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BIO-PROTECTIVE ACTIVITY OF GRAPE MELANIN IN RELATION TO ANTIBIOTICS

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In this study, melanin was shown to have a bioprotective effect on lactic acid bacteria (LAB) belonging to different genera and species. In particular, melanin has been shown to increase the resistance of LAB to antibiotics. It was shown, that the effectiveness of melanin depends on the species of LAB and duration of exposure. In the case of *Enterococcus faecium* KE-5 and *Lactobacillus rhamnosus* 20-12 strains, the effectiveness of melanin is more obvious. At a concentration of 30 *mg/mL*, melanin reduces the cell count by 1 log unit, but it does not affect the rate of milk fermentation by LAB. In the case of two other strains, *Lactobacillus acidophilus* 1991 and *Lactobacillus fermentum* 27-2, it is less pronounced. *Lactobacillus acidophilus* 1991 showed the highest sensitivity, displaying high sensitivity to both the antibiotics and the indicated concentration of melanin.

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***Keywords*:** melanin, lactic acid bacteria, bioprotective agent, antibiotic.

Introduction. In recent years, numerous biopolymers have been investigated for their potential as drug delivery systems. Despite this research, many of these polymers require additional structural or morphological modifications, which are often challenging to achieve and yield limited results [1, 2]. In contrast, melanin offers a practical advantage due to its high yield and ease of extraction from wine waste [3].

Melanin, a diverse group of polymeric pigments, is widely present in various biological systems [4]. Recent studies have explored the use of melanin and similar compounds in biomedical fields, including bioimaging, photothermal therapy, and drug delivery systems [5, 6]. These compounds are valued for their biocompatibility and biodegradability, making them useful in numerous applications [7, 8]. The appeal of melanin as a drug delivery agent stems from its natural abundance and biopolymeric nature. Additionally, melanin may have protective roles against antimicrobial drugs, with some microorganisms producing melanin linked to their virulence and pathogen city [9, 10]. Research by Minasyan et al. has shown that melanin extracted from grape peels exhibits antibacterial properties against both gram-positive and gram-negative bacteria [11].

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The rise of antimicrobial resistance (AMR) is a significant concern, driven by the widespread use of antimicrobials in humans and animals. This resistance occurs when microorganisms acquire mutations or resistance genes, rendering standard treatments ineffective [12]. AMR poses a global threat to treating infectious diseases, with annual deaths attributed to AMR estimated at around 750 000 and projected to reach 10 million by 2050 [13, 14]. This resistance can affect bacteria, fungi, viruses, and protozoa [12].

Antibiotics target a broad range of bacteria, including both pathogenic and beneficial ones. Their use can disrupt the balance of gut microbiota, potentially leading to issues such as antibiotic-associated diarrhea [15, 16]. To mitigate resistance, it is essential to use antibiotics judiciously and only when necessary, thereby preserving their effectiveness and maintaining healthy gut microbiota [17].

Recent advancements in antimicrobial technology focus on biological antimicrobial agents (AMPs), which have either bactericidal or bacteriostatic properties. These agents, including phytochemical flavonoids (such as galangin, quercetin, and baicalein), bacteriocins, metabiotics, and peptides from probiotic lactic acid bacteria (LAB), offer a broad spectrum of activity and may serve as viable alternatives to traditional antibiotics [18].

Probiotics, which are known to avoid causing antibiotic resistance, are often recommended during antibiotic treatments to support beneficial bacteria and counteract adverse effects like abdominal pain, gas, diarrhea, and Candida infections [19]. Despite their benefits, probiotic bacteria can also be affected by antibiotics, highlighting the need for protective strategies. Some LAB produce compounds with intrinsic antibacterial activity or that modify antibiotic resistance, potentially aiding in overcoming bacterial resistance when used in combination with antibiotics [20].

Given the challenges of developing new AMPs and the need to prevent antibiotic resistance, melanin presents a potential solution. Investigating melanin's role as a bioprotective agent against antibiotics could offer new avenues for maintaining the efficacy of antibiotics and protecting beneficial probiotic LAB.

Materials and Methods.

