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The *Brettanomyces bruxellensis* Contamination of Wines: A Case Study of Moldovan Micro-Winery

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Abstract: *Brettanomyces bruxellensis* yeasts cause wine spoilage by producing volatile phenol compounds with specific off-odors. Assessing the propagation of this species is challenging, especially for micro-wineries. In this study, wines produced in a micro-winery from the grapes of different varieties collected from three PGI regions of Moldova over three years were studied for the presence and infection level of *Brettanomyces* spoilage yeasts, using traditional microbiological and molecular methods. The results of *Brettanomyces* infection monitoring in mature wines might speak in favor of the hypothesis that grape berries can be a potential source of *B. bruxellensis* in wine. The contamination levels of mature wines with respective species fluctuated in accordance with the year of grape cultivation, being the highest during the 2023 vintage. This study shows the potential of applying sequencing analysis for tracking the source of *Brettanomyces* contamination in wineries.

Keywords: *Brettanomyces* yeasts; wine spoilage; primers; real-time PCR; sequencing

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1. Introduction

One of the trends in modern wine-making is the development of micro-wineries. Since micro-wineries are categorized as small and medium enterprises (SMEs), they are drivers for local economic development and are of crucial importance for a country's wealth as they furnish employment [1]. For Moldova, this trend is especially relevant, since the Moldovan wine sector is dominated by micro, small and medium-sized wineries. Most wineries (51 or 46%) process up to 1000 tons of wine grapes each year [2]. According to Moldova's legislation, vines and wine law no. 57-XVI from 10 March 2006 [3], small wine producers are those who produce up to 100,000 L of wine per year and own from one to twenty hectares of vineyards. The technological process in micro-wineries is the same as in a traditional winery and is regulated by the same law. Thus, micro-wineries face the same challenges as traditional wineries throughout the technological process, but they do not have the same resources as bigger wineries to overcome them.

Brettanomyces/Dekkera yeasts of the *Pichiaceae* family are generally considered one of the main spoilage agents of wine [4,5]. This genus includes five anamorphic (asexual) species (*B. custersianus*, *B. naardenesis*, *B. nanus*, *B. anomalus* and *B. bruxellensis*) and two teleomorphic (sexual) forms (*D. anomala* and *D. bruxellensis*) [6]. *Brettanomyces bruxellensis*

is ubiquitous and is the most studied representative species found in almost every wine-producing area of the world with the highest spoilage potential among other *Brettanomyces* yeasts [7–9]. The spoilage potential of *B. bruxellensis* in wine is strongly connected with its ability to enter a Viable But Non-Culturable state (VBNC) when exposed to harsh wine conditions [10]. Another important property of *Brettanomyces*/*Dekkera* yeasts is their ability to form biofilms [11,12]. As biofilms, microorganisms exhibit increased resistance to various chemicals, heat, light and drying. *Brettanomyces* yeasts have the ability to tolerate environmental stresses such as high ethanol concentrations (up to 14.5–15%) and sulfur dioxide, low pH and oxygen, low sugar (lower than 300 mg/L) and fermentable nitrogen concentrations [13–15]. These yeasts are facultative anaerobes and can produce high amounts of acetic acid and ethanol under aerobic conditions. They contribute to both wine's volatile acidity [6] and volatile phenol content [16].

Volatile phenols found in wines are microbial derivatives of hydroxycinnamic acids naturally present in grape berries [16,17]. Volatile phenols are recognized for their specific aromas and low sensory thresholds. The phenolic off-odors have been described as "medicinal", "phenolic", "rancid", "sweaty", "smoke", "band-aid", "barnyard" or "horse sweat" [17] and are regarded as olfactory defects in wines. The production of different undesirable flavors in wines can cause high economic losses since such wines are rejected by consumers [6,15,18–20]. There are six compounds responsible for the phenolic flavor: 4-ethylguaiacol, 4-ethylphenol, 4-ethylcatechol and their precursors 4-vinylguiacol, 4-vinylphenol and 4-vinylcatechol [16,17,21,22]. *B. bruxellensis* is able to produce 4-vinylphenol and 4-ethylphenol from *p*-coumaric acid, whereas 4-vinylguaiacol and 4-ethylguaiacol stem from ferulic acid [8]. The threshold levels in red wines for 4-ethylphenol (4-EP) is 620 µg/L and 426 µg/L for the mixture (10:1) of 4-EP and 4-ethylguaiacol [17,23]. The studies show that these values are exceeded worldwide in red wines [24] and are influenced by several factors, the non-volatile wine matrix being one of the key ones [25], while the others are the level of free sulfites, level of dissolved oxygen, storage temperature and enzymatic activity of cinnamate decarboxylase, leading to vinyl derivatives and reduction by a vinyl phenol reductase, originating in the ethyl derivatives [17].

