Journal of Engineering Science *Vol. XXIX, no. 4 (2022), pp. 176 - 189*
Fascicle *Food Engineering* *ISSN 2587-3474*
Topic *Biotechnologies, Food Chemistry and Food Safety* *eISSN 2587-3482*

https://doi.org/10.52326/jes.utm.2022.29(4).14
UDC 634.743:632(478)

PHYTOPATHOGENIC MICROBIOTE OF SEA BUCKTHORN AND IMPACT ON STORAGE

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Received: 08. 31. 2022
Accepted: 10. 15. 2022

Abstract. Sea buckthorn (*Hippophae rhamnoides L.*) has considerable potential for the agri-food, pharmaceutical and cosmetic industries. This article presents an international and experimental research conducted on plantations in Dubasari district, Pohrebea village in the Republic of Moldova of the microbiota which is able to attack sea buckthorn plantations and reduce the harvest. This research includes changes in the microbiota that is able to attack sea buckthorn plants to reduce the harvest and shelf life of the berries. Information on potential diseases of sea buckthorn plantations, the causative agent and recommendations to reduce this risk are included in the article. The information presented is important for the development of sea buckthorn plant cultivation strategies and disease management also for improving the quality and preservation of post-harvest fruit.

Keywords: *Hippophae rhamnoides L., microbiota of sea buckthorn, microbiological investigation, safety indicators*

Rezumat. Cătina albă (*Hippophae rhamnoides L.*) are un potențial considerabil pentru sectorul agro-alimentar, farmaceutic și cosmetic. În acest articol este prezentat un studiu de sinteză internațional și experimental realizat pe plantațiile din raionul Dubăsari, satul Pohrebea din Republica Moldova al microbiotei fitopatogene care poate ataca plantațiile de cătină, diminua recolta și calitatea fructelor. Studiul include identificarea microbiotei fitopatogene (fungice și bacteriene) de pe suprafața organelor cătinei albe (a frunzelor, fructelor în diferite stadii de coacere), folosind metode de izolare și obținere a culturilot pure în condiții de laborator. În baza studiilor efectuate a fost identificată microbiota fitopatogenă de origine micotică, bacteriană și elaborate recomandări de prevenire și combatere a bolilor cătinei albe. Informațiile prezentate sunt importante pentru dezvoltarea strategiilor de cultivare a plantelor de cătină, gestionarea posibilelor boli, precum și pentru îmbunătățirea calității și conservării fructelor post-recoltare.

Cuvinte cheie: *Hippophae rhamnoides L., microbiota cătinii, investigații microbiologice*, *indicatori de siguranță.*

1. Introduction

In recent years, sea buckthorn has attracted considerable attention from researchers around the world, especially for its relevant nutritional and medicinal value. Berries contain many bioactive substances, including organic acids, amino acids, flavones and vitamins [1, 2]. It should be noted that few scientific papers are published that address the issue related to the impact of the phytopathogenic microbiota associated with the sea buckthorn *(Hippophae rhamnoides L.)*. The authors of the publications [3-5] reported that the yeasts *Cryptococcus, Rhodotorula, Sporobolomyces* and *Aureobasidium pullulans* were associated with the early stage of fruit ripening. According to the information presented by Juliana Lukša et al. [6] depending on the ripening stage, sea buckthorn showed a significantly different fungal microbiota. Fungi from the genera *Aureobasidium, Taphrina* and *Cladosporium* were present on unripe berries, while ripe berries were dominated by *Aureobasidium* and *Metschnikowia*. In another study by Xue Zhou [7], the authors report that in the sea buckthorn microbiota, fungi from the genera *Ascomycota, Basidiomycota* and *Zygomycota* had the dominant weight. The distribution of rhizospheric fungi in sea buckthorn is determined by both environmental and geographic factors. The authors of the studies [7, 8] mention that of all the soil characteristics examined, altitude and pH had a significant effect ($p < 0.05$) on the rhizospheric fungal microbiota

2. Bacterial and fungal mycobiota associated with sea buckthorn fruits

Sea buckthorn is a fruit plant with a high content of complex nutrients that compete in the natural nutraceutical market sold internationally. Sea buckthorn (*Hirrorhae rhamnoides* L.) has been cultivated in Europe since the 1970s [9, 10]. Due to the high demand of sea buckthorn fruits in the food industry and the increased demand for non-thermally processed foods, plant microbiota may have an important role in evaluating the impact of fruits on food quality and human health.

