Inhibition of germfree extracts derived from fermented milk on flaA σ^{28} promoter in Campylobacter jejuni

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[Abstract]: To study the inhibition of germfree extracts derived from milk fermented by lactic acid bacteria (LAB) on virulence gene *flaA* expression of foodborne pathogen bacteria *Campylobacter jejuni*, a luminescent phenotype fusion between the *flaA* σ^{28} promoter and a promoterless *lux*CDABE cassette carried on the plasmid pRYluxCDABE in *Campylobacter jejuni* was used. Through evaluation of the bioluminescence, the effect of germfree extracts derived from milk fermented by lactic acid bacteria (LAB) (5 *Bifidobacterium* strains and 6 Lactobacillus strains) was assessed on *flaA* σ^{28} promoter activity. The results showed that gremfree extracts of milk fermented by LAB were found to have a significant ($P < 0.05$) inhibitive effect on the expression of *flaA* σ^{28} promoter, and down-regulate virulence gene *flaA* expression.

[Key words] flaA; promoter; bioluminescence; germfree extracts of milk fermented by probiotics

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益生菌发酵乳无菌提取物对空肠弯曲杆菌 flaA σ^{28} 启动子的抑制作用

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[摘 要] 为了研究益生菌发酵乳无菌提取物对食源性有害微生物(空肠弯曲杆菌)有毒基因表达的抑制作用,以利用空肠弯曲杆菌的有毒基因 *flaA* σ^{28} 启动子和无启动子的质粒 pRYluxCDABE 联结构建的生物冷光转基因模型为试验材料,通过检测生物发光特性的方法,研究了空肠弯曲杆菌有毒基因 *flaA* 表达与益生菌(5 种双岐杆菌,6 种乳酸杆菌)发酵乳无菌提取物之间的关系。结果表明,益生菌发酵乳无菌提取物对空肠弯曲杆菌 *flaA* σ^{28} 启动子的活性有显著的抑制作用($P < 0.05$),并可显著抑制空肠弯曲杆菌有害基因 *flaA* 的表达。

[关键词] *flaA* 基因;启动子;生物冷光;发酵乳无菌提取

The concept of probiotics has been around for almost a century, yet the science behind it is still sparse[1-4]. The much of the research into the efficacy of probiotics has been questioned (or goes unpublished because of commercial secrecy)[5-8]; their impact on human nutrition and therapeutic treatment is still only vaguely understood[9-12]; the selection of desirable microbial strain is an uncertain

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process and the necessary tools to monitor their performance in vivo are still being developed[13-15]. Therefore, methods to assess the efficacy of probiotics and to help understand their mode of action are urgently needed.

Campylobacter spp. possesses a single polar flagellum at one or both ends of the cell. The flagellum consists of two proteins, *flaA* and *flaB*, with significantly more *flaA* being incorporated into the flagellar filament[16]. Correspondingly, numerous studies have identified the flagella as a virulence determinant[17]. A classical σ^{28} flagellar promoter controls transcription of the *flaA* gene. The effects of environmental stimuli and chemotactic effectors on the activity of the *Campylobacter jejuni flaA* σ^{28} have been determined[18].

The goal of our study was to assess the effect of milk fermented by different LAB strains effectors on *flaA* σ^{28} promoter activity. A luminescent phenotype in *Campylobacter jejuni* ATCC33291 and ATCC35921, generated by a transcriptional fusion between the *C. jejuni flaA* σ^{28} promoter and luxCDABE genes of Xenorhabdus luminecens on plasmid pRYLux CDABE was used to study the interaction of lactobacilli and bifidobacteria with the pathogen. We report here that *Campylobacter jejuni flaA* σ^{28} promoter responds to probiotics.

1 Materials and Methods

1.1 Growth of Campylobacter

Campylobacter. Jejuni ATCC33291 and ATCC35921 were human isolates. The strains were grown at 37 ℃ on Blood Agar Base No2 (BAB) (Oxoid, Nepean, ON, Canada) supplemented with 100 g/mL of kanamycin (BABK). A microaerophilic environment was created using the CampyPak Microaerophilic system (BBL, Cockeysville, MD, USA).

1.2 Construction of pRYLuxCDABE

The construction of pRYLuxCDABE was described previously (Fig 1)[19].

