

Lactococcus garvieae に対する 食品および乳酸菌由来抗菌性物質の影響

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論文要約

乳酸菌の一種の *Lactococcus garvieae* は、魚病や乳房炎の起因菌としてのみならず、ヒトに対しても不顕性感染を引き起こしていることが判明している。本菌に対して、食品由来抗菌性物質である茶カテキン4種（エピカテキン、エピカテキンガレート、エピガロカテキン、エピガロカテキンガレート）と、乳酸菌由来バクテリオシンのナイシンおよびガセリシンの抗菌効果について、寒天拡散法とマイクロプレート法を用いて検証した。その結果、緑茶カテキンでは抗菌効果が認められなかったが、乳酸菌由来バクテリオシンでは条件次第で抗菌効果が認められた。今回使用した乳酸菌由来バクテリオシンのナイシンは、正に帯電して細胞膜に静電結合して孔を形成し、ATPなどの細胞内容物を細胞外に流出させて抗菌活性を示すことから、*Lc. garvieae* の細胞膜は帯電性の抗菌性物質の影響を受けやすい構造を有していることが示唆された。また2種の方法での活性の違いがみられ、抗菌活性は自由水の割合や酸素分圧に影響されるのではないかと考えられた。今後、より詳細な研究を行い、安全な抗菌性物質の開発を考えている。

キーワード：ガルピエ菌，緑茶カテキン，ナイシン，ガセリシン，抗菌活性

The Effects of Antibacterial Substances from Foods and Lactic Acid Bacteria on *Lactococcus garvieae*

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ABSTRACT

It has been demonstrated that *Lactococcus garvieae* causes not only diseases in fish but also mastitis in cows and subclinical infections in humans. In this study, we examined the antibacterial effects of green tea catechins and bacteriocins produced from lactic acid bacteria on *Lc. garvieae*. The results showed that an antibacterial effect was recognized on bacteriocin from lactic acid bacteria, *i.e.*, nisin in the agar well diffusion assay and *Lactobacillus gasserii* LA39 supernatant in the microplate assay, whereas green tea catechins didn't. It was suggested that the cell membrane of *Lc. garvieae* was easily affected by the electrostatic charged antibacterial substances. Furthermore, the antibacterial effects differed between agar well diffusion and microplate assay, so the ration of free water and oxygen tension might be affected.

Keywords: *Lactococcus garvieae*, green tea catechin, nisin, gassericin, antibacterial activity

1. Introduction

It is well known that lactic acid bacteria is the general term for bacteria, which can produce lactic acid as the final product of carbohydrate metabolism, and contributes to health as probiotics, on the other hand, there are some strains indicating the pathogenicity.

Lactococcus garvieae, which is a type of lactic acid bacteria, not only causes epidemic infectious disease in cultured fish, but also infects latency in humans¹⁾. It is considered that capsular polysaccharide or exopolysaccharide encoding on plasmid is related to the pathogenicity¹⁾. The clinical isolated strain possesses five plasmids, and three of them relate to bacteriocin production²⁾. At present, there is concern about outbreaks of hospital infection by healthy carrier humans and the presence of multiple varieties of drug resistant bacteria¹⁾.

Recently, there has been interest in the function of foods. Catechins, which is including in Japanese green tea, has been authenticated as Generally Recognized As Safe (GRAS). Representative Japanese green tea catechins are epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, and it been proven that these catechins have antibacterial and antiviral properties and have the effect of inhibiting toxin production³⁾. The antibacterial activities of catechins are mainly reported against gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus mutans*, and *Str. sanguinis*^{4,5)}. It has been suggested that the sensitivity for catechins depends on the chemical structure of the bacterial cell surface^{6,7)}.

The anti-bacterial function of lactic acid bacteria has also been a focus for the safety of food. Peptides or proteins, which indicate the antibacterial effects on related strain and synthesized on ribosome, are called "bacteriocin". Especially, nisin produced from *Lactococcus lactis* subsp. *lactis* has been used as a food additive in over 50 countries in the world and was officially authorized in 2009 in Japan⁸⁾. It has been found that nisin could effectively inhibit the growth of gram-positive food pathogenic bacteria such as *Bacillus*, *Clostridium*, *Staphylococcus*, and *Listeria* genus⁹⁻¹¹⁾, resist high temperatures and low pH, and is easily digested in stomach and intestinal tract^{8,12)}. Under these specific conditions, it is considered that resistant bacteria rarely appear.

