Brief Report

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Human gut associated *Bacteroides* and *Akkermansia* bacteria exhibit immunostimulatory activity in the silkworm muscle contraction assay

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SUMMARY: The immunoregulatory activity of human gut bacteria has attracted attention in recent years. To assess the innate immune-stimulatory activity of various samples *in vivo* efficiently, we previously introduced a silkworm-based assay as a novel alternative method. The method has been used for over a decade to screen for substances with potential physiological activity. In this study, we prepared heat-killed cells of four strains of human gut bacteria (*Bacteroides ovatus*, *B. thetaiotaomicron*, *B. uniformis*, and *Akkermansia muciniphila*) and assessed their innate immune-stimulatory activity within the silkworm model. Our findings indicate that the sample from either *B. ovatus* or *B. thetaiotaomicron* has immunostimulatory activity in the silkworm, in contrast to *B. uniformis* and *A. muciniphila*. Moreover, a pathogenicity assessment using the silkworm infection model was conducted to determine the safety of these bacterial strains for human consumption when considered as food ingredients. None of the four gut bacterial strains exhibited pathogenic effects in silkworms, with *Pseudomonas aeruginosa* serving as a positive control of the pathogenicity test. These results suggest that the silkworm-based assay can distinguish between the immunostimulatory effects of different human gut microbes and may enhance the safety evaluation of microbial ingredients.

Keywords: gut bacteria, silkworm model, muscle contraction assay, innate immune system

1. Introduction

Throughout history, humanity has achieved significant advancements in medicine and health sciences by discovering and developing numerous beneficial foods and pharmaceuticals. Such progress has been made under the rigorous evaluation of physiological functionality and safety at the developmental phases of foods and pharmaceuticals. It is believed that the enhancement of evaluation systems at various stages, from basic research to applied research, will lead to the accumulation of further knowledge and the efficient derivation of products contributing to health promotion. In this study, we primarily focus on the immune system and explore the potential of using silkworms as a model organism for physiological function assessment.

The challenge of applying silkworms to health science is one that we have been proposing for approximately 20 years (*1-12*). Our research group has demonstrated that human pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* can cause fatal infections in silkworms, and we have also shown that the therapeutic effects of antibiotics against bacterial infections can be evaluated using this model. Furthermore, we have been exploring compounds with therapeutic effects against *S. aureus* infections from a soil bacterial library. As a result, we have developed a novel antibiotic lysocin E, which has shown excellent therapeutic efficacy in mammals and is currently put in clinical development pipeline (*9,13*). Conventionally, mammalian animals such as mice have been commonly used for *in vivo* assays. However, such evaluations incur significantly higher costs compared to *in vitro* assays and alternative methods using invertebrate species.

We propose that this issue can be overcome by using silkworms, which offers the advantage of easy administration of quantitative samples and allow for the evaluation of pharmacological effects of compounds at the organismal level (*11-13*). Furthermore, silkworms have the advantage of requiring less housing space compared to mice and other mammals. Moreover, in the average-sized research laboratory at universities and

national research institutions, compound screening using silkworms allows for the evaluation of approximately 100 compounds per day (with one dose, using 300 silkworms with n = 3/compound) (*14*). Hence, assessments using silkworms offer significant economic advantages. To elucidate the effects of various compounds and food materials on the immune system, we have also been working on understanding the innate immune system using silkworms. Our studies have indicated that the contraction of silkworm muscles is synchronized with the triggering of their innate immunity (*15-17*). A peptide known as the paralytic peptide (PP) found in silkworms causes paralysis and prompts muscle contraction, while stimulating the silkworm's hemocytes (*18,19*). The activation of this peptide can be gauged by observing the resultant muscle contractions in the silkworms, using these as a physiological marker of the peptide's activity. Utilizing this information, we have developed a method using silkworms to identify substances in food that activate the innate immune response. Our findings include the identification of certain polysaccharides, like β-glucans, which exhibit a high activity (*17,20*). These polysaccharides are believed to not only improve the health of silkworms but also hold potential as indicators for evaluating similar health benefits in humans.