1.3 Milk fermentation and fractionation

Strains of lactic acid bacteria (LAB) used in this study are documented in Table 1.

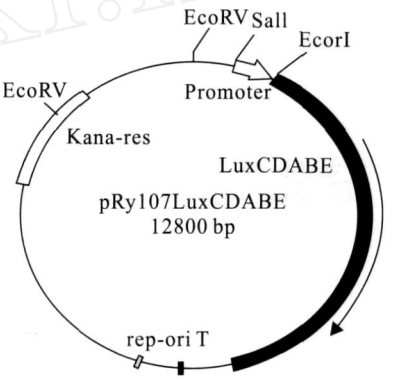

Fig. 1 Construction of Plasmid pRYLuxCDABE

Table 1 Lactic acid bacteria used in the study

Strains	Source or reference	Strains	Source or reference
Bifidobacteriun longum	ATCC15707	*Lactobacillus acidophilus*	LA-5
Bifidobacteriun infantis	ATCC15697	*Lactobacillus rhamnosus*	GR-1
Bifidobacteriun breve	ATCC15700	*Lactobacillus*	MG-1
Bifidobacteriun bifidum	ATCC15696	*Lactobacillus fermentum*	RC-14
Bifidobacteriun adolescentis	ATCC15703	*Lactobacillus acidophilus*	ATCC13648
Lactobacillus rhamnosus	GG		

Bifidobacterium strains were grown in Cm149 (Oxoid, Nepean, ON, Canada) and *Lactobacillus* strains in MRS (Oxoid, Nepean, ON, Canada). Both were incubated in anaerobic jars under an atmosphere developed using GasPak anaerobic system envelopes (BBL, Cockeysville, MD) at 37 ℃ for 16 h. Pasteurized, reconstituted skim milk (10%, V/V) was inoculated with each of these LAB strains separately and fermented at 37 ℃ for 24 h. *Bifidobacterium* and *Lactobacillus* were added at a dose rate of 4%, just like the processing of yoghourt. Fermented milk samples were centrifuged with MC centrifuge (Beckman Instruments) at 28 000 g for 5 min at 25 ℃. Following centrifugation, the suspension was filtered through a 0.22 μm pore size filter (Millipore Nepean, ON, Canada), and the pH was adjusted to approximately 7.0 with 1 M NaOH. Centrifugal ultra filtration fractionated these extracts crudely by molecular weight. Samples from each successive step of fraction were as-

sayed for C. jejuni flaA s28 promoter activity.

1.4 Quantification of bioluminescence output

Both strains possessing pRYLux CDABE were incubated in BABK for 24 h at 37 ℃ and harvested in 1.5 mL of MHB. In a test tube, the optical density at 600nm (OD_{600}) was adjusted to 1.0 and 100 μL were spread on BAB plates containing 10% concentration of extracts of milk fermented by different LAB strains. Plates were grown anaerobically for 24 h at 37 ℃ and were harvested in 1ml of MH broth (one plate = one replicate). The OD_{600} was adjusted to 0.6. From each replicate, 1 mL of adjusted bacterial suspension was placed into a cuvette, vortexed for 10 s and placed into a Multi-Lite luminometer (Biotrace, Bridgend, UK) to determine the relative light units (RLU).

As a control, unfermented, pasteurized and reconstituted skim milk was centrifuged under the same conditions described above, and supernatants were filter (Supernatant filtered with 0.22 mm pore Fisher). pH was adjusted to approximately 7.0 with 1 M NaOH. BAB plates were prepared containing 10% of the unfermented milk extracts.

1.5 Data analysis

The statistical significance of differences among treatments was determined by variance analysis, and by either Fisher's protected least significant difference (LSD) test or Dunnet's t test. Differences were considered statistically significant when $P \leq 0.05$. Each experiment was repeated in triplicate.

2 Results and analysis

By adjusting the pH level of the experimental and control sets, the effect of the gremfree extracts of milk fermented by LAB on promoter activity was studied independent of pH. The LAB was all able to modulate light production of both *Campylobacter constructs* (Tables 2 & 3). A statistically significant difference in light output was observed for both *Campylobacter constructs* in the presence of gremfree milks fermented by all LAB compared with control milks with pH adjusted to the same value as the experimental group with lactic acid. RLU in the control samples were 17 425 to 30 041 for *C. jejuni* ATCC35921 and 12 388 to 24 678 for *C. jejuni* ATCC33291. The corresponding values for the experimental group were 3 433 to 9 989 and 2 646 to 9 472, respectively. Overall, both *C. jejuni* strains responded to all LAB in a similar manner with both strains significantly suppressing the *flaA* s28 promoter.