Taking into account the huge impact of *Brettanomyces* on wine quality, a number of research papers were focused on identifying the source of Brettanomyces contamination in wineries. For a long time, *Brettanomyces* was considered to be the issue of mature wines associated with poor sanitary practices in cellars, the presence of cellobiose and micro-oxygenation related to the use of wooden barrels, together with high ethanol content [26–28]. It was not until 2007 that the presence of *B. bruxellensis* on the grape berry surface was clearly demonstrated [18]. Albertin et al. [29] showed the connection between *the B. bruxellensis* strains present on grape berries and in the cellar. This conclusion was supported by the findings of Oro et al. [30], whose data strongly suggest the flux of *B. bruxellensis* from grape berries to the wineries, indicating grape berries as a source of contamination. Once in the cellar, *Brettanomyces* showed an amazing ability to persist in the winery over decades [31], surviving harsh wine conditions [13–15]. This emphasizes the risk of initial contamination of the winery equipment with *Brettanomyces* as well as the challenges and importance of *Brettanomyces* management and control. Being present in vineyards, these yeasts propagate more actively on damaged berries and berries at later stages of ripening [27]. Therefore, grape integrity and harvest time may affect the risk of introducing *Brettanomyces* to the cellar. *Brettanomyces* can survive for a long time in grape pomace, which, when left in vineyards, leads to infestation of a new grape harvest [32]. Insects and air dust can also be a means of yeast dissemination [17].

There are several approaches for the rapid detection of *B. bruxellensis*, with selective plating being considered the gold standard [33]. Different molecular techniques proved to be effective in *Brettanomyces* identification [34,35], both directly from wine [7,36] and from different grape varieties [8,18,37,38]. Molecular assays such as random amplified polymorphism DNA (RAPD) PCR [39–41], mitochondrial DNA (mtDNA) restriction analysis [38], amplified fragment length polymorphism (AFLP) analysis [42–44] and restriction enzyme analysis and pulse field gel electrophoresis (REA–PFGE) [41] have been applied to characterize *Brettanomyces* isolates from different parts of the world [8]. Advanced molecular techniques steadily uncover the knowledge gaps in *Brettanomyces* physiology and systematics explained by the lack of genetic studies on these microorganisms [45].

Controlling the propagation of the yeast during wine-making includes different strategies: by sulfitation, chitosan addition, changing physicochemical variables (temperature, pH, oxygen and alcohol), reducing the precursors of ethylphenols, protein clarification and alcoholic fermentation management (using selected yeast during fermentation, proper rehydration of active dry yeast, alcoholic and malolactic fermentation co-inoculation, etc.) [18,19]. Other than the conventional methods, some novel/experimental physical techniques have been proposed: high hydrostatic pressure (HHP) technology, pulsed electric field (PEF) treatment, high-power ultrasound (HPU) waves, UV-C radiation (254 nm), the application of microwaves, the use of killer toxins and ozone and electrolyzed water use for the disinfection of berries [15,19,46]. All of these methods proved to be effective for diminishing the negative impact of *Brettanomyces* contamination in wines. However, these strategies do not guarantee the complete elimination of *Brettanomyces*, leaving viable cells of the spoilage yeast in the wine. In this regard, monitoring the contamination status of wine during the initial stages and throughout the production process, as well as determining the source of contamination, may help the producer to make informed decisions for applying targeted measures of *Brettanomyces* control, thus reducing the negative impact *of Brettanomyces* and minimizing economic costs. So, the option of choice for wineries is the early detection of wine spoilage as an integrative part of a complex strategy for assuring the quality of the final product. In this context, the objectives of this study were to observe the dynamics and to trace a possible source of *Brettanomyces* contamination in the conditions of a micro-winery, as well as to assess the genetic diversity of *Brettanomyces* isolates in the micro-winery.

2. Materials and Methods

2.1. Collection of Samples

Grape samples were collected from different regions of the Republic of Moldova with Protected Geographical Indication (PGI)—Codru, Stefan Voda and Valul lui Traian. Grapes belong to four major groups (Table 1): international varieties (Merlot, Cabernet Sauvignon), Georgian varieties grown in Moldova (Rkatsiteli), local Moldavian–Romanian varieties (Feteasca Neagra, Feteasca Alba, Feteasca Regala, Rara Neagra) and local Moldavian new selection varieties (Augustina, Ametist, Malena, Alexandrina).

Table 1. Origin of wine samples used in the study.

Nr.	Grape Variety	PGI	Variety Origin
1	Feteasca Neagra	Codru	Local Moldavian–Romanian variety
2	Feteasca Alba	Codru	Local Moldavian–Romanian variety
3	Feteasca Regala	Codru	Local Moldavian–Romanian variety
4	Rkatsiteli	Codru	Georgian variety
5	Merlot	Codru	International variety
6	Feteasca Neagra	Valul lui Traian	Local Moldavian–Romanian variety

Table 1. *Cont.*

Nr.	Grape Variety	PGI	Variety Origin
7	Ametist	Codru	Local Moldavian variety of new selection
8	Augustina	Codru	Local Moldavian variety of new selection
9	Malena	Codru	Local Moldavian variety of new selection
10	Rara Neagra	Stefan Voda	Local Moldavian–Romanian variety
11	Cabernet Sauvignon	Codru	International variety
12	Alexandrina	Codru	Local Moldavian variety of new selection
13	Feteasca Alba/ FeteascaRegala	Codru	Mixed local white Moldavian–Romanian varieties
14	Feteasca Neagra/ Rara Neagra	Codru	Mixed local red Moldavian–Romanian varieties

Samples were collected during three vintage years, 2021, 2022 and 2023, at two stages of wine-making: must production and wine production after clarification and stabilization, and before wine filtering and bottling.

