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Preliminary identification of gut microbes between normal and diseased *Dorcus titanus castanicolor* (Coleoptera: Lucanidae)

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Abstract

The popularity of keeping stag beetles (*Dorcus titanus castanicolor* Motschulsky 1861, *Coleoptera: Lucanidae*) as pets has increased. Consistent with the rise in the number of insect farms using these beetles, the number of contaminated or diseased *D. titanus castanicolor* has also increased. This investigation was conducted to analyze the cause of *D. titanus castanicolor* disease. The contaminated larvae of *D. titanus castanicolor* showed *Allomyrina* nudivirus infection symptoms similar to those of *Allomyrina* nudivirus infection. However, the disease carried by of *D. titanus castanicolor* is not derived from the virus infecting *Allomyrina*, as determined by PCR. Our study revealed that the major gut microbes of infectious *D. titanus castanicolor* belonged to the phylum Proteobacteria, and specifically, *Pseudomonas knackmussi* (Symptom 1 - 39.62% to Symptom 2 - 41.50% to Symptom 3 - 76.76% as the disease progressed severely) and *Citrobacter koseri* (Symptom 1 - 1.48% to Symptom 2 - 6.04% to Symptom 3 - 6.16% as the disease progressed severely) were detected. Additionally, a high proportion of larvae from the uninfected group were found to harbor bacteria belonging to the phylum Firmicutes (72%). However, as the disease progressed severely in these beetles, the proportion of Firmicutes decreased (Symptom 1 - 72.03% to Symptom 2 - 44.7% to Symptom 3 - 26.3%). These findings imply that colonization by Firmicutes was inversely proportional to Proteobacteria colonization in the gut. This was found to be true for both the normal and disease conditions of *D. titanus castanicolor*. In this study, we examined the distribution of intestinal microbial communities in normal and contaminated larvae. We observed a correlation between these contaminated microbes and the overall health of the beetle, and our findings suggest that there may be a link between disease progression and the gut microbiome.

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Introduction

In the field of agriculture, the value of insect resources is being increasingly recognized in the field of agriculture, and correspondingly, the market for insects is growing. In addition, insects are now being widely used for pollination, animal feeding, medicine development, educational purposes, and as pets. *Dorcus titanus castanicolor* (Motschulsky, 1861; Hong and Lee, 2014) have recently been included in the insect market as pets and for educational purposes along with the Japanese rhinoceros beetle (*Allomyrina dichotoma*). *D. titanus castanicolor* (Coleoptera: Lucanidae) is one of the largest species of stag beetles in Korea and can be found throughout the country. The body length including the big jaw is 38–85 mm (male) or 28–44 mm (female), and their life span is approximately 1–2 years. The adult stag beetles appear in April–September and feed on the sap of oak trees, while their larvae grow on rotten oak wood and leaf litter.

As the number of insect industry farms growing, beetle farms have suffered severe economic losses, with the spread of the beetle disease in Korea including virus and entomological fungi (Kwak *et al*., 2018; Yun *et al*., 2017; Lee *et al*., 2015a). The stag beetle disease commonly occurs in the insect farms and has been spreading since 2017 in South Korea. Also, *Allomyrina* virus disease continues to occur since April 2014 (Lee *et al*., 2015a) until now in Gyungi, Chungcheong, Gangwon, Jeolla, Gyungbuk province in South Korea (Lee *et al*., 2015a; Lee *et al*., 2015b). Therefore, to identify the cause of the disease, diseased insects were collected from breeding farms, expecting that they would be infected by viruses or bacteria. After the experiments, it is possible to determine the specificity of the breeding environment by comparing the intestinal bacterial community between diseased and healthy beetles. Therefore, this study was conducted to examine the distribution of intestinal microorganisms of insects with these symptoms through the analysis of intestinal microorganisms of diseased insects and to confirm the distribution of the total flora according to the severity of symptoms.

