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D. Kairat, A. Smagulova, V. Kiyan*

Research Platform of Agricultural Biotechnology,
S. Seifullin Kazakh Agrotechnical University, Kazakhstan, Nur-Sultan
*e-mail: vskiyan@gmail.com

INVESTIGATION OF *GALLERIA MELLONELLA* MICROBIOME TO DETERMINE ITS SPECIES COMPOSITION

Microbiome research is a key important method of microbiological research that can be beneficial in solving modern problems. Organisms with a normal microbiome are less susceptible to pathogens, as well as, probiotic features of symbiotic bacteria in the microbiome positively act in their sustainable development and survival. The current study aimed to characterize the gut microbiome of greater wax moth larvae – Galleria mellonella and its species composition. A total of 38 bacterial isolates from the gut microbiome of greater wax moth larvae were identified by using 16S rRNA gene analysis. Isolates of microorganism from G. mellonella larvae could be grouped into three phyla: Bacillus (60%), Rhizobium (20%), and Pseudomonas (20%). Morphological and phylogenetic analysis showed that bacterial strains belonging to Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus subtilis, Rhizobium pusense and Pseudomonas parafulva and were dominant in the gut microbiome Galleria mellonella. Bacterial strains isolated from larvae gut separately can be used in biotechnology, agriculture, and ecology.

Key words: Galleria mellonella, gut microbiome, 16S rRNA sequencing.

Д. Қайрат, А. Смағұлова, В. Киян*

Ауылшаруашылық биотехнологиясының ғылыми платформасы,
С. Сейфуллин атындағы Қазақ агротехникалық университеті, Қазақстан, Нұр-Сұлтан қ.
*e-mail: vskiyan@gmail.com

Galleria mellonella баланқұртының микробиомын зерттеу және оның құрамын анықтау

Микробиомды зерттеу – заманауи мәселелерді шешуде пайдалы болуы мүмкін микробиологиялық зерттеулердің негізгі әдісі. Қалыпты микробиомы бар ағзалар патогендерге аз сезімтал, ал микробиомдағы симбиотикалық бактериялардың пробиотикалық қасиеттері олардың тұрақты дамуы мен өмір сүруіне оң әсер етеді. Баланқұрттардың симбиотикалық ішек микроағзалары бейтаныс микроағзаларды және токсиндерді жоюға көмектеседі, сондай-ақ жәндіктің иммундық жүйесінің жұмысын арттырады. Ішек микроағзалары баланқұрттардың метаболизміне қатысып ферментативті белсенділігін арттырады, осыған орай ішек микроағзаларының және жәндіктің ферменттерінің синергизмі пластикалық полимерлерді биологиялық ыдырату мүмкіншілігі зерттелуде. Осы зерттеу Galleria mellonella балауыз көбелегі дернәсілдерінің ішек микробиомын және оның түрлік құрамын сипаттауға бағытталған. Балауыз көбелегі дернәсілдерінің ішек микробиомынан барлығы 38 бактериялық изолят 16S rRNA гендік анализімен анықталды. G. mellonella дернәсілдерінен оқшауланған микроорганизмдерді үш негізгі тұқымға бөлуге болады: Bacillus (60%), Rhizobium (20%) және Pseudomonas (20%). Морфологиялық және филогенетикалық талдау көрсеткендей, Galleria mellonella ішек микробиомында Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus subtilis, Rhizobium pusense және Pseudomonas parafulva түрлеріне жататын бактериялық штамдар басым болған. Баланқұрттардың ішектерінен бөлек оқшауланған бактериялық штамдарды биотехнологияда, ауыл шаруашылығында және экологияда қолдануға болады.

Түйін сөздер: Galleria mellonella, ішек микробиомы, 16S рРНҚ секвенирлеу.