Bacteroides ovatus and *Bacteroides thetaiotaomicron*, gut bacteria in humans, have been reported to maintain skin condition (*21*) and improve sleep quality (*22*) by ingesting prebiotics that increase the abundance of these bacteria (*23*) . Additionally, other effects on human health, such as immune system, have also been anticipated, although details have remained unclear. In this study, we used four bacterial species of the genera *Bacteroides* and *Akkermansia* present in the human gut and evaluated their immunostimulatory capacity. Furthermore, in order to assess the potential safety risk of these gut bacteria for humans, we conducted pathogenicity tests for these bacteria using silkworms.

2. Materials and Methods

2.1. The bacterial strains used in the experiment and their cultivation conditions

In this study, we obtained *Bacteroides ovatus* JCM5824, *B. thetaiotaomicron* JCM5827, *B. uniformis* JCM5828, and *Akkermansia muciniphila* JCM33894 from the Japan Collection of Microorganisms (JCM; Ibaraki, Japan) and used them in the experiments. The above-mentioned three strains of *Bacteroides* were inoculated into Gifu anaerobic medium (GAM) (Nissui Pharmaceutical Co., Ltd.; Tokyo, Japan) and cultured anaerobically at 37°C for 48 hours. Additionally, *A. muciniphila* was inoculated into GAM medium supplemented with 0.4% mucin (Fujifilm; Osaka, Japan) and cultured anaerobically at 37°C for 48 hours. *Pseudomonas aeruginosa* strain PAO1 was inoculated into Luria-Bertani (LB) 10 medium and cultured aerobically at 37°C overnight.

2.2. The rearing of silkworms

The silkworm eggs used in the experiment were obtained from the Ehime Sanshu (Ehime, Japan). Silkworm rearing followed the previously reported method (*6*). The hatched silkworms were reared at 27°C. Silkworms were fed Silkmate 2S (Nihon Nosan Kogyo; Kanagawa, Japan). Approximately 1 gram of feed per individual was provided to the silkworms on the first day of the fifth instar, and the silkworms on the second day of fifth instar were used in the experiment.

2.3. The muscle contraction assay using silkworms

The silkworm muscle contraction assay followed the experimental method reported previously (*17,24*). In brief, decapitated individual silkworm specimens were suspended with thread, and the contraction of silkworm muscles was measured when 50 μL of the sample was injected into the haemocoel using a 1 mL syringe with a 27-gauge needle (Terumo; Tokyo, Japan). The muscle contraction was calculated as the C-value, which is the percentage decrease in muscle length from its maximum length after injection of the sample. Additionally, following the report by Fujiyuki *et al*., the activity of inducing 15% contraction in silkworm muscles was defined as 1 Unit (*17*).

2.4. The pathogenicity tests of each bacterial strain on silkworms

Fifty μL of ten-fold concentrated anaerobic culture of the four gut bacterial strains and a serial-diluted solution of the *P. aeruginosa* culture were injected into the body fluids of silkworms on the second day of fifth instar using a 1 mL syringe with a 27-gauge needle and the survival of silkworms was observed. Each group of silkworms used in this experiment consisted of 5 individuals per dose. The 50% lethal dose (LD_{50}) of each bacterial strain on silkworms was estimated from the survival rate of silkworms in each group 24 hours after injection and the number of viable bacteria for each strain.

3. Results and Discussion

3.1. Quantification of the innate immunostimulatory capacity of human gut bacteria

The insect cytokine PP (paralytic peptide) is known to induce the muscle contraction and activation of the innate immune system in silkworms. Therefore, measuring muscle contractions in silkworms injected with the test substance can provide insight into the response of the innate immune system. Utilizing this assay system, the ability of microorganisms from the

human gut to induce the silkworm paralytic peptides (insect cytokines) was measured in this study (Figure 1). As a result, among *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis,* and *A. muciniphila*, two bacteria, *B. ovatus* and *B. thetaiotaomicron*, exhibited muscle contraction-inducing activity in silkworms (Table 1). On the other hand, *B. uniformis* and *A. muciniphila* did not show the activity (Table 1). These results suggest that substances present in *B. ovatus* and *B. thetaiotaomicron,* among the genus *Bacteroides*, may have innate immune-stimulatory effect with potential to human application.