Earlier investigations of internal microorganisms associated with various insects led to identification of diverse bacteria. Microorganisms were present in the insect epidermis and midgut, in addition to the symbiotic bacteria and opportunistic resistant symbiotic bacteria inside cells (Gurung *et al*., 2019; Kikuchi, 2009). These protective microbial symbiotic species are not unique to stag beetles but are found in a variety of insects (Boucias *et al*., 2018; Gurung *et al*., 2019). Furthermore, symbiotic bacterial communities are known to be important for nutrient utilization, digestion, development of host organisms, second generation sex ratios, defense against external environmental stresses, and interactions with pathogens (Douglas, 2011; Feldhaar, 2011; Cartagena *et al*., 2014; Wernegreen, 2012). Therefore, the aim of this study was to confirm the intestinal bacterial distribution in relation to the degree of disease by analyzing the intestinal bacteria of normal *D. titanus castanicolor* and comparing the bacterial communities to those in infected larvae of *D. titanus castanicolor.*

Materials and Methods

Collection of *Dorcus titanus castanicolor* larvae and isolation of midgut

Disease occurrence in larvae of *D. titanus castanicolor* was first identified at insect rearing farms in Daejeon, Korea, in August 2018. The normal and contaminated larvae of *D. titanus castanicolor* were collected from August 2018 to August 2019. *D. titanus castanicolor* larvae were collected along with the sawdust surrounding them and placed one by one in a plastic bucket. Both healthy and infected larvae were starved of feed for approximately 5 days, and then washed with tertiary distilled water, sterilized with 70% alcohol, and the midgut dissected. The segment of midguts of infected insects and normal insects were stored separately at -70 °C. Further, next generation sequencing and analysis was performed on the midgut segments. The analysis of the intestinal microbial community was commissioned to Macrogen (Seoul, Koera). The library preparation kit used was Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 and the 16S Metagenomic Sequencing Library was used to confirm bacterial community distribution.

Diagnosis of *Allomyrina* virus using Polymerase Chain Reaction (PCR)

To diagnose diseased larvae, viral DNA was isolated from the larval sample and amplified using PCR with a pair of primers, AdV-F1 and -R1 (Lee *et al*., 2015b). The hemolymph was also analyzed in this study. Hemolymph was extracted

from the beetles, centrifuged at 2,000 × *g* for 15 min at 4 °C, and DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, USA). The viral DNA was amplified via PCR using AccuPower PCR premix (Bioneer, Daejoen, Korea) under the following conditions: denaturation at 95 °C for 3 min, 35 cycles of 1 min at 94 °C, 30 s at 57 °C, and finally, 45 s at 72 °C (Lee *et al*., 2015b). The primer sequences were as follows: Primer set 1: Forward Primer1 5′-TCCGGAAATTACACGAGCCAC-3′; Reverse Primer1 5′-ATGCCGTACGAGAGTATGGTCG-3′. The loading volume of PCR product was 15μL per well in 1% agarose gel.

MiSeq-metagenomic sequencing from sample Quality Control (QC) to sequencing

DNA was extracted using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, USA) according to the manufacturer's instructions. Each sequenced sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols. The quantification of DNA and the DNA quality was measured using PicoGreen and NanoDrop. The 16S rRNA genes were amplified using 16S V3-V4 primers. The primer sequences were as follows: 16S V3-V4 primer: 16S Amplicon PCR Forward Primer 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGCCTACGGGNGGCWGCAG-3′; 16S Amplicon PCR Reverse Primer 5′- GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGACTACHVGGGTATCTAATCC-3′.

Input gDNA was amplified with 16S V3-V4 primers and a subsequent limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. The final products were normalized and pooled using PicoGreen, and the size of libraries were verified using the TapeStation DNA ScreenTape D1000 (Agilent, USA). Thereafter, we sequenced the DNA using the MiSeq™ platform (Illumina, San Diego, USA).