Д. Кайрат, А. Смагулова, В. Киян*

Исследовательская платформа сельскохозяйственной биотехнологии,
Казахский агротехнический университет им. С. Сейфуллина, Казахстан, г. Нур-Султан
*e-mail: vskiyan@gmail.com

Исследование микробиома *Galleria mellonella* для определения его видового состава

Исследование микробиома – это ключевой метод микробиологического исследования, который может быть полезен при решении современных проблем. Организмы с нормальным микробиомом менее восприимчивы к патогенам, а пробиотические свойства симбиотических

бактерий в микробиоме положительно влияют на их устойчивое развитие и выживание. Симбиотические кишечные микроорганизмы личинок способствуют нейтрализации инородных патогенных микроорганизмов и их токсинов, а также повышают активность иммунной системы насекомого. Кишечные микроорганизмы участвуют в метаболизме личинок и повышают их ферментативную активность, в связи с чем изучается синергизм кишечных микроорганизмов и ферментов насекомых для определения возможности биоразложения пластичных полимеров. Настоящее исследование направлено на характеристику микробиома кишечника личинок восковой моли – Galleria mellonella и его видовой состав. Всего 38 бактериальных изолятов из микробиома кишечника личинок восковой моли было идентифицировано с помощью анализа гена 16S рРНК. Выделенные избличинок G. mellonella микроорганизмы микроорганизмов можно разделить на три основных рода: Bacillus (60%), Rhizobium (20%) и Pseudomonas (20%). Морфологический и филогенетический анализ показал, что штаммы бактерий, принадлежащие к видам Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus subtilis, Rhizobium pusense и Pseudomonas parafulva, доминировали в микробиоме кишечника Galleria mellonella. Штаммы бактерий, выделенные отдельно из кишечника личинок, могут быть использованы в биотехнологии, сельском хозяйстве и экологии.

Ключевые слова: Galleria mellonella, микробиом кишечника, секвенирование 16S рРНК.

Introduction

Microorganisms are ubiquitous organisms found almost everywhere on our planet; further more, they can be found in living organisms. The community of microorganisms living in and on bodies of living organisms is called “microbiome”. The definition of the term “microbiome” first given by Joshua Lederberg, states: “the ecological community of commensal, symbiotic and pathogenic microorganisms that share our body space” [1]. Microorganisms not only present in the gut, moreover, but they also found on the body surface of different plants and animals, including humans, and are capable of carrying out several metabolic tasks, which ordinary body cells do not perform [2].

Microbiome research of insects is increasing importance in understanding their vital functions and communication with other branches of life; hence, an enormous number of insects are involved in symbiotic, parasitic or commensal interactions. For instance, *Drosophila melanogaster* gut microbiome research, carried out by Angela E. Douglas illustrates that insects and their microbiome haveundeniable value as a model organism for microbiome research, including genetic and genomic investigations in microbiome manipulations accomplished in different conditions [3].

Microbial interactions occur not only within one organism but also in natural communities, involved in synergism and antagonism between different species. Microbiomes of insects, in case, represent uninvestigated interactions that can be used in different areas of biotechnology and medicine, for example, in antimicrobial drug discovery. Recent scientific research that was done by Marc G. Chevretteet al. showed insect microbiome-derived *Streptomyces* antimicrobial metabolites tend to be more active than soil-derived *Streptomyces* strains [4]. Exploration of the insect microbiome compositions has great potential as a valuable source of new substances and interactions with other species in the environment.

Several usages of *Galleria mellonella* in biology and medicine had been reported [5-8]. For the past two decades, microbiologists have searched alternatives to mammals for studying the molecular basis of virulence and for testing antimicrobial drugs. Tsai et al. Made a literature review which reported the value of *G. mellonella* larvae as a model for investigating bacterial pathogens. The authors highlight many of the attractive features of this model: when compared with mammals, *G. mellonella* larvae are cheaper and easier to maintain, they do not require specialized laboratories or equipment and work with *G. mellonella* does not require ethical approval. Unlike many alternative models, *G. mellonella* can be maintained at 37°C. It can be an essential feature of this model is the ease with which the larvae can be injected with precise doses of a pathogen, allowing the relative virulence of strains and mutants to be compared [9].

In a limited number of studies done by Péchy-Tarr M. et al. showed that preparations from either bacteria or fungi that have been injected into *G. mellonella* to study their toxicity were less virulent to the larvae. In many cases, the toxins studied are known to be insecticidal, and *G. mellonella* larvae provide an excellent model to investigate toxicity [10].

