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Microbiological characteristic of microflora of *(Mytilus galloprovincialis Lam.*) in The Bulgarian Black Sea aquatory.

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ABSTRACT

In the present study we present the results from the isolation and study of microorganisms from *M. galloprovincialis* Lam. from the Bulgarian Black Sea aquatory. The Black Sea mussel (*M. galloprovincialis* Lam.) is in fact the only marine species grown as aquaculture in the Bulgarian Black Sea aquatory. Mussels have an exceptional nutritional value, making them suitable for the human diet. The cultivation of sea products, in particular the Black Sea clam, is becoming an important part of the Western Black Sea industry. With increasing tourist pressure and construction activities on the Black Sea coast, maritime pollution increased in the last decades. These pollutants also include various pathogenic microorganisms - *Escherichia coli, Staphylococcus aureus, Salmonella thyphimurium, Hymeniacidon perlevis*, incl. the cholera cause (*Vibrioo cholera*). Such pathogenic microorganisms are often present in the mussels and can cause the spread of various diseases. We investigated for pathogens *M. galloprovincialis* Lam. gathered from northern part of The Bulgarian Black Sea aquatory. For the isolation of the various microorganisms, different nutritious media were used. The isolated microorganisms were macro-morphologically and micro-morphologically studied.
Keywords: *Mytilus galloprovincialis* Lam; microbiological; probiotic bacteria; Black Sea

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INTRODUCTION

Monitoring and remediation actions of areas impacted by human activities had become the main cornerstones of the national strategies for territory recovery and management. This problem is particularly significant in sites affected by an intense industrialization and urbanization process along the coastline [8].The Black Sea mussel (*M. galloprovincialis* Lam.) is the only species of mussels grown as aquaculture in the Bulgarian Black Sea aquatory. Mussels have an exceptional nutritional value making them suitable for the human diet. Mussel flesh is rich in selenium, calcium, iron, magnesium, phosphorous and vitamins (A, B1, B2, B6, B12 and C). Mussel fat is also rich in polyunsaturated fatty acids (PUFA, 37–48% of total fatty acids) mainly ω-3 PUFA. These fatty acids are biologically important and have been associated with a decreased risk of cardiovascular diseases. Mussel farming has an important economic impact in many European countries. In Bulgaria the production in 2005 amounted up to 170 tons, which accounted for 5.4% of the total aquaculture production in the country. From a social point of view, safe food forms a significant problem for modern civilization. The cultivation of sea products, in particular the Black Sea clam, had becom an important part of the Western Black Sea industry. With increasing tourist pressure on the Bulgarian Black Sea coast, maritime pollution had increased. These pollutant include various pathogenic microorganisms - *Escherichia coli*, *Staphylococcus aureus*, *Salmonella thyphimurium*, *Hymeniacidon perlevis*, incl. the cholera cause (*Vibrioo cholera*). Such pathogenic microorganisms are often present in the mussels and can cause the spread of various diseases. Bivalve molluscs can accumulate microorganisms, including pathogens, from seawater [5] and the number and type of microorganisms present in the water depend on several seasonal, climatic and anthropogenic factors. The mussels are, flter-feeding organisms and can concentrate bacteria in high number [24]. The microbiota found in shellfishes can be divided into autochtonous and allochtonous microorganisms, and reflects the microbial population of the water in which they grow [27]. On the other hand, it has been found that seafood is a source of lactic acid bacteria (LAB), which synthesize substances with antimicrobial activity against many pathogenic microorganisms [1; 14;16;23; 28]. Consuming foods containing high quantities of chemical conservants provokeds the interest in the use of natural and minimally processed foods, with a focus on the use of naturally produced antimicrobial agents - bioconversants such as bacteriocins [4;9;10;15;29] . Bacteriocinproducing LABs can improve the aquatic environment of shrimp and fish aqua-culture [2;3]. The development of bacteriocinogenic strains and the isolation of bacteriocins from seafood and products may result in valuable strains that are used both as bioconversants and as fooeed additives in aquaculture animals [7; 21;25]. In the present study we present the results from the isolation and study of microorganisms from *M. galloprovincialis* Lam. in The Bulgarian Black Sea aquatory. After harvesting, the samples were analysed in order to determine the presence of fecal coliforms FC including *E. coli*, the presence of *Salmonella* and other pathogens (*Pseudomonas aeroginosa, Listeria monocytogenes*), and isolation of lactic acid bacteria.