* Amplicon PCR (1st PCR): a. 3 min at 95 °C; b. 25 cycles of: 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; c. 5 min at 72 °C; d. Hold at 4 °C

* Index PCR (2nd PCR): a. 3 min at 95 °C; b. 8 cycles of: 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; c. 5 min at 72 °C; d. Hold at 4 °C

Results and Discussion

Disease symptom

The normal and diseased larvae of *Dorcus titanus castanicoloar* larvae were shown in Fig.1. The color of the head in normal larval is bright orange and the surface of the epidermis is light and creamy-white (Fig. 1A). The cracks on the larvae are highly distinct, and inside of the crack line is a round groove and the jaw is slightly curved inwards (Fig. 1A). Infected *D. titanus castanicolor* larvae presented the following severe symptoms: all tissue in the body melted, and the cuticle became transparent and filled with pale ivory lymph with no smell. The pale-colored lymph was visible either over the entire body or just a part of it (Fig. 1B). These symptoms were similar to those reported for the larvae infected with the *Allomyrina* virus. Korean horn beetles (*Allomyrina dichotoma*) were killed nationwide by infection with *Allomyrina* virus (AV) in Korea (Lee *et al*., 2015a; Lee *et al*., 2015b).

The diseased larvae samples of *D. titanus castanicolor* were collected from insect farm in Daejeon and shown in Fig. 2. The larvae could be categorized three groups; Symptom 1, Symptom 2, and Symptom 3 (Fig. 2). The diseased larvae appear pale, clear lymph and fairly translucent. The diseased larvae bodies become very soft and juicy. Severity of symptoms in infected *D. titanus castanicolor* larvae were devided into three categories (1, 2, and 3) depending on the distribution area of pale, milky, and transparenct in the whole body. Symptoms 1, 2, and 3 were defined as degree of soft and juicy of the whole body, respectively, and the symptom degree was divided according to the translucent part filled with clear lymph.

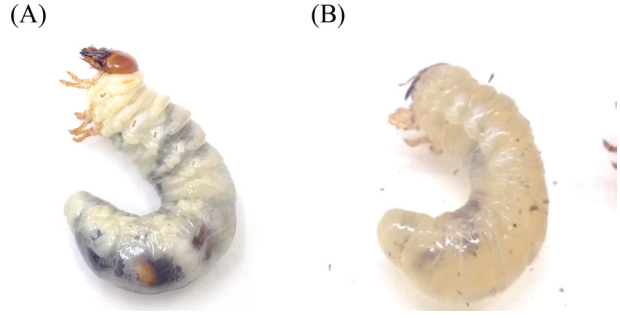

Fig. 1. Photographs of normal and diseased *Dorcus titanus castanicolor* larvae (A) healthy larva (B) the one contaminated larva.

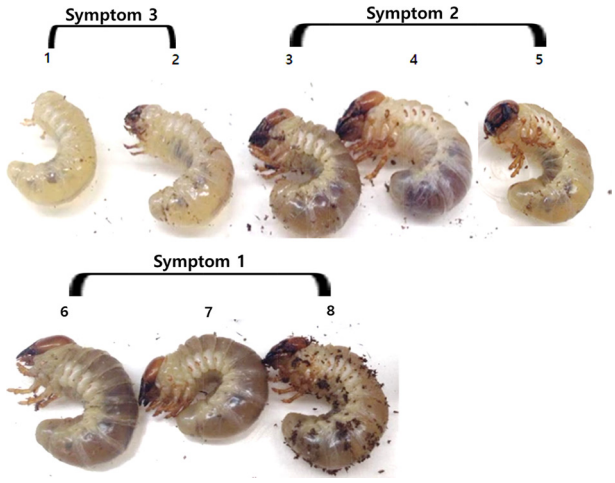

Fig. 2. Photograph of the infected larvae of *Dorcus titanus castanicolor* (Coleoptera: Lucanidae) collected from insect rearing farm in Daejeon, South Korea (Symptom 1: larvae 6 - 8; Symptom 2: 3 - 5; Symptom3: 1, 2).

Virus diagnosis

The following experiments were performed, because the contaminated larvae of *D. titanus castanicolor* showed symptoms similar to those of *Allomyrina* virus infection and as *D. titanus castanicolor* belongs to the same family Coleoptera. AV was confirmed in anticipation of the *Allomyrina* viral infection in the stag beetles. Therefore, to identify *Allomyrina* virus (AV), PCR was conducted using AV sensitive primers 1 (Lee *et al*., 2015b). AV primer set 1 was used to amplify the DNA from the hemolymph extracted from eight larvae with pathological symptoms, and one normal larva. PCR result of larvae that had pathological symptom was negative for AV virus (Lee *et al*., 2015b) (Fig. 3).