The authors Lukša, J. [6] and Yang, M. [11] studied the predominant microbial taxonomic units on sea buckthorn fruits, presented by bacteria and fungi. The effect of plant age, season, and weather conditions was studied only in sea buckthorn plants on the microbiota of the rhizosphere [6, 12]. The fungal pathogen tolerates a period of low temperatures in the form of spores around the kidneys and in closed areas of the cortex. In early spring, under favorable conditions, primary infection occurs. Mushrooms intensively develop and grow until the appearance of buds and the first leaves. The most intense infection occurs at elevated and stable humidity for more than two nights [13].

- ***Aureobasidium***

Aureobasidium pullulans, the most common saprophytic mold, is a major source of environmental pollution. The mold of the genus *Aureobasidium* is mainly found in places with a temperate climate - in the British Isles, the USA, Canada, Alaska, Antarctica, Europe and Russia [14]. The main habitat of this type of mold is forest soil, fresh water, air and leaf surfaces. *Aureobasidium* is a spoilage agent in fruits and fruit drinks, vegetables such as tomatoes, cereals. For the growth of this type of fungus, the optimal growth temperature is 25 °C, and high humidity - a_w (available water) 0.89–0.90, but they are able to grow in a fairly wide temperature range from 2 to 35 °C [14, 15]. *Aureobasidium pullulans* can usually be separated from permanently wet surfaces. These fungi produce d-gluconic acid and d-glucono-1,4-lactone from saccharides, mainly glucose [16].

The study and analysis of the types and structure of molds on the surface of the berries makes it possible to determine a clear difference between immature sea buckthorn fruits and fruits that have reached full maturity. According to the data reported in the work [6], unripe sea buckthorn fruits were dominated by microorganisms from the genera *Aureobasidium, Taphrina, Cladosporium and Filobasidium (Cryptococcus),* while *Aureobasidium and Metschnikowia* predominated on ripe fruits. Juliana Lukša et al. [17], reported that fungal microorganisms of the *Dothioraceae* family predominated on sea buckthorn fruits, which at the lower taxonomic level were assigned to uncultivated *Aureobasidium*. The results of the study [17] are consistent with the conclusions of other authors [18, 19], which confirm that among the diversity of mold microflora on sea buckthorn, the most common representative is Aureobasidium sp.

- ***Cryptococcus neoformans***

Crytococcus neoformans and *Crytococcus gattii* are pathogenic microorganisms, namely yeasts, that cause diseases that are life-threatening to humans [20]. The yeast cell is covered with a capsule consisting of polysaccharides; for this reason, cryptococcus is often referred to as "sugar yeast" [21]. *Crytocuccus neoformans* is a fungus that occurs almost everywhere in the environment, most often in the soil, which produces extracellular enzymes. Lasses and phospholipase B are localized in the cell wall and ensure the formation of melanin and the integrity of the cell wall. The phospholipase enzyme also contributes to the fungal infection of the host's lung tissues and the rapid spread of an infectious and inflammatory disease [22].

- ***Rhodotorula***

Rhodotorula spp. belong to basidiomycete fungi, that is widely distributed in nature - in the air, soil, water, colonizes plants, fruits, humans and other mammals. The genus *Rhodotorula* includes eight species, some of which are pathogenic to humans - *Rhodotorula musilaginosa*, *Rhodotorula glutinis* and *Rhodotorula minuta* [23, 24]. Unlike ascomycetous yeasts such as *Saccharomyces spp.* and *Candida spp., Rhodotorula spp.* belong to the *Sporidiobolale* family. Typically, *Rhodotorula spp.* produce carotenoids that form pink to red colonies on agar [25].