MATERIALS AND METHODS

Place and duration of the study: The study was conducted at the Department of Biology, University of Shumen, Bulgaria, from June 2017 to September 2017.

Collection of samples

The mussels were harvested from northern part of the Bulgarian Black Sea aquatory. After collection, the samples (about 10 kg) were immediately refrigerated (4 °C) and transported to the laboratory for the analyses.

Microbiological analyses

Three subsamples (each of about 1 kg of mussels) were used for the microbiological analyses. The mussels were scrubbed free of dirt, washed in hypochlorite solution (20 mg l^{-1}), rinsed with sterile distilled water, and shucked with a sterile knife. Tissue liquor samples (about 100 g) were homogenized.

Fecal coliforms were enumerated through a five tubes per dilution most probable number (MPN) series [12]. After 3 h at 37 °C plus 21 h at 44 °C, gas positive tubes were recorded for FC. From each FC gas positive tubes, 0.1 ml were transferred in tubes with 10 ml of Tryptone Water (Oxoid, Basingstoke, UK) and then incubated for 24 h at 44 °C.

E. coli was enumerated by MacConkey agar (Merck, Darmstadt, Germany). The plates very incubated aerobically at 35-37ºC. for 18-24 hours. *E. coli* growthed; pink to red colonies with salt precipitate surrounding the colonies.

For the evaluation of the presence of ***Salmonella*** spp. in 25 g of mussels homogenates the method of the International Organisation for Standardisation (ISO 6579, 1993) [13] was followed.

The presence of ***L. monocytogenes*** was determined according to McClain and Lee (1989).

Pseudomonas aeroginosa was enumerated by Cetrimide Agar (Merck KGaA, 64271 Darmstadt, Germany).

Lactic acid bacteria (LAB) were isolated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia).

RESULTS

The microorganisms were isolated from *M. galloprovincialis* Lam. (figure 1) in the Bulgarian Black Sea aquatory. The microscopic pictures were performed using stereomicroscope OPTIKA (Italy) with a camera Canon EOS 60D.

Figure 1: *M. galloprovincialis* Lam.

After 24 h of cultivated on the different culture media various microbial colonies were obtained (Table 1) and Figure 2 and 3.

Table 1: Number of obtained colonies on the different media

Media/strain	FC	*E.coli*	*Salmonella* spp.	*L. monocytogenes*	*P. aeroginosa*	LAB
Tryptone Water	72.10^7	38.10^7	-	-	34.10^7	-
MacConkey agar	-	36.10^7	-	-	-	-
Ceramide agar	-	-	-	-	42.10^6	-
MRS	-	-	-	-	-	83.10^8
McClain agar	-	-	-	-	-	-

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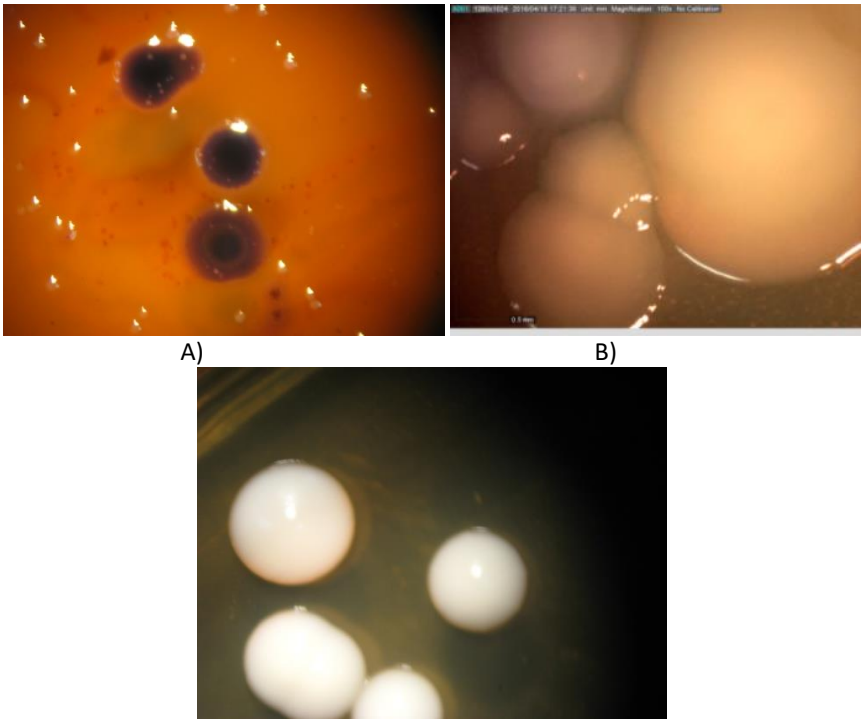