Wojda et al. made researches about *G. mellonella* immunity, describing anatomical and physiological barriers of insects, protecting them against invasion by microorganisms [11]. While *D. melanogaster* is used to study the genetic aspect of insect immunity,

G. mellonella can serve as a good model for biochemical research [12]. According to the size of the insect, it is possible to easily obtainhemolymph and other tissues as a source of many immune-relevant polypeptides. Therefore, larvae serve as a model to study the virulence mechanisms of human pathogens. Besides, Wojda et al. affirm that antibacterial and antifungal peptides derived from insects and proteins can be considered and applied as alternatives to antibiotics according to their potential [13].

According to the research done by Paolo Bombelli et al. biodegradation of polyethylene is possible by larvae of the wax moth *G. mellonella*, producing ethylene glycol [14]. However, the question that whether the hydrocarbon-digesting activity of *G. mellonella* derives from the organism itself, or enzymatic activity of larval gut microbiome remains unsolved.

A recent scientific study carried out by MélyssaCambronelet al. describes a successful implementation of *G. mellonella* larvae as a model for the *P. aeruginosa* H103 virulence demonstration that had been treated with epinephrine [15].

G. mellonella is one of the common testing organisms in the investigation of several pesticides, insect pathogens, and biologically active substances. However, most properties of this organism remain unstudied. Immunity and highsurviving abilities of organisms closely related to their microbiome and symbiosis. Digestive tract (gut) of *G. mellonella* had been studied to obtain knowledge about its microbiological composition via microbiology and molecular genetic analysis techniques.

Materials and methods

Sample collection and microbiome isolation

The samples of *G. mellonella* larvae were collected from the family apiary in the Akmola region, Kazakhstan (Fig.1). Honeycombs contaminated with larvae were used for further larvae proliferation in laboratory conditions. A larvae sample was treated with 70% ethanol for 2-3 min, to avoid contamination from the caterpillar surface. Thereafter larvae samples were treated with sterile 1× PBS (pH 7.2-7.4) and moved them to a glass slide for the preparation, isolating the insect intestine. The isolated intestine then was moved to a 1 ml sterile tube with 1× PBS with 0.9% sodium chloride and centrifuged at 3000 RPM for 5 min. After the centrifugation, larvae gut tissues were carefully removed from the tube, remained suspension was vortexed and used as inoculum.

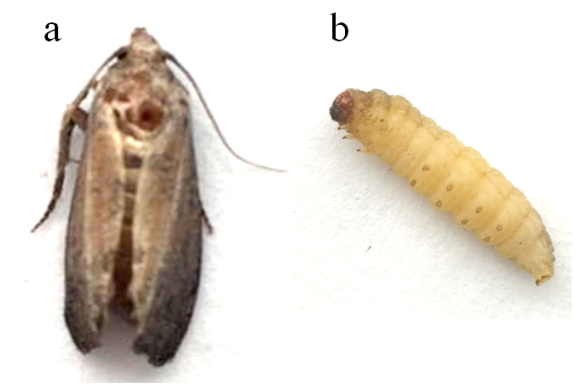


Figure 1 – Wax moth *G. mellonella***:** a – adult moth, b – larva

Luria-Bertani (LB) broth (Sigma-Aldrich, USA) (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 2.2 g/L inert binding agents, and pH 7.2) was used for the isolation and culture of bacteria present in larvae gut tissues. Isolated gut bacteria were inoculated to culture media under sterile conditions and cultivated at 37°C in an incubator for 24 hours under 150 RPM on a laboratory shaker. After the 24-hour cultivation of gut bacteria, we made a smear, to carry out microscopy of cells via Gram staining. After the 24-hour cultivation of gut bacteria, did a smear and carry out microscopy of cells via Gram staining. Serial dilutions (10^{-1} – 10^{-10}) were made for larvae gut tissue samples. The dilutions from 10^{-3} to 10^{-6} were inoculated on LB agar plates to isolate single colonies. Plates were incubated at 37°C until the appearance of bacterial colonies. Bacterial colonies were studied by morphological properties and using microscopy. The bacteria were purified by repeated sub-culturing of single colonies.

