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Escherichia coli GutM4 produces 2,5-diketopiperazines and inhibits human pathogens *in vitro*

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ABSTRACT

Background: It has been a very common practice to use probiotics or their metabolites as alternative antimicrobial strategies for the treatment and prevention of infections as rampant and indiscriminate use of antibiotics causes the development of antibiotic-resistant pathogens. The objective of this study was to select a potential antimicrobial probiotic strain of *Escherichia coli* from the human gastrointestinal tract and investigate the production of diketopiperazines that contribute to the antimicrobial activity.
Results: E. coli GutM4 was isolated from the feces of a healthy adult. *E. coli* GutM4 showed significant antagonistic activity against 10 indicator pathogens, and this activity was no less than that of the reference strain *E. coli* Nissle 1917 against eight of the indicator pathogens. Moreover, *E. coli* GutM4 produced antagonistic substances containing trypsin-targeted peptide bonds because the inhibitory effects of *E. coli* GutM4 supernatant significantly decreased upon treatment with trypsin. Consistent with the antagonistic activity and peptide compounds of *E. coli* GutM4, 14 2,5-diketopiperazines were isolated from the fermented broth of *E. coli* GutM4, including 12 cyclo(Pro-Phe), 3 cyclo(Pro-Tyr), and 5 cyclo(4-hydroxyl-Pro-Leu), which are reported to have antipathogenic activity.
Conclusion: E. coli GutM4 produces 2,5-diketopiperazines that are partly involved in antagonistic action against human pathogens *in vitro*.

1. Introduction

Some infections and disorders in the body, such as irritable bowel syndrome, inflammatory bowel disease, and antibiotic-induced diarrhea, could be caused by deficient or compromised intestinal microflora. To overcome such disorders, the use of probiotics with various beneficial activities has been considered as one of the disease control strategies. Of all kinds of antimicrobial activities, the use of microorganisms against pathogens has an important potential. In fact, it has been a very common practice to use probiotics or their metabolites as alternative antimicrobial strategies for the treatment and prevention of infections as rampant and indiscriminate use of antibiotics for disease treatments causes the development of antibiotic-resistant pathogens [1].

To select a probiotic candidate, the most attractive strategy is to identify them from the human gastrointestinal tract (GIT), where a large number of microorganism have been reported as potential probiotics, *e.g.*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Enterococcus faecium*, and *Escherichia coli* [2]. Strains from human internal cavities would be better adapted to colonize the GIT and have an enormous effect on the nutritional and health status of the host [3]. Of particular interest, *E. coli* is one of the largely present bacterial species and most of the strains do not cause disease. Moreover, several commensal nonpathogenic *E. coli* strains have been reported for their probiotic nature. *E. coli* Nissle 1917 is widely used as an efficient probiotic in therapy and in the prevention of human intestinal disease [4]. Probiotic *E. coli* G3/10 can produce microcin S, which can inhibit the adherence of enteropathogenic strains to intestinal epithelial cells [5]. *E. coli* H22 can inhibit some enteric pathogens both *in vitro* and *in vivo* by producing microcin, variants of colicins, and bacteriophage particles [6]. *E. coli* EM0, a human fecal strain, and JM105 can protect germ-free mice against *Salmonella typhimurium* infection [7]. In brief, the promising activities and tolerance of *E. coli* strains to the human GIT have drawn more attention as potential probiotics.

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2,5-Diketopiperazines (DKPs) have been known for a long time as microbial natural products and exhibit a wide range of bioactivities as antitumor, antiviral, antifungal, antibacterial, and antihyperglycemic agents. Their excellent actions are because of their significant structural features: small size, conformational rigidity that makes them excellent peptidomimetics, and very well-defined and controlled stereochemistry. In addition, DKPs have both proton donor and proton acceptor groups that allow them to interact favorably with many biological targets, and they are stable to proteolysis [8].

In the present study, a single *E. coli* strain inhabiting in the GIT was selected from human feces as a potential probiotic with antimicrobial activity against human pathogens. In addition, the production of antimicrobial DKPs by the strain was investigated.

