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DETECTION OF PHYTOPATHOGENS *AGROBACTERIUM SPP.* AND THEIR ANTAGONISTS *BACILLUS THURINGIENSIS, ALCALIGENES FAECALIS* AND *LACTOBACILLUS PLANTARUM* IN FLOWERS AND BERRIES OF GRAPE

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Aim. *Detection of representatives of saprophytic (Bacillus thuringiensis, Alcaligenes faecalis, Lactobacillus plantarum) and pathogenic (Agrobacterium spp.) microbiota in flowers and berries of grape.* ***Materials and Methods.*** *Flowers, non-damaged green and ripe berries of grape were selected from a vineyard of Vitis vinifera L. cv Pinot noir located in Odessa region in May, July and September 2018, accordingly. Flowers or berries were homogenized and left for autofermentation process for 7 days. Real-Time polymerase chain reaction to detect species B. thuringiensis, L. plantarum, A. faecalis and Agrobacterium tumefaciens, Agrobacterium vitis was carried out with DNA isolated from the resulted fermented homogenate.* ***Results.*** *B. thuringiensis was prevalent among the tested species: these bacteria were identified in 50% of samples of grape flowers, in 90% of green berries and 20% of samples of ripe berries. L. plantarum was not detected in flowers, but these bacteria were identified in green and ripe berries (10% of tested samples). Results confirm literature data about the presence of B. thuringiensis and L. plantarum on grape. For the first time, we detected A. faecalis and Agrobacterium spp. in flowers and berries of grape. A. faecalis was found in 20% of flower samples, 60% of green berries and 20% of ripe berries. Agrobacterium spp. were detected in flowers and green berries (10% of samples), and in ripe berries these microorganisms were not found. Amount of detected species increased in green berries compared with the flowers, and decreased in ripe berries.* ***Conclusions.*** *For the first time, we detected A. faecalis and Agrobacterium spp. in flowers and berries of grape. Coexistence of phytopathogenic agrobacteria and their potential antagonists – Bacillus thuringiensis, Alcaligenes faecalis and Lactobacillus plantarum in one ecological niche (flowers and green grape berries) was revealed.*

Keywords: Bacillus thuringiensis, Lactobacillus plantarum, Agrobacterium tumefaciens, Agrobacterium vitis, Alcaligenes faecalis, saprophytic microbiota, phytopathogens, Vitis vinifera.

Microbiota of plants is a source of new strains perspective for biotechnology. Bacteria with strong antagonistic activity could be used for development of biological preparations for plant protection [1; 2]. Bacteria with GRAS (Generally Recognized As Safe) status and beneficial technological characteristics could be selected as starter cultures for fermentation of food products and beverages [3; 4].

Microbiota of grape is usually studied from the point of its possible influence on fermentation process and quality of wine [5; 6]. But some representatives of grape microbiota could be studied not only from the point of view of oenology, but also as perspective microorganisms for plant protection. According to investigations of Mezzasalma et al. (2017), grape microbiome includes 44 orders and 73 families of microorganisms. Bacteria from order *Bacillales* [7; 8; 9; 10; 11] and family *Pseudomonadaceae* [12; 13; 14; 15; 16] were found most often. Many representatives of these groups are characterized by strong antagonistic activity against phytopathogens and stimulation of plant growth [17; 18; 19]. For instance, some *Bacillus spp.* are known for their antagonistic effect on pathogenic agrobacteria – crown gall agents [20; 21; 22]. The prevalent species of bacilli on grape is *Bacillus thuringiensis* [8; 14; 23].

Besides the mentioned bacteria, in grape berries representatives of ß-*Proteobacteria* Class – microorganisms of order *Burkholderiales* [7] were found. One species from this order – *Alcaligenes faecalis* is known for its antagonistic activity against some phytopathogens [24; 25] including pathogenic *Agrobacterium tumefaciens* [26].