- ***Sporobolomyces***

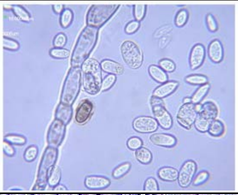
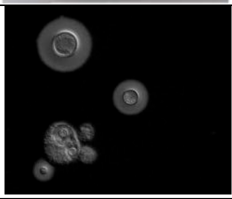
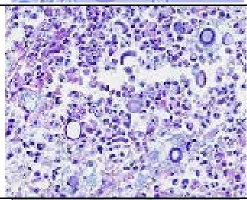
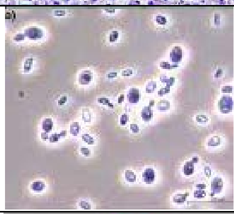
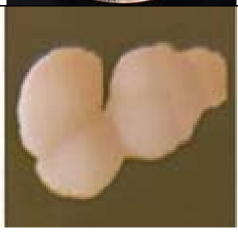
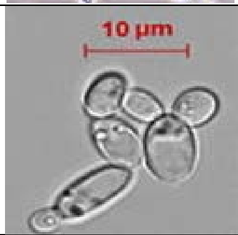
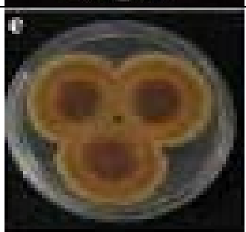
Sbololomyses is a yeast that naturally inhabits most environmental objects - air, tree leaves, many plants, birds, humans, and other mammals [26]. Microorganisms of the genus *Sporobolomyces*, like many fungi, are mesophilic, with an optimal growth and development temperature of 25-30 °C. Colonies are smooth, often wrinkled, from shiny to dull. Genus *Sporobolomyces* contains about 20 species that can cause infections [27].

Table 1 shows the results regarding the identification of microorganisms isolated from the sea buckthorn surface.

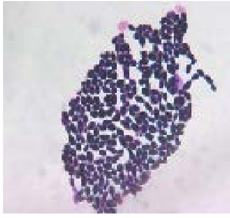
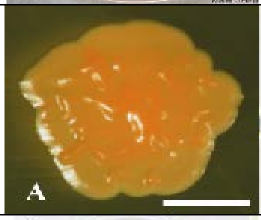
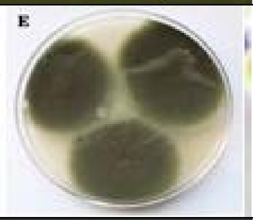
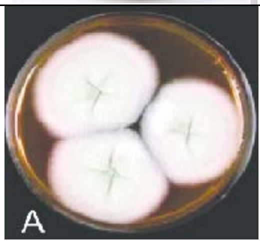
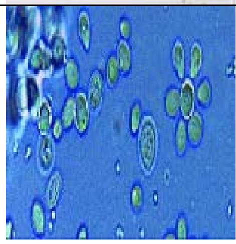
The purpose of the study by Maas P.W.Th. [30] was to evaluate the analysis of the influence of soil microflora on the root system and growth of sea buckthorn. The results of studies [31, 32] have shown that parasitic roundworms living in the soil alone or in combination with soil fungi can help retard the growth of sea buckthorn in the field, especially when the soil content and availability of nitrogen is low.