2. Materials and methods

2.1. Isolation of E. coli GutM4

Feces (0.98 g) of a healthy adult female were homogenized in 10 mL saline, and this solution was serially diluted. For each dilution, 1.0 mL solution was added to 9 mL of preheated (37°C for 30 min) simulated gastric juice (NaCl 2 g/L and pepsin 3.2 g/L adjusted to pH 1.5 with 1 M HCl) and incubated at 37°C for 24 h to obtain culture broth for screening. Then, 200 μL of screening culture broth was spread on de Man–Rogosa–Sharpe agar, brain heart infusion (BHI) agar, and reinforced clostridial agar plates and incubated for 24 h at 37°C. Finally, from the plates on which isolated microbial colonies grew, individual strains were isolated according to the diversity of colony types including the size, shape, color, and transparency.

Later, from the several isolated individual bacterial strains, *E. coli* GutM4 was preliminarily selected as a probiotic candidate after performing biochemical tests according to a handbook of microbiology (data not shown).

2.2. Antagonistic assay

Ten human pathogens were used as test strains to investigate the antagonistic activity of the candidate *E. coli* GutM4. *Candida albicans*, *Staphylococcus epidermidis*, *Propionibacterium acnes*, *E. coli* O104:H4, *E. coli* O157:H7, and *Helicobacter pylori* were obtained from the Center for Disease Control and Prevention of Xiamen, China; *Enterobacter cloacae*, *Listeria monocytogenes*, *Klebsiella pneumoniae* (K36), and *Staphylococcus aureus* (S244) were obtained from the People's Second Hospital of Xiamen, China. *E. coli* Nissle 1917, stored in the Laboratory of Food Nutrition and Molecular Mechanism, College of Food and Biological Engineering, Jimei University, Xiamen, China, was used as a reference strain.

The antagonistic activities of the probiotic were evaluated using the agar spot test described by Toure et al. [9], with modifications. Briefly, 2 μL of overnight culture of *E. coli* GutM4 (final concentration of 7 log CFU/mL) was spotted on BHI agar plates. The plates were dried for 30 min at room temperature and then incubated anaerobically at 37°C for 18 h in an anaerobic incubator (Oxoid, UK). After colony development, the plates were overlaid with 10 mL soft microorganism-specific medium (0.8% (w/v) agar) seeded with 1% (v/v) active overnight culture of the target pathogenic strain (final concentration of 7 log CFU/mL) and incubated anaerobically at 37°C, except for *C. albicans*, which was incubated at 24°C. *C. albicans* was cultured at 24°C by mistake, and this was realized later, long after the experiments; however, the data were still included, and the right procedure of culturing at 37°C should be kept in mind. The microorganism-specific media were yeast mold broth for *C. albicans*, trypticase soy broth for *S. aureus*, BHI broth for *L. monocytogenes*, and nutrient broth for the other pathogenic strains. After 48 h of incubation, measurements of inhibition zones around the *E. coli* colonies were taken from the outer edge of the colonies to the outer edge of the clear zones. Inhibition zones of >20 mm, 10–20 mm, and <10 mm were considered strong, good, and low inhibitions, respectively. The test was performed twice, each in triplicate.

