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## LACTOBACILLUS PLANTARUM FROM BERRIES OF GRAPE CULTIVATED IN THE SOUTH OF UKRAINE

The **aim** of the investigation was the isolation of *Lactobacillus plantarum* from berries of grape and the study of their genetic diversity. **Materials and Methods.** Lactic acid bacteria were isolated from berries of 14 grape cultivars grown in the south of Ukraine. PCR-assay and RAPD-PCR were used for *L. plantarum* identification and the study of their diversity. The reconstruction of phylogenetic tree of the studied strains was carried out with Matlab 7.0 program. **Results.** Lactobacilli have been isolated from 7 samples of must among 14 of the tested grape cultivars. 80% of the studied strains belonged to *L. plantarum* species. Groups of genetic similarity between *L. plantarum* strains have been revealed. In the phylogenetic tree four clusters of the strains grouped by their similarity were formed. The mentioned results allowed to **conclude** that *L. plantarum* strains from must of grape of different cultivars and territories of grape cultivation could show the genetic similarity. Opposite, some *L. plantarum* strains from one source of origin could demonstrate the genetic diversity.

*Key words:* lactic acid bacteria, grape must, RAPD-PCR.

Lactic acid bacteria of *Lactobacillus* genus inhabit various biotopes, including plant surfaces, where they survive as the representatives of epiphytic microbiota and can be characterized by the genetic diversity [3; 9; 13; 15]. Insects fed on flowering grape are the important source of berry colonization [6]. Bacteria of *Lactococcus*, *Leuconostoc* and *Lactobacillus* genera have been revealed in gut of bees and wasps [11]. *L. plantarum* and *Oenococcus oeni* from the surfaces of grape berries cause malolactic fermentation in wine and in cases of uncontrolled fermentation they are responsible for “grape diseases”. Usually malolactic fermentation begins in two-four weeks after the end of alcohol fermentation [2]. One of the consequences of this process is less acidity that occurs as a result of decarboxylation of malic acid to lactic acid. The controlled process of malolactic fermentation is desirable in wine-making, especially for acid wines, because it gives them



more soft taste. For controlled malolactic fermentation, bacteria *Oenococcus oeni* are used as starter cultures. In case of using lactobacilli, there is a risk of their rapid growth resulting in non-desirable taste of wine [7].

The aim of the investigation was the isolation of *Lactobacillus plantarum* from grape must and the study of their genetic diversity.

### Materials and Methods

Must from berries of 14 grape cultivars grown in the south of Ukraine in 2011-2012 (Pinot noir, Chardonnay, Riesling, Cardinal, Kishmish, Moldova, Dnistrovskiy rozhevyi, Kesha, Vostorg, Odesa souvenir, Lidia, Isabella, Cabernet Sauvignon and Muscat Hamburg) was incubated under the room temperature (18–20 °C) for the natural fermentation process. The isolation of lactobacilli from grape must was carried out on 2–28<sup>th</sup> days of fermentation. The quantity of lactobacilli (CFU/ml) in must was evaluated by inoculating MRS agar with serial dilutions of must, incubation at 30 °C and counting the grown colonies [8]. All the inoculations have been carried out in 5 repeats.

The colonies with the typical for lactic acid bacteria morphology have been selected for the standard tests revealing their belonging to *Lactobacillus* genus. The next identification of *Lactobacillus plantarum* was carried out by the polymerase chain reaction (PCR) [5]. DNA was isolated by the heat lysis method in modification of Szegedi and Bottka (2002) [14]. PCR mixture contained 0.8 µl of Mg<sup>++</sup> (50 mM, AmpliSens, Russia), 2.0 µl of dNTP (4 mM each, SibEnzyme, Russia), 1.0 µl of each primer (10 pmol), and 0.4 µl of Taq-polymerase (5 U/µl) (Primetech, Belorussia) in a standard PCR buffer (10x) (Taq-polymerase buffer, SibEnzyme, Russia).