Table 1

Cultural and morphological properties of some microorganisms isolated from the surface of sea buckthorn fruits

Microorganism	Cultured cells	Microscope image	Year, country	Source
Aureobasidium pullulans			2020, Lithuania; 2018 Republic of Corea	[6] [28]
Cryptococcus wieringaie			2016, USA; 2012, USA	[20] [21]
Metschnikowia pulcherrima			2016, Basilicata	[29]
Pichia kudriavzevii			2020, Sweden	[30]
\Ascomycota			2017, China	[7]
Basidiomycota			2017, China	[7]
Zygomycot			2017, China	[7]
Taphrina			2020, Lithuania	[6]

Continuation Table 1

Rhodotorula			1995, USA	[23, 24]
Sporobolomyces			1924, USA	[26]
Cladosporium			2020, Lithuania	[6]
Filobasidium			2020, Lithuania	[6]

Sea buckthorn grows best in sandy soils and requires abundant watering. Lack of moisture can lead to loss of foliage, damage to branches, shedding of fruits and, as a result, death of the tree.

The lack of water in the soil reduces the resistance of sea buckthorn trees to cold, increases the susceptibility to winter temperature changes, and also increases the level of attack by pests and diseases [33].

For a long time, farmers believed that sea buckthorn was not problematic in cultivation. To date, it has been proven that there are many health problems with sea buckthorn. Verticillosis and other fungi pose a great danger to the health and life of the plant, especially for sea buckthorn cultivated in Russia and Western Europe [9]. On the Figure 1 shows an analysis of healthy and diseased parts of sea buckthorn plants [33].

Verticillium was detected in only 2% of the analyzed samples. In moderately damaged and diseased parts of the plant, verticillosis was not found. The more the parts of the plant were affected, the more was the infection with microorganisms of the genera *Alternaria* and *Phytophora*.

Among other maintenance works of sea buckthorn plantations, special attention must be paid to the supply of water and nutrients to the plants. In Lithuania, sea buckthorn is harvested mainly on sites with irrigation and controlled fertilization [33]. Global standards for sea buckthorn are currently being developed. Consumers in Europe demand quality, so farmers must ensure that sea buckthorn has a clear identity based on combined product and service standards [9, 10].