Lactic acid bacteria from genus *Lactobacillus* could be found during fermentation of grape must, but without preliminary enrichment they were detected just in few samples of berries in amount not exceeding 1,8% from total bacterial population [7]. Opposite, fermentation of crushed berries resulted in increase of quantity of lactobacilli (10^2–10^4 CFU/ml) and allowed to identify the species [10; 23; 27; 28]. Lactobacilli with their strong antagonistic activity were proposed for organic agriculture as antagonists against phytopathogens [29; 30] including crown gall agent *A. tumefaciens* [31].

Representatives of genus *Agrobacterium* were isolated from stems, roots, bark, rhizosphere [12; 15; 32; 33], leaves and dormant buds of grape [34]. Till this time agrobacteria have not been detected in flowers and berries of grape, such data were described only for tomato fruits [35].

Taking into account the wide spectrum of representatives of plant microbiota, isolation of strains antagonistic against certain phytopathogens should be carried out from plants – hosts of these phytopathogens. Coexistence in one ecological niche could result in increase of antagonistic reactions due to competition [36], and isolated strains could exhibit stronger potential for biotechnology of plant protection. Taking into account the results of previous investigations that showed antagonistic effect of *Bacillus* spp*., A. faecalis* and *L.plantarum* on phytopathogens *Agrobacterium vitis* and *A. tumefaciens* under laboratory conditions and on test-plants [20; 21; 26; 31], it was necessary to study the possibility of coexistence of all these microorganisms in one ecological niche on a plant.

The aim of this work was to detect representatives of saprophytic (*Bacillus thuringiensis, Alcaligenes faecalis, Lactobacillus plantarum*) and pathogenic (*Agrobacterium* spp.) microbiota in flowers and berries of grape.

Materials and Methods. Flowers, green and ripe berries of grape were selected from a vineyard of *Vitis vinifera* L. cv Pinot noir located in Odessa region in May, July and September 2018, accordingly. Chemical, biological pesticides or fertilizers have never been applied on this plot. Non-damaged flowers and berries, without any symptoms of diseases or insect damage were selected from 10 plants growing on a distance of 2 m from each other.

Flowers or berries were homogenized in sterile plastic tubes and left for autofermentation process without addition of any solutions [9] for 7 days at 24 – 25°C. Investigations were carried out in three repeats.

After 7 days, DNA from enriched bacterial cultures was isolated by test kit SureFast Prep Bacteria (Congen, Germany) according to manufacturer instruction. Real-Time polymerase chain reaction (PCR) was carried out with reaction mixture ssoFast Master Mix SYBER green (Bio-Rad, USA), which contained all the components except the primers. Real-Time PCR assay was used instead of classic PCR taking into account the possible little quantity of DNA of tested microorganisms in samples of plant material [37].

L. plantarum was detected with primers planF and pREV according to Torriani et al. (2001) [38]. Bacteria of *A. faecalis* species were found with primers 1F/2R according to Nakano et al. (2014) [39]. *B. thuringiensis* was identified using primers gro2F and gro2R according to Park et al. (2007) [40]. Detection of *Agrobacterium spp.* was carried out with primers VCF and VCR according to Suzaki et al. (2004) [41]. Use of this primer pair allowed to detect presence of two phytopathogenic species inhabiting grape – *A. vitis* and *A. tumefaciens* – simultaneously [41].

Real-Time PCR assay was carried out in a thermocycler CFX96 Real-Time System Bio-Rad (USA) using the following parameters: initial denaturation – 95°C – 8 min, after 40 cycles (95°C – 10 s, 60°C – 30 s, 72°C – 1 s). DNA of *L. plantarum* was detected using annealing temperature 56°C [38].

Results. Real-Time PCR data showed the presence of pathogenic *Agrobacteria* spp. and commensal *L. plantarum* in two samples of plant material from all tested (Fig. 1, A, D), saprophytic *A. faecalis* – in 10 samples (Fig. 1, B), and ubiquitous *B. thuringiensis* – in 16 samples (Fig. 1, C).