Isolation of Melanin from Wine Waste. Squeezed black grapes were obtained from a local winery located in the Armavir region of the Republic of Armenia. The process for isolating and purifying water-soluble melanin from the grape peels followed the procedure outlined by Aghajanyan et al. [3].

Microbial Strain and Growth Media. Probiotic LAB strains, including *Lactobacillus rhamnosus* 20-12, *Lactobacillus acidophilus* 1991*, Enterococcus faecium* KE-5, and *Lactobacillus fermentum* 27-2, were sourced from the Microbial Depository Center (MDC) of SPC "Armbiotechnology" NAS RA. These strains were cultured in skim milk (1.5% fat), as well as in Man-Rogosa-Sharpe (MRS) agar and broth ("Merck", Germany; "ISO", Italy; "HiMedia", India). For preservation, the LAB strains were stored as frozen stocks at –20°C in MRS broth containing 40% glycerol. Prior to use, the strains were subcultured twice into the respective growth media and incubated at 37°C for 24 *h* [21].

The Bioprotective Effect of Melanin on LAB was investigated by the serial dilution method with simultaneous application of melanin, Amoxiclav and Ceftriaxone antibiotics. For the growing and determination of survival of LAB strains,

the selective plating medium MRS and milk agar ("Himedia", India) were used. LAB cultures were grown in MRS broth containing appropriate amount of melanin and antibiotics for 24 *h* at 37°C. The melanin without antibiotics, melanin with antibiotics, and antibiotics without melanin samples were taken as controls. At the end of culture growth count of survived cells (colony forming unit, CFU/*mL*) was determined by the serial dilution method. By spread plate technique the LAB cultures were sown on the surface of agar plates and then incubated at 37°C for 48 *h* and then the count of grown colonies was calculated. Influence of melanin on the growth of LAB was determined by their growing in the skim milk (1.5 % of fat) and MRS broth containing appropriate amount of melanin (*mg*/*mL*). The milk fermentation ability during 24–48 *h* was detected as control. Clot formation indicated no effect of melanin on LAB growth.

Determination of Resistance to Antibiotics. To assess the antibiotic resistance of probiotic LAB strains, the disk diffusion method was employed [22]. Each strain was cultured overnight in the appropriate broth at 37°C. Upon reaching a cell concentration of $(7.0 \pm 2.0)10^8$ CFU/*mL*, LAB cultures were spread onto agar plates using a sterile swab. Antibiotic disks, including Ofloxacin (5 μg), Doxycycline (10 μg), Ampicillin (10 μg), Ciprofloxacin (5 μg), Gentamicin (120 μg), Azithromycin (15 μg), Tetracycline (30 μg), Piperacillin (110 μg), Amoxicillin (10 μg), Rifampicin (5 μg), and Penicillin G (10 IU, Oxford, UK), were placed on the agar plates. The plates were then incubated at 37°C for 48 *h*. After incubation, the diameters of the inhibition zones were measured. The results were expressed as Ø, *mm* (Tab. 1).

Table 1

Antibiotic resistance of some LAB strains

Antibiotics (antibiotic sensitivity disks)	Disk content	LAB strains	
		L. rhamnosus 20-12	*L. acidophilus* 1991
		growth inhibition zone, Ø, *mm*	growth inhibition zone, Ø, *mm*
Doxycyclin	30 μg	0	25 ± 1
Azithromycin	15 μg	15 ± 1	14.0 ± 0.5
Tetracycline	30 μg	10.0 ± 0.5	10.0 ± 0.5
Ampicillin	10 μg	0	10.0 ± 0.5
Ofloxacin	5 μg	0	0
Piperacillin (Tazobactam)	110 μg	0	0
Amoxicillin	10 μg	28 ± 2	0
Amoxiclav	30 μg	28 ± 2	25 ± 2
Gentamicin	120 μg	15 ± 1	14.0 ± 0.5
Ciprofloksacin	5 μg	0	0
Rifampicin	5 μg	15 ± 1	12.0 ± 0.5
Penicillin G	10 IU	10.0 ± 0.5	12.0 ± 0.5
Ceftriaxone	30 μg	30 ± 2	26 ± 2

Results and Discussion. Probiotics have shown promise in treating certain chronic infections that are resistant to most antibiotics. When used alongside traditional antibiotics, probiotics have the potential to help overcome antimicrobial resistance and address complex infections [23]. However, this approach presents significant technical challenges, as many probiotics are bacteria themselves and are

therefore susceptible to antibiotics, which can hinder their survival and effectiveness when administered together [24].