2.2. Wine Production

Wine production was done in the new micro-winery section of the Technical University of Moldova, created in 2015. The TM INOX steam generator was used to sterilize the winery equipment. Wine samples were stored in the production section at 10–12 °C and were taken for *Brettanomyces* detection three months after clarification and stabilization before wine filtering and bottling. Both white and red wines were produced following traditional technology [47]. Detailed information on the wine production procedure is given in Table 2 for white wines and Table 3 for red wines.

Table 2. Technological stages and oenological materials used for production of dry white wines.

Technological Process	Oenological Material/ Equipment	Dose	t °C	Time, Days
Crushing and destemming grapes	Roller crusher/destemmer (Zambelli Enotech)			
Sulfitation of grape must	Potassium metabisulfite, $K_2S_2O_5$	150 mg/dm^3		1
Pressing grape must	Pneumatic press (Zambelli Enotech)			
Clarification of must	Bentonite	0.5–1 g/dm^3	10–14 °C	1
Fermentation of grape must	Selected yeast EnartisFerm SC/ stainless steel tanks (TM INOX)	20 g/100 dm^3	16 °C ± 1	8
Feteasca Alba				8
Feteasca Regala				8
Rkatsiteli				7
Augustina				8
Malena				8
Alexandrina				8
Post-fermentation and wine formation			10–12 °C	30

Table 2. *Cont.*

Technological Process	Oenological Material/ Equipment	Dose	t °C	Time, Days
Removal of wine from the yeast lees with wine equalization and sulfitation	H_2SO_3/centrifugal pump (Zambelli Enotech)	10–20 mg/dm^3	12 °C	1
Integrated wine treatment	Bentonite	0.5 g/dm^3		12
	Fish glue	0.7 g/100 dm^3		
	H_2SO_3	20 mg/dm^3		
Decanting wine from the sediment	Depth filter sheets			1
Cold treatment			−3–5 °C	5–7
Wine filtering	Cartridge membrane filter with pore size 0.65 μm (HOBRAFILT S20 N)		−3–5 °C	1
Wine resting and storage			10–12 °C	10
Sulfitation	H_2SO_3	30 mg/dm^3	10–12 °C	1
Wine bottling and corking	Semi-automatic bottling and topping machine (Zambelli Enotech)			1

For white wine production, harvesting grapes was done manually in 15–20 kg boxes, destemming and crushing grape berries was done by roller crusher/destemmer (Zambelli Enotech) with a capacity of 0.8 tons per hour.

Must separation was carried out with pneumatic press (Zambelli Enotech) (1 ton per hour), with separation of free-run must and the first press fraction in the quantity of 600 dm^3 per one ton of grapes, followed by sulfitation with potassium metabisulfite, $K_2S_2O_5$ at 150 mg/dm^3. Clarification of the must was conducted for 18 h with bentonite administration at 0.5–1.0 g/dm^3, at temperatures of 10–14 °C, followed by decanting the must with centrifugal pump (Zambelli Enotech). Selected yeast EnartisFerm SC (Enartis©, Italy, Galliate) was added to the must at a concentration of 20 g/100 dm^3. Must samples were taken for analysis before yeast inoculation.

The alcoholic fermentation took place at 16 °C for 7–10 days in stainless steel tanks (TM INOX) with a capacity of 500 and 2000 L and temperature regulator. The post-fermentation and wine formation was done at temperatures of 10–12 °C in the production section. Removal of the wine from the yeast lees with wine equalization and sulfitation at 10–20 mg/dm^3 was done with a centrifugal pump (Zambelli Enotech). The storage took place in the production section at 10–12 °C. Integrated wine treatment included fining, racking, bentonite treatment at 0.5 g/dm^3 and treatment with fish glue at 0.7 g/100 dm^3 for 12 days followed by decanting from the sediment with depth filter sheets. Cold treatment lasted 5–7 days in a cold room at temperatures of −3–5 °C with filtering at the same temperature with cartridge membrane filter; wine resting took place for 10 days in the production section followed by bottling in 0.75 dm^3 glass bottles and corking with a semi-automatic bottling and topping machine, (Zambelli Enotech).

For red wine production, grapes were harvested manually in 15–20 kg boxes, followed by destemming and crushing grape berries using roller crusher/destemmer (Zambelli Enotech) with a capacity of 0.8 tons per hour. Selected yeast EnartisFerm SC was added to the crushed grapes at a concentration of 20 g/100 dm^3 along with potassium metabisulfite—$K_2S_2O_5$ at 150 mg/dm^3.

Maceration and fermentation took place in stainless steel TM INOX tanks with a capacity of 500 and 2000 dm^3 and with temperature regulator at 26–28 °C for 4–6 days.

Table 3. Technological stages and oenological materials used for production of dry red wines.

