**Effect of divalent metal cations on production of gassericin T by**

*Lactobacillus gasseri* SBT2055

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**Abstract**

Gassericin T, a class IIb bacteriocin with two complementary peptides, is produced by *Lactobacillus gasseri* commonly found in human intestines. *L. gasseri* strains containing bacteriocinogenic LA158 isolated from a human infant in our laboratory grew well in modified milk-based media, but the gassericin T production by LA158 was inhibited by high concentrations of divalent metal cations in the milk-based media. In this study, it was confirmed that gassericin T production by *L. gasseri* SBT2055, which has an antiobesity effect, was specifically inhibited in MRS broth by adding divalent metal cations (Mg2+, Ca2+, Mn2+, Fe2+, and Zn2+) depending on the concentration of cations, although the effective concentrations were different from those in LA158 tested (Mg2+ and Ca2+). The addition of 200 mM divalent metal cations resulted in the almost complete disappearance of gassericin T despite a good cell growth. Furthermore, gassericin T production was restored by adding trisodium citrate dihydrate (TSC), which is a food-grade chelator of divalent cations. These findings may contribute to the effective use of bacteriocinogenic *L. gasseri* strains as probiotics for yogurt manufacture without the inhibition of growth of starter strain(s).

**Key words:** *Lactobacillus gasseri*, bacteriocin, gassericin T

**Introduction**

Bacteriocins, which are ribosomal antimicrobial peptides or proteins synthesized by bacteria, are active against other bacteria, either of the same species (narrow spectrum) or across genera (broad spectrum).1 Many bacteriocins are produced by food-grade lactic acid bacteria (LAB), which have attracted much attention as novel tools for controlling contamination in foods and provide new insights into food biopreservation using them either alone or in combination with other methods of preservation applied to packaging films and food surfaces.2,3 Moreover, ingestion of probiotic bacteria may allow the *in vivo* production of the bacteriocin in the small and large intestines and positively affect the microbiota of the host.4–6

In our laboratory, we have researched the bacteriocins produced by *Lactobacillus gasseri*, which belong to the *Lactobacillus acidophilus* group of LAB and are natural inhabitants of the human intestinal tract and have probiotic effects.7,8 Gassericin T (GT) produced by the probiotic strains *L. gasseri* SBT2055 and LA158 is a two-peptide bacteriocin (class IIb) consisting of hydrophobic GatA and GatX molecules.9 GT has a broad antibacterial spectra against LAB and food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*.10 It may be an attractive candidate as a biopreservative.
Many *L. gasseri* strains including JCM 1131\(^T\) and GT producers can grow well in food-grade milk-based media, such as skim milk and cheese whey, with proteose peptone as a nitrogen source. However, GT production by *L. gasseri* LA158 in milk-based media is inhibited\(^{11}\).

In 2009, Arakawa *et al.* reported that various soluble divalent metal cations such as Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\) inhibit GT production by *L. gasseri* LA158 cultured in artificial composition nutrient medium, such as MRS broth, and the addition of trisodium citrate dihydrate (TSC), which is a food-grade chelator of divalent cations extensively used as a food additive with no legal additive limitation in Japan, prevents the decrease of GT production in either MRS broth containing divalent cations or food-grade milk-based media with proteose peptone\(^{12}\). Divalent metal cations have been known as a factor affecting bacteriocin production, but their effects empirically differ from each other and could be strain-specific\(^{13}\). In this study, we investigated the inhibitory effect of available divalent metal cations on GT production by another GT producing strain, *L. gasseri* SBT2055, determined the effective concentrations of divalent metal cations suppressing GT production, and examined the specificity of the strain other than LA158.