Nevertheless, the bacteriocin, with the exception of nisin, are rarely used in the industry at present. If

they become available, the control of harmful bacteria will be easier. In this study, we examined the effect of green tea catechins and bacteriosins from lactic acid bacteria to prevent the infection of *Lc. garvieae* and aimed for the establishment of an effective antibacterial approach.

2. Materials and Methods

2.1 Bacterial strain and culture

Lactococcus garvieae JCM10343^T was purchased from Japan Collection of Microorganisms (Tsukuba, Japan). This strain was cultivated with MRS broth (DifcoTM Lactobacilli MRS broth, BD, NJ, USA) and stored at -80°C until use.

The strain culture was inoculated into 5 ml of MRS broth with a ratio of 1% (v/v) and cultivated at 30°C for 24 hr. This procedure was repeated twice before the examination.

2.2 Preparation of sample solutions

Epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) were purchased from Wako Industrial Co. Ltd. (Osaka, Japan). Catechins were dissolved into distilled water with a concentration of 1 mg/ml, and sterilized through a 0.20µm pore size membrane filter (DISMIC 13CP, ADVANTEC, Tokyo, Japan).

Nisin (from *Lactococcus lactis*, Sigma-Aldrich, USA) was dissolved into 20% ethanol with a concentration of 2 mg/10ml, and stored at 4°C after sterilizing filtration.

Lactobacillus gasseri LA39 and LA158 were cultured twice in MRS broth and inoculated 1% (v/v) was inoculated into 5 ml of MRS broth. After cultivation (37°C, 24 hr), the culture was centrifuged (5,000 rpm, 10 min, room temperature) to remove bacterial cells. The culture supernatants were sterilized by filtration and stored at 4°C.

Just before the experiment, each solution was diluted serially using distilled water for catechins, 20% ethanol for nisin, and 0.85% NaCl for *Lb. gasseri* supernatant.

2.3 Agar well diffusion assay

Anti-bacterial activity was determined with the agar well diffusion method. According to previous reports¹³⁾, MRS agar plates were overlaid with MRS soft agar (0.75%) lawn prepared *Lc. garvieae* JCM10343^T and wells (d.m. 5.5 mm) were made, then 65 µl of the sample solutions was put into each well.

The plates were incubated at 30°C for 24 hr and a

hallo formation inhibiting the growth of *Lc. garvieae* JCM10343^T was observed with direct transmitting lighting installation.

2.4 Microplate assay

Anti-bacterial activity was also determined using the microplate method. MRS broth (160 μ l) was put into a well on a 96-well microplate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan), the culture of *Lc. garvieae* JCM10343^T (20 μ l) and each sample solutions (20 μ l) were added to the well, and the microplate was incubated at 30°C for 24 hr.

After cultivation, the turbidity, which had been diluted 10 times with distilled water, was measured at 600 nm with a spectrophotometer (U-1800 Hitachi, Tokyo, Japan).

2.5 Statistics

The turbidity with a triplicate of each sample was measured. And then, the means, standard deviations, and significant difference by ANOVA analysis and multiple comparison authorization (Sheffe's) were calculated (* <0.05).

3. Results and Discussion

3.1 The effect of green tea catechins on *Lc. garvieae* JCM10343^T

The anti-microbial activity of green tea catechins was evaluated by agar well diffusion and microplate assay (Fig. 1-3). On both assays, inhibitions with significant differences were not observed at all. These results showed that the anti-bacterial activity of green tea catechins could not be expected for *Lc. garvieae*. It has been demonstrated that green tea catechins acts to Lys-type peptidoglycan such as *Lactobacillus acidophilus*, while it had no almost antibacterial effect to DAP-type peptidoglycan on *Lactobacillus plantarum*¹⁴⁾. It was considered that green tea catechins were not able to exhibit antibacterial activity because crosslink peptide of peptidoglycan on *Lc. garvieae* combined with Lys-Ala-Gly-Ala, not generally L-Lys-D-Asp^{12, 15)}. In addition, cloudy circles were recognized at the concentration of 1-2 AU/ml ECg on the agar well diffusion assay. This phenomenon was observed before¹⁶⁾, and it seemed that MRS medium ingredient and ECg caused aggregation.