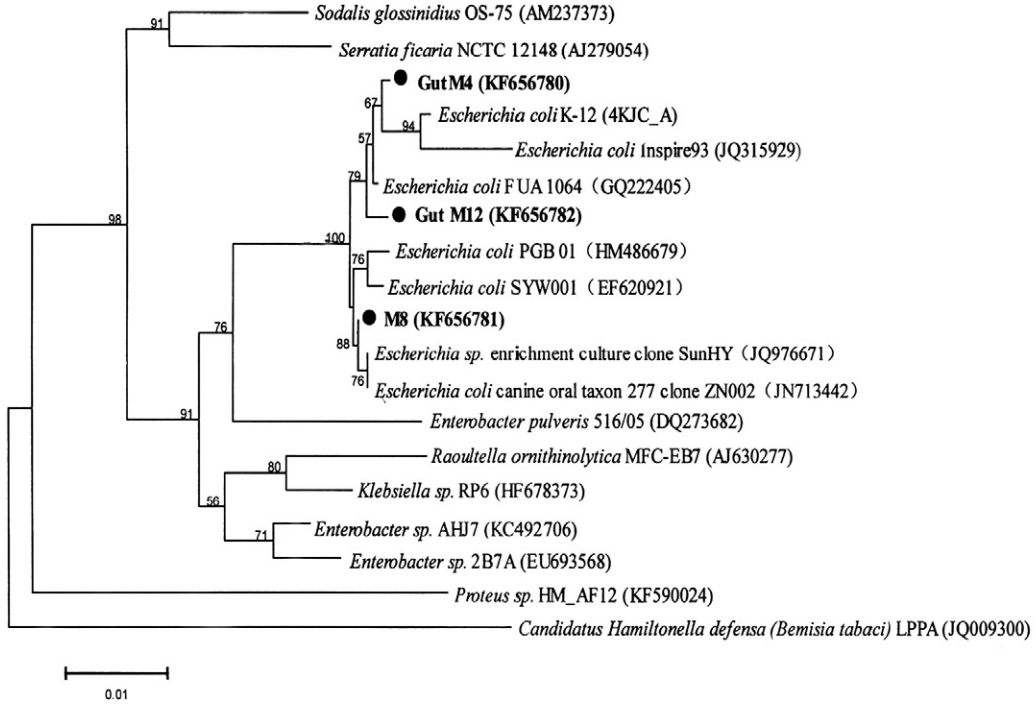


Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of GutM4 (1314 bp) and reference sequences. Numbers at nodes are bootstrap percentages (from 1000 replications). Only values above 50% are reported. The scale bar represents 0.01 nucleotide substitution per site.

Table 1
Good antagonistic activity of *Escherichia coli* GutM4.

	Helicobacter pylori	*Staphylococcus epidermidis*	*Enterobacter cloacae*	*E. coli* O104:H4	*E. coli* O157:H7	*Staphylococcus aureus*
E. coli GutM4	11.4 ± 1.2^{b}	11.6 ± 0.5^{b}	17.1 ± 0.6^{a}	13.4 ± 1.0^{a}	11.9 ± 0.6^{a}	12.0 ± 0.5^{b}
E. coli Nissle 1917	13.5 ± 1.6^{a}	12.8 ± 0.5^{b}	16.1 ± 0.2^{b}	8.1 ± 0.8^{b}	8.6 ± 0.2^{b}	14.2 ± 1.1^{a}

[a,b]Means with different superscripts within a column are significantly different ($P < 0.05$). *E. coli* Nissle 1917 was used as a reference probiotic strain. Values are inhibition zone (from the outer edge of *E. coli* colony to the outer edge of the clear zone) (mm). Means are from two independent experiments, each in triplicate, and differences were considered significant at $P < 0.05$.

2.3. Characterization of antimicrobial substances

E. coli GutM4 strains were assayed to determine the characteristics of antimicrobial substances such as bacteriocin, hydrogen peroxide, and organic acids using the agar well diffusion technique described by Toure et al. [9], with modifications. *E. coli* GutM4 grown in 25 mL of BHI broth at 37°C overnight was centrifuged at 4000 × g for 10 min at 4°C; then the supernatant was divided into equal portions of 5 mL for different assays. For bacteriocin assay, the supernatant was treated with 1 mg/mL trypsin or 1 mg/mL pronase (Sigma, USA). For organic acid assay, the pH of the supernatant was adjusted to 6.5 ± 0.1 using 1 N NaOH. For hydrogen peroxide assay, the supernatant was treated with 0.5 mg/mL catalase (Sigma, USA). All the treated supernatants were filter sterilized through 0.22-μm pore-size filters (Pall, USA).