According to the recent literature data, melanin has not only a photoprotective effect, but also a bioprotective property, so there has been interest to study the bioprotective property of melanin, particularly as an antibiotic resistance enhancer [25, 26].

A preliminary assessment of LAB sensitivity to commonly prescribed antibiotics was conducted. The data for two representative strains are presented in Tab. 1.

The data reveal that antibiotics impact LAB growth differently. Given that Ceftriaxone and Amoxiclav are frequently used for treating a range of diseases, these two antibiotics were chosen for further research to explore the bioprotective properties of melanin. Additionally, the effect of melanin on LAB was initially assessed by evaluating milk fermentation capacity and growth in MRS broth. The results of this investigation are summarized in Tab. 2.

Table 2

Growth of LAB in the presence of melanin

Growth media + melanin	*mg*/*mL*	LAB cultures							
		L. rhamnosus 20-12		*L. acidophilus* 1991		*L. fermentum* 27-2		*Ent. faecium* KE-5	
		cell count, CFU/*mL*							
		MRS	milk	MRS	milk	MRS	milk	MRS	milk
	0	$2.6 \cdot 10^{10}$	$1.6 \cdot 10^{9}$	$3.2 \cdot 10^{10}$	$1.8 \cdot 10^{9}$	$2.6 \cdot 10^{10}$	$1.5 \cdot 10^{9}$	$3.2 \cdot 10^{10}$	$1.3 \cdot 10^{9}$
	10	$1.2 \cdot 10^{10}$	$3.5 \cdot 10^{9}$	$1.2 \cdot 10^{9}$	$1.5 \cdot 10^{8}$	$2.2 \cdot 10^{9}$	$1.5 \cdot 10^{9}$	$1.2 \cdot 10^{10}$	$3.1 \cdot 10^{9}$
	20	$1.2 \cdot 10^{9}$	$3.5 \cdot 10^{9}$	$1.2 \cdot 10^{8}$	$3.5 \cdot 10^{7}$	$1.1 \cdot 10^{9}$	$3.5 \cdot 10^{8}$	$2.5 \cdot 10^{9}$	$3.5 \cdot 10^{9}$
	30	$1.2 \cdot 10^{8}$	$1.6 \cdot 10^{8}$	$1.2 \cdot 10^{6}$	$1.5 \cdot 10^{7}$	$1.3 \cdot 10^{8}$	$1.3 \cdot 10^{7}$	$1.5 \cdot 10^{9}$	$1.5 \cdot 10^{8}$

As observed from the given results, the addition of 10 *mg* of melanin led to vigorous growth of all studied LAB strains in both media. In the case of 20 *mg* and 30 *mg* of melanin, the strain *L. acidophilus* 1991 showed the highest sensitivity. The remaining 3 LAB were resistant to the indicated concentrations of melanin and had only 1 log unit decrease in titer, which, however, did not affect the rate of milk fermentation by LAB. Further studies were conducted using a melanin concentration of 10 *mg*/*mL* in the medium (final concentration).

LAB was shown to be sensitive to antibiotics Ceftriaxone and Amoxiclav. Fig. 1 shows the results of studies on the bioprotective effect of melanin against Ceftriaxone and Amoxiclav among four LAB strains belonging to different species. As can be seen from the presented data, the bioprotective effects of melanin depend on the species of LAB and the duration of exposure. The highest bioprotective effect was shown for *Ent. Faecium* KE-5 and *L. rhamnosus* 20-12. *L. acidophilus* 1991 showed high sensitivity to both the antibiotic and the indicated concentration of melanin. *L. acidophilus* 1991 showed a sharp 80 percent decrease in growth after 24 *h*, and almost no growth after 48 *h* for both antibiotics tested.