3.2. Pathogenicity test

Either of the four human gut bacteria was injected into the hemolymph of silkworms, which were subsequently raised at 37°C. As a result, all silkworms survived after 24 hours, and the LD_{50} values were: > 1.3×10^9 (*B. ovatus*), > 2.5×10^8 (*B. thetaiotaomicron*), > 4.6×10^8 (*B. uniformis*), and > 1.7×10^8 (*A. muciniphila*) colony forming units (CFU) /larva. In contrast, silkworms injected with the *P. aeruginosa* died within 24 hours, with an LD_{50} value of 1.1 CFU/larva (Table 2). These results suggest that the aforementioned gut bacteria do not possess pathogenicity against silkworms and are of minimal risk for human consumption.

In this study, we found that among the four strains of human gut-derived bacteria (*B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, and *A. muciniphila*), *B. ovatus* and *B. thetaiotaomicron* induced muscle contractions in silkworms. Previous research has shown that silkworm muscle contractions are induced by the activation of paralytic peptide (PP) in the hemolymph (*18,19*). Furthermore, since the activation of PP has been shown to contribute to host resistance against pathogenic microorganisms (*15,19*), *B. ovatus* and *B. thetaiotaomicron* were suggested to activate the innate immune system of silkworms. Additionally, none of the four strains of gut-derived bacteria used in this study were lethal to silkworms even when administered, suggesting that these strains do not possess pathogenicity against silkworms.

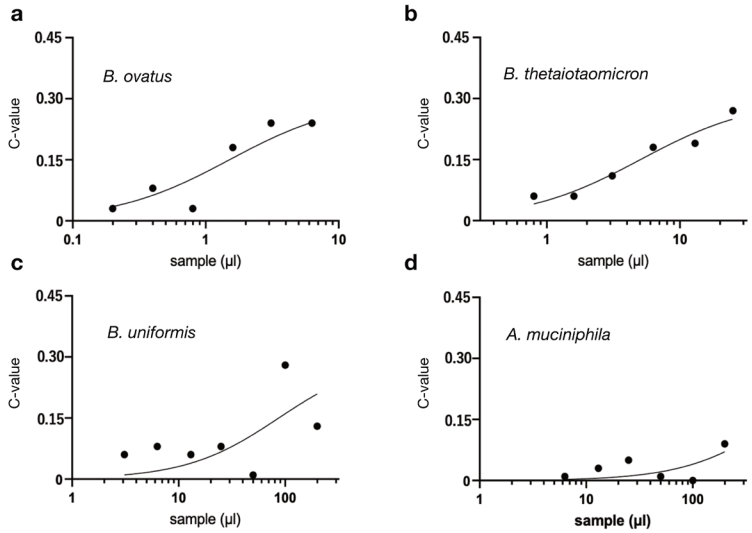

Figure 1. Activation of insect cytokines (paralytic peptides) by human gut-derived bacteria. A dilution series of heat-killed cells of each strain was prepared, and muscle contraction was measured when injected into silkworm muscle specimens. Samples injected into silkworms were (**a**) *B. ovatus*, (**b**) *B. thetaiotaomicron*, (**c**) *B. uniformis*, and (**d**) A. *muciniphila*. The C-value indicates the percentage of the muscle specimen that contracted and shortened from its original state after the sample was injected.

Table 1. Muscle contraction activity of the *Bacteroides* and *Akkermansia* strains

	Sample Conc. [mg/mL]	Activity per volume [U/mL]	Specific Activity [U/mg]	Reference
B. ovatus JCM5824	8.9	660	74	This study
B. thetaiotaomicron JCM5827	11	200	17	
B. uniformis JCM5828	0.88	< 5.0	< 5.7	
A. muciniphila JCM33894	3.5	< 5.0	< 1.4	
Curdlan	-	-	100	Fujiyuki *et al*. (2012)
Yeast β-glucan (Sigma)	-	-	33	
Lichenan	-	-	6	
Laminaran	-	-	< 1	

The muscle contraction activity of each gut-bacterial sample was measured when injected into silkworm muscle. One unit (U) is defined as the activity of a sample that causes a silkworm muscle specimen to contract by 15%. The specific activity was determined from the concentration of each sample and the muscle contraction activity per volume.