The genus specific primers LbLMA-1/R-161 to the sequence of 16s rRNA: 5'-CTC AAA ACT AAA CAA AGT TTC-3' and 5'-CTT GTA CAC ACC GCC CGT TCA-3' were used [5]. For identification of *Lactobacillus plantarum* species the specific primers to gene *recA* of *L. plantarum* – planF (5'-CCG TTT ATG CGG AAC ACC TA-3') and pREV (5'-TCG GGA TTA CCA AAC ATC AC-3') were used [15]. The parameters of amplification were the next: 94 °C – 3 min for the initial denaturation, after 30 cycles at 94 °C – 30 s, 55 °C – 10 s, 72 °C – 30 s, and final elongation at 72°C for 5 min [5]. PCR products were analyzed by electrophoresis in 1.5% agarose gel using TBE buffer with ethidium bromide (AmpliSens, Russia). Markers of molecular weight – pUC19 DNA/MspI 110, 147, 190, 242, 331, 404, 489, 501 b.p.; Lambda DNA/EcoRI+HindIII 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 b.p. (Fermentas, Lithuania) were used.

The control strains *L. plantarum* UCM B-2709 and *L. plantarum* UCM B-2694 from the Collection of Microorganisms of Zabolotny Institute of Microbiology and Virology were kindly provided by Professor Kovalenko N.K.

RAPD-PCR were carried out with *L. plantarum* strains isolated from must and from the control reference strains *L. plantarum* UCM B-2709 and *L. plantarum* UCM B-2694.



The mixture for RAPD-PCR contained the next components: 1.0  $\mu\text{l}$  of a primer M13 (10 pmol), 0.8  $\mu\text{l}$  of  $\text{Mg}^{++}$  (50 mM, AmpliSens, Russia), 2.0  $\mu\text{l}$  of dNTP (4 mM of each, SibEnzyme, Russia), and 0.4  $\mu\text{l}$  of Taq-polymerase (5 U/ $\mu\text{l}$ ) (Primetech, Belorussia) in a standart PCR-buffer (10x) (Taq-polymerase buffer, SibEnzyme, Russia). Amplification was carried by the parameters described in Ben Omar et al. (2008). PCR-products were analyzed in 1% agarose gel using TAE buffer (40 V, 4 hours). Gels were photographed with GelDoc system (BioRad, USA).

The reconstruction of phylogenetic tree of the studied strains was carried out with Matlab 7.0 program. Cluster analysis based on Spearman's correlation coefficient for measuring the distance between the objects and the method of Ward for constructing a hierarchical tree of binary clusters were used.

### Results and Discussion

Lactobacilli were isolated from 7 samples of must among 14 tested grape cultivars. The quantity of lactobacilli increased at the end of the 4<sup>th</sup> week of fermentation (Table 1).

Table 1

Concentration of bacteria from *Lactobacillus* genus in grape must

Grape cultivar	Year of investigation	Place of grapevine cultivation	Day of must fermentation	Concentration of lactobacilli, CFU/ml
Chardonnay	2011	Odesa	2	$(4,3 \pm 2,8) \times 10^3$
			3	$(4,5 \pm 1,4) \times 10^3$
			28	$(2,3 \pm 1,6) \times 10^4$
Lidia	2011	Odesa	2	$(2,3 \pm 1,8) \times 10^3$
			3	$(4,3 \pm 1,7) \times 10^4$
	2012	Odesa	4	$(1,2 \pm 0,5) \times 10^5$
Pinot noir	2011	Odesa	2	$(2,4 \pm 1,6) \times 10^4$
			3	$(4,2 \pm 2,6) \times 10^4$
			28	$(4,3 \pm 1,6) \times 10^4$
Isabella	2011	Odesa	3	$(4,8 \pm 2,6) \times 10^4$
Moldova	2011	Odesa	3	$(4,3 \pm 2,3) \times 10^2$
Dnistrovskiyi rozhevyi	2012	Kiliya district, Odesa region	12	$(3,7 \pm 3,6) \times 10^5$
Cardinal	2012	Odesa	12	$(4,1 \pm 1,2) \times 10^5$



As it is known from the literature, after some period of time individual for every certain wine material, cells survived after ethanol fermentation, start to multiply. Non desirable multiplication of such microorganisms could happen in vines with pH higher than 3.5 [2]. Under the conditions of low pH, lactic acid bacteria die rapidly, but when pH is higher than 3.5, they can continue to multiply and in concentrations  $10^6$ – $10^8$  cause the spoilage of wines [2, 4]. In our experiment, fermentation of wine was uncontrolled and carried out under the conditions that favor the proliferation of lactic acid bacteria in must (temperature 18–20 °C, absence of anaerobic conditions necessary for ethanol fermentation).