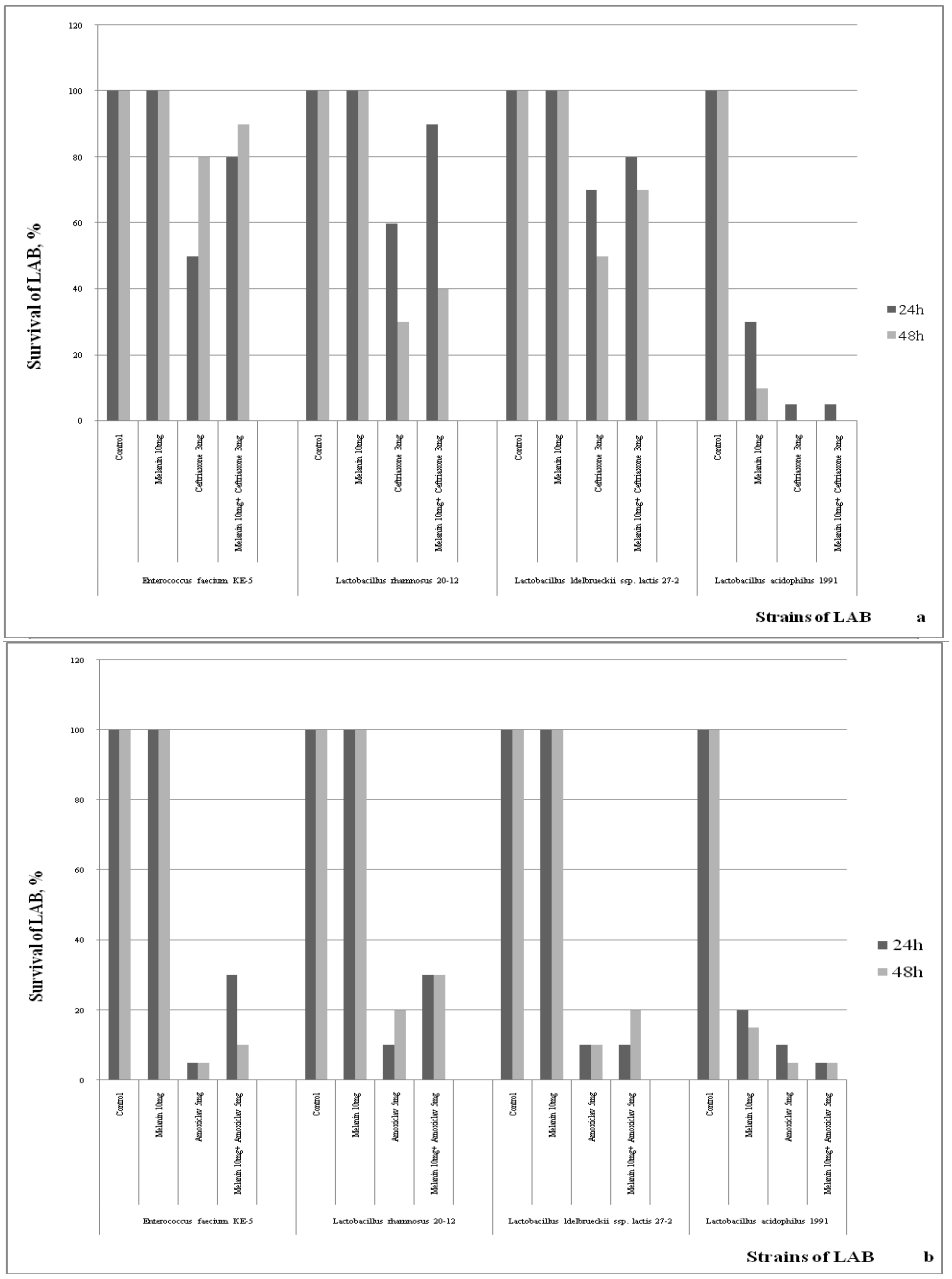

Fig. 1. Bioprotective activity of melanin in relation to antibiotics: a) Ceftriaxone, b) Amoxiclav.

For investigated three strains, melanin increased resistance to Amoxiclav for about 10%. Resistance to Ceftriaxone increased up to 20%. It is evident from the data presented that the pattern was maintained by the application of two antibiotics at 24 *h* and 48 *h* of cultivation. Thus, melanin has been shown to increase the resistance of LAB to antibiotics.