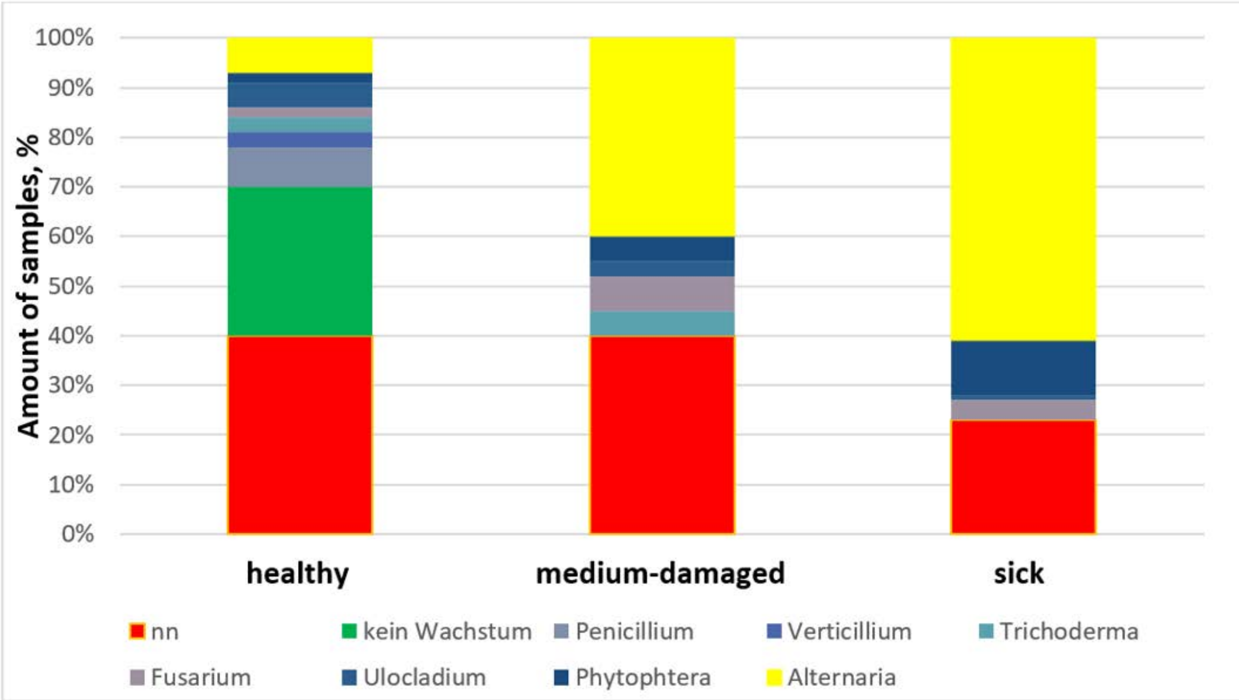


Figure 1. Species of fungi that were found from healthy, medium-damaged and diseased parts of sea buckthorn plants from Lithuania [33].

3. Diseases of the sea buckthorn culture and responsible agents

There are many studies on the relevant properties of *Hippophae rhamnoides* L. (chemical composition [34-38], antioxidant properties [39-42], antimicrobial properties [43-48]), but the study and research of sea buckthorn plantation diseases is limited. In general, only a few diseases were recorded, such as cancer caused by *Stigmina sp.,* wilt *(Verticillium spp.*), bud blight *(Pseudomonas syringae)* and dry shrinkage disease *(Fusarium spp.)*.

The most common diseases of sea buckthorn, scientists call verticillium wilt caused by soil fungi *Verticillium albo atrum* and *Verticillium dahliae*, fusarium wilt and rot caused by microorganisms *Pythium sp., Alternaria sp., Fusarium sp., Botrytis sp*. Verticillium wilt of sea buckthorn is a very dangerous disease and is widespread in both temperate and and in tropical regions of the world [49]. This disease usually appears in sea buckthorn plantations 5−8 years after planting [50, 51]. *Verticillium sresis* is widespread in many soils and more often affects tomatoes, peppers, strawberries, blackberries, sea buckthorn, etc. [52].

Fusarium wilt and rot are fungal diseases caused by *Fusarium oxysporum* that affect many perennial and annual plants. The causative agent, *Fusarium oxysporum*, overwinters in the soil. When the conditions for its development are favorable - moist, poorly drained soil - the fungus infects the roots of plants. It is extremely persistent in soil. For this reason it is necessary to extract the plant and it is also proposed to replace or sterilize the soil [53].

Another problem for sea buckthorn plantations is the fruit fly. Across Europe, for example, in Lithuania and Belarus, the sea buckthorn fruit fly is a big problem, while in Finland, Sweden and Estonia this problem does not exist [9, 49].

In recent years, growers are increasingly concerned about the problems related to diseases spreading in sea buckthorn plantations [51]. Since most of the infections that affect this plant are fungal, the diseases can be detected visually. Signs of disease onset include [54, 55]:

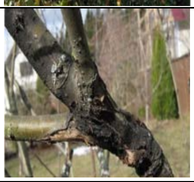
- yellowing, blackening, premature wilting and falling of leaves;

- changes in the color of the bark of a tree, the appearance of stains, plaque, mold, outbreaks of rot on it;
- premature fall of berries, change in their consistency, drying, deterioration of quality;
- appearance of growths, neoplasms.

Table 2 shows the most common sea buckthorn diseases and the agents that cause these diseases.