For the detection of the antimicrobial activity of the treated supernatant, 100 μL supernatant was placed into wells (7 mm diameter) in BHI agar plates, and the plates were incubated at 4°C for 3 h for better diffusion of the treated supernatant. Next, the plates were overlaid with 10 mL soft nutrient agar (Merck, Germany), and the agar was then inoculated with 1% (v/v) overnight culture of *L. monocytogenes* as test pathogen, followed by incubation for 48 h at 37°C. Finally, the diameters of the inhibition zones (including the 7 mm well diameter) were measured. The assays were conducted twice, each in triplicate.

2.4. Elucidation of compounds produced by E. coli GutM4

Extracts were prepared using equal volumes of petroleum ether and ethyl acetate successively for three times with the fermented broth of *E. coli* GutM4 cultured at 37°C for 3 d. Then the ethyl acetate extract was chromatographed first on a Sephadex LH-20 column (1200 × 35 mm i.d.) eluted with CH_2Cl_2–MeOH (1:1, v/v), then on an ODS column with MeOH–H_2O (5:95 to 100:0, v/v), and, subsequently, on a silica gel column. Finally, purified substances were collected by reversed-phase HPLC (Waters 1525/2996. YMC-Pack Pro C18 RS) eluted with CH_3CN–H_2O at a flow rate of 2 mL/min.

To identify the metabolites, high resolution electrospray ionization mass spectroscopy and electrospray ionization mass spectroscopy spectra were acquired using a Waters Q-Tof micro YA019 mass spectrometer. All the analyses were carried out in the positive ion mode and negative ion mode. Moreover, ^{1}H (600 MHz), ^{13}C (150 MHz), and DEPT 135° NMR spectra of all HPLC fractions were recorded at room temperature on Bruker AVANCE-600 and Bruker AMX-500 instruments using TMS as the internal standard.

2.5. Identification of E. coli GutM4

E. coli GutM4 was identified by 16S rRNA gene sequencing. Bacterial genomic DNA was extracted using the Genomic RNA Extraction Kit for bacteria according to the manufacturer's protocol. The 16S rRNA gene fragments were amplified by PCR using the universal primers Eubac 27F (5-AGAGTTTGATCCTGGCTCAG-3) and Eubac 1492R (5-GGTTAC CTTGTTACGACTT-3). The PCR reaction mixture (final volume, 20 μL) comprised 10× Ex Taq buffer 2.0 μL, 2.5 mM dNTP mix 1.6 μL, 5p Primer 1 0.8 μL, 5p Primer 2 0.8 μL, template 0.5 μL, 5 U Ex Taq 0.2 μL, and ddH_2O 14.1 μL. The amplification program consisted of one cycle of 95°C for 5 min; followed by 24 cycles of 95°C for 30 s, 55°C for 30 s, and 55°C for 90 s; and finally one cycle of 72°C for 10 min. The PCR products were verified by agarose gel electrophoresis and observed after they were photographed in the gel imaging system. The cloned 16S rRNA genes were sequenced using ABI 3730XL sequencer.

The 16S rRNA sequences were matched against sequences in the GenBank database using nucleotide BLAST with default parameters in National Center for Biotechnology Information (NCBI) BLAST. Sequences were aligned against reference sequences using CLUSTAL X, and then the aligned dataset was used as input for phylogenetic analysis, which was performed using the Molecular Evolutionary Genetics Analysis software (version 5.2). Tree topology was evaluated by bootstrap analyses based on 1000 replicates, and phylogenetic tree was inferred using the neighbor-joining (NJ) method.

2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the SAS (Statistical Analysis System, 2008) program version 9.2. Comparison among treatment means was performed using Duncan's new multiple range test. Differences were considered significant at $P < 0.05$.

Table 2
Strong and low antagonistic activity of *Escherichia coli* GutM4.