Table 2. Lack of virulence in the *Bacteroides* and *Akkermansia* strains (intra-hemocoel infection)

	LD_{50} [CFU/larva]	Fold
B. ovatus JCM5824	$> 1.3 \times 10^9$	$> 1.2 \times 10^9$
B. thetaiotaomicron JCM5827	$> 2.5 \times 10^8$	$> 2.3 \times 10^8$
B. uniformis JCM5828	$> 4.6 \times 10^8$	$> 4.2 \times 10^8$
A. muciniphila JCM33894	$> 1.7 \times 10^8$	$> 1.5 \times 10^8$
P. aeruginosa PAO1	1.1	1

Samples obtained from cultures of bacteria of the genera *Bacteroides* and *Akkermansia* and *P. aeruginosa* PAO1 were injected into silkworms, and the 50% lethal dose (LD_{50}) of each bacterium to the silkworm was determined from the survival rate of the silkworm after 24 hours. Fold indicates the magnification of each strain when the LD_{50} of *P. aeruginosa* is set as 1.

In previous studies using silkworms, various polysaccharides have been evaluated for their muscle contraction activity. Fujiyuki *et al*. compared the activities of structurally different β-glucans and reported the activity ranging from 0-100 units/mg using the same assay system as in the present study (*17*). The activity values of the samples examined in the present study that exhibited activity also fell within this range, consistent with previous results. Additionally, we have recently reported that neutral polysaccharides derived from broccoli exhibit higher activity (*20*). However, it is important to note that the broccoli-derived neutral polysaccharides used were semi-purified fractions obtained through ethanol precipitation or organic solvent extraction, making direct comparisons of activity levels difficult.

The human gut bacteria, which exhibited innate immune-stimulatory activity in silkworms in this study, have been reported to possess various functions such as anti-rotavirus (*25*) and anti-inflammatory effects (*26,27*). The prebiotics which selectively propagate *B. ovatus* and *B. thetaiotaomicron*, in particular, are known (*23*). These bacterial species are expected to be potent candidates of novel probiotics, and as their beneficial effects on humans become clearer, it is likely that their utilization in functional foods and supplements will be further explored.

In the development of functional foods, safety assessment is indispensable. Currently, *in vitro* assays using cultured cells are predominantly employed alternative methods for studying the safety and functionality of food materials. For example, the *in vitro* 3T3 neutral red uptake (NRU) phototoxicity test evaluates the toxicity of substances excited by light based on the uptake of neutral red by Balb/c 3T3 cells, and it is widely used for testing food additives and similar substances (*28*). Additionally, analyzing the genomic sequences of organisms used as food materials leads to infer factors that may be harmful to the human body or understand the functions of functional components (*29*). However, safety assessments at the cellular level may not fully capture the effects of metabolism that food components undergo in the body, leading to insufficient verification of their toxicity. Moreover, the information obtained from genome sequences is limited to known factors, thus proving inadequate when exploring unknown functionalities and toxicities. From the perspective of complementing these data, there is a need for new experimental models capable of alternative organisms with multiple organs as evaluation systems. Therefore, we propose the use of silkworms, an insect species, as an experimental model for investigating the functionality and safety of food components (*6,12,20,24,29*).

The human gut bacteria discovered in this study hold promise for use in food development research as probiotics and for research and development aimed at isolating active substances to obtain useful compounds. The differences in the innate immune-stimulatory ability in the silkworm among the bacterial strains discovered in this study (based on muscle contraction assay) are presumed to stem from differences in cell wall composition among the strains. For example, bacterial cell walls contain a polysaccharide-based structure called peptidoglycan. Peptidoglycan has a structure in which glycan chains consisting of repeating units of *N*-acetylglucosamine and *N*-acetylmuramic acid are cross-linked by short peptide chains (*30*). It is known that the structure of peptidoglycan varies among bacterial species (*30,31*). Furthermore, it has been reported that peptidoglycan, through peptidoglycan recognition proteins (PGRPs), is recognized by insects, triggering their innate immune response (*32*). Although the differences in peptidoglycan structure among the gut bacteria used in this study may potentially influence the differences in muscle contraction activity, the investigations into such structure-activity relationships are future tasks. Especially, for such bacterial-derived polysaccharides, it is considered useful to conduct biochemical purification of active substances based on physiological activity, as well as verification using chemically synthesized compounds of the respective substances (verification based on partial structures).