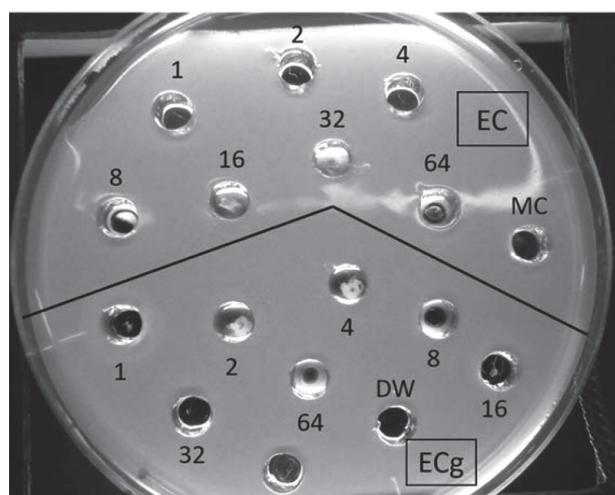


Figure 1 Anti-microbial activity of epicatechin and epicatechin gallate by agar well diffusion assay (Epicatechin (EC), Epicatechin gallate (ECg))

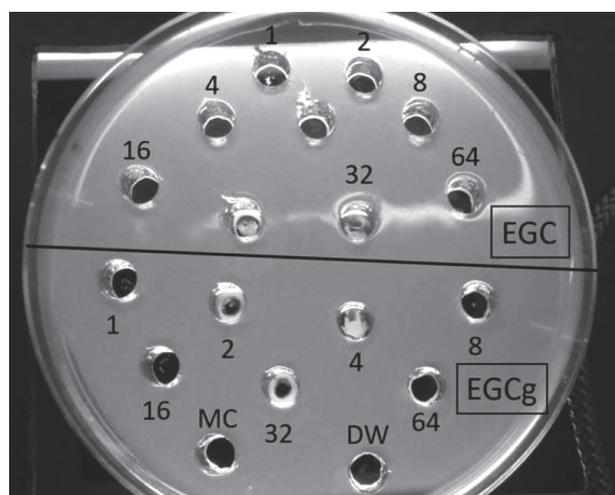
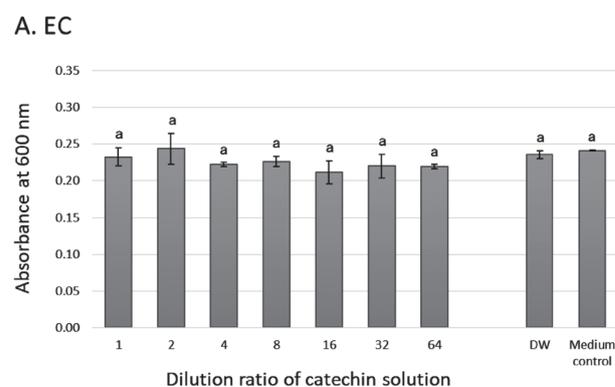


Figure 2 Anti-microbial activity of epigallocatechin and epigallocatechin gallate by agar well diffusion assay (Epigallocatechin (EGC), Epigallocatechin gallate (EGCg))