Table 2

Sea buckthorn diseases

The Name	Image	Pathogen	Result	Source
Endomycosis. Sea buckthorn fruit rot		Soil fungi g. *Phytium, alternaria* and *Botrytis*	Affected berries soften, lesions appear on the fruit, fungal spores affect healthy fruits.	[9, 54, 55, 57, 66]
Wilting		g. *Fusarium Verticillium spp.*	Complete wilting of the plant. The shoots are affected, the leaves dry up, the fruits do not ripen, although they have a characteristic color.	[42, 54, 56-58]
Vertical wilting. Verticillosis		*Verticillium spp. Pseudomonas syringae*	Plants stop growing, dry up and wither prematurely. Intense rotting of the roots occurs.	[53, 55]
Sea buckthorn stem rot		*Coriolus hirsutus (Fr.) Quel*	Affected wood disintegrates into thin plates. Infection occurs through cracks, dry bays, mechanical damage to the bark.	[54]
Black cancer		*Stigmina sp.*	Characteristic black spots appear on the trunk, the affected bark cracks and falls off, and the wood underneath turns black.	[54]
Rust		g. *Puccinia*	The first symptoms - the appearance of chlorotic spots on the upper part of the leaves. The disease evolves, and orange colored pustules (fungal colonies) appear on the lower part, causing a severe deformation of the leaves.	[56, 59]
Brown spot		*Coniothyrium olivaceum Bon*	*Irregular light brown spots appear on the leaves, which grow rapidly. The disease manifests itself both on the fruit and on the bark of the branches.*	[54]

Continuation Table 2

Ulcerative necrosis of the cortex		A fungus that grows in the bark of a tree	At the site of the injury, an accumulation appears, which then breaks, the fungal spores affect the tree.	[54, 57]
Nectric necrosis		*Nectria cinnabarina (Tode) Wint*	*The bark of the tree is affected. Clumps of red spores appear throughout the film. The shoot affected by fungi dries up and dies over time.*	[54]
Blackleg		Soil fungi	*A disease caused by soil fungi affects seedlings. The subcotyledonous knee becomes thinner at the point of contact with the ground. The hawthorn stem simply spins and falls to the ground.*	[54]
Septoria stain		*Septoria hippophaes Desm. et Rob. Sacc.*	*On the upper side of the leaves, rounded, dark brown spots appear, surrounded by a discolored ring of leaf blade tissue. The frost resistance of sea buckthorn drops sharply.*	[54]
Scab Heterospory		*Heterosporiu m*	*Dark spots with a purple border appear on the surface of the leaves. Over time, they grow, the leaf completely fades.*	[54]

4. Materials and Methods

The study was carried out as part of the project: *Development of the production technology of white sea buckthorn in an ecological system and the processing of fruits and biomass* at the Technical University of Moldova.

Object of study. The purpose of this case study was to investigate the specific and phytopathogenic microbiota of 8 varieties of sea buckthorn fruits: R1, R2, R4, R5, L1, C6, AGG, AGA, harvested in September 2020 originating from Dubăsari district, Pohrebea village, Republic of Moldova [60, 61]. The harvesting of the white sea buckthorn fruits was carried out in the complete sweeping phase.

Methods. Sampling of sea buckthorn fruits was carried out in accordance with the SM SR ISO 874:2006 standard [62]. The microbiological investigation of the sea buckthorn samples was conducted according to the methods referred to in the sources [63-66]. Ten sea buckthorn fruits of each variety were placed in 50 cm^3 centrifuge tubes (three samples from each sea buckthorn variety) with 25 cm^3 of physiological solution and stored in a dark and cool place for further analysis (24 samples). For microbiological analysis, the sea buckthorn samples were kept in physiological solution no more than 24 hours after harvesting. The dilution method was usedfor the quantitative calculation of colony forming units (CFU) in determining the corresponding groups of microorganisms in 1 cm^3 of bacterial solution. Basic dilutions (10^1, 10^2, 10^3) were prepared as follows: 1 cm^3 of sample was added to a test tube containing 9 cm^3 of saline (0.85%). In Petri dishes with a gel-like nutrient substrate, 1 cm^3 of a bacterial solution from the surface of sea buckthorn was inoculated in triplicate. For the cultivation of microorganisms, two types of nutrient media were used to separate groups of microorganisms: Sabouraud for yeast (5 days of incubation at 25 °C, aerobic cultivation method) and agar for bacteria (48 hours of incubation at 28 °C). After incubation, the results were analyzed and different types of microorganisms were identified. Colonies grown after preliminary microscopy were isolated in cultures and subjected to diagnostic testing for cultural and morphological characteristics, according to which yeast identification was performed. The bacterial culture was further identified by Gram staining.