Additionally, an important research question remaining is whether the innate immune-stimulatory effect of gut bacteria administration demonstrated in this study can induce immune priming response of silkworms, as we have previously reported, and thereby confer resistance to human pathogenic microbial infections (*6,33*). Through such investigations, it is anticipated that the full extent and mechanism of action of the innate immune-stimulatory function of the samples identified in this study will be elucidated at the molecular level, leading to the creation of functional food ingredients that can be extrapolated to humans.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Kaito C, Akimitsu N, Watanabe H, Sekimizu K. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. Microb Pathog. 2002; 32:183-190.
2. Hamamoto H, Kurokawa K, Kaito C, Kamura K, Manitra Razanajatovo I, Kusuhara H, Santa T, Sekimizu K. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. Antimicrob Agents Chemother. 2004; 48:774-779.
3. Fujiyuki T, Imamura K, Hamamoto H, Sekimizu K. Evaluation of therapeutic effects and pharmacokinetics of antibacterial chromogenic agents in a silkworm model of Staphylococcus aureus infection. Drug Discov Ther. 2010; 4:349-354.
4. Miyashita A, Iyoda S, Ishii K, Hamamoto H, Sekimizu K, Kaito C. Lipopolysaccharide O-antigen of enterohemorrhagic *Escherichia coli* O157:H7 is required for killing both insects and mammals. FEMS Microbiol Lett. 2012; 333:59-68.
5. Miyashita A, Kizaki H, Kawasaki K, Sekimizu K, Kaito C. Primed immune responses to gram-negative peptidoglycans confer infection resistance in silkworms. J Biol Chem. 2014; 289:14412-14421.
6. Miyashita A, Takahashi S, Ishii K, Sekimizu K, Kaito C. Primed Immune Responses Triggered by Ingested Bacteria Lead to Systemic Infection Tolerance in Silkworms. PLoS One. 2015; 10:e0130486.
7. Tabuchi F, Matsumoto Y, Ishii M, Tatsuno K, Okazaki M, Sato T, Moriya K, Sekimizu K. Synergistic effects of vancomycin and β-lactams against vancomycin highly resistant *Staphylococcus aureus*. J Antibiot (Tokyo). 2017; 70:771-774.
8. Tabuchi F, Matsumoto Y, Ishii M, Tatsuno K, Okazaki M, Sato T, Moriya K, Sekimizu K. D-cycloserine increases the effectiveness of vancomycin against vancomycin-highly resistant *Staphylococcus aureus*. J Antibiot (Tokyo). 2017; 70:907-910.
9. Hamamoto H, Panthee S, Paudel A, Ishii K, Yasukawa J, Su J, Miyashita A, Itoh H, Tokumoto K, Inoue M, Sekimizu K. Serum apolipoprotein A-I potentiates the therapeutic efficacy of lysocin E against *Staphylococcus aureus.* Nat Commun. 2021; 12:6364.
10. Mikami K, Sonobe K, Ishino K, Noda T, Kato M, Hanao M, Hamamoto H, Sekimizu K, Okazaki M. Evaluation of pathogenicity and therapeutic effectiveness of antibiotics using silkworm *Nocardia* infection model. Drug Discov Ther. 2021; 15:73-77.
11. Miyashita A, Hamamoto H, Sekimizu K. Applying the silkworm model for the search of immunosuppressants. Drug Discov Ther. 2021; 15:139-142.
12. Miyashita A, Sekimizu K. Using silkworms to search for lactic acid bacteria that contribute to infection prevention and improvement of hyperglycemia. Drug Discov Ther. 2021; 15:51-54.
13. Hamamoto H, Urai M, Ishii K, *et al.* Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. Nat Chem Biol. 2015; 11:127-133.
14. Miyashita A, Mitsutomi S, Mizushima T, Sekimizu K. Repurposing the PDMA-approved drugs in Japan using an insect model of staphylococcal infection. FEMS Microbes. 2022; 3:xtac014.
15. Ishii K, Hamamoto H, Kamimura M, Nakamura Y, Noda H, Imamura K, Mita K, Sekimizu K. Insect cytokine paralytic peptide (PP) induces cellular and humoral immune responses in the silkworm *Bombyx mori*. J Biol Chem. 2010; 285:28635-28642.
16. Dhital S, Hamamoto H, Urai M, Ishii K, Sekimizu K. Purification of innate immunostimulant from green tea using a silkworm muscle contraction assay. Drug Discov Ther. 2011; 5:18-25.
17. Fujiyuki T, Hamamoto H, Ishii K, Urai M, Kataoka K, Takeda T, Shibata S, Sekimizu K. Evaluation of innate immune stimulating activity of polysaccharides using a silkworm (*Bombyx mori*) muscle contraction assay. Drug Discov Ther. 2012; 6:88-93.
18. Ha SD, Nagata S, Suzuki A, Kataoka H. Isolation and structure determination of a paralytic peptide from the hemolymph of the silkworm, *Bombyx mori*. Peptides. 1999; 20:561-568.
19. Ishii K, Hamamoto H, Kamimura M, Sekimizu K. Activation of the silkworm cytokine by bacterial and fungal cell wall components *via* a reactive oxygen species-triggered mechanism. J Biol Chem. 2008; 283:2185-2191.
20. Miyashita A, Kataoka K, Tsuchida T, Ogasawara AA, Nakajima H, Takahashi M, Sekimizu K. High molecular weight glucose homopolymer of broccoli (*Brassica oleracea* var. *italica*) stimulates both invertebrate and mammalian immune systems. Front Food Sci Technol. 2023; 3. https://doi.org/10.3389/frfst.2023.1012121
21. Tanihiro R, Sakano K, Oba S, Nakamura C, Ohki K, Hirota T, Sugiyama H, Ebihara S, Nakamura Y. Effects of Yeast Mannan Which Promotes Beneficial Bacteroides on the Intestinal Environment and Skin Condition: A Randomized, Double-Blind, Placebo-Controlled Study. Nutrients. 2020; 12:3673.
22. Tanihiro R, Yuki M, Sasai M, Haseda A, Kagami-Katsuyama H, Hirota T, Honma N, Nishihira J. Effects of Prebiotic Yeast Mannan on Gut Health and Sleep Quality in Healthy Adults: A Randomized, Double-Blind, Placebo-Controlled Study. Nutrients. 2023; 16:141.
23. Oba S, Sunagawa T, Tanihiro R, Awashima K, Sugiyama H, Odani T, Nakamura Y, Kondo A, Sasaki D, Sasaki K. Prebiotic effects of yeast mannan, which selectively promotes *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* in a human colonic microbiota model. Sci Rep. 2020; 10:17351.
24. Li W, Miyashita A, Sekimizu K. Peanut triacylglycerols activate innate immunity both in insects and mammals. Sci Rep. 2022; 12:7464.
25. Varyukhina S, Freitas M, Bardin S, Robillard E, Tavan E, Sapin C, Grill JP, Trugnan G. Glycan-modifying bacteria-derived soluble factors from *Bacteroides thetaiotaomicron* and *Lactobacillus casei* inhibit rotavirus infection in human intestinal cells. Microbes Infect. 2012; 14:273-278.
26. Delday M, Mulder I, Logan ET, Grant G. *Bacteroides thetaiotaomicron* ameliorates colon inflammation in preclinical models of Crohn's disease. Inflamm Bowel Dis. 2019; 25:85-96.