Figure 2: Pictures of the colonies of isolated species: A) colonies of *E. coli* on media MacConkey agar B) colonies of *P. aeroginosa* on media Cetrimide Agar; C) colonies of Lactic acid bacteria on media MRS agar. The pictures were taken using stereomicroscope OPTIKA (Italy) and DinoEye, Eyepiece camera, USB, 1.3 megapixsel, up to 5 megapixel.

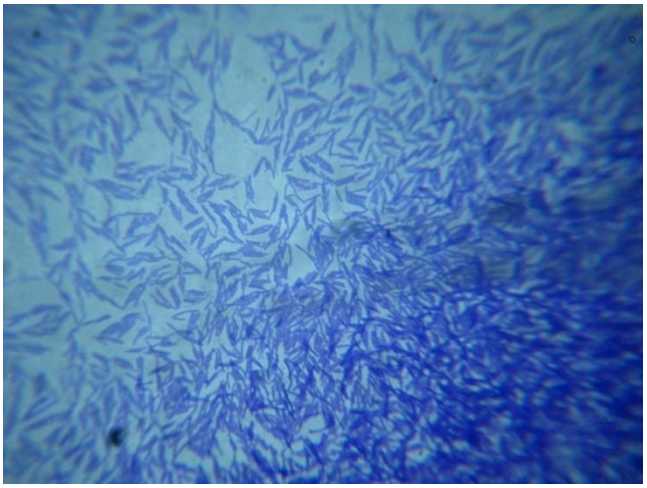

Figure 3: Light microscopic visualizations of Gram staining of Lactic acid bacteria. The picture were taken using microscope OPTIKA (Italy) at magnification 1000 by immersion and DinoEye, Eyepiece camera, USB, 1.3 megapixsel, up to 5 megapixel.

The behaviour observed for FC was conformed also by *E. coli* counts (table 1 and fig.2 A). In particular, *E. coli* was the responsible for the FC peak in July (the two counts coincided), suggesting the presence of particular environmental conditions which influenced the quality of mussels harvested in that month. In addition, the same samples were also tested for the presence of *L. monocytogenes, Salmonella spp*. and *P. aeroginosa.* While *L. monocytogenes* and *Salmonella* spp. were not detected in all the samples analysed, samples (July) were positive for the presence of *P. aeroginosa* (table 1 and fig.2 B).

Lactic acid bacteria were isolated over MRS agar. The results are represented on table 1 and fig.2 C.The micromorphologic characteristic demonstrated that the cells possess elongated form and staina as Gram

positive cells. The cells gave negative probe for catalase and we propose that these are representatives of the *Lactobacillus sp.*

DISCUSSION

The high FC counts observed in the warmer month July, can be attributed to the increase of the metabolic activity of the molluscs, depending prevalently on the elevated temperature of seawater and to their biological cycle which decreases their filtering capacity [17;22]. In addition, the seawater can also influence the microbial counts through its nutrient concentration [11]. It was reported by [27] that in the samples with microbial concentration higher than the limits imposed by the Italian regulation, the FC and *E. coli* counts coincided, indicating that the latter species is the responsible for the high microbial proliferation in these mussels. The peak of *E. coli* observed in July can be related to some particular environmental conditions. In fact, the characteristics of the mussels can be affected by a variety of extrinsic and intrinsic factors, such as water temperature and salinity, food availability and gametogenic cycle of the animals [19].