Sample 1 to 8 represent pathological symptoms (Fig. 3), and sample 9 is a healthy sample (Fig. 1A). Regardless of the severity of symptoms, the *Allomyrina* virus was found to be negative in the results of Polymerase Chain Reaction (PCR) of all *D. titanus castanicolor* larvae, including normal ones. The AV was negative in all individuals of *A. dichotoma* larvae, but also more than 95 % negative for Entomopoxviruses, Iridoviruses, Nodaviruses and Cypoviruses that recorded host order to Coleoptera (data not shown). Therefore, this data was confirmed that the contaminated larvae of *D. titanus castanicolor* were not infected AV viruses because of the host specificity.

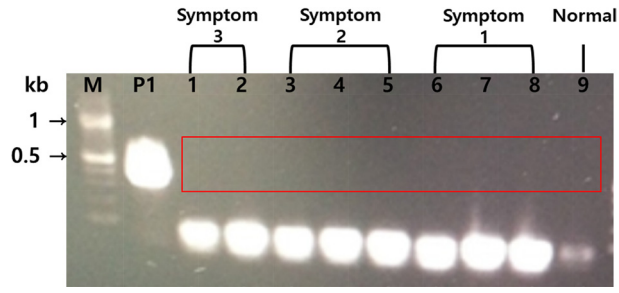

Fig. 3. Diagnosis of *Allomyrina* virus by PCR using primer set (M: Marker, P1: Positive control using primer set 1; 644 bp, Lane 1 – 9: experimental hemolymph from diseased *Dorcus titanus castanicolor* sample 1 – 8, Lane 9: normal larva hemolymph sample 9).

Classification of gut microbes

Symptoms 1, 2, and 3 indicate the severity of disease progression from 1 to 3 (Fig. 2). Symptom 1, 2, and 3 were defined as conditions where degree of the translucent body filled with clear lymph, respectively (Fig. 2). To find the disease cause of *D. titanus castanicolor*, gut microbe of normal and contaminated larvae was screened and identified through BLAST analysis of Mi-seq data (Table 1, Fig. 4). Eight phylum including Euryarchaeota, Actinobacteria, Bacteroidia, Chloroflexi, Firmicutes, Proteobacteria, Tenepicutes, and Verruomicrobia were detected.

In the Archaea kingdom, Euryarchaeota phylum was present as 0.01, 0.04, 0.11, and 0.01 % in normal conditions and symptoms1, 2, and 3 respectively. The Actinobacteria phylum was identified as 0.12, 0.20, 0.05, and 0.01 % in normal subjects, and those with symptoms1, 2, and 3, respectively. Bacteroidetes was 27.11, 7.29, 19.83, and 0.28 % in normal conditions and, symptoms 1, 2, and 3, respectively. The Chloroflex phylum absent in healthy subjects, were detected as 0.01, 0.00, and 0.01 % in symptoms 1, 2, and 3, respectively. The Firmicutes phylum showed a normal distribution of 72.03, 44.70, 26.33 and 11.38 % in normal and Symptom 1, 2, and 3, respectively. The Firmicute phylum showed a normal and Symptoms 1, 2, and 3groups, respectively. On the other hands, the distribution of Proteobacteria phylum increased with disease progression and was found to be 0.12, 42.18, 51.52, and 88.29 % in normal conditions and, Symptoms 1, 2, and 3, respectively. The Tenericutes phylum was detected only in healthy subjects (0.36 %), symptom 2 – 0.00 %, Symptom 3 – 0.00 % were found to be distributed only in the normal group,

Table 1. Closest sequences relatives identified through BLAST analysis of the Mi-seq sequence data from *Dorcus titanus castanicolor* larvae.