The isolated strains of lactobacilli were first identified by morphological and biochemical properties. After, PCR-assay with bacterial DNA was carried out. Amplification product of 250 b.p. size was synthesized in the samples of DNA from all the tested strains, and this result revealed presence of 16S rRNA fragment specific for the bacteria of *Lactobacillus* genus. PCR with primers planF/pREV [15] resulted in amplicons 318 b.p. in size in case of 80% of the tested *Lactobacillus* strains that confirmed their belonging to the species *L. plantarum*.

It is known that in the beginning of ethanol fermentation in grape must *L. plantarum* and *L. casei* are mostly occurred [16]. Besides, the representatives of such species as *L. brevis*, *L. hilgardii*, *L. curvatus* and *L. buchneri* can also be revealed in must [13].

Totally 23 strains of lactic acid bacteria have been isolated from grape must during the investigations; they were identified and studied. In 2011, strains *L. plantarum* ONU 12, 311, 312 and 313 were isolated from must of Pinot noir cultivated in Odesa. In 2012, strains *L. plantarum* ONU 341–350 were isolated from grape of Lidia cultivar grown in Odesa. From berries of Cardinal cultivar (Odesa) strains *L. plantarum* ONU 332–336 and ONU 339, 340 were isolated, and must of Dnistrovskiy rozhevyyi cultivated in Kiliya district of Odesa region was the source of the strains *L. plantarum* ONU 337, 338.

The results of RAPD-PCR showed presence of groups of genetic similarity and diversity between the strains of *L. plantarum*. As it could be seen from the electrophoretic analysis, amplification products with DNA of the strains isolated from one source (grapevine of Lidia cultivar) were very similar (fig. 1).

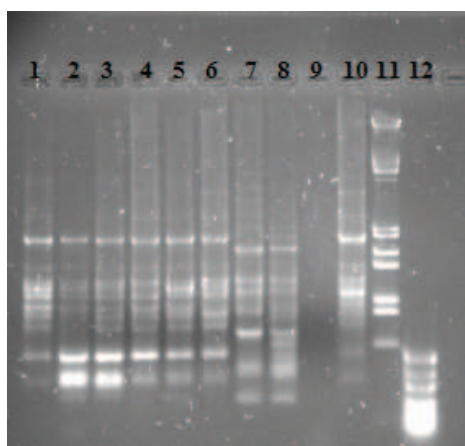
The results of cluster analysis based on Spearman's correlation coefficient and the method of Ward used for evaluation the similarity between the studied lactic acid are presented in fig. 2.

As it could be seen from the presented data, all the strains formed 4 basic clusters grouped by the level of their similarity.

Basing on the obtained data, it can be said that the maximal similarity showed the strains *L. plantarum* ONU 338 and *L. plantarum* ONU 340 forming cluster G3 (the percentage of similarity between the strains –



65.0%) although these strains were isolated from different sources. Strain *L. plantarum* ONU 338 was isolated from grape of Dnistrovskiy rozhevyi cultivar grown in Kiliya district of Odesa region, and strain *L. plantarum* ONU 340 – from grape of Cardinal cultivar from Odesa. This allowed us to conclude that the genetic similarity of the strains not always coincided with the territory of grape origin.



**Fig. 1. The electrophoretic analysis of amplicons resulted from RAPD-PCR**  
1–8,10 – amplicons of RAPD-PCR with DNA from *L. plantarum* ONU 341, 342, 343, 344, 345, 346, 347, 348, 349 and 350, respectively; 11, 12 – the markers of molecular weight.

Cluster G1 was formed by the strains from wine material of one cultivar and place of cultivation (Lidia, Odesa) (fig. 2). These are the strains *L. plantarum* ONU 341, 344, 345, 343, 342, 346 and 350. Three more strains from the same origin were arranged to other clusters. Strains *L. plantarum* ONU 347 and 348 together with the strains *L. plantarum* ONU 335 and ONU 334 isolated from wine material of Cardinal cultivar (Odesa) formed the separate cluster G4 with the similarity between the strains from 48.0 to 52.0%.

Strain *L. plantarum* ONU 349 also isolated from must of Lidia cultivar (Odesa) showed the similarity to the strains forming subcluster G2.4 in a cluster G2. Thus, among the strains *L. plantarum* from must of Lidia cultivar grown in Odesa three groups of genetic diversity were revealed.