Table 2　Effect of LAB on flaA σ^{28} promoter activity in C. jejuni ATCC 35921

LAB	Control group bioluminescence RLU	*C. jejuni* ATCC 35921 bioluminescence RLU
ATCC15707	23 054 ±3 974 *	4 478 ±2 183 ab
ATCC15697	17 425 ±2 087 *	3 538 ±1 486 a
ATCC15700	19 464 ±2 810 *	7 116 ±4 056 ab
ATCC15696	18 498 ±2 263 *	7 671 ±1 093 b
ATCC15703	20 411 ±3 252 *	8 243 ±1 847 b
GG	24 971 ±1 515 *	3 433 ±123 a
LA-5	29 633 ±4 846 *	8 769 ±1 854 c
GR-1	24 098 ±1 515 *	3 994 ±221 b
MG-1	20 073 ±1 658 *	6 241 ±716 c
RC-14	27 976 ±3 269 *	7 547 ±772 c
ATCC13648	30 041 ±2 080 *	9 989 ±1 593 c

Note: * means RLU values of the control group are significantly different from experimental group ($P < 0.05$); different letter means the differences are significant ($P < 0.05$); Next table is similar.

However, some differences were observed. The effect of *Bifidobacterium infantis* ATCC15697, *Bifidobacterium bifidum* ATCC15696 and *Bifidobacterium adolescentis* ATCC15703 on light production by *Campylobacter jejuni* ATCC35921 differed significantly ($P < 0.05$). Similarly for *Campylobacter jejuni* ATCC33291, light output was significantly different ($P < 0.05$) in the presence of germfree fractions of milks fermented by *Bifidobacterium longum* ATCC15707, *Bifidobacterium bifidum* ATCC15696 and *Bifidobacterium adolescentis* ATCC15703.

Similarly, for *Lactobacillus* spp. significant differences resulted in light production when *C. jejuni* ATCC35921 was exposed to milk fermented by *L. rhamnosus* GG and *L. acidophilus* ATCC13648 ($P < 0.05$). These two organisms also affected light production by *C. jejuni* ATCC33291 in a different ways ($P < 0.01$).

Further, in *Lactobacillus* spp. modulation of the light output of light by the two *Campylobacter*

constructs was strain dependent, with significant differences ($P < 0.05$) being seen for *L. acidophilus* strains LA-5 and ATCC13648 and *L. rhamnosus* GG and GR-1. Light output by *C. jejuni* ATCC33291 was consistently higher than *C. jejuni* ATCC 33291.

Table 3 Effect of LAB on flaA σ^{28} promoter activity in in C. jejuni ATCC 33291

LAB	Control group bioluminescence RLU	*C. jejuni* ATCC 33291 bioluminescence RLU
ATCC15707	13 486 ±2 371 *	2 946 ±1 189 a
ATCC15697	12 332 ±1 824 *	3 903 ±917 ab
ATCC15700	14 003 ±1 290 *	4 762 ±203 ab
ATCC15696	14 111 ±1 275 *	4 994 ±54 b
ATCC15703	15 217 ±4 463 *	5 672 ±83 6b
GG	17 162 ±2 253 *	2 941 ±272 a
LA-5	19 944 ±4 590 *	7 043 ±1 534 d
GR-1	18 476 ±1 302 *	3 017 ±112 ab
MG-1	16 432 ±3 716 *	4 177 ±260 c
RC-14	19 465 ±4 777 *	4 417 ±543 c
ATCC13648	20 004 ±6 024 *	9 472 ±1 787 e

3 Discussion

For an enteric pathogen to be successful, it must be able to survive, grow and ultimately exert pathogenicity in a highly competitive environment. This requires that the pathogen identify environmental factors so that appropriate genes are expressed and/or repressed. Current research on the health benefits of probiotics mostly focus on the mechanisms of anti-infection, competitive attachment and colonization and immunomodulation.