Kingdom	Taxon (rank)	Organism
Archaea	Euryarchaeota (Phylum)	*Methanobrevibacter arboriphilus*
Bacteria	Actinobacteria (Phylum)	*Chryseoglobus flavigena*
		Plantactinospora endophytica
	Bacteroidia (Phylum)	*Dysgonomonas alginatilytica*
		Dysgonomonas gadei
	Chloroflexi (Phylum)	*Ktedonobacter racemifer*
	Firmicutes (Phylum)	*Acetanaerobacterium elongatum*
		Anaerotignum aminivorans
		Anaerotruncus colihominis
		Anaerotruncus rubiinfantis
		Christensenella massiliensis
		Clostridium colinum
		Clostridium propionicum
		Desulfosporosinus fructosivorans
		**Desulfitobacterium metallireducens*
		Ethanoligenes harbinese
		Harryflintia acetispora
		Intestinimonas butyriciproducens
		Lustipora thermophile
		Monoglobus pectinilyticus
		Natranaerovirga pectinivora
		**Ruthenibacterium lactatiformans*
		Sporomusa acidovorans
	Proteobacteria (Phylum)	*Aquincola tertiaricarbonis*
		Acidovorax wautersii
		Diaphorobacter aerolatus
	Gammaproteobacteria (Class)	*Citrobacter koseri*
		** *Pseudomonas entomophila*
		**Pseudomonas knackmussii*
		Serratia fonticola
		Serratia marcescens
		Stenotrophomonas rhizophila
	Tenepicutes (Phlyum)	*Acholeplasma brassicae*
	Mollicute (Class)	*Spiroplasma helicoides*
	Verruomicrobia (Phylum)	*Terrimicobium sacchariphilum*

* separated from contaminated ground water (Finneran *et al*., 2002, Stolz *et al*., 2007, Shkoporov *et al*., 2016)
** known as insect pathogenic bacteria (Vodovar *et al*., 2006, Dieppois *et al*., 2015)

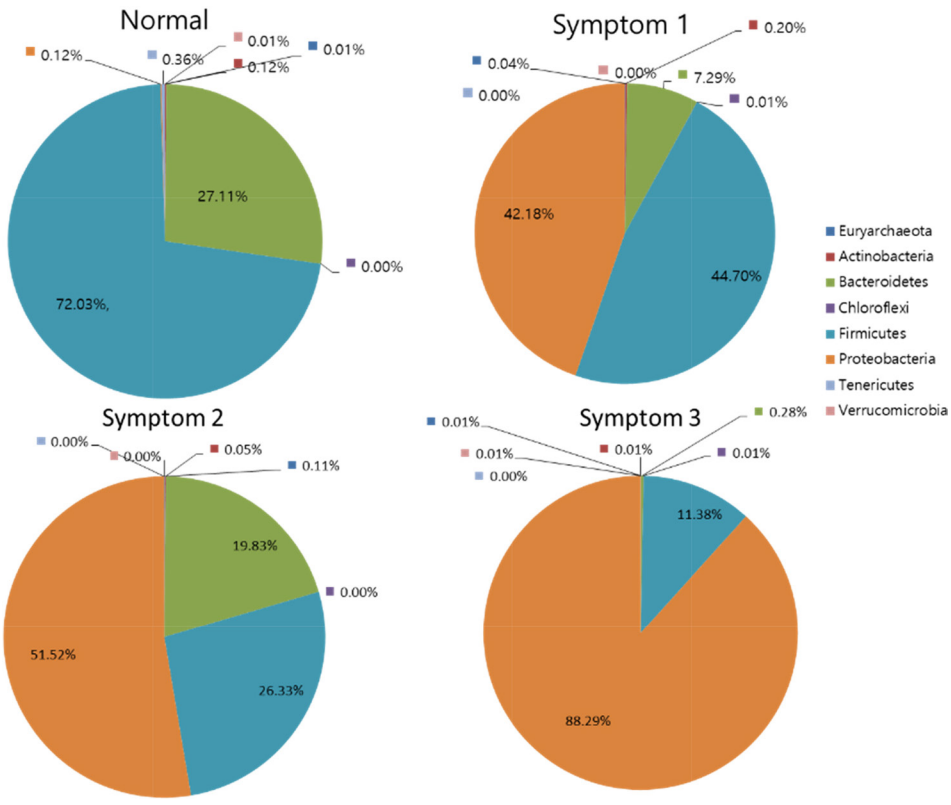

Fig. 4. Summary of bacterial groups of phylum from the larvae of *Dorcus titanus castanicolor* (Coleoptera: Lucanidae) derived from categorized as diseased and normal colonies. BLAST hit for over 99% of sequences from 16S rRNA clones was a sequence obtained in previous studies on bacterial associates. The sequenced bacterial categories are: Euyarchaeota, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Tenericutes, Verrucomicrobia.

and the Verrucomicrobia phylum was identified in healthy subjects (0.01 %), and absent in diseased groups.