Escherichia coli is a food borne enteropathogen belonging to the group of pathogens known as Shiga toxin-producing *E. coli* (STEC), and it is an important cause of severe gastrointestinal disease in humans, including haemorrhagic colitis, which may lead to the life threatening haemolytic uraemic syndrome. *E. coli* had caused numerous outbreaks worldwide due to consumption of raw or undercooked contaminated foods. After excretion, *E. coli* can persist for months in the environment, including in fresh and seawater. However, *E. coli* can survive for 2 weeks in marine water and there is a high probability of infection following low of less than 100 cells exposure (Griffin & Tauxe 1991; http://www.cfsph.iastate.edu/Factsheets/pdfs/e_coli.pdf). Therefore consumption of live shellfish should not be discounted as a potential route of disease transmission to humans [20]. The new results obtained in the present study in relation to those found by other authors [20] could be linked to several factors such as the different identification and cultural methodology used or the climatic and geographical areas where samples were collected.

Grimes et al. [11] pointed out that many investigators erroneously interpret the wastewater discharge as the source of the pathogens rather than as a source of nutrients, which may stimulate the growth of the autochthonous pathogens.

The anthropogenic disturbance is acting on ecosystems with serious consequences for the environment and the human health, driving strong social and economic impacts on the community. Often, the case of chemical and petrochemical productions, the areas exposed to the most intensive industrialization are located along the coastline, being a severe threat to the marine and costal environment.

Each year, food-borne and water-borne zoonotic diseases affect tens of millions of people. The prevalent etiological agents involved in the outbreaks are bacteria (*Camplylobacter jejuni, Salmonella enteritidis and S. typhimurium, verocytotoxigenic E. coli, Listeria monocytogenes*), viruses (caliciviruses) and parasites (*Cryptosporidium parvum, Cryptosporidium hominis*) EFSA and ECDC, 2015 [6]. Despite advances in food safety, in some cases, the causative agent associated with disease may be underestimated or unknown due to the lack of a specific protocol for their detection and identification in clinical laboratories [18].

Even though the legislation regulating food safety requires assessment of *E. coli* and *Salmonella* contamination (EC Regulation no. 1441/2007), the increase in foodborne disease related to the consumption of raw shellfish suggests that further epidemiological data are required to establish more specific microbiological criteria in seafood and to apply new depuration technologies in order to guarantee food safety. Therefore, effective national and European food control systems are essential to protect consumer's health by implementing routine research into the emerging pathogens in this food chain [18].

Probiotic applications, in biological control of seafood associated pathogens can be an alternative solution, providing consumer with a product of good quality owing to the use of nontoxic compounds. Based on our results, LAB could be used as a bioprotective culture in *M. galloprovincialis* Lam. depuration to prevent patogenic growth. Most of the consumed shellfish in Bulgaria is produced on aquaculture farms. Because much of the shellfish is grown and harvested as aquacultures, this offers an opportunity to monitor and improve microbiological safety of the product both preharvest and postharvest. Several processes, including freezing, low-temperature pasteurization, high pressure processing and irradiation have been reported to be

capable of reducing patogenics bacteria. The massive use of antibiotics may lead to the emergence of resistant bacteria, which can spread in the environment and jeopardize human health (WHO, 2006). Probiotics are among the most promising alternatives to antibiotics and the application in aquaculture is now widely accepted. It may be possible to reduce the load of pathogens in seafood by improving water quality and also by introducing biocontrol bacteria capable of excluding pathogens from shellfishassociated microbial communities [14;26;29]. The molluscs are filter feeders, capable of filtering up to 10 L of water per hour. This feeding behaviour leads to the accumulation (at least transient) of human pathogens within shellfish. The consumption of raw bivalve molluscs is associated high risk of food poisoning.

CONCLUSIONS

In summary, the results in the present study demonstrate that bivalve molluscs are potential pathogenic hosts.

Furthermore, additional studies are needed to provide data that will help extend knowledge and confirm the role played by contaminated shellfish consumption in human diseases. Based on our results, LAB could be used as a bioprotective culture in *M. galloprovincialis* Lam. depuration to prevent patogenic growth. The LAB colonization mechanism in *M. galloprovincialis* remains to be investigated.

In the future, the probiotic potential of LAB will be investigated.

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