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Research Article

MOLECULAR GENETIC STUDY OF POTENTIALLY BACTERIO-CINOGENIC AND NON-VIRULENT *ENTEROCOCCUS* SPP. ISOLATES FROM BEEHIVES IN BULGARIA

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Summary: A total of 7 *Enterococcus spp*. beehives isolates from Pirdop region, Bulgaria, were studied in order to evaluate the incidence of bacteriocin gene determinants and genes for antibiotic resistance and virulence factors. Structural genes coding for enterocin A (entA), enterocin P (entP), enterocin B (entB), enterocin AS-48 (as-48), enterolysin A (entlA), collagen-binding protein (ace), invasins - hyaluronidase (*hyl*) and gelatinase (*gelE*), as well for vancomycin-resistance genes, were detected by polymerase chain reaction (PCR) with specific primers. PCR amplifications with specific primers for enterocin A, enterocin P, enterocin B, enterocin AS-48, enterolysin L50A revealed the presence of entB, entA, entAS-48, entP and entL50A genes among two of the investigated strains, IMD01 and IMD02, while another one, IMD05, revealed gene determinants for three enterocins - P, AS-48 and L50A. The performed PCR amplifications with specific primers for virulence genes gave negative results for the presence of virulence factors with all three primer sets that had been used. PCR amplification with specific primers for vancomycin-resistance genes showed that the investigated *enterococci* do not belong to the vancomycin-resistant types.

Keywords: *Enterococcus* spp, beehives, bacteriocin-encoding genes, vancomycin-resistance genes, virulence factors.

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INTRODUCTION

Enterococci are Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria, which usually inhabit the alimentary tract of humans in addition to being isolated from environmental and animal sources. (Fisher K., Phillips C, 2009). *Enterococci* belong to the lactic acid bacteria (LAB) species. Before they were assigned their own genus, *enterococci* were classified as group D *streptococci*. They are also important nosocomial pathogens that cause bacteraemia, endocarditis and other infections. Some strains are resistant to many antibiotics and possess virulence factors such as the synthesis of adhesins, invasins, pili and haemolysin. On the other side, some *enterococci* have many positive characteristics that have been appreciated in food fermentation and preservation, and may also serve as probiotics to promote human health (Moreno, 2006). They occur as nonstarter LAB in a variety of cheeses and sausages. In addition, *enterococci* have been scrutinized for bacteriocin production.

Bacteriocins are ribosomally synthesized, extracellular released antimicrobial peptides that show activity against closely related bacterial species. Numerous strains of *enterococci* associated with food systems, mainly *E. faecalis* and *E. faecium*, are capable of producing a variety of bacteriocins, called enterocins, some of them with activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium spp.* (including *Clostridium botulinum* and *Clostridium perfringens*), and *Vibrio cholerae*. (Giraffa, 2003). Enterocins usually belong to class II bacteriocins. They are small, heat-stable nonlantibiotics (Klaenhammer, 1993). Such strains which do not express virulence characteristics can be considered as probiotic.

In recent years, an attention was payed on the presence of probiotic strains in beehives due to great importance of the honey industry not only as food production but also because of the importance of the bees for natural pollination, including pollination in agriculture. Nowadays, the worldwide decrease in the number of bees, including the colony collapse disorder, represents a real risk for the production of many fruits in different geographic area. So, investigations of the factors affecting the overall beehive health is of great importance. In this research we focused our attention on the presence of probiotic *Enterococcus* strains which have been previously reported by other authors (Soria et al., 2014).

MATERIALS AND METHODS

Samples origins and sampling

Beehives in Dushantzi village (Pirdop region), Mid-Western Bulgaria, were used for sampling in this work. Freshly collected pollen granules agglutinated by the bees' saliva (about 30-40 mg) were used for the isolation of the bacterial strains. Two hours after their collection all samples were re-suspended by intensive vortexing in 1 ml of sterile peptone water, followed by plating of 100 μl aliquots on selective 1,5% agar medium D-coccosel (Biomérieux). Thus, enterococci formed black colonies after incubation at 37°C. Random single black colonies were inoculated in liquid BHI media, and after 18 h of incubation at

37°C they were re-plated on 1,5 % BHI agar. Some of the formed single colonies were picked up randomly and used for further analyses by giving them isolates codes "IMD" + a corresponding number.