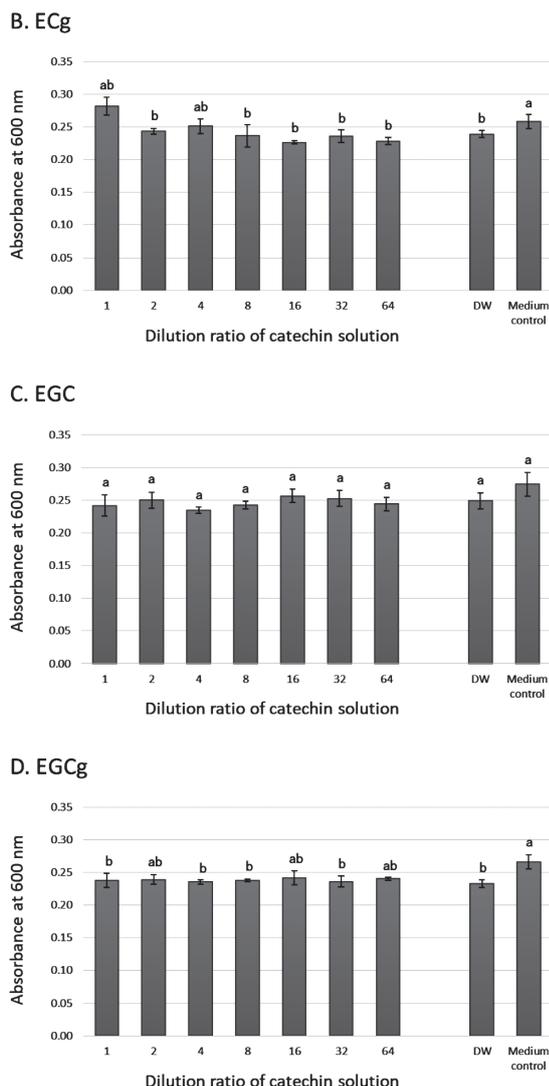


Figure 3 Anti-microbial activity of green tea catechins by microplate assay (A: Epicatechin (EC), B: Epicatechin gallate (ECg), C: Epigallocatechin (EGC), D: Epigallocatechin gallate (EGCg))

3.2 The effects of bacteriocins produced lactic acid bacteria on *Lc. garvieae* JCM10343^T

The anti-microbial activity of bacteriocins produced from lactic acid bacteria was evaluated by agar well diffusion and microplate assay (Fig. 4-7). In the agar well diffusion assay, anti-bacterial activity was found in nisin (2 AU/ml), nevertheless neither *Lb. gasseri* LA39 nor LA158. On the other hand, anti-microbial activity of nisin didn't determine significant differences in the microplate assay. In addition, *Lb. gasseri* LA39 supernatant indicated anti-bacterial activity on microplate assay (2 AU/ml).

It has been demonstrated that there are loops in nisin molecules, because unusual amino acids such as rantonin exists within its structure. Gassericin A produced from *Lb. gasseri* LA39 is a cyclic peptide with

strong hydrophobicity¹³⁾. Furthermore, nisin takes a positive charge in the solution, perforates bacterial cell membrane, and leads to run off cellular contents such as ATP²⁾. Two types of bacteriocin, which had anti-bacterial activity, might act to cell membrane of *Lc. garvieae* by the biochemical characteristic similarity. In addition, it was suggested that anti-bacterial differences between the agar well diffusion assay and the microplate assay were related to the proportions of free water and oxygen partial pressure.

Based on these results, it is considered that *Lc. garvieae* is able to resist existing anti-bacterial substances on behalf of the chemical characterization of its peptide-glycan or cell membrane. To develop secure antibiotics, it is necessary to conduct research in detail on bacterial mechanisms and bacterial cell surface chemical structures.