Technological Process	Oenological Material/Equipment	Dose	t °C	Time, Days
Crushing and destemming grapes	Roller crusher/destemmer (Zambelli Enotech)			1
Sulfitation of grape must	Potassium metabisulfite , $K_2S_2O_5$	150 mg/dm^3		
Maceration and fermentation of must	Selected yeast EnartisFerm SC/stainless steel tanks (TM INOX)	20 g/100 dm^3	26–28 °C	4–6
Feteasca neagra				6
Merlot				6
Ametist				4
Rara Neagra				6
Cabernet Sauvignon				6
Pressing fermented grape must	Pneumatic press (Zambelli Enotech)			1
Malolactic fermentation with sugar content of no more 7g/dm^3	Viniflora CH16 (*Oenococcus oeni*)	20 g/100 dm^3	20 °C ± 1	6–8, depending on content of malic acid
The wine aging on yeast lees	Potassium metabisulfite , $K_2S_2O_5$	60 mg/dm^3	10–12 °C	30
Remove wine from yeast lees with equalization and sulfitation	H_2SO_3/centrifugal pump (Zambelli Enotech)	20 mg/dm^3	10–12 °C	1
Integrated wine treatment	Bentonite	0.5 g/dm^3		12
	Fish glue	0.7 g/100 dm^3		
	H_2SO_3	20 mg/dm^3		
Decanting wine from the sediment	Depth filter sheets			1
Cold treatment			−3–5 °C	5–7
Wine filtering	Cartridge membrane filter with pore size 0.65 µm (HOBRAFILT S20 N)		−3–5 °C	1
Wine resting and storage			10–12 °C	
Sulfitation	H_2SO_3	20 mg/dm^3	10–12 °C	1
Wine bottling and corking	Semi-automatic bottling and topping machine, (Zambelli Enotech)			1

Pressing grape mash was done by pneumatic press (Zambelli Enotech) (1 ton per hour), with separation of free-run must and the first press fraction for additional fermentation. For malolactic fermentation the bacterial strain Viniflora CH16 (*Oenococcus oeni*) was used. The wine was aged on yeast lees and removed from yeast lees with equalization and sulfitation (20 mg/dm^3). The storage took place in the production section at 10–12 °C. Integrated wine treatment included fining, racking, bentonite treatment at 0.5 g/dm^3 and treatment with fish glue at 0.7 g/100 dm^3 for 12 days followed by decanting from the sediment with cardboard filters. Cold treatment lasted for 5–7 days in a cold room at −3–5 °C with filtering at the same temperature with cartridge membrane filter with pore size of 0.65 µm (HOBRAFILT S20 N); wine resting took place for 10 days in the production section followed by bottling in 0.75 dm^3 glass bottles and corking with semi-automatic bottling and topping machine (Zambelli Enotech).

Density of must at 20 °C was between 1.076 and 1.097 kg/m^3, according to standard OIV-MA-AS2-01B: R2009 [48]; sugar content in must was 175–233 g/dm^3, according to standard OIV-MA-AS311-01A: R2009 [49]; pH of must was in the range of 3.2–3.5, according to standard OIV-MA-AS313-15: R2011 [50]; titratable acidity of wine was in the range of 5.6–8.0, recalculated in tartaric acid g/dm^3 according to standard OIV-MA-AS313-01: R2015 [51]. Alcohol content in wine was in the range of 10.00–13.60% vol. Detailed information about wine physicochemical properties is given in Table 4.

Table 4. Physicochemical characteristics of wine 2021–2023 vintage **.

Nr.	Grape Variety	Vintage	Density, kg/m^3	Sugar, g/dm^3	Alcohol, %	pH	Titrable Acidity, * g/dm^3
1	Feteasca Neagra	2021	1.094	223	13.4	3.35	6.20
		2022	1.095	228	13.6	3.35	6.00
		2023	1.093	220	13.2	3.45	5.90
2	Feteasca Alba	2021	1.090	212	12.3	3.30	7.00
		2022	1.089	210	12.2	3.25	7.10
		2023	1.091	215	12.5	3.30	6.60
3	Feteasca Regala	2021	1.091	215	12.5	3.30	6.80
		2022	1.090	212	12.3	3.29	6.90
		2023	1.092	218	12.6	3.30	6.50
4	Rkatsiteli	2021	1.080	186	10.8	3.20	7.90
		2022	1.076	175	10.1	3.20	8.00
		2023	1.078	180	10.0	3.20	8.00
5	Merlot	2021	1.096	228	13.3	3.50	6.10
		2022	1.094	223	13.4	3.50	6.10
		2023	1.094	233	13.4	3.40	6.20
6	Feteasca Neagra	2021	1.097	231	13.4	3.50	5.70
		2022	1.094	223	13.3	3.50	5.80
		2023	1.094	222	13.2	3.50	5.60
7	Ametist	2021	1.089	210	12.2	3.25	6.50
		2022	1.091	215	12.5	3.25	6.50
		2023	1.090	212	12.3	3.30	6.40
8	Augustina	2021	1.085	199	11.5	3.30	6.90
		2022	1.087	204	11.9	3.20	6.70
		2023	1.086	202	11.7	3.25	6.80
9	Malena	2021	1.079	183	10.6	3.2	7.10
		2022	1.082	191	11.0	3.25	7.00
		2023	1.080	186	10.8	3.20	7.00
10	Rara Neagra	2021	1.093	220	12.8	3.35	6.00
		2022	1.095	226	13.1	3.30	6.10
		2023	1.094	220	13.1	3.30	6.50
11	Cabernet Sauvignon	2021	1.097	231	13.4	3.40	6.10
		2022	1.097	231	13.4	3.45	5.90
		2023	1.094	223	13.4	3.50	6.00
12	Alexandrina	2021	1.085	199	11.5	3.25	6.90
		2022	1.085	199	11.5	3.35	6.80
		2023	1.087	204	11.9	3.25	6.80
13	Feteasca Alba/Feteasca Regala	2021	1.090	212	12.3	3.30	6.90
		2022	1.091	215	12.5	3.30	6.70
		2023	1.091	215	12.5	3.30	6.60
14	Feteasca Neagra/Rara Neagra	2021	1.094	223	13.4	3.30	6.00
		2022	1.095	228	13.5	3.35	6.00
		2023	1.094	223	13.4	3.40	6.50

*—recalculated in tartaric acid; **—data are presented for wines that were analyzed for all three years.