27. Ihekweazu FD, Fofanova TY, Queliza K, Nagy-Szakal D, Stewart CJ, Engevik MA, Hulten KG, Tatevian N, Graham DY, Versalovic J. *Bacteroides ovatus* ATCC 8483 monotherapy is superior to traditional fecal transplant and multi-strain bacteriotherapy in a murine colitis model. Gut microbes. 2019; 10:504-520.
28. Lynch AM, Wilcox P. Review of the performance of the 3T3 NRU *in vitro* phototoxicity assay in the pharmaceutical industry. Exp Toxicol Pathol. 2011; 63:209-214.
29. Matsumoto Y, Ishii M, Hasegawa S, Sekimizu K. *Enterococcus faecalis* YM0831 suppresses sucrose-induced hyperglycemia in a silkworm model and in humans. Commun Biol. 2019; 2:157.
30. Vollmer W, Blanot D, De Pedro MA. Peptidoglycan structure and architecture. FEMS Microbiol Rev. 2008; 32:149-167.
31. Vollmer W. Structural variation in the glycan strands of bacterial peptidoglycan. FEMS Microbiol Rev. 2008; 32:287-306.
32. Kordaczuk J, Sułek M, Wojda I. General overview on the role of Peptidoglycan Recognition Proteins in insect immunity. Acta Biochim Pol. 2020; 67:319-326.
33. Miyashita A, Kizaki H, Kawasaki K, Sekimizu K, Kaito C. Primed immune responses to gram-negative peptidoglycans confer infection resistance in silkworms. J Biol Chem. 2014; 289:14412-14421.

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