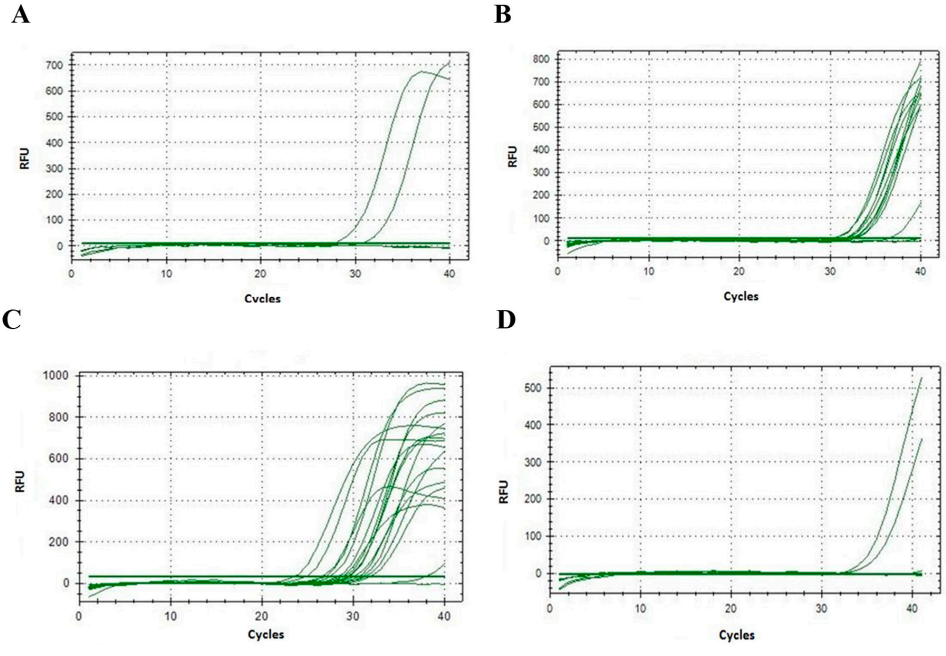

Fig. 1. Graphical representation of Real-Time PCR data showing relative fluorescence units measured during amplification of total DNA from flowers and berries of grape with primers specific to gene sequences of: A – *Agrobacterium* spp., B – *Alcaligenes faecalis*, C – *Bacillus thuringiensis*, D – *Lactobacillus plantarum*

According to the results of Real-Time PCR, Table 1 describing the occurrence of microorganisms on flowers, green berries and ripe berries was created. Presence of detected bacteria was not regular. Thus, tested microorganisms were found on grape in different vegetation phases – on flowers and berries (Table 1, Fig. 1).

Table 1

Results of detection of the tested bacterial species in grape samples

Type of samples	**Number of plant samples**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Flowers	**B.t.**	**B.t.**	**B.t.**						**B.t.**	**B.t.**
			A.f.						**A.f.**	
			A.spp.							
Green berries	**B.t.**	**B.t.**	**B.t.**		**B.t.**	**B.t.**	**B.t.**	**B.t.**	**L.p.**	**B.t.**
	A.f.		**A.f.**			**A.f.**		**A.f.**	**B.t.**	**A.f.**
			A.spp.						**A.f.**	
Ripe berries	**B.t.**		**A.f.**			**B.t.**			**L.p.**	**A.f.**

Note: B.t. – *Bacillus thuringiensis*; L.p. – *Lactobacillus plantarum*; A.f. – *Alcaligenes faecalis*; A. spp. – *Agrobacterium* spp.

There was an exception: none tested microorganisms were found on flowers and berries of plant №4. In plants №3 and №9 the majority of tested species were identified. But all four bacterial species were detected in none of the plants.