Cluster G2 joined the biggest amount of the studied strains with different levels of similarity forming subclusters G2.1, G2.2, G2.3 and G2.4 (fig. 2). Thus, to subcluster G2.1 strains *L. plantarum* ONU 313 and 312 from wine material of Pinot noir (Odesa) and strains *L. plantarum* ONU 332 and 339 from Cardinal cultivar (Odesa) were arranged. Percentage of similarity among the strains in this subcluster was relatively high (from 49.0 to 52.0%). Strains *L. plantarum* ONU 333 and 336 also isolated from must of Cardinal cultivar (Odesa) formed subcluster G2.2 with the percentage of similarity between the strains 50.0%. Subcluster G2.4 joined the

strains *L. plantarum* ONU 12 and 311 (Pinot noir, Odesa), *L. plantarum* ONU 349 (Lidia, Odesa) and *L. plantarum* ONU 337 (Dnistrovskiyi rozhevyi, Kiliya district, Odesa region). Similarity between the strains in this case was evaluated as 36.0%.

Such grouping of the strains one more confirmed that the genetic similarity of the strains was not always related to the territorial origin. The control strains *L. plantarum* UCM B-2694 and *L. plantarum* UCM B-2709 were ranged to the same cluster G2 (subcluster G2.3), and the level of similarity between them was established as 49.0% (fig. 2).

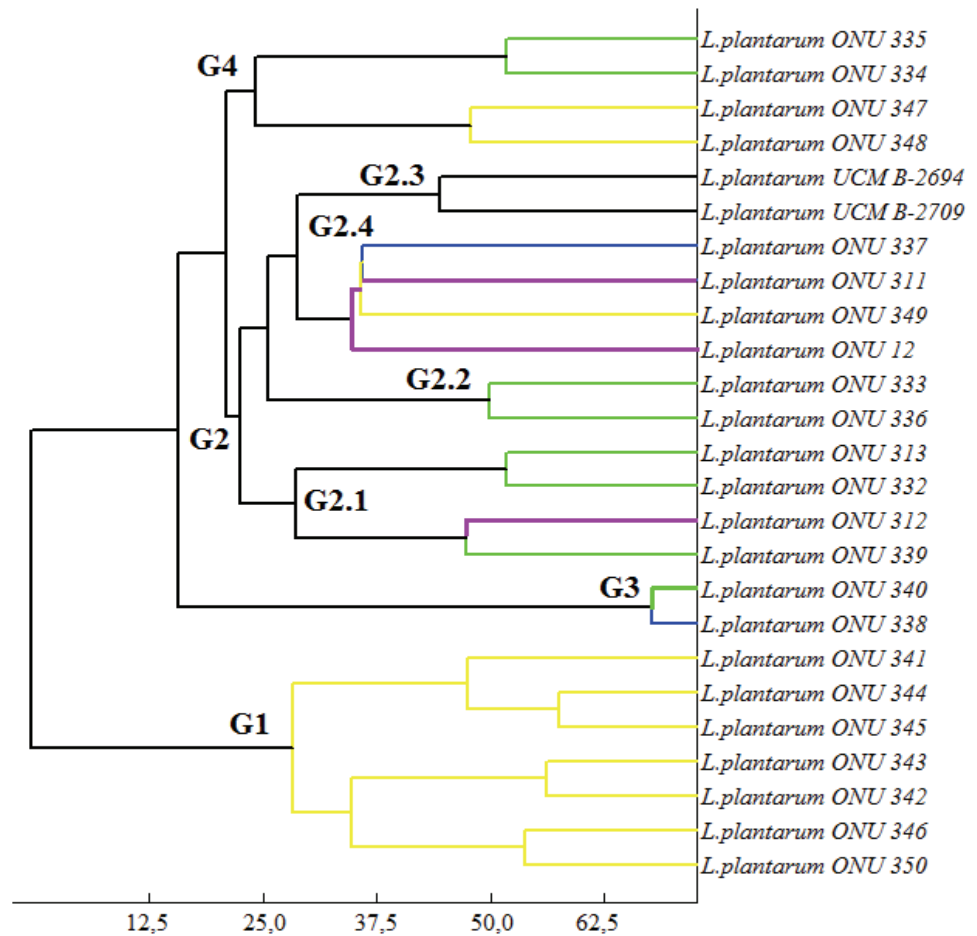


Fig. 2. The results of RAPD-PCR analysis with the primer M13 presented as the phylogenetic reconstruction and grouped by cluster analysis based on Spearman's correlation coefficient and method of Ward (Matlab 7.0).