4. Results

Table 3 shows the results of the microbiological analysis of the 8 varieties of sea buckthorn fruits: R1, R2, R4, R5, L1, C6, AGG, AGA, harvested in September 2020 originating from Dubăsari district, Pohrebea village, Republic of Moldova. The harvesting of the sea buckthorn fruits was carried out in the complete sweeping phase.

Table 3

The results of testing the microbiota of sea buckthorn on the surface of fruits in laboratory conditions

Varieties of sea buckthorn fruits	Detected microorganisms									
	P		Cl		As		Rh		M	
	+/total	%	+/total	%	+/total	%	+/total	%	+/total	%
R1	1/24	4.1	0/24	0	0/24	0	1/24	4.1	0/24	0
R2	0/24	0	0/24	0	0/24	0	0/24	0	1/24	4.1
R4	0/24	0	1/24	4.1	0/24	0	1/24	4.1	0/24	0
R5	0/24	0	0/24	0	0/24	0	0/24	0	0/24	0
L1	0/24	0	0/24	0	1/24	4.1	1/24	4.1	0/24	0
C6	0/24	0	1/24	4.1	0/24	0	1/24	4.1	1/24	4.1
AGG	1/24	4.1	0/24	0	0/24	0	1/24	4.1	0/24	0
AGA	0/24	0	0/24	0	0/24	0	0/24	0	0/24	0
Sea buckthorn fruits	**2/198**	**1.0**	**2/198**	**1.0**	**1/198**	**0.5**	**5/198**	**2.5**	**2/198**	**1.0**

Varieties of sea buckthorn fruits	Detected microorganisms							
	Al		Ge		C		Pi	
	+/total	%	+/total	%	+/total	%	+/total	%
R1	0/24	0	0/24	0	0/24	0	0/24	0
R2	0/24	0	0/24	0	0/24	0	1/24	4.1
R4	0/24	0	1/24	4.1	0/24	0	0/24	0
R5	0/24	0	0/24	0	1/24	4.1	1/24	4.1
L1	0/24	0	1/24	4.1	0/24	0	0/24	0
C6	1/24	4.1	1/24	4.1	1/24	4.1	1/24	4.1
AGG	0/24	0	1/24	4.1	0/24	0	0/24	0
AGA	1/24	4.1	0/24	0	0/24	0	0/24	0
Sea buckthorn fruits	**2/198**	**1.0**	**4/198**	**2.0**	**2/198**	**1.0**	**3/198**	**1.5**

P- *Penicillium:*
Cl- *Cladospurium*
As-*Aspergillus niger*
Rh-*Rhodotorula*
M- *Mucor*
Al-*Alternaria*
Ge-*Geotrichum*
C- *Candida*
Pi- *Pichia*

In the selected samples of sea buckthorn, the following were determined: the quantity of mesophilic aerobic and facultative anaerobic microorganisms – QMAFAnM (CFU)*, the total number of yeasts and molds (CFU), the presence/absence of coliform bacteria and *Salmonella*. The results of microbiological control of sea buckthorn fruits are included in Table 4.

Table 4

Quality and safety indicators of sea buckthorn

Microbiological indicators	Admitted level [67]	Experimental data
QMAFAnM, CFU, max.	$5*10^4$	$<1*10^2$
Coliform bacteria	Not allowed in 0.1 g	Were not found
Pathogenic microorganisms, including *Salmonella*	Not allowed in 25 g	Were not found
Yeast, CFU, max.	$2*10^2$	<10
Fungi, CFU, max.	$5*10^2$	<50

Note. QMAFAnM – quantity of mesophilic aerobic and facultative anaerobic microorganisms; CFU – colony-forming unit.