It was shown by us that Melanin has a bioprotective effect on LAB belonging to different genera and species. In particular, melanin has been shown to increase the

resistance of LAB to antibiotics. The effectiveness of melanin depends on the species. In the case of probiotic LAB strains *Ent. Faecium* KE-5 and *L. rhamnosus* 20-12, the effectiveness of melanin is more obvious, and in the case of the other two strains, it is less pronounced.

Conclusion. This study demonstrated that melanin, easily extracted from wine waste, holds potential as a valuable bioprotective agent against the side effects of antibiotics. From the literature it is known that melanin has a bioprotective property against UV and some chemicals, particularly antibiotics. Therefore, it was of interest to study the bioprotective property of melanin against LAB. In particular, we studied the change in the sensitivity of LAB against antibiotics Amoxiclav and Ceftriaxone during their growth in media containing melanin. Based on the obtained data, melanin can be successfully used as a protective agent in the production of probiotics, for example as an additive in the production of probiotic capsules.

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Է. Վ. ՄԻՆԱՍՅԱՆ

ԽԱՂՈՂԻ ՄԵԼԱՆԻՆԻ ԿԵՆՍԱՊԱՇՏՊԱՆԻՉ ԱԿՏԻՎՈՒԹՅՈՒՆԸ ՀԱԿԱԲԻՈՏԻԿՆԵՐԻ ՀԱՆԴԵՊ

Հետևյալ հետազոտության ընթացքում ցույց է տրվել, որ մելանինը ունի կենսապաշտպանիչ ազդեցություն տարբեր ցեղերին և տեսակներին պատկանող ԿԹԲ-երի վրա: Մասնավորապես ցույց է տրվել, որ մելանինը բարձրացնում է ԿԹԲ-ների կայունությունը հակաբիոտիկների նկատմամբ: Մելանինի արդյունավետությունը կախված է տեսակային պատկանելիությունից և ազդեցության տևողությունից: *Enterococcus faecium* KE-5 և *Lactobacillus rhamnosus* 20-12 շտամների դեպքում մելանինի արդյունավետությունը ավելի ակնհայտ է, 30 *մգ/մլ*, մելանինի դեպքում նրանց մոտ նկատվում է տիտրի նվազում ընդամենը 1 log միավորով, որը սակայն չի ազդում կաթի մերման արագության վրա ԿԹԲ-երի կողմից: Իսկ մյուս երկու՝ *Lactobacillus acidophilus* 1991 և *Lactobacillus fermentum* 27-2 շտամների դեպքում ավելի թույլ է արտահայտված: Առավել բարձր զգայունություն դրսևորել է *Lactobacillus acidophilus* 1991 շտամը, որը բարձր զգայունություն է դրսևորում ինչպես հակաբիոտիկի, այնպես էլ մելանինի նշված կոնցենտրացիայի հանդեպ:

Э. В. МИНАСЯН

БИОЗАЩИТНАЯ АКТИВНОСТЬ МЕЛАНИНА ИЗ ВИНОГРАДА ПО ОТНОШЕНИЮ К АНТИБИОТИКАМ

В этом исследовании было показано, что меланин оказывает биозащитное действие на молочнокислые бактерии, принадлежащие к разным родам и видам. В частности было показано, что меланин повышает устойчивость этих бактерий к антибиотикам. Показано, что эффективность меланина зависит от вида молочнокислых бактерий и продолжительности воздействия. В случае штаммов *Enterococcus faecium* KE-5 и *Lactobacillus rhamnosus* 20-12 эффективность меланина более очевидна. Меланин в концентрации 30 *мг/мл* приводит к уменьшению количества клеток только на 1 log единицу, но это не влияет на скорость сквашивания молока молочнокислыми бактериями. У двух других штаммов – *Lactobacillus acidophilus* 1991 и *Lactobacillus Fermentum* 27-2 – это менее выражено. Наибольшую чувствительность проявил штамм *Lactobacillus acidophilus* 1991, показав высокую чувствительность как к антибиотикам, так и к указанной концентрации меланина.