Bacterial strains and growth conditions

All bacterial strains used in this study were cultivated on BHI media at 37°C. All seven strains belonging to the genus *Enterococcus* were determined by polyphase approach including growth on the selective medium D-coccosel and genus-specific PCR.

Molecular techniques

DNA isolation

DNA from all used strains was isolated with GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's instructions from a 3 ml overnight culture inoculated with a respective single colony. Unless it was used immediately, the DNA was stored for several weeks at -20°C.

PCR techniques

Genus-specific PCR

In order to confirm that our isolates belong to *Enterococcus* genus, PCR amplification was performed with genus-specific primers, Ent1/Ent2 (*Ke et al, 1999)* (Table 1). PCR was carried out in a total volume of 15 µl containing 10 ng of template DNA, 1× PCR Master Mix (Thermo Scientific) comprising 1× reaction buffer, 0.2 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 0.2 mM $MgCl_2$ and 0.3 U Taq polymerase.

The DNA was amplified using the following protocol: after an initial denaturation step for 5 min at 94°C, 35 amplification cycles were performed. They consisted of incubation for 15 sec at 94°C for denaturation, 15 sec. for hybridization at 55°C, and 30 sec. at 72°C for synthesis. A final extension step for 1 min at 72°C completed the amplification. 8 µl of the PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer system, followed by staining with GelRed™.

Detection of antibiotic resistance genes and virulence factors by specific primers

All oligonucleotides used as amplification primers are shown in Table 1. In order to confirm the presence of genes coding for different virulent factors, PCR amplifications were performed with primers targeting the collagen-binding protein (ace), the hyaluronidase (*hyl*), the gelatinase (*gelE*) and the vancomycin-resistance genes. PCR were carried out in a total volume of 10 µl containing 10 ng of template DNA, 1× PCR Master Mix (Thermo Scientific) comprising 1× reaction buffer, 0.2 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 0.2 mM $MgCl_2$ and 0.2 U Taq polymerase.

The DNA was amplified using the following protocol: after an initial denaturation step for 5 min at 94°C, 35 amplification cycles were performed. They consisted of incubation for 3 min at 94°Cfor denaturation, 1 min. for hybridization at the corresponding temperature, and 1 min. at 72°C for synthesis. A final extension step for 3 min at 72°C completed the amplification. The annealing temperature was different for each primer pairs. Annealing

Table 1. Oligonucleotides used as primers in this study and PCR conditions (Part 1).

Primer pairs	Sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Source
Genus-specific primers				
Ent1	5’-TACTGACAAACCATTCATGATG-3’	112 bp	55°C	*(Ke et al)*
Ent2	5’-AACTTCGTCACCAACGCGAAC-3’			
Primers for virulence genes				
hyl n1	5’ACA GAA GAG CTG CAG GAA ATG-3’	276 bp	55°C	*(Vankerckhoven et al)*
hyl n2	5’-GAC TGA CGTCCA AGT TTC CAA-3’			
vanA-F	5′-GGGAAAACGACAATTGC-3’	733 bp	61°C	*(Dutka-Malen et al)*
vanA-R	5′-GTACAATGCGGCCGT-3’			
vanB-F	5′-ATGGGAAGCCGATAGTC-3’	635 bp	61°C	*(Dutka-Malen et al)*
vanB-R	5′-GATTTCGTTCCTCGACC -3’			
vanC1-F	5′-GGTATCAAGGAAACCTC -3’	822 bp	61°C	*(Dutka-Malen et al)*
vanC1-R	5′-CTTCCGCCATCATAGCT -3’			
vanC2-F	5′-CTCCTACGATTCTCTTG-3’	439 bp	61°C	*(Dutka-Malen , et al)*
vanC2-R	5′-CGAGCAAGACCTTTAAG-3’			
gelEF	5’-ACC CCG TAT CAT TGG TTT-3’	213 bp	50°C	*(Vankerckhoven et al)*
gelER	5’-ACG CAT TGC TTT TCC ATC-3’			
ACE 1	5′-AAA GTA GAA TTA GAT CCA CAC-3’	1008 bp	50°C	(*Hadji-Sfaxi et al)*
ACE 2	5′-TCT ATC ACA TTC GGT TGC G-3’			

Table 1. Oligonucleotides used as primers in this study and PCR conditions (Part 2).