DNA extraction and molecular characterization

The genomic DNA of separate colonies of the microorganisms was isolated using the bacterial DNA isolation kit (“Biosilica”). The isolation was performed according to the kit instructions. The quality of genomic DNA was monitored by electrophoresis on a 1 % agarose gel. Electrophoresis was carried out in a Max Fill HU10 horizontal electrophoresis chamber and a Consort EV 243 current source. 1× TAE buffer was used as an electrode buffer. The 16S rRNA was amplified using the primer pair: forward *16SrRNA-8F* (5’-AGAGTTTGATCCTGGCTCAG-3’) and reverse *16SrRNA-806R* (5’- GGACTACCAGGGTATCTAAT-3’) (Sigma-Aldrich, USA).For all used primers, we prepared 20 µl mixture that contained 25 ng of each target DNA. The mixture also contained Taq DNA

Polymerase (Fermentas), 0.2 mM of each dNTP, 1× PCR buffer, 2.5 mM $MgCl_2$, and 10 pmol of each primer. The PCR program was run on a Master cycler Gradient, (Eppendorf) amplificator.

PCR samples purification

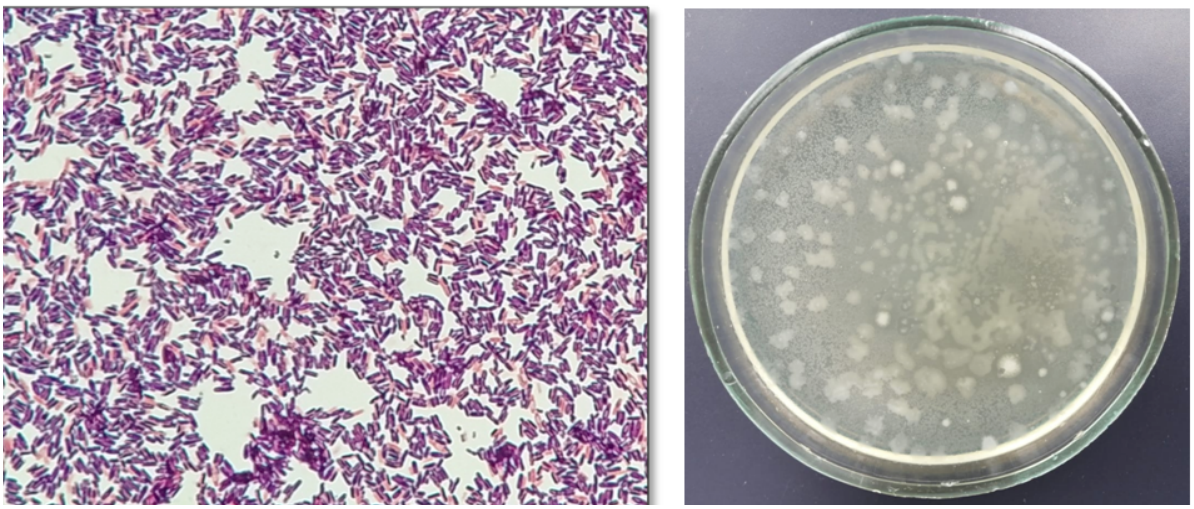
PCR samples were purified from oligonucleotide residues by dephosphorylation using alkaline phosphatase (SAP – shrimp alkaline phosphatase) and endonuclease. A mixture was prepared in a total volume of 10 µl for each sample – dH_2O – 7.25 µL, 10× PCR Buffer – 1.0 µl, $MgCl_2$ – 1.0 µl, SAP (5 mM) – 2.5 µl, Exonuclease I (5 units/µL) – 0.125 µl. The resulting mixture was added to each PCR product, placed in a thermal cycler under the following conditions: 37°C – 30 min, 85°C – 15 min, 4°C – ∞. Sample preparation for sequencing carried out by precipitation with an alcohol-acetate mixture.