	Listeria monocytogenes	*Candida albicans*	*Propionibacterium acnes*	*Klebsiella pneumoniae*
E coli GutM4	23.4 ± 0.8^{a}	5.6 ± 0.7^{a}	9.1 ± 1.8^{a}	7.6 ± 0.5^{a}
E coli Nissle 1917	15.8 ± 0.5^{b}	3.8 ± 0.7^{b}	7.2 ± 1.3^{b}	6.5 ± 0.2^{b}

[a,b]Means with different superscripts within a column are significantly different ($P < 0.05$). *E. coli* Nissle 1917 was used as a reference probiotic strain. Values are inhibition zone (from the outer edge of *E. coli* colony to the outer edge of the clear zone) (mm). Means are from two independent experiments, each in triplicate, and differences were considered significant at $P < 0.05$.

Table 3
Inhibitory diameter of supernatants treated or untreated with *Escherichia coli* GutM4.

	Inhibition zone
Control	26.94 ± 0.5^{a}
Pronase (1 mg/mL)	25.34 ± 1.4^{a}
Trypsin (1 mg/mL)	15.49 ± 0.5^{b}
pH 6.5	26.79 ± 0.6^{a}
Catalase (0.5 mg/mL)	24.4 ± 0.8^{a}

[a–b]Means with different superscripts are significantly different compared to the control ($P < 0.05$). The control is untreated supernatant. Diameter of the inhibition zone (mm) includes 7 mm well diameter, and the values are means ± SD of two independent experiments, each in triplicate, using *Listeria monocytogenes* as indicator strain. Differences were considered significant at $P < 0.05$.

Table 4
Diketopiperazines of *Escherichia coli* GutM4. Fifteen compounds were isolated from the fermented broth of *E. coli* GutM4 and elucidated using high resolution electrospray ionization mass spectroscopy, electrospray ionization mass spectroscopy spectra, ^{1}H (600 MHz), ^{13}C (150 MHz), and DEPT 135° NMR spectra.

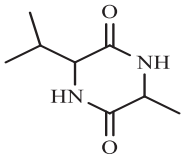
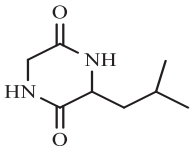
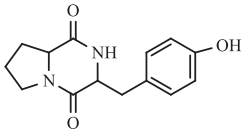
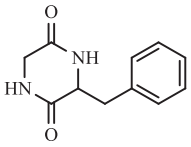
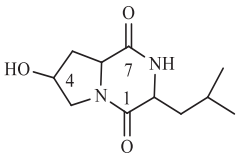
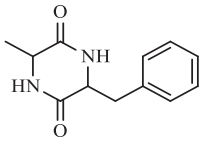
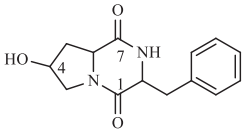
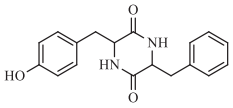
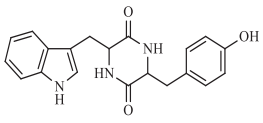
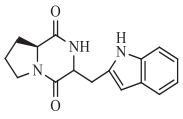
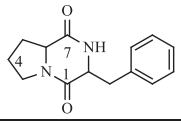
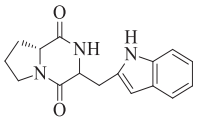
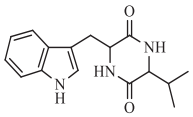
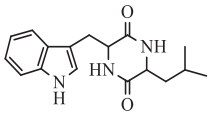
Compounds	Structure	Reference(s)
1. Cyclo(Val-Ala)		Wang et al. [17]
2. Cyclo(Gly-Leu)		Guo et al. [18]
3. Cyclo(Pro-Tyr)		Kamikawa et al. [19]
4. Cyclo(Phe-Gly)		Chen et al. [20]
5. Cyclo(4-OH-Pro-Leu)		Cronan et al. [21]
6. Cyclo(Phe-Ala)		Wang et al. [22]
7, 8. Cyclo(4-OH-Pro-Phe)		Cronan et al. [21]
9. Cyclo(Tyr-Phe)		Wang et al. [22]
10. Cyclo(Trp-Tyr)		Koleva et al. [23]
11. Cyclo(L-Pro-L-Trp)		Sanz-Cervera et al. [24]
12. Cyclo(Pro-Phe)		Fdhila et al. [14]