4. Discussions

The surface of the sea buckthorn fruit was inhabited by various types of bacteria, molds and yeast. The descriptions obtained from the microscopy were compared with the descriptions of other authors.The analyzed sea buckthorn microbiota is represented by Gram pozitive bacteria of the genus *Micrococcus, Staphylococcus, Lactobacillus*, *Streptococcus* and Gram negative bacteria of the genus *Acetobacter*. There were isolated and identified 9 different species of eukaryotes of the genius *Penicillium, Cladospurium, Aspergillus niger, Rhodotorula, Mucor, Alternaria, Geotrichum, Candida, Pichia*.

The results of the study of phytopathogenic microbiota of 8 varieties of sea buckthorn, collected in the phase of full ripeness, are the average values for 3 determinations for each variety. The percentage of detected microorganisms was calculated. The results of the study showed that the phytopathogenic microbiota identified on the studied sea buckthorn fruits formed the following percentage sequence: *Rhodotorula* (2.5%) > *Geotrichum* (2%) > *Pichia* (1.5%) > *Penicillium, Cladosporium, Mucor, Candida, Alternaria* (1%) > *Aspergillus niger* (0.5%).

Proper agricultural technology and timely disease prevention are the key to an abundant and healthy sea buckthorn harvest. The main cause of many diseases of sea buckthorn plantations *(Hippophae rhamnoides L.)* lies in the ignorance of the biological characteristics and the improper technology of growing horticultural crops.

According to the bibliographic study, in both Asia and Europe, sea buckthorn losses occur mainly due to environmental stress, wilt disease and insect pests.

Specialists [68-70] recommend a set of preventive measures against sea buckthorn diseases. Most sea buckthorn diseases are fungal infections, which thrive best in high humidity and temperature conditions. Therefore, the general rule is sanitary care for sea buckthorn, which consists of cleaning fallen leaves, thinning plantings, cutting dry, broken and diseased branches. Do not allow water to accumulate at the roots of the shrubs.

According to specialists in the field, an important step in preventing the development of parasitic fungi would be to spray sea buckthorn plantings with 1% Bordeaux liquid solution or its analogues. This should be done in early spring, before the growing season, and in autumn, after leaf fall. After the procedure of cleaning sea buckthorn trees, all sections should be treated with a solution containing copper, then processed with natural oil paint. This will prevent the development of infection.

On dense plantations, the probability of fungal infection increases significantly, the danger is greater when soil and air humidity are higher.

5. Conclusions

Based on the bibliographic study, it was found that the phytopathogenic microbiota of the sea buckthorn plantations varies depending on the climatic factors, the fruit harvesting period, the location of the sea buckthorn plantation, etc. Monitoring the microbiota present on the berry surface could be a suitable tool for the control of phytopathogenic and potentially antagonistic microorganisms that affect the development and quality of sea buckthorn fruits. This information is highly relevant for developing strategies for organic plant cultivation, disease management and prevention.

Based on the experimental study, it was found that the surface of sea buckthorn fruits is dominated by microorganisms of the species *Rhodotorula, Geotrichum, Pichia*. These types of microorganisms are part of the specific microflora of sea buckthorn fruits and can be carried by vector insects such as fruit flies, bees and wasps. Microbiological control of sea buckthorn fruits showed that the phytopathogenic microbiota does not exceed the established sanitary standards. Thus, the tested varieties of sea buckthorn have good post-harvest properties, can be recommended for storage and can be used throughout the year for a variety of food products.

Acknowledgments: The authors would like to thank the Moldova State Project no. 20.80009.5107.13 *Development technology of sea buckthorn production in the ecological system and processing of fruits and biomass*, running at Technical University of Moldova.

Conflicts of Interest: The authors declare no conflict of interest.

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Citation: Sandulachi, E.; Netreba, N.; Macari, A.; Sandu, Iu.; Boestean, O.; Dianu, I. Phytopathogenic microbiote of sea buckthorn and impact on storage. *Journal of Engineering Science* 2022, 29 (4), pp. 176-189. https://doi.org/10.52326/jes.utm.2022.29(4).14.

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