2.3. Primer Design for Brettanomyces Detection

Initially, two pairs of primers recognizing the large subunit ribosomal RNA gene sequence specific to *Brettanomyces* were developed. Primers were designed using free Primer-BLAST software Primer3 [52].

The default values were used for most parameters except for PCR product size, which was set to the value of 70–200. Primer melting temperature (Tm) was set up to be within 60 for Min, 61 for Opt, 63 for Max; Max GC in primer 3′ end was set to the value of 2; Max Self Complementarity and Max Pair Complementarity-5 were set to the parameter Any. The template used was OP846637.1 *Brettanomyces bruxellensis* strain Bb_5B large subunit ribosomal RNA gene, partial sequence. The amplified product length was 165 bp for the p33 and p34 primer pair and 134 bp for the p35 and p36 primer pair. The sequences and main characteristics of the primers are shown in Table 5.

Table 5. Primers for detection and quantification of *Brettanomyces*.

Name	Primer Orientation	Primer Sequence 5′→3′	Length	Tm	GC%	Self-Complementation	
						5′	3′
p33	Forward primer	AAGCGGCAAGAGCCCAAAT	19	60.61	52.63	3.00	2.00
p34	Reverse primer	ACTCTTCGGCGGGCACTA	18	60.68	61.11	3.00	2.00
p35	Forward primer	TTGATCCGACATGGTGTTTAGCA	23	60.56	43.48	4.00	3.00
p36	Reverse primer	ACACCCTCCGACAGAATCGAA	21	61.16	52.38	4.00	2.00

2.4. Isolation of Brettanomyces from Wine Samples and Wine Inoculation

For isolation of *Brettanomyces* from wine, 10 cm^3 of wine was vortexed for 1 min. Then, 1 cm^3 and 0.1 cm^3 of the vortexed wine sample were spread on two corresponding Petri dishes containing the selection media (*Brettanomyces* Agar, Liofilchem 163942, Roseto degli Abruzzi, Italy). The plates were incubated at 30 °C for five days, and two individual colonies from each plate were picked and regrown on Petri dishes with the *Brettanomyces Agar* [53]. A clump of approximately 2 mm in diameter was picked by a sterile inoculation loop and resuspended in DNA extraction buffer for further DNA extraction from *Brettanomyces* culture.

For checking the efficiency of DNA extraction, the *Brettanomyces* culture grown from blended red wine (Table 1, sample 14) was diluted to 2.5 × 10^3 cfu/cm^3 (confirmed by colony counting) and used to make four consecutive 10-fold dilutions. *Brettanomyces*-free wine was sterilized by autoclaving and inoculated with the dilutions. DNA from the inoculated wine was extracted and *Brettanomyces* concentration was measured by qPCR. The experiment was performed in duplicate.

2.5. Isolation of DNA

DNA isolation from wine and must was performed as previously described with minor modifications [54].

For DNA isolation from wine and must, 10 cm^3 of each wine or must sample was centrifuged at 5000× *g* for 30 min. The pellet was resuspended in 0.6 cm^3 of the extraction buffer (Tris-HCl 0.2 M pH 8.0, NaCl 0.25 M, Na_2EDTA 0.025 M, SDS 5% *w*/*v*), supplied with 0.5 µL of glass beads (Sigma-Aldrich, G8772, Steinheim, Germany), vortexed vigorously for one minute and heated at 65 °C for 30 min. All reagents were of molecular biology grade (Sigma-Aldrich, Burlington, Massachusetts, USA). Then, 60 mg of polyvinylpyrrolidone (PVP) powder and a 0.5 volume of ammonium acetate solution (7.5 M) were added to the sample and incubated on ice for 30 min. After 10 min of centrifugation at 10,000× *g* the supernatant was transferred to a fresh tube, mixed with an equal volume of chloroform,

vortexed and centrifuged again at 10,000× g. The upper phase was transferred to the new tube, mixed with equal volume of isopropanol and incubated at −20 °C for 30 min. The samples were centrifuged, and the pellet was washed twice with 70% ethanol, air dried and dissolved in 50 µL of water; then, 2 µL of the resulting DNA solution was used for each PCR reaction. DNA quality and concentration were checked spectrophotometrically using a Genova Nano micro-volume spectrophotometer. Three aliquots of each sample were taken for DNA extraction.

For DNA isolation from *Brettanomyces* culture, the same protocol was used, with the omission of PVP step.

2.6. Quantification of Brettanomyces

Polymerase chain reactions (PCR) were conducted via the real-time PCR detection system CFX96 TouchTM BIORAD. For SYBRGreen-based analysis, the PCR cycling conditions were used as recommended by SYBRGreen's manufacturer (Applied Biosystems A25741, Vilnius, Lithuania): 95 °C for two minutes as the initial denaturation step followed by alternations of 95 °C for 15 s and 60 °C for 1 min for 40 cycles. For melting curve construction, samples were heated to 95 °C for 15 s, and then incubated at 60 °C for 1 min (1.6 °C/s ramp rate) and heated to 95 °C for 15 s (0.15 °C/s ramp rate). The detection of the amplified product was performed via the SYBR channel.