In the four most severely infected larvae, 88.3% of marker sequences belonged to the Proteobacteria phylum-level group, whereas in the other two infected larvae, 51.3% and 42.2% were Proteobacteria and it was 0.1% in normal larvae (Fig. 4). Conversely, in the normal larvae, the Firmicutes phylum-level group, constituted 72% of marker sequences, and as the severity of pathological symptoms increased, the population of Firmicutes decreased (Symptom 1 – 11.4%; Symptom 2 – 26.3%; Symptom 3 – 44.7%; Normal – 72.03%; Fig. 4).

The analysis of microbial populations identified in normal *D. titanus castanicolor* larvae showed that Firmicutes accounted for 72% of marker sequences, Bacteroidetes accounted for 27.1%, and Proteobacteria accounted for 0.1%. In contrast, the infected insects showed 99.3%, 11.4%, and 0.3% of marker sequences belonging to Proteobacteria, Firmicutes, and Bacteroidetes, respectively. In other words, the Firmicutes group decreased rapidly and the Proteobacteria group increased rapidly from the normal to the diseased group (Fig. 4).

The Actinobacteria group, that accounted for 0.1 – 0.2% of marker sequences in the normal, early (Symptom 1), and middle stages (Symptom 2) of infection, was absent (0%) in the most severely infected beetles (Symptom 3). Tenericutes was detected only in normal subjects, and not in the diseased groups. Therefore, the phylum Tenericutes may be a marker that distinguishes between normal and diseased individuals (Symptom 1, 2, and 3). *Acholeplasma brassicae* and *Spiroplasma helicoides* from the phylum Tenericutes were identified only in the normal subjects.

Screening of gut microbes in normal and contaminated larvae

According to the health status of *D. titanus castanicolor*, the distribution of gut microbes are classified four types. Intestinal microbial analysis was performed according to intensification of the symptoms (Fig. 4). Intestinal microorganisms were identified in the normal group and infected groups according to

the symptoms. The identified microorganisms were distributed as follows according to each type. In the Archea kingdom, *Methanobrevibacter arboriphilus* was identified in both normal and diseased individuals, with a slightly increased occurrence (0.01 - 0.4%) in diseased individuals, compared to normal ones (0.01%). However, the difference was not large. In the Bacteria kingdom, *Chryseoglobus flavigena* was found to be present in 0.06% abundance in the Actinobacteria phylum, but the proportion decreased to 0.00 – 0.01% in diseased individuals. In addition, *Plantactinospora endophytica* was found to have a relative proportion of 0.04% and 0.00% in normal and diseased individuals, respectively.

In addition, *Dysgonomonas alginatilytica* belonging to the phylum Bacteroidetes were more common in normal beetles compared to in diseased beetles, (Symptom 1 – 0.06%; Symptom 2 – 10.46%; Symptom 3 – 3.03%; Normal – 27.11%). Meanwhile, *Anaerotignum aminivorans* and *Lutispora thermophila* from the phylum Firmicutes were 150 – 200 times more common in diseased individuals (Fig. 4, Table 1). On the other hand, *Dysgomonas gadei* was absent in healthy subjects. However, in diseased subjects, it was detected as 8.26 and 2.96% corresponding to symptoms 1 and symptom 2, respectively. These observations confirmed the initial increase in distribution of bacteria.

The Chloroplexi phylum absent in healthy larvae, were detected as 0.01, 0.00, and 0.01 in symptoms 1, 2, and 3, respectively.