### Materials and Methods

#### Bacterial strains and culture conditions

*L. gasseri* SBT2055, a GT producer, was isolated from human adult (female) feces by Snow Brand Milk Products Co., Ltd. (Kawagoe, Japan). *Lactobacillus delbrueckii* subsp. *bulgaricus* JCM 1002\(^T\), which was used as an indicator in antibacterial activity assays, was purchased from the Japan Collection of Microorganisms (JCM, Riken, Tsukuba, Japan). Both the *Lactobacillus* strains were propagated three times in Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, USA) at a 10% inoculum rate at 37°C for 18–24 h. The culture supernatants of SBT2055 were prepared by centrifugation (6,000 × g, 5 min, 4°C) and sterile filtration through a 0.20 μm pore size membrane filter (Toyo Roshi Kaisha Ltd., Tokyo, Japan).

#### Bacteriocin activity assay

Bacteriocin activity was determined by agar-well diffusion assay\(^{12}\). MRS agar plates (9 cm × 4 mm, 15 mL) were overlaid with an MRS soft-agar lawn (10 mL) prepared with a 10⁻¹-diluted overnight culture (250 μL) of the indicator strain, *L. delbrueckii* subsp. *bulgaricus* JCM 1002\(^T\). Wells of 6 mm diameter were filled with 65 μL of the sample solution. The plates were incubated for 18 h at 37°C. A clear halo without cell growth around the well indicated the presence of bacteriocin activity. To determine bacteriocin titer, the samples were serially diluted twofold using sterile 0.85% (w/v) sodium chloride. The unit of bacteriocin activity (AU: arbitrary unit) was defined as the reciprocal of the highest dilution inhibiting the growth of the indicator strain. The results presented are mean values of three independent determinations.

#### Effect of divalent metal cations on gassericin T production

The concentration-dependent effect of divalent metal cations, such as Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), and Zn\(^{2+}\), on GT production was determined by the agar-well diffusion assay of the culture supernatants of *L. gasseri* SBT2055 cultivated in “MRS broth supplemented with 10, 50, 100, or 200 mM of each of the following soluble divalent metal salts: MgSO\(_4\) (pH 6.1–5.8), CaCl\(_2\) (pH 6.1–5.7), MnSO\(_4\) (pH 6.1–5.6), FeSO\(_4\) (pH 6.0–5.2), and ZnCl\(_2\) (pH 5.9–4.7)”\(^{14}\). The growth of cells was monitored by measuring pH of each medium.

#### Restoration of gassericin T production using trisodium citrate dihydrate

The restoration of GT production was investigated by adding TSC, a chelator of divalent cations, to each MRS broth containing MgSO\(_4\), CaCl\(_2\), MnSO\(_4\), FeSO\(_4\), or ZnCl\(_2\). The culture supernatants of SBT2055 cultivated in “MRS broth supplemented with a certain concentration of 10, 50, 100, or 200 mM TSC and a divalent metal cation such as Mg\(^{2+}\) (pH 6.1–5.8), Ca\(^{2+}\) (pH 6.1–5.7), Mn\(^{2+}\) (pH 6.1–5.6), Fe\(^{2+}\) (pH 6.0–5.2), and Zn\(^{2+}\) (pH 5.8–4.7)”\(^{14}\)” were examined by agar-well diffusion assay.
Results

Effect of divalent metal cations on gassericin T production

To investigate the effect of divalent metal cations on GT production, *L. gasseri* SBT2055 was cultivated in MRS broth supplemented with 10, 50, 100, or 200 mM of each of the following metal salts: MgSO₄, CaCl₂, MnSO₄, FeSO₄, and ZnCl₂. GT production was measured as GT activity determined by agar-well diffusion assay, because divalent metal cations do not directly affect GT activity. Figure 1 shows that all the tested divalent cations markedly inhibited GT production in the culture supernatants from MRS broth supplemented with these divalent cations in a concentration-dependent manner (Fig. 1). With regard to the effectiveness of these inhibitors, 10 mM, the minimum concentration tested, was sufficient for decreasing GT production from the native activity of 31,508 AU/mL to 1,969 (Mg²⁺, Fe²⁺), 3,938 (Ca²⁺), 15,754 (Mn²⁺) and 31 (Zn²⁺) AU/mL. Mg²⁺ and Ca²⁺ at 200 mM each maximally decreased the GT activity to 123 and 31 AU/mL, respectively, and Fe²⁺, Mn²⁺ and Zn²⁺ at 50 mM each resulted in the almost complete disappearance of active GT.

