57	11.05±0.28	15	0.000	27.42±0.38 ^{a}
L6+K88	0.3	32.14	10.77±0.86	17	0.000	17.87±0.36 ^{c}
L7+K88	0.3	35.94	11.36±0.18	18	0.000	24.58±0.12 ^{b}

Note: ✦:indicates the 12th day survival rate of *C.elegans* after challenging by ETEC K88; ▪: indicates OP50+K88 vs other groups' significant level of DT50.

4. Conclusion

In this study, a ETEC K88 strain CVCC 225 challenge model based on of *C.elegans* was established, and the challenge concentration of K88 was 2×10^9 cfu/mL. On the basis of this model, screening of anti-K88 *Lactobacillus* was carried out, and a *Lactobacillus* L7 capable of antagonizing ETEC K88-infected *nematode* was obtained. Using the *Lactobacillus*, the survival rate of *nematode* increased by 24 % and the DT50 time was prolonged 3.11 days, on the 12th day, the protection effect is extremely significant. At the same time, the antibacterial performance of most strains is similar to that of the *in vitro*, but some strains are also quite different. In general, the screening results of antagonistic ETEC K88-infected *nematode* based on *C.elegans* are reliable and efficient, and can be used for the screening of probiotics.

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