The Firmicutes phylum showed normal distribution of 72.03, 44.70, 26.33, and 11.38% in normal and symptom 1, 2, and 3 groups, respectively. In the Firmicutes phylum, these gut microbes were identified; *Acetanaerobacterium elongatum, Anaerotignum aminivorans, Anaerotruncus colihominis, Anaerotruncus rubiinfantis, Christensenella massiliensis, Clostridium colinum, Clostridium propionicum, Desulfosporosinus fructosivorans, Desulfitobacterium metallireducens, Ethanoligenes harbinese, Harryflintia acetispora, Intestinimonas butyriciproducens, Lustipora thermophile, Monoglobus pectinilyticus, Natranaerovirga pectinivora, Ruthenibacterium lactatiformans,* and *Sporomusa acidovorans.*

The specific bacteria among these microbiobes, the Firmicutes phylum*, Desulfitobacterium metallireducens* (22.11%) and *Desulfosporosinus fructosivorans* (3.47%), were identified in normal individuals, of which *D. metallireducens* is the first known isolate from uranium-contaminated groundwater. The food and sawdust that the insects were in contact with should be inspected more closely for traces of radioactive substances. However, in diseased individuals, the proportion of these bacteria decreased considerably, and was present at only 0.1%.

Ruthenibacterium lactatiformans in the Firmicutes phylum was found to be absent in normal individuals. But Symptom 1 (early stage), 2, and 3 (middle stage) were associated with respective fractions of 25.47 %, 3.68 %, and 1.64 %, and 0.00 % in the normal stage. Therefore, the disease increased significantly in the early stages of the disease, but the fraction decreased as the disease worsened. The difference in fractions confirmed that the distribution of bacteria increased by 25 times in infected subjects, compared to normal ones. In addition, *Ruthenibacterium lactatiformans* (25.47 %) that occurred in the early stages of the disease may be used for early diagnosis. Importantly, *R. lactatiformans* was first isolated from the caecum of chickens, and whether the species is entomopathogenic needs to be determined in future studies (Shkoporov *et al*., 2016). Furthermore, byproducts associated with this bacterium have the potential to make breeding food inedible for insects. In the severest manifestation of the disease, the bacteria detected were the following species: *Aquincola tertiaricarbonis*, *Acidovorax wautersii*, *Diaphorobacter aerolatus*, and *Serrtia fonticola* from the phylum Proteobacteria.

In the Proteobacteria phylum, these gut microbes were identified; *Aquincola tertiaricarbonis, Acidovorax wautersii, Diaphorobacter aerolatus, Gammaproteobacteria class*: *Citrobacter koseri, Pseudomonas entomophila, Pseudomonas knackmussii, Serratia fonticola, Serratia marcescens,* and *Stenotrophomonas rhizophila.* Especially, *Pseudomonas knackmussii* (Symptom 1 – 39.62%; Symptom 2 – 41.50%; Symptom 3 – 76.76%), and *Citrobacter koseri* (Symptom 1 – 1.48%; Symptom 2 – 6.16%; Symptom 3 – 6.04%), belonging to the phylum Proteobacteria were found only in diseased beetles (Table 1).

Citrobacter koseri, a Proteobacteria known as an opportunistic bacterium, was 0% in normal insects but was 1.48% in the less infected beetles (Symptom 2) and reached 6.04% in the severely infected larvae (Symptom 3). Gram-negative bacteria, *Pseudomonas knackmussii*, isolated from sewage treatment plants (Stolz *et al*., 2007), were found in very high abundance in all diseased individuals, 39.62–76.76%, with the highest values in the most severely infected beetles. Therefore, this bacterium can be considered as the main pathogenic bacterium. Similar to the case of *D. metallireducens*, the identification of *P. knackmussii* suggested that contaminated water (Stolz *et al.*, 2007) may have been used

at the farm. Subsequently, it was confirmed that the groundwater used as a food source in the farms, contained wild insects. It was important to determine this because *P. knackmussii* is known to degrade haloaromatic compounds.