The growth of *L. gasseri* SBT2055 was monitored by measuring pH of each test medium. The SBT2055 strain grew well (final pH, ≤4.3) in the MRS broth supplemented with Mg²⁺, Ca²⁺, and Fe²⁺ at 10–200 mM, but the growth was slightly inhibited in the MRS broth with 50–100 mM Mn²⁺ and 50–200 mM Zn²⁺ (final pH, 4.3–4.9). The activity of the culture supernatant from the MRS broth with 200 mM Mn²⁺ was not determined because of the poor cell growth (final pH, 6.2, data not shown in Fig. 1).

Restoration of gassericin T production using trisodium citrate dihydrate

GT production inhibited in each modified MRS broth containing a divalent metal cation (100 mM Mg²⁺, 100 mM Ca²⁺, 50 mM Fe²⁺, 50 mM Mn²⁺, and 50 mM Zn²⁺, which maximally decreased the GT production and did not cause significant growth inhibition of the cells) was restored by chelation.
with TSC in a concentration-dependent manner (Fig. 2). In the case of adding 200 mM TSC to the medium containing 100 mM Mg\(^{2+}\) or 50 mM Fe\(^{2+}\), the activity of the culture supernatant was restored to its native value (31,508 AU/mL). It is likely that following the adding of 100 mM TSC to the medium containing 50 mM Mn\(^{2+}\) or 50 mM Zn\(^{2+}\), the inhibition of the activity of the culture supernatant by metal ions was released. Moreover, the SBT2055 culture supernatant from the medium containing 50 mM TSC and 100 mM Ca\(^{2+}\) also showed an activity equivalent to the native value. All the cells grew well (final pH, ≤ 4.8) in each test medium except for the MRS broth supplemented with 50 mM Zn\(^{2+}\) and 200 mM TSC, which showed no activity.

**Discussion**

Food-grade media should be essential to utilize LAB and LAB antimicrobial agents such as bacteriocins in the food industry. In our previous reports, the GT-producing LA158 strain grew well in proteose-peptone-containing milk-based media we developed\(^{11}\). However, GT production was inhibited by divalent metal cations existing at high concentrations in these milk-based media (about 28.25 mM Ca\(^{2+}\) and 4.17 mM Mg\(^{2+}\) in common milk)\(^{12}\); the contents of metal ions are markedly higher than in MRS broth (0.83 mM Mg\(^{2+}\) and 0.33 mM Mn\(^{2+}\)). Another GT producer, *L. gasseri* SBT2055 isolated from human intestines, has the ability to establish itself in the human gastrointestinal tract, may affect the composition and metabolism of the intestinal microflora\(^{14}\), and attenuates abdominal adiposity\(^{15}\), suggesting it is an excellent candidate probiotic. In this study, we showed that all the tested divalent metal cations contained in milk (Mg\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\), Mn\(^{2+}\) and Zn\(^{2+}\)) exert an inhibitory effect on GT production by *L. gasseri* SBT2055 in a concentration-dependent manner. Although the negative effects of divalent metal cations on bacteriocin production were considered to differ from each other and be strain-specific\(^{13}\), our results are the first to suggest that GT production by *L. gasseri* strains may be extensively inhibited by divalent metal cations. However, the concentrations of divalent metal...
cations at which GT production was inhibited to the same degree differed, indicating strain specificity. For example, 10 mM Mg²⁺ decreased the production (activity) of GT from LA158 by 64-fold (from 15,754 to 246 AU/mL)¹² and decreased that from SBT2055 by 16-fold (from 31,508 to 1,969 AU/mL, Fig. 1). On the other hand, five tested cations exhibited different inhibitory activities; in particular, 10 mM Zn²⁺ exerted the maximum inhibitory effect on GT production by SBT2055 (from 31,508 to 31 AU/mL). The effect of anions such as SO₄²⁻ and Cl⁻ used in this study on GT production would be extremely small, because we proved that 100 mM NaCl did not decrease the GT production of SBT2055 (data not shown) and both of MgCl₂ and MgSO₄ had no obvious difference on the GT production of LA158¹⁵.