Cultivars of grapevine are marked in colors:

- Lidia cultivar, Odesa
- Cardinal, Odesa
- Pinot noir, Odesa
- Dnistrovskiyi rozhevyi, Kiliya district, Odesa region



Besides this, it is needed to be mentioned that the year of isolation of lactobacilli didn't influence the topology of cluster formation. For instance, strains *L. plantarum* ONU 313 and 311 were isolated from wine material of Pinot noir (Odesa) in 2011, but according to their genetic properties they were ranged into different subclusters of the cluster G2 (fig. 2).

Genetic diversity of the strains is a common feature of *L. plantarum* [9]. Thus, it is known that the strains inside the species *L. plantarum* can differ in presence of various genes responsible for bacteriocin productions and in their variations [10]. Besides, the strains of *L. plantarum* can also be characterized by the spectrum of antimicrobial activity [1]. The investigators have shown the presence of 7 genetic groups and 18 subgroups in oenological *L. plantarum* strains from one geographical territory (Spain) [12].

In our case the results of RAPD-PCR with the primer M13 have demonstrated the preferential genetic similarity among the strains of *L. plantarum* isolated from the certain sample of grape must, but the strains from grape collected in distant territories could also be similar. At the same time, some *L. plantarum* strains from one source of origin could demonstrate the genetic diversity.

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## **LACTOBACILLUS PLANTARUM ІЗ ЯГІД ВИНОГРАДУ, ЯКИЙ КУЛЬТИВУЄТЬСЯ НА ПІВДНІ УКРАЇНИ**

### **Реферат**

**Метою** дослідження було виділення бактерій *Lactobacillus plantarum* із ягід винограду та вивчення їх генетичної різноманітності. **Матеріали і методи.** Молочнокислі бактерії були виділені з ягід 14 сортів винограду, який культивувався на півдні України. Для ідентифікації *L. plantarum* та вивчення різноманітності штамів застосовували класичний метод ПЛР та RAPD-ПЛР. Для реконструкції філогенетичного дерева досліджених штамів було використано програму Matlab 7.0. **Результати.** Бактерії роду *Lactobacillus* були виділені з 7 проб виноградного суслу серед 14 протестованих сортів винограду. 80% досліджених штамів лактобацил належали до виду *L. plantarum*. Виявлено групи генетичної схожості між штамми *L. plantarum*. На філогенетичному дереві штамми формували чотири кластери, згруповані за ступенем їх подібності. Вказані результати дозволили зробити **висновок** про те, що генетично подібними могли бути штамми *L. plantarum*, виділені з виноградного суслу різних сортів та місцевостей



культивування винограду. У той же час штами *L. plantarum* з однакового джерела походження могли виявляти генетичну різноманітність.

Ключові слова: молочнокислі бактерії, *Lactobacillus plantarum*, виноградне сусло, RAPD-ПЛР.

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## ЛАКТОБАЦИЛЛУС ПЛАНТАРИУМ ИЗ ЯГОД ВИНОГРАДА, КУЛЬТИВИРУЕМОГО НА ЮГЕ УКРАИНЫ

### Реферат

**Целью** исследования было выделение *Lactobacillus plantarum* из ягод винограда и изучение их генетического разнообразия. **Материалы и методы.** Молочнокислые бактерии были выделены из ягод 14 сортов винограда, выращенного на юге Украины. Для идентификации *L. plantarum* и изучения их разнообразия была использована полимеразная цепная реакция и RAPD-ПЦР. Реконструкция филогенетического дерева была проведена при помощи программы Matlab 7.0. **Результаты.** Лактобациллы были выделены в 7 пробах сусла из 14 протестированных сортов винограда. 80% изученных штаммов принадлежали к виду *L. plantarum*. Были выявлены группы генетического сходства между штаммами *L. plantarum*. На филогенетическом дереве штаммы образовывали четыре кластера, сгруппированных в соответствии с их подобием. Указанные результаты позволили сделать **вывод** о том, что штаммы *L. plantarum* из сусла винограда различных сортов и территорий культивирования винограда могли проявлять генетическое сходство. Напротив, некоторые штаммы *L. plantarum* из одного источника происхождения могли демонстрировать генетическое разнообразие.

Ключевые слова: молочнокислые бактерии, виноградное сусло, RAPD-PCR.

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