For Taqman-based analysis, the commercial kit QuickGen PCR kit *Dekkera bruxellensis* quantitative (Gen-AL, Q372, Troisdorf, Germany) was used. PCR cycling conditions were followed as recommended by manufacturer: 95 °C for 15 min, 95 °C for 10 s, 63 °C for 35 s. The detection of *Brettanomyces bruxellensis* fragments was performed via FAM channel, while inhibition control was performed via HEX channel.

For standard curve construction, *Dekkera bruxelensis* Standard DNA 200000 cfu (Gen-AL, Q360, Troisdorf, Germany) was used according to manufacturer's instructions.

2.7. Sequencing and Sequence Analysis

For sequencing, DNA extracted from *Brettamonyces* cultures from the two independent colonies was PCR amplified using ITS1 and ITS4 primers with DreamTaq PCR Master Mix (Thermo Fisher Scientific, K1071, Vilnius, Lithuania) as recommended by the manufacturer. The annealing temperature was adjusted individually from 47 °C to 55 °C, depending on the PCR results. The amplified fragment was visualized on 1.5% agarose gel. In case a single band was observed, PCR product was cleaned up using GeneJet Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, K0831 Vilnius, Lithuania) according to the manufacturer's protocol for PCR fragment cleanup. In case of multiple bands, the band of expected size was excised from the gel and purified using the same kit according to the manufacturer's protocol for gel extraction. The purified fragment was sent for sequencing to CeMIA S.A., Greece. Sequences were processed and BLAST aligned using UGENE 51.0 software; dendrogram was built using the same software with PHYML Maximum Likelihood method with the default parameters.

2.8. Statistical Analysis

The experiments for determining primer efficiency, LOD and LOQ in this study were performed in triplicate. Assessing the efficiency of DNA extraction protocol was performed in duplicate. Assessing *B. bruxellensis* quantity in must and wine was performed in three biological replicates; variances were compared using one-way analysis of variance (ANOVA) followed by Tukey's test at a significance level of $p \leq 0.05$ using the Statgraphics software Centurion XVI 16.1.17 (Statgraphics Technologies, Inc., The Plains, VA, USA) and GraphPad Prism (Boston, MA, USA).

3. Results and Discussion

3.1. Primer Testing and Validation

Primer specificity, efficiency, LOD (Limit of Detection) and LOQ (Limit of Quantification) were tested (Table 3, Figure 1). The specificity of the primers was checked both bioinformatically (in silico) to ensure that no overlapping with other species was detected by BLAST and in vitro by PCR with an inoculation culture of yeast for red wines and white wines, routinely used in the micro-winery facility for wine-making, as well as some grape and wine-associated fungi from an internal collection (*Hanseniaspora uvarum*, *Penicillium* sp., *Meyerozyma* sp.), standard *Brettanomyces* DNA and *Brettanomyces* culture from an internal collection. Blast analysis of the primers, counting the number of hits of the primer sequences against GeneBank accessions, showed high specificity of all primers to *Brettanomyces bruxellensis*, with a limited ability to recognize *Brettanomyces anomalus* (Table 6).

Experimental testing showed that neither of the primer pairs amplified the irrelevant DNA sequence. The results of the primer specificity tests are shown in Figure 1a.