B. thuringiensis was identified most often – totally in 16 samples from 30 investigated (53,3% of samples). Species *A. faecalis* was found in 10 samples from 30 (33,3%), that also point out the wide spread of these bacteria on grape. Lactobacilli and agrobacteria were detected in 6,7% of samples (Fig. 2).

Amount of detected species increased in green berries compared with the flowers, and decreased in ripe berries. Thus, in ripe berries of the plants №2, №5, №7, №8 none tested species were found, and in the plants №1, №3, №6, №9, №10 amount of detected species decreased to one. In flowers tested microorganisms were present in 50% of samples.

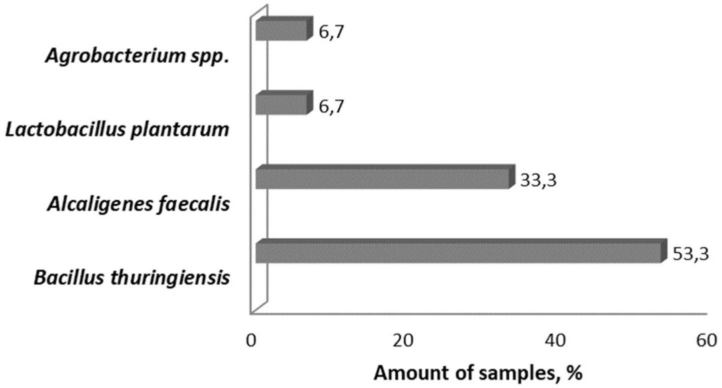

Fig. 2. Amount of samples (%) with detected microorganisms

In grape flowers *B. thuringiensis* was prevalent among the tested species: it was detected in 50% of samples (Fig. 3).

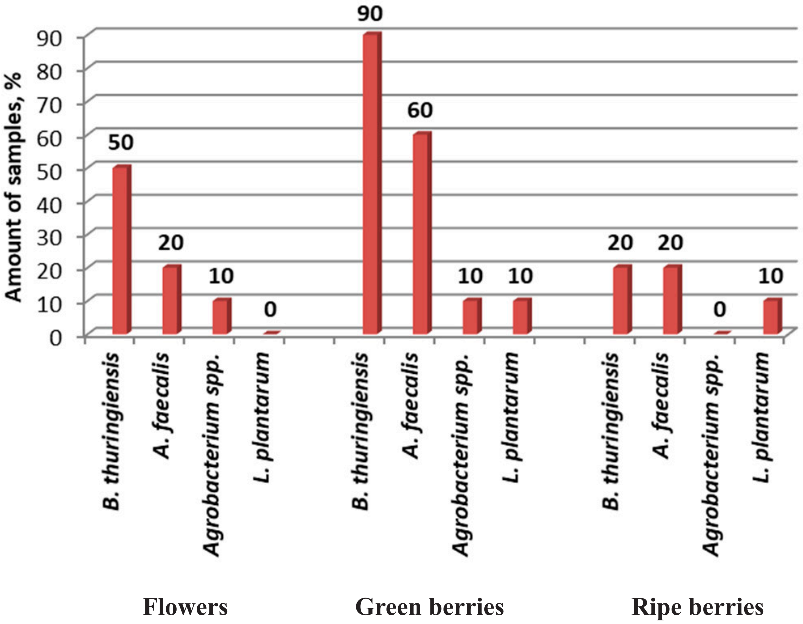

Fig. 3. Amount of samples (%) of flowers, green berries and ripe berries with detected microorganisms

In flowers from two plants (№3 and №9) *A. faecalis* was detected (20% of samples). Besides, on plant №3 *Agrobacterium* spp. were found in green berries together with bacilli and *A. faecalis*, but after, in ripe berries they were no longer detected.

In green berries, bacilli were also prevalent among the tested species – 90% of samples contained these bacteria, and *A. faecalis* was found also in significant amount of samples – in 60%.