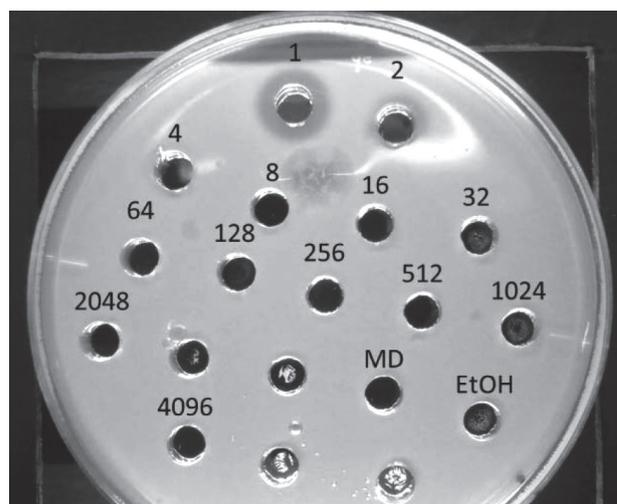


Figure 4 Anti-microbial activity of nisin in agar well diffusion assay

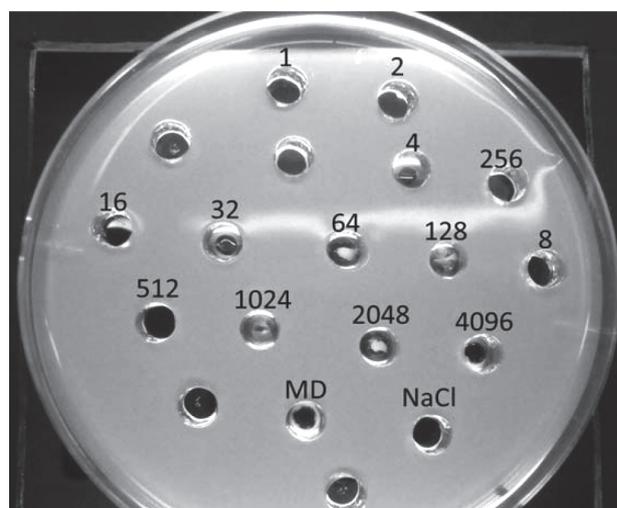


Figure 5 Anti-microbial activity of *Lb. gasseri* LA39 supernatant in agar well diffusion assay

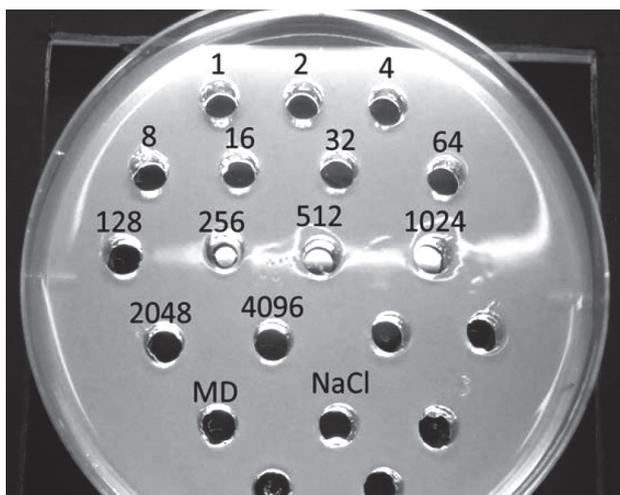
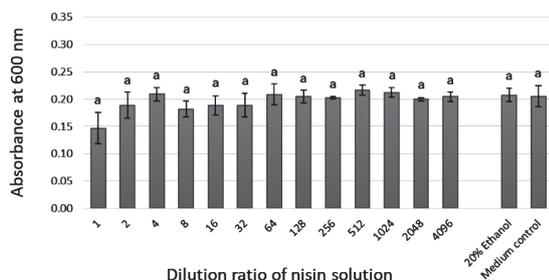
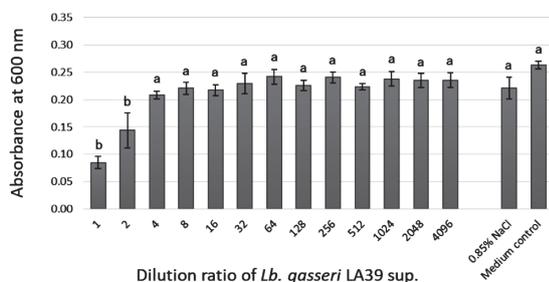


Figure 6 Anti-microbial activity of *Lb. gasseri* LA158 supernatant in agar well diffusion assay

A. Nisin



B. *Lb. gasseri* LA39 sup.



C. *Lb. gasseri* LA158 sup.

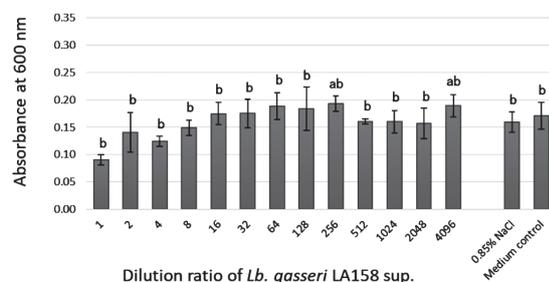


Figure 7 Anti-microbial activity of bacteriocins in microplate assay (A : nisin, B : *Lb. gasseri* LA39 supernatant, C : *Lb. gasseri* LA158 supernatant)

4. Conclusion

The antimicrobial effects of green tea catechin and bacteriocin produced from lactic acid bacteria on *Lactococcus garvieae* were investigated. As a result, bacteriocin from lactic acid bacteria showed antimicrobial activity depending on a condition, whereas green tea catechins didn't. On this study, it was suggested that the antimicrobial activity to *Lc. garvieae* was influenced chemical characteristics of bacterial surface and ratio of free water and/or oxygen in environmental condition.

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