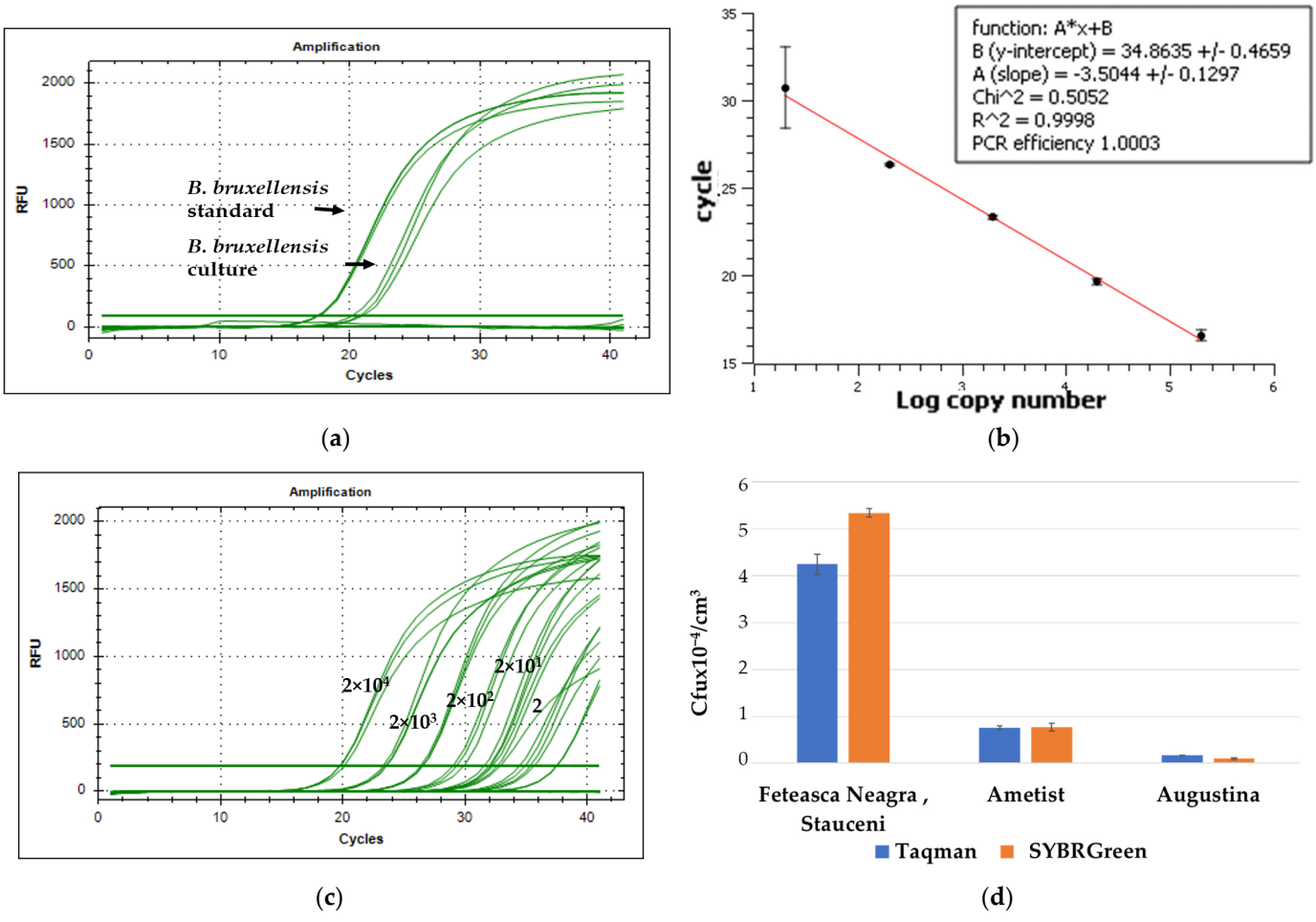

Figure 1. Validation of the primers for *Brettanomyces* detection and quantification. (**a**) Primer specificity of primers p35–36. The amplification is observed only in the samples with *B. bruxellensis* standard DNA or culture DNA. (**b**) Standard curve for primers p35–36. (**c**) LOD and LOQ of the primer pair p35–36. (**d**) The results of quantification of *B. bruxellensis* by Taqman commercial kit and SYBR with home-designed primers.

Table 6. Blast analysis of the primers.

Name	*Brettanomyces*	*Brettanomyces bruxellensis*	*Brettanomyces anomalus*	Uncultured *Dekkera*
p33	250	183	64	-
p34	267	208	56	-
p35	267	209	55	42
p36	267	208	56	19

If a primer pair has 100% efficiency, the DNA amount will double after each PCR cycle. Practically, primer pairs with an efficiency ranging between 90 and 110% are acceptable for real-time PCR. Primer efficiency was calculated from the slope of the standard curve, depicting the dependence of the Cq value of the log of the initial copy number (Figure 1b), and constituted 100% for the p35–36 primer pair. So, the efficiency of this primer pair was acceptable for *B. bruxellensis* detection and quantification by real-time PCR.

LOD (limit of detection) is generally considered the minimum concentration of nucleic acid or the number of cells, which gives a positive PCR result in over 95% of replicas tested [55]. In this case, the amount of 2 cfu consistently gave a positive PCR result with standard DNA (Figure 1c).

The LOQ (limit of quantification) was defined as the smallest amount of analyte that can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation of the International Organization for Standardization [56]. For qPCR, the LOQ is determined as the lowest concentration of analyte, which gives a variability (CV, coefficient of variation) below 25%. In this case, LOQ was 20 cfu for both pairs of primers using a standard DNA template (Figure 1c).

To confirm the correct quantification of *B. bruxellensis* with home-designed primers, three wines of the 2023 vintage were tested with both a commercial kit for *B. bruxellensis* quantification and SYBR green with the primers. One wine sample (Feteasca Neagra, Straseni) had a high infection level, one sample (Ametist) had a medium infection level and one sample (Augustina) had a low infection level. Similar results were obtained by both the commercial kit and primers. The results obtained by both methods are shown in Figure 1d.

Thus, the primers were considered suitable for *B. bruxellensis* detection and quantification by real-time PCR. For the first time, two new primer pairs were tested for Brettanomyces detection; one (p35–36) was shown to have better performance, and its performance was compared to that of the commercial kit.

3.2. Efficiency of DNA Extraction from Wine and Must

Next, the efficiency of DNA extraction followed by *B. bruxellensis* real-time PCR quantification was evaluated. DNA extraction is a crucial yet challenging step of molecular detection of wine contamination since the low DNA quality extracted form wine can compromise the final results [57]. Figure 2 shows the comparison between the cfu number in the sample deduced by colony count (Figure 2a) and by real-time PCR (Figure 2b).