In ripe berries species *B. thuringiensis* and *A. faecalis* were detected in equal quantity of samples (20%).

L. plantarum was not found in flowers, but this species was detected in green, and after – in ripe berries of the plant №9.

Thus, green berries retained the highest amount of the tested species, and ripe berries – the lowest.

Discussion. Our results confirm literature data about the presence of *B. thuringiensis* and *L. plantarum* on grape [7; 8; 9; 10]. As for *Alcaligenes* spp., there are just few works about isolation of these bacteria from grape, and they are mentioned as inhabitants of plant vessels [26; 42]. For the first time, we detected *A. faecalis* in flowers and berries of grape.

Presence of agrobacteria in flowers and fruits was earlier described for tomatoes [35], and for the grape we detected *Agrobacterium* spp. in flowers and berries for the first time.

Microbiota has a lot of influence on plants. Thus, it was shown that on vigorous plants high amount of microorganisms with phytostimulating activity were found [43]. It could be suggested that presence of *B. thuringiensis*, *L. plantarum* and *A. faecalis* – species with described phytostimulating activity [18; 22; 44; 45], has a favorable effect on grape plants. Presence of the phytopathogens *Agrobacterium* spp. in the same ecological niche indicates the possibility of coexistence of the studied species despite the fact that under laboratory conditions and on test-plants the mentioned saprophytic bacteria inhibited these pathogens [20; 21; 26; 31].

Few investigations on microbiota of flowers are described [14; 35; 46; 47]. It is known that flower microbiome is more diverse than microbiome of leaves [47]. Some bacterial taxons were specific for flowers and fruits and could not be found in other parts of the plants as it was described for tomatoes [35]. High occurrence of representatives of *Lactobacillus* genus was detected in flowers of apple on the third day after the start of blooming [47]. Compant et al. (2011) found that amount of endophytic cultivated microbiota in flowers and berries of grape gathered in Austria reached 2,77 ± 1,08 lg/g. High diversity in species of bacilli was revealed, including *B. thuringiensis* inhabiting both flowers and berries and seeds of grape [14]. For the first time, we found that grape flowers and berries could be inhabited by such species as *A. faecalis* and *Agrobacterium* spp. Pinto et al. (2014) showed the presence of bacteria from the family *Rhizobiaceae* on grape leaves, but the species in this study were not detailed [13]. Agrobacteria – the representatives of this family – have been previously found only in flowers and fruits of tomato by Ottesen et al. (2013).

Species diversity of grape berries microbiota is influenced by a large number of factors such as geographical location, climate, cultivation technique, presence of insects, microbial interactions, cultivar of grape, maturity stages of berries and their damage [5; 48]. Amount of microorganisms on green grape berries is usually higher (10^3 – 10^6 CFU/g) than on ripe berries (10^2 – 10^4 CFU/g) [9]. Species *B. thuringiensis* was prevalent on grape berries in investigations of previous authors, and amount of these bacteria on a phase of green berries reached 10^2 - 10^6 CFU/g depending on a vineyard and cultivar, and decreased till 10 – 10^2 CFU/g on a phase of ripe berries [9]. In our investigations bacilli were also prevalent among the tested species, inhabiting green berries of 90% of the plants (Fig. 3), and their occurrence significantly decreased (till 20%) in ripe grape berries that confirmed the data of previous authors.

Lactobacilli have not been found before in green grape berries [9; 23]. Occurrence of *Lactobacillus* spp. on ripe berries is widely studied due to possible effect of these microorganisms on fermentation of must [7; 10; 23; 27; 28]. Amount of lactic acid bacteria on grape berries usually does not exceed 10^2–10^4 CFU/g, and represented by such species as *L. plantarum, L. hilgardii, L. casei, L. sanfranciscansis, L. lindneri, L. kunkeei, L. mali, L. brevis, L. parabuchneri, L. paracasei, L. acidophilus, L. bifermentans, L. nageli* [5; 10; 23; 49]. We detected the presence of *L. plantarum* in green berries of one plant in which these bacteria retained also on a phase of ripe berries (Table 1). In grape flowers lactobacilli were not found. Our results confirm literature data about the non-regular occurrence of *Lactobacillus* spp. in grape samples [7; 9].