DNA sequencing

The components of a standard set of reagents for the sequencing reaction were prepared in a 0.2-ml thin-walled thermocycler tube. A standard set of reagents for cyclic sequencing using *CEQ WellRED* terminator dyes (partially mixed). The following thermal cycle program was chosen: 96℃ – 20 sec, 50℃ – 20 sec, 60℃ – 4 min for 30 cycles and followed by aging at 4℃. The sequencing was done by using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the sequence was deposited in GenBank. These sequences were compared with other sequences in the GenBank by using the BLAST analysis. The phylogenetic analysis was carried out with MEGA 6 software.

Results

The phenotypic characteristics of the isolated bacterial strains. Microorganisms obtained from the *Galleria mellonella* intestine showed a multitude of different strains of microorganisms. The total culture samples containing various types of microorganisms having morphological and microscopic characteristics (Fig. 2). The nature of the growth of colonies on LB broth nutrient medium and the results of staining total culture samples showed that their microorganisms belong to bacterial strains.



a b

Figure 2 – Microscopy ofthe sample from larvae gut tissues

The intestinal microbiome of wax moth larvae showed a great many species of microorganisms, which subsequently were divided into five groups according to their main properties and characteristics. Each group is distinguished by the features of culture growth and data of microscopic analysis (Fig. 3). The most common bacterial species included *Bacillus* strains. Three of five groups (strain #1, 6, 8) were characterized by spore formation and similar growth patterns, which allowed these strains of microorganisms to be classified as *Bacillus* spp. (Fig. 4). The fourth and fifth groups of bacteria were characterized by the presence of pronounced properties for bacteria of the genus *Rhizobium spp.* (strain #3) and *Pseudomonas spp.*, (strain #5) respectively (Fig. 3, 5).

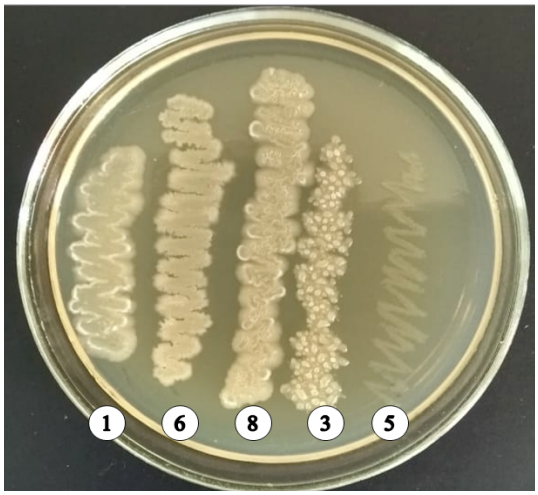


Figure 3 – The growth of colonies of microorganisms in the LB medium

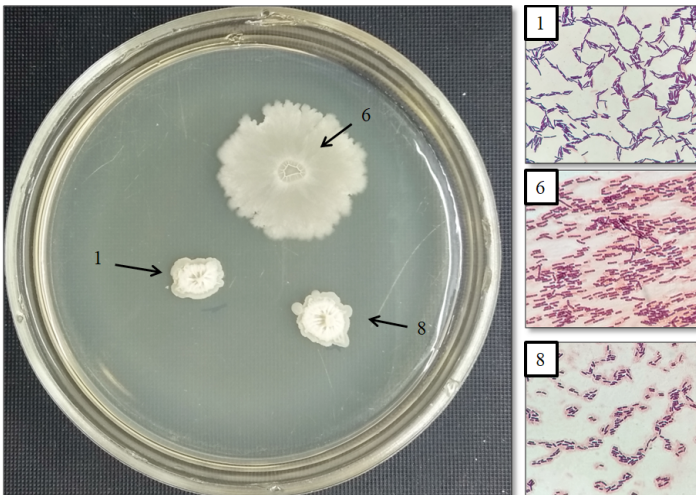


Figure 4 – The growth of colonies and microscopy analysis the strains of *Bacillus spp.*