Primer pairs	Sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Source
Primers for bacteriocin structural genes				
As48-1	5′-AATAAACTACATGGGT-3′	377 bp	50°C	*(Sabia et al)*
As-48-5	5′-CCAAGCAATAACTGCTCTTT-3′			
EntB(F)	5′-GAAAATGATCACAGAATGCCTA -3′	160 bp	60°C	*(du Toit et al)*
EntB(R)	5′-GTTGCATTTAGAGTATACATTTG-3′			
EntP1	5′-ATGAGAAAAAAATTATTTAGTTT-3′	215 bp	60°C	*(du Toit et al)*
EntP2	5′-TTAATGTCCCATACCTGCCAAACC-3′			
EntA(F)	5′-AAATATTATGGAAATGGAGTGTAT-3′	123 bp	55°C	*(du Toit et al)*
EntA(R)	5′-GCACTTCCCTGGAATTGCTC-3′			
EntL50A[f]	5′-GAT TGG AGG AGT TAT ATT ATG GG-3’	135 bp	51°C	*(Hadji-Sfaxi et al*)
EntL50A[r]	5′-CAA ATT ATA AAG AAA TAA TTA CCT ATC ATT AAC-3’			

temperatures are shown in Table 1. 8 µl of the PCR products were analyzed by electrophoresis in a 1.5% agarose gel using TBE buffer system, followed by staining with GelRed™.

Detection of bacteriocin genes by specific primers

Oligonucleotides used as amplification primers are shown in Table 1. PCR amplifications were performed in order to confirm the presence of genes coding for different class bacteriocins: class II.1. [enterocin A (entA) and enterocin P (entP)], class II.3. [enterocin B (entB)], class III [enterocin AS-48 (as-48)] and class IV [enterolysin A (entlA)] bacteriocins (Franz et al.,2007). PCR were carried out in a total volume of 10 µl containing 10 ng of template DNA, 1× PCR Master Mix (Thermo Scientific) comprising of 1× reaction buffer, 0.2 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 0.2 mM $MgCl_2$ and 0.2 U Taq polymerase in a total volume of 10 µl.

The DNA was amplified using the following protocol: after an initial denaturation step for 5 min at 94°C, 35 amplification cycles were performed. They consisted of incubation (3 min at 94°C, for denaturation), 1 min. for hybridization at the corresponding temperature, and 1 min. at 72°C for synthesis. A final extension step for 3 min at 72°C completed the amplification. The annealing temperature was different for each pair of primers. Annealing temperatures are shown in Table 1. 8 µl of the PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer system, followed by staining with GelRed™.

RESULTS AND DISCUSSION

Bile Esculin Agar, known also as D-coccosel medium, was used primarily to differentiate *Enterococcus spp.* from *Streptococcus spp.*, thus allowing at the same time the growth of the members only of these two genera. Members of the genus *Enterococcus* are capable of growing in the presence of 4% bile (oxgall) and hydrolyzing esculin to glucose and esculetin. Esculetin combines with ferric ions to produce a black colored complex. After 24 hours of incubation at 37°C we observed the typical black coloring. This allowed us to accept that our isolates belong to *Enterococcus spp.*

Using genus-specific primers to confirm that our isolates belong to *Enterococcus spp,* the performed PCR gave positive results for all seven investigated strains.

However, PCR amplification performed with specific primers for structural genes coding for the collagen-binding protein (*ace*), as well as for the invasins, hyaluronidase (*hyl*) and gelatinase (*gelE*), gave negative results for the presence of these genes in all tested seven strains (Table 2). Moreover, PCR amplifications with specific primers for vancomycin-resistance genes showed that the investigated *enterococci* do not belong to the vancomycin-resistant types (Table 2). These findings allow us to speculate that our isolates are not a result of some animal faecal contaminations but they constitute an integral part of the normal beehive microbiota participating in the processes of fermentation of the honey bread.