The improvement of host acquired immunity, especially immunocompetence, has been proven to inhibit translocation, survival and proliferation in extra-intestinal tissues (such as the liver and spleen) and prevented bacterium. These effects have been established mainly by measuring immunological parameters and correlating these parameters with pathogen counts in visceral tissues. It has been established that probiotics exert their immunoenhancing effects by augmenting both non-specific (e.g. phagocyte function, natural-killer-cell activity) and specific (e.g. antibody production, cytokine production, lymphocyte proliferation, delayed-type hypersensitivity) host immune responses[20]. Although recent reports have revealed some immunoenhancing mechanisms, the precise mechanisms by which LAB act on the immune system are not fully understood[21].

The role of probiotics in disease prevention may also involve antagonistic effects on the adhesion, colonization, growth and translocation of pathogens, such as *Staphylococcus aureus*, *Salmonella* Typhimurium, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Clostridium perfringens*, *Escherichia coli* O157 ∶H7 and rotavirus. To date, there has been no direct evidence demonstrating competitive attachment and colonization in vivo. However, recent studies showed that probiotics inhibited enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression to modulate the barrier effect of the gut[22].

Lactobacillus acidophilus and *Bifidobacteria* also exert antagonistic effects on the growth of pathogens such as *Staphylococcus aureus*, *Salmonella* Typhimurium, *Yersinia enterocolitica* and *Clostridium perfringens* In this study, we found that the gremfree extracts of milk fermented by LAB *Lactobacillus acidophilus* and *Bifidobacteria* have an inhibitory effect on the growth of the *Campylobacter, jejuni*, and this effect is genus, species and strain dependent. These results are similar to those reporting that probiotic bacteria enhance resistance against intestinal pathogens via antimicrobial mechanisms. These include competitive colonization and production of organic acids, such as lactic and acetic acids, bacteriocins and other primary metabolites, such as hydrogen peroxide carbon dioxide and diacetyl. A recent study using a luminescent phenotype of *Salmonella Enteritidis* to monitor infection in live mice has demonstrated that oral ingestion of fermented milks and germfree preparations of fermented milk can prevent infection[23].

Anti-infection mechanisms of LAB may include the production of substances directly microbicidal for pathogens, including organic acids, bacteriocins and other primary metabolites (such as H_2O_2, CO_2); inter-microbial competition with pathogens for intestinal attachment sites; and effec-

tive enhancement of host immunity against pathogen infection[24].

In this study, we present evidence that probiotics influence the expression of virulence genes of *Campylobacter*. Light production by a construct carrying a transcriptional gene fusion between the *C. jejuni flaA* σ^{28} promoter and luxCDABE genes was influenced by LAB, indicating that the *flaA* gene is suppressed in the presence of germfree extracts of milk fermented by the LAB. If pathogens cannot express their virulence genes, then competitive attachment and colonization will be inhibited and pathogenicity in this highly competitive environment will not be exerted.

In conclusion, the results have indicated that probiotics suppress virulence gene expression in pathogens, which reveals an important and so far relatively unknown aspect of their anti-disease mechanisms. Furthermore, using the reporting system of Lux gene will provide a relatively simple means of investigating functions of functional food and selection of desirable microbial. However, what can be attributed to the inhibitive effect of probiotics on virulence gene expression is yet to be explored in future studies.

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可改善酒的色泽。但进一步试验得知,热处理会加速甜橙干酒的氧化褐变,影响果酒的色泽而不宜采用。

3 结 论

不同澄清剂对甜橙干酒的澄清效果不同,琼脂的澄清效果最好,当其加量为 0.15 g/L 时,甜橙干酒的透光率为 98.8 %,吸光度为 0.032,酒体澄清透明,色泽浅黄;明胶的加量为 1.5 g/L 时,甜橙干酒的透光率为 94.7 %,吸光度为 0.054;壳聚糖的澄清效果也较理想,酒液色泽较好,澄清速度很快,最适加量为 0.4 g/L。但综合考虑澄清效果及速度,作者建议以 0.15 g/L 琼脂作为甜橙干酒的首选澄清剂。将甜橙干酒在 0～2 ℃冷处理 5 d 或在 40～45 ℃热处理 5 d 均能大幅度提高酒的澄清度,且冷处理还可改善酒的色泽,而热处理会加速酒液的氧化褐变,使酒液色泽变深。故作者建议,在甜橙干酒的生产中,可用冷处理来提高甜橙干酒的稳定性。

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