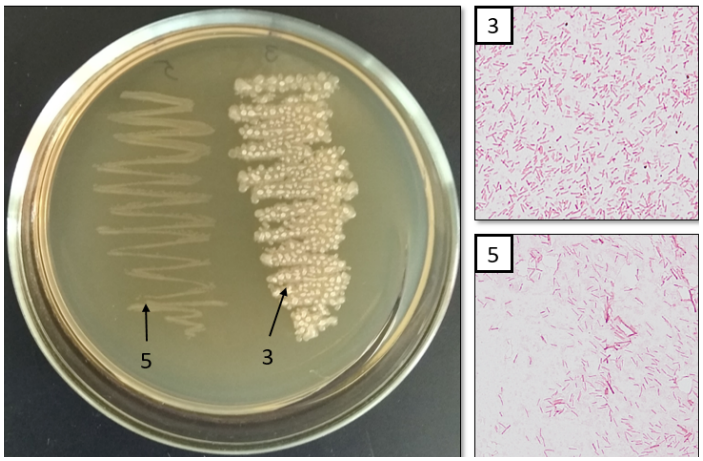


Figure 5 – The *Rhizobium spp.* and *Pseudomonas spp.* strain growth patterns in LB agar medium

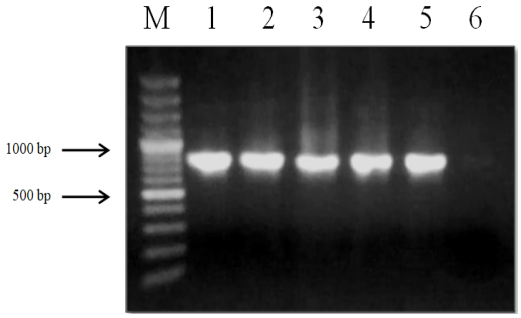


Figure 6 – Electrophoretic analysis of PCR products obtained with DNA of the bacterial strains: Lane M, DNA ladder (bp); lane 1-3, *Bacillus spp.* DNA; lane **4,** *Pseudomonas spp.* DNA; lane 5, *Rhizobium spp***.** DNA; lane 6, negative control.

The phylogenetic analysis of the bacterial strains by gene sequences. The amplification of the genome DNA from the bacterial strains allowed obtaining products of approximately 800-900 bp when using species-specific primers (Fig. 6).

The PCR products of bacterial strains were subjected to sequence analysis. The nucleotide sequences of the studied species were deposited in NCBI GenBank database (*B. amyloliquefaciens* isolate MT015597.1, *B. velezensis* isolate MT022411.1, *B. subtilis* isolate MT498848.1 and *R. pusense* isolate MT022412.1. The phylogenetic analysis showed that isolates from *G. mellonella* larvae could be grouped into three phyla: *Bacillus* (60%), *Rhizobium* (20%), and *Pseudomonas* (20%).

Discussion

Historically, microbiome researches have always been not sufficient enough to investigate it more deeply. Methods based on ordinary cultivation are not capable of growing different communities of bacteria from a variety of different taxonomic groups; certain types of bacteria are unable to cultivate either. However, with the development of Sanger's sequencing technology, bacterial identification in the microbiome became easier and cost-effective. This scientific breakthrough, with the support of bioinformatics, has unveiled several new frontiers in the analysis of microbiome, including its community structure, pathogenic microorganisms detection in the microbiome with their virulence

mechanisms and whole bacterial community interactions, like commensalism, mutualism, and amensalism [16].

Insects are the most diversified and plentiful life formon our planet, found almost in every ecological niche. Widespread success and evolutionary progress of insects are connected with their close communication and cooperation with beneficial bacteria. As a result, microorganisms facilitate the digestion of nutrient-poor food sources, protect from pathogens and parasites, take part in intraspecific communication, and regulate their reproductive processes. Microorganisms, primarily located in the gut, also contribute to certain relevant functions that are connected with medicine, ecology, and agriculture. Besides, several species of insects can be implemented as laboratory models for microbial interactions investigation between different bacteria or with their hosts in metabolic or immunity cases [17].