Study of succession of species in must from grape gathered in German vineyards showed that *Bacillus* spp. could be isolated on nutritional media in the fourth week of spontaneous fermentation, and lactobacilli (*L. buchneri, L. paracasei*) could be isolated after two weeks of fermentation [27]. Bae et al. (2006) revealed *L. plantarum* in berries from Australian vineyards on the 5th day of autofermentation when concentration of bacteria of this species reached 2,8 x 10^6 CFU/g and increased on the 10th day till 1,4 x 10^7 CFU/g [23]. We found lactobacilli after seven days of autofermentation of samples gathered both in July and September, which indicated that *L. plantarum* occured both on green and ripe berries.

Representatives of order *Burkholderiales* were found in grape berries by Mezzasalma et al. (2017), but species inside this order were not identified [7]. We found one species from the order *Burkholderiales – A. faecalis*, in green and ripe berries, and these bacteria were detected in 60% of samples of green berries that indicates their significant spread on grape plants. *A. faecalis* was also found in must where the quantity of samples with this microorganism decreased to 20% – the same as for bacilli.

Different content of bacterial species on grape plants probably depends on different inoculation sources. It is known that insects play important role in microbial colonization of grape berries [50]. We found the differences in species content of the tested microorganisms for flowers and green and ripe berries of grape cultivated in the south of Ukraine.

For the first time, we detected *A. faecalis* and *Agrobacterium* spp. in flowers and berries of grape. Coexistence of phytopathogenic agrobacteria and their potential antagonists – *Bacillus thuringiensis, Alcaligenes faecalis* and *Lactobacillus plantarum* in one ecological niche (flowers and green grape berries) was revealed. If phytopathogens and antagonistic saprophytes are able to coexist, it makes it necessary to monitor the effectivity of biological preparations after inoculation of plants.

ВИЯВЛЕННЯ ФІТОПАТОГЕНІВ *AGROBACTERIUM* SPP. ТА ЇХ АНТАГОНІСТІВ *BACILLUS THURINGIENSIS, ALCALIGENES FAECALIS* ТА *LACTOBACILLUS PLANTARUM* У КВІТАХ ТА ЯГОДАХ ВИНОГРАДУ

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Резюме

Мета. Виявлення представників сапрофітної (*Bacillus thuringiensis, Alcaligenes faecalis, Lactobacillus plantarum*) та патогенної (*Agrobacterium* spp.) мікробіоти у квітах та ягодах винограду. **Матеріали і методи**. Квіти, неушкоджені зелені та стиглі ягоди відбирали на виноградинку *Vitis vinifera* L. сорту Піно чорний в Одеській області у травні, липні та вересні 2018 р. відповідно. Квіти або ягоди гомогенізували і залишали для процесу аутоферментації на 7 днів. З утвореного ферментованого гомогенату виділяли ДНК і з нею проводили полімеразну ланцюгову реакцію у реальному часі для детекції видів *B. thuringiensis*, *L. plantarum*, *A. faecalis* та *Agrobacterium* spp. **Результати**. Серед досліджених видів переважав *B. thuringiensis*: ці бактерії були ідентифіковані у 50% зразків квітів винограду, у 90% зразків зелених ягід та у 20% стиглих ягід. *L. plantarum* у квітах не виявлявся, але був наявним у зелених, а потім – у достиглих ягодах винограду (10% досліджених зразків). Результати підтверджують дані літератури, які вказують на зустрічальність *B. thuringiensis* і *L. plantarum* на винограді. Наявність *A. faecalis* і *Agrobacterium* spp. у квітах та ягодах винограду показана нами вперше. *A. faecalis* було виявлено у 20% зразків квітів, у 60% зелених ягід та у 20% достиглих ягід. *Agrobacterium* spp. виявляли у квітах і зелених ягодах (10% зразків), а у стиглих ягодах цих мікроорганізмів знайдено не було. Кількість видів, що виявлялися, збільшувалася у зелених ягодах у порівнянні з квітами і зменшувалася у стиглих ягодах. **Висновки**. Наявність *A. faecalis* і *Agrobacterium* spp. у квітах та ягодах винограду показана нами вперше. Виявлено можливість співіснування в одній екологічній ніші (квіти і зелені ягоди винограду) фітопатогенних агробактерій та їх потенційних антагоністів – *Bacillus thuringiensis, Alcaligenes faecalis* та *Lactobacillus plantarum*.