Pseudomonas entomophila in the Proteobacteria phylum, related to *P. putida*, is a bacterium that can infect insects (Vodovar *et al*., 2006). In the present study, this bacterial strain was shown to be present as 0.01% in both normal and early disease (Symptom 1). However, *P. entomophila* was absent in both mid- and late stages of the disease (Symptom 2 and 3). Therefore, *P. entomophila* may be a major cause of the disease, but it does not appear fatal, so it is not considered to be caused by this species. However, since it was detected in normal condition and early disease stages, it may be necessary to confirm the cause and progression of disease through *in vivo* experiments.

The Tenericutes phylum, *Acholeplasma brassicae* and *Spiroplasma elicoides* (Mollicute class) identified distribution of 0.21% and 0.15% in normal larvae respectively, and absent in the diseased larvae (Table 1).

On the other hands, the intestinal bacteria species composition depends on the host's diet and its nutritional balance (Choi *et al*., 2015). Digestive organs are divided into the cuticle-lined foregut (full length), midgut, and hindgut, and it is known that there are differences in bacterial cluster complexity depending on the organ site and age (Boucias *et al*., 2018; Ohbayashi *et al*., 2015; Dillon and Dillon 2004). A correlation exists between food specificity and symbiotic bacteria (McLean *et al*., 2016; Skidmore and Hansen 2017; Rook *et al*., 2017). Intestinal bacteria also serve to protect the host from pathogenic microorganisms and inhibit infection by opportunistic bacteria. Therefore, depending on the effect of infected pathogens, the number of beneficial intestinal bacteria may increase or decrease. Consequently, based on these theories, symbiotic bacteria were decreased due to increasing of opportunistic and pathogenic bacteria in this research. According to many studies on microbes, almost all insects are known to have symbiotic bacteria, which can potentially defend them against other pathogens (Kituchi, 2009). These symbiotic bacteria can survive in the presence of antimicrobial chemicals or modulators. They are also known to inhibit opportunistic pathogenic microorganisms, thus, further strengthening the host's immune system and protecting the surrounding microbial system. (Boucias *et al*., 2018; Douglas, 2011). Therefore, it is necessary to investigate the relationship between gut microbes related with immune system and entomopathogenic pathogens in further researches.

In other insect such as honeybees, extensive research is being conducted on intestinal microbes. In the case of honeybees, a study on the gut microbe community of insects by Engel and Moran (2013) showed that the intestinal microbes of bees comprising *Snodgrassella alvi*, *Gilliamella apicola*, and *Lactobacillus* spp. were mainly from the pollen and honey in their diet (Engel and Moran 2013; Gilliam, 1997). Honeybees contain 8 to 10 colonies of bacteria that belong to two species, *Snodgrassella alvi* and *Gilliamella apicola*, as well as species belonging to *Bifidobacterium* and *Lactobacillus* (Raymann and Moran 2018; Cox-Foster *et al*., 2007). Groups known as opportunistic or secondary pathogenic bacteria include *Serratia*, *Hamiltonella*, *Spiroplasma,* and *Regiella* spp. Among these bacteria, the occurrence of harmful substances produced in *Pseudomonas aeruginosa* is inhibited by organobromine (2-(4-bromophenyl)-2-oxoethyl benzoate) produced by the pathogenic bacterium *Aspergillus parasiticus* (Moreno Romo *et al*. 1986; Cartagena *et al*., 2014). Based on the above studies, the Proteobacteria occupied in the gut microbes of Honey bee larvae compared to those in the *D. titanus castanicolor* or adult honey bee guts; in the adult honey bee guts, the order Lactobacillales generally dominates. The data showed dissimilar results regarding the characteristics of Proteobacteria and Firmicutes in *D. titanus castanicolor*.

This study confirms the distribution of gut microbes in *D. titanus castanicolor* larvae bred using contaminated groundwater in a specific area. By identifying the bacterial communities according to the severity of the disease, it may be possible earlier to control and suppress the outbreak of the disease. The characteristics and functions of the gut microbes identified from *D. titanus castanicolor* larvae can be used as a basis for identifying microorganisms that can be used in other fields.

In conclusion, despite sharing similarities with the symptoms of *Allomyrina* nudivirus disease, the disease under consideration is not a viral infection. Systemic research would be required in a precisely controlled environment to understand the observed mechanism of *D. titanus castanicolor* disease occurrence.

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