Recent microbiome studies of the *Galleria mellonella* microbiome in the investigation of polyethylene and polystyrene revealed *Bacillus* and *Pseudomonas* strains contributing to larvae in the digestion of represented plastic polymers [18]. Isolated *Bacillus* and *Pseudomonas* strains can colonize and partly degrade polystyreneand polyethylene, causing plastic weight loss in the range of 0.5-1.5 percent for *Bacillus* [19] and 23 percent HIPS (High Impact Polystyrene) film degradation for *Pseudomonas* after treatment with bromine-containing compounds respectively [20]. In our studies, we were also able to isolate and identify these two types of bacteria (Fig. 3-4). In addition, by species sequencing via 16S rRNA primers, the species affiliation with *B. amyloliquefaciens, B. velezensis, B. subtilis*, and *Pseudomonas* was shown.

Isolated *Bacillus amyloliquefaciens* strains are commonly used in the production of amylases and proteases in industrial scales; further more current bacteria also have specific probiotic properties with no harmful effect for humans or animals [21, 22]. Besides, *Bacillus* genus bacteria often act as a plant growth-promoting bacteria (PGPR) found in soil, simultaneously acting as a biocontrol agent regarding several phytopathogenic fungi that cause plant diseases. Recent studies have shown that *Bacillus amyloliquefaciens* strain has antagonistic activity against *Fusarium graminearum* phytopathogenic fungus, commonly recognized as Fusarium Head Blight (FHB) inducing agent [23]. Furthermore, *Bacillus* species can be a struggle factor for pathogenic fungi nutrition.

Bacillus subtilis is a ubiquitous organism that can be found and isolated from soil, air, water, and dead plant matter. Bacteria belonging to the genus *Bacillus* are grampositive, rod-shaped, straight cells often arranged in chains ranging in size from 0.5 to 2.5 × 1.2-10 μm. According to the Bergey Manual of Systematic Bacteriology, strains belonging to the genus *Bacillus* are chemo-organotrophs, express respiratory or enzymatic metabolism, ferment glucose, leading to acid production, are positive in the catalase test and do not reduce sulfates until sulfides. Several biochemical features of the genus, for example, nitrate reduction and oxidase formation, can vary as well as species-dependent [24]. Numerous *Bacillus subtlils* species have also been found in the gastrointestinal tract of animals and insects, possibly, as an indirect result of the consumption of plants [25]. The *Bacillus* strain spores impact by which, especially those of *B. subtilis* species, act as probiotics, is not entirely understood. Bacillus subtilis thought to have beneficial probiotic effects, including antimicrobial production, stimulation of the immune system, and an overall improvement in intestinal microflora [26].

Bacterial strains isolated from the *Galleria mellonella* intestine have a tremendous biotechnological implementation, for further commercial goods production in agriculture or healthcare purposes.

Conclusion

The scientific study of *Galleria mellonella* gut microbiome strains and their morphological and molecular genetic properties. The gut microbiome of wax moth larvae showed a great multitude. Different metabolic pathways of gut microorganisms and their enzymatic differences give an ability to degrade several molecular complex substances like honeycomb wax. Microbial species multiplicity also helps host organisms to counter internal invasions by extracting antimicrobial metabolites and maintaining conditions in an interior of the organism, which is harmful to others. Such properties of wax moth larvae microbiome can act as valuable tools for the study of host and pathogen interactions. Using insect larvae can facilitate the identification of bacterial pathogens and give possibilities to discover new components that are involved in host innate immune responses and bacterial interactions.

Bacterial strains isolated from larvae gut separately can be used in biotechnology, agriculture, and ecology. Further investigations of bacterial properties must be performed. Our results show that wax moth larvae gut composition characteristics included a variety of microorganisms. Using 16

s rRNA sequencing, we obtained a result that the *Galleria mellonella* microbiome consists of the following microorganisms: *Bacillus amyloliquefaciens, Bacillus velezensis, Bacillus subtilis, Pseudomonas parafulva, Rhizobium pusense.*

More detailed studies of *Galleria mellonella* gut microbiome and possibly useful properties of microorganisms and the whole organism itself need further investigations.

Acknowledgments

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