Ключові слова: Bacillus thuringiensis, Lactobacillus plantarum, Agrobacterium tumefaciens, Agrobacterium vitis, Alcaligenes faecalis, сапрофітна мікробіота, фітопатогени, *Vitis vinifera.*

ВЫЯВЛЕНИЕ ФИТОПАТОГЕНОВ *AGROBACTERIUM* SPP. И ИХ АНТАГОНИСТОВ *BACILLUS THURINGIENSIS, ALCALIGENES FAECALIS* И *LACTOBACILLUS PLANTARUM* В ЦВЕТАХ И ЯГОДАХ ВИНОГРАДА

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Резюме

Цель. Выявление представителей сапрофитной (*Bacillus thuringiensis, Alcaligenes faecalis, Lactobacillus plantarum*) и патогенной (*Agrobacterium* spp.) микробиоты в цветах и ягодах винограда. **Материалы и методы**. Цветы, неповрежденные зеленые и спелые ягоды отбирали на винограднике *Vitis vinifera* L. сорта Пино черный в Одесской области в мае, июле и сентябре 2018 г. соответственно. Цветы или ягоды гомогенизировали и подвергали процессу аутоферментации на протяжении 7 дней. Из образовавшегося ферментированного гомогената выделяли ДНК и с ней проводили полимеразную цепную реакцию в реальном времени для детекции видов *B. thuringiensis*, *L. plantarum*, *A. faecalis* и *Agrobacterium* spp. **Результаты.** Среди исследуемых видов преобладал *B. thuringiensis*: эти бактерии были идентифицированы в 50% образцов цветов винограда, в 90% образцов зеленых ягод и в 20% созревших ягод. *L. plantarum* в цветах не выявлялся, но присутствовал в зеленых, а потом – в спелых ягодах винограда (10% исследованных образцов). Результаты подтверждают данные литературы, указывающие на встречаемость *B. thuringiensis* и *L. plantarum* на винограде. Присутствие *A. faecalis* и *Agrobacterium* spp. на цветах и ягодах винограда показано нами впервые. *A. faecalis* был выявлен в 20% образцов цветов, в 60% зеленых ягод и в 20% спелых ягод. *Agrobacterium* spp. выявляли в цветах и зеленых ягодах (10% образцов), а в созревших ягодах эти микроорганизмы обнаружены не были. Количество выявленных видов увеличивалось в зеленых ягодах по сравнению с цветами и уменьшалось в спелых ягодах. **Выводы**. Присутствие *A. faecalis* и *Agrobacterium* spp. в цветах и ягодах винограда показано нами впервые. Выявлена возможность сосуществования в одной экологической нише (цветы и зеленые ягоды винограда) фитопатогенных агробактерий и их потенциальных антагонистов – *Bacillus thuringiensis, Alcaligenes faecalis* и *Lactobacillus plantarum*.

Ключевые слова: Bacillus thuringiensis, Lactobacillus plantarum, Agrobacterium tumefaciens, Agrobacterium vitis, Alcaligenes faecalis, сапрофитная микробиота, фитопатогены, *Vitis vinifera*.

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