In order to clarify the function of the

Table 2. Results from PCR with specific primers for bacteriocin structural genes, antibiotic resistance genes and virulence factors in *Enterococcus* spp.

Primers	*Enterococcus* spp. strains						
	IMD01	IMD02	IMD03	IMD04	IMD05	IMD06	1444
As-48	+	+	-	-	+	-	-
EntL50A	+	+	-	-	+	-	-
EntA	+	+	-	-	-	-	-
EntP	+	+	-	-	+	-	-
EntB	+	+	-	-	-	-	-
hyl n1	-	-	-	-	-	-	-
hyl n2	-	-	-	-	-	-	-
vanA-F	-	-	-	-	-	-	-
vanA-R	-	-	-	-	-	-	-
vanB-F	-	-	-	-	-	-	-
vanB-R	-	-	-	-	-	-	-
vanC1-F	-	-	-	-	-	-	-
vanC1-R	-	-	-	-	-	-	-
vanC2-F	-	-	-	-	-	-	-
vanC2-R	-	-	-	-	-	-	-
gelEF	-	-	-	-	-	-	-
gelER	-	-	-	-	-	-	-
ACE 1	-	-	-	-	-	-	-
ACE 2	-	-	-	-	-	-	-

isolated non-pathogen strains within the beehive, we decided to test them for the presence of some bacteriocins commonly found among the probiotic *enterococci*. We selected in total five bacteriocins – AS-48, EntL50A, EntA, EntB and EntP. (*Soria et al., 2014, Audisio et al., 2011*). The results of the PCR amplifications with specific primers for enterocin AS-48 and enterocin B are shown in Table 2. Three of the strains, IMD01, IMD02 and IMD05, revealed the presence the corresponding gene determinants. PCR-amplifications with specific primers for enterocin A and enterocin P, revealed the presence of the entA and entP genes among two of the investigated strains, IMD01 and IMD02, and presence of entP gene in IMD05. The amplification results for genes encoding enterolysin L50A revealed the presence of the gene in three of all seven investigated strains, IMD01, IMD02 and IMD05.

The lack of virulence factors among

the seven studied *Enterococcus spp*. isolates, and the presence of bacteriocins' genes within at least three of them, allowed us to assume that these strains are selected and maintained within the bee hive not only because of their possible specific functions in the honey bred fermentation but also for the protection of the hive against pathogens, the later explaining the presence of bacteriocin gene determinants in at least three of them. It should be noted, that the bacteriocinogenic strains reveal more than one bacteriocin, namely one of the strains presenting three of the five selected bacteriocins and the other two – all five of them. Although we did not investigate the expression of these bacteriocins, it is highly doubtful that bacteriocin-positive strains are maintained if not to expresse purely by chance within the hive, especially because of the presence of multiple genes. As long as the strains to express more than one bacteriocin is not an uncommon fact, two of our isolates revealed the existence of five genes, an observation which is quite exceptional. This assumption confirm our suggestion of long-time maintenance of probiotic bacteria within the bee hive which can create the appropriate conditions and favour a horizontal genetic transfer among them.

CONCLUSION

Accordingly to the "World's Top Exports" (WTEx) statistics for 2017, Bulgaria ranks the 15th place in honey export with 2% of the world's production. However, the scientific research on the beehives' probiotic microbiota, which directly affects the overall health conditions of the hives and their resistance to pathogens, are very limited and practically absent in our country. In this respect, it is difficult to explain this fact regarding the role of the honey production for Bulgarian export economy. In order to make up for this deficiency, we started this work as the first step for characterizing the role of the probiotic strains maintained within the beehives. The lack of virulence factors and the presence of multiple bacteriocin gene determinants revealed purposeful maintenance of non-pathogenic and *enterococci* which may play a specific role in the hive overall health. These bacterial strains remain to be further characterized as probiotics, and their potential for possible use as food and feed additives to be studied.

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