Table 4 (*continued*)

Compounds	Structure	Reference(s)
13. Cyclo(D-Pro-L-Trp)		Lu et al. [25]
14. Cyclo(Trp-Val)		Soledade et al. [26]
15. Cyclo(Trp-Leu)		Shiba and Nunami [27]

3. Results

3.1. Genus and biochemical characteristics of E. coli GutM4

E. coli GutM4 was identified to the species level by both phenotypic and genotypic investigations. The strains showed phenotypic characteristics typical of *E. coli*: gram negative; facultative anaerobic; rod shape; positive for MR and catalase tests; and negative for VP, H_2S production, arginine decarboxylase, and phenylalanine deaminase tests.

The identification was then further confirmed by 16S rRNA, as shown in the NJ phylogenetic tree (Fig. 1). Moreover, the BLAST program indicated that the 16S rRNA gene sequence of *E. coli* GutM4 showed 99% similarity to that of *E. coli* K12 (4KJC_A).

The obtained 16S rRNA gene sequences were deposited in GenBank under the accession number **KF656780** with the given bacterial name as *E. coli* GutM4.

3.2. Antagonistic activity of E. coli GutM4

E. coli GutM4 showed good inhibition against *S. epidermidis*, *E cloacae*, *H. pylori*, *E. coli O104:H4*, *E. coli O157:H7*, and *S. aureus* (inhibition zones of 10 to 20 mm) ($P < 0.05$) (Table 1). When compared with other tested pathogens, *E. coli* GutM4 exhibited strong inhibition on the growth of *L. monocytogenes* (inhibition zones >20 mm), while low inhibitory activities against *K. pneumonia*, *C. albicans*, and *P. acnes* (inhibition zones <10 mm) ($P < 0.05$) (Table 2). Further and more importantly, *E. coli* GutM4 showed more effective inhibition than the reference strain *E. coli* Nissle 1917 of most of the tested pathogens, except *H. pylori* and *S. aureus* ($P < 0.05$) (Table 1 and Table 2).

3.3. Antagonistic substance produced by E. coli GutM4

3.3.1. Preliminary characterization of substance produced by E. coli GutM4

The antimicrobial substance produced by *E. coli* GutM4 was characterized by the agar well diffusion assay against the indicator strain *L. monocytogenes*. Supernatants of *E. coli* GutM4 treated with catalase, pronase, and NaOH did not show any change in inhibitory activities against the indicator strain. However, trypsin-treated supernatants exhibited significantly decreased inhibitory activities, which indicated that the inhibitory effects of *E. coli* GutM4 were partly and more possibly due to active compounds containing trypsin-targeted peptide bond (Table 3).

3.3.2. Diketopiperazines produced by E. coli GutM4

In total, 15 DKPs, which are cyclic dipeptides, were isolated from the fermented broth of *E. coli* GutM4. All the compounds were reported before but never from *E. coli* strains. Most of the isolated

compounds contained benzene ring, except compounds 1, 2, and 5. Compounds 7 and 8 were identified as the same in the end; they were hydroxyl-substituted (C4) DKPs, which was also the case with compound 5 too. Furthermore, compounds 11 and 13 were isomers (Table 4).

4. Discussion

In the present study, *E. coli* GutM4 was isolated from human feces and was determined *in vitro* to have antagonistic activity against human pathogens. *E. coli* GutM4 produced 14 DKPs, which possibly contribute to its antagonistic activity.