We focused on the chelation effect of TSC on divalent metal cations. TSC is a trisubic salt of citric acid produced by complete neutralization of citric acid with a highly pure sodium source and subsequent crystallization, and it is widely used in foods and beverages as a food-grade chelator of divalent cations and has various technical applications mainly as buffering, sequestering, and emulsifying agents. The inhibition effect of divalent cations on GT production was counteracted by TSC concentration-dependently, which provides a route to realizing GT application as a food additive. Recently, it has been found that TSC has antimicrobial activity against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa, and its antimicrobial mechanism may be related to degradation of the cell wall membrane, inhibition of biogenesis, or the increase in the permeability of bacterial cells¹⁶–¹⁸. Scannell et al. have reported the synergistic effects of bacteriocins such as nisin and lacticin 3147 and citrate against Gram-positive bacteria¹²,¹⁹. In this study, the maximum 2-folds synergistic antibacterial effects of GT produced by SBT2055 with 200 mM TSC were detected (data not shown) as same as those of GT produced by LA158 was increased 2.0–2.7 times following the addition of 50 and 100 mM TSC, indicating the actual GT amounts in the culture supernatants with TSC might be estimated to 1/2 by antibacterial activity measurement.

These divalent cations may act on the cell membrane to affect bacteriocin secretion²⁰, stimulate the synthesis of prepeptides and/or the activation of prepeptide maturation enzymes, and displace bacteriocins absorbed on the cell wall²¹. However, the precise mechanism of inhibition of bacteriocin production by divalent metal cations has never been reported until now. The inhibition and restoration of GT production by divalent cations and TSC, respectively, shown in our study may contribute to the utilization of not only GT as a food additive for biopreservation but also GT producers as probiotics for human health maintenance by cocultivation with dairy GT-sensitive LAB starter (s) in fermented dairy products such as yogurt. However, GT production by the producer strains and the GT activity in the actual human gastrointestinal tract have never been researched. The function of GT and the effect on the intestinal microbiota in the human intestines should be further clarified.

References

7) Reuter, G.: The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and


二価金属イオンが *Lactobacillus gasseri* SBT2055の「ガセリシン T」生産に及ぼす影響

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ガセリシン T (GT) は, ヒト腸管より検出される乳酸桿菌 *Lactobacillus gasseri* により生産される二成分性バクテリアルオシンである。また, 当研究室で分離したヒト乳児由来の GT 生産 LA158株を含む *L. gasseri* は, 改良乳培地で良好に生育するものの, 乳中に豊富に存在する二価金属イオンにより LA158株の GT 生産は抑制されることが知られている。本研究により, GT 生産株として最初に見出された, 抗肥満効果を有する *L. gasseri* SBT2055は, MRS 培地にて二価金属イオン (Mg2+, Ca2+, Mn2+, Fe2+ および Zn2+) の添加により濃度依存的に GT 生産が抑制されることは判ったが, LA158株における抑制濃度 (Mg2+ および Ca2+) とは異なっていた。また, 200 mM の二価金属イオン添加では, 良好に生育するにもかかわらず, GT の生産は完全に消失した。さらに, 二価金属イオンのキレート剤で食品添加物であるクエン酸三ナトリウム (TSC) の添加により GT 生産は回復した。以上の結果から, ヨーグルト製造においてスターターの生育を阻害せずに, GT 生産性のプロバイオティック *L. gasseri* 株を効率的に添加・利用出来るものと考えられた。