Enteric diseases are mostly due to bacterial toxin and competitive growth of pathogens. For example, *H. pylori* contributes to diseases such as duodenal/gastric ulcer disease, gastritis, gastric adenocarcinoma, mucosa-associated tissue lymphoma, and primary B-cell gastric lymphoma. However, patients who receive *H. pylori* eradication therapy (proton pump inhibitor, amoxicillin, and clarithromycin) often experience eradication failure, which is mainly related to the development of antibiotic-resistant strains of *H. pylori* [10]. The concerns on the low treatment success against pathogens and the development of antibiotic-resistant pathogens have led to increased interest in the application of probiotics and their metabolites as alternative antimicrobial strategies for the treatment and prevention of enteric diseases. The use of enteric microorganisms to inhibit pathogens has great potential because these bacteria are natural competitors of pathogens; in addition, they are usually well adapted to the gastrointestinal environment and are easy to administration [11]. In this study, *E. coli* GutM4 was isolated from the feces of a healthy adult and showed antagonistic activity against all indicator pathogens. Moreover, *E. coli* GutM4 showed stronger or the same level of antimicrobial activity as the reference strain *E. coli Nissle 1917* against the indicator strains, except *H. pylori*, and *S. aureus*. Although the antagonistic activity was illustrated *in vitro*, but not *in vivo*, it is plausible that *E. coli* GutM4 eradicates enteric pathogens, given that *E. coli* GutM4 is capable of gastrointestinal inhabitation.

Furthermore, this study endeavored to explore the mechanisms of *E. coli* GutM4 in the prevention of colonization in the intestine by pathogens. Generally, prevention of pathogen colonization results from the production of antimicrobial substances or metabolites by probiotics and competitive exclusion. Of the antimicrobial substances produced by probiotic strains, bacteriocin, hydrogen peroxide, and organic acids are the most commonly reported [12]. In this study, the relationship between the inhibitory effects of *E. coli* GutM4 and the production of hydrogen peroxide and organic acids could not be established. This is probably due to the existence of other antagonistic mechanism. For example, other antimicrobial substances such as ethanol, diacetyl, acetaldehyde, acetoin, carbon dioxide, reuterin, and reutericyclin could be produced by microorganism. Moreover, competitive exclusion, in which probiotic strains compete with pathogens for nutrients and attachment sites, would prevent colonization in intestine by pathogens [12]. However, trypsin-treated supernatant of *E. coli* GutM4 showed significantly decreased inhibitory effects, which indicated that the antagonistic activity of *E. coli* GutM4 was partly and more likely due to active compounds with trypsin-targeted peptides. Trypsin cleaves peptide chains mainly at the carboxyl end of the amino acids lysine and arginine, except when either is followed by proline.

The hint on the structural type of the antagonistic substance from *E. coli* GutM4 prompted the exploration of its metabolites with a presumptive chemical structure. In the present study, 14 DKPs, which are cyclic dipeptides, were isolated from the fermented broth of *E. coli* GutM4. It is obvious that *E. coli* GutM4 produces a great number of DKPs, some of which may have the amino acids lysine and arginine. This partly explains why the inhibitory effects of *E. coli* GutM4 significantly decreased after treatment with trypsin. Consistent with the antagonistic activity of *E. coli* GutM4, three of the isolated DKPs in this study were reported to have antipathogenic activity; they are 12 cyclo(Pro-Phe) [13], 3 cyclo(Pro-Tyr), and 5 cyclo(4-hydroxyl-Pro-Leu) [14]. Of other 11 DKPs, 11 cyclo(L-Pro-L-Trp), 13 cyclo(D-Pro-L-Trp) [15], 10 cyclo(Trp-Tyr), and 9 cyclo(Phe-Tyr) [16] were reported to have health benefits in chronic disease such as angiocardiopathy and cancer. Taken together, it is certainly worth studying the production and activity of DKPs from *E. coli* GutM4 in the future.

The biological identification of *E. coli* GutM4 was completed correctly by phenotypic and genotypic methods. The accurate identification of *E. coli* GutM4 also revealed a predictable safety and its potential as a probiotic. Moreover, *E. coli* GutM4 has 99% similarity to *E. coli* K12 that was not reported in relation to human diseases. Furthermore, probiotic *E. coli* JM105, which is a strain of *E. coli* K12, can inhibit *S. typhimurium* [7].

5. Conclusions

To conclude, *E. coli* GutM4 produces DKPs that are partly involved in antagonistic action against human pathogens *in vitro*.

Conflicts of interest

The authors declare that there is no conflict of interest in this work.

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