Thus, in the first dilution, where 2.5×10^4 cfu was introduced to the wine, 2.5×10^4 cfu was detected using real-time PCR (81.9% recovery); in the second dilution, when 2.5×10^3 cfu was introduced, 1.9×10^3 cfu was detected using real-time PCR (75.2% recovery); in the third dilution, when 2.5×10^2 cfu was introduced, 2.6×10^2 cfu was detected (104% recovery); and in the fourth dilution, when 25 cfu was introduced, 40 cfu was detected (158.6% recovery). In general, though there is a tendency of underestimation of the actual number of cfu in the case of higher *B. bruxellensis* content and overestimation in the case of lower *B. bruxellensis* content, this precision is good enough for routine *B. bruxellensis* contamination control in the conditions of a micro-winery.

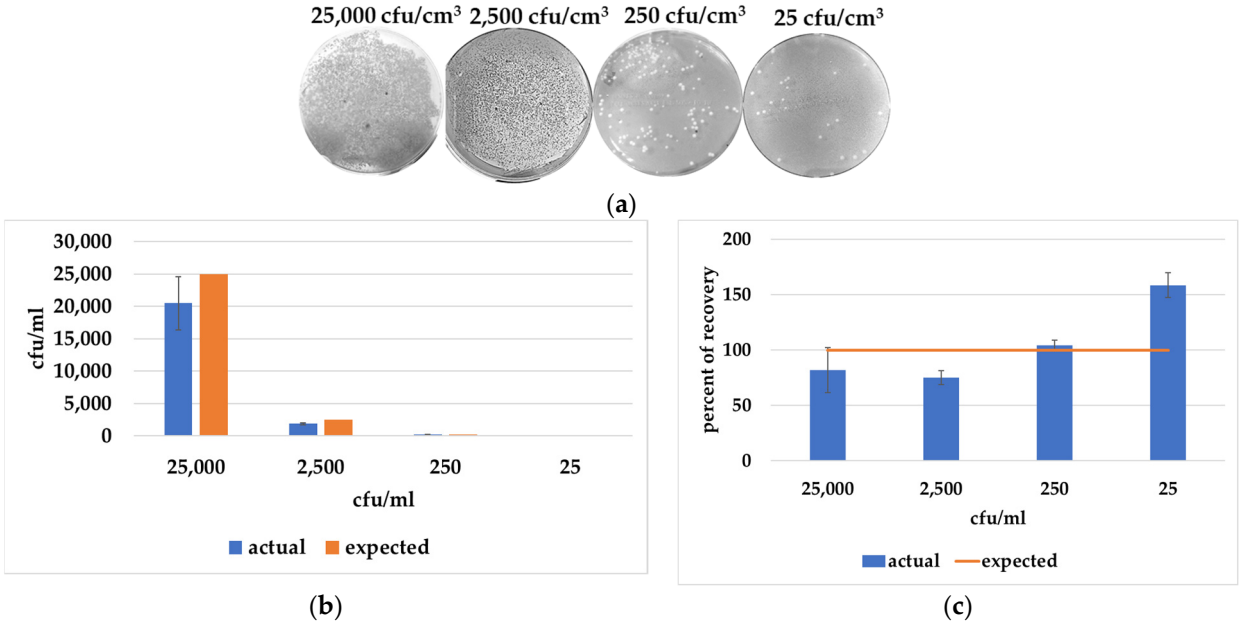

Figure 2. The quantification of *B. bruxellensis* in artificially infected sterile wine by colony count (**a**) and SYBR-based real-time PCR calculated in cfu/cm^3 (**b**) and percent of recovery (**c**).

3.3. Wine Monitoring for B. bruxellensis Infection and Quantification

Wines produced in the micro-winery were monitored for the presence of *B. bruxellensis* at the stage of must and mature wine. Figure 3 shows the dynamics of the infection of musts and wines by year.

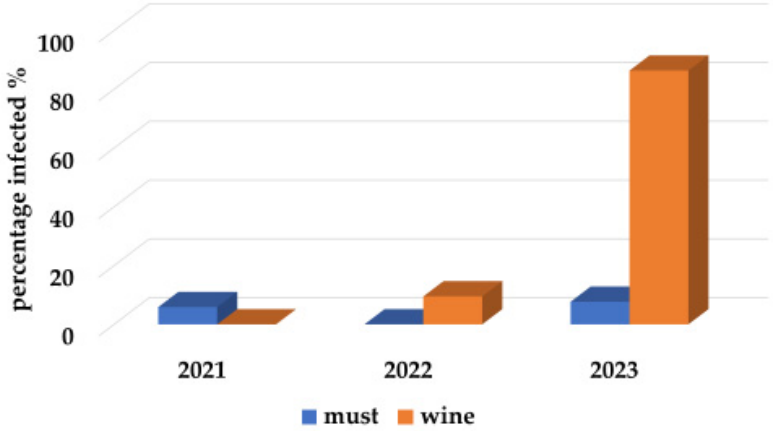

Figure 3. The dynamics of *B. bruxellensis* infection of musts and wines during three years of observation in the micro-winery. *B. bruxellensis* content was monitored in the wines produced in the micro-winery at different stages of wine-making.

There was no significant correlation detected between *B. bruxellensis* must infection and further wine contamination with *B. bruxellensis*. The infection rate of must and wine samples was uneven in different years.

For those samples having a Cq below those corresponding to LOQ (Cq < 32), the quantity of *B. bruxellensis* was determined.

In 2021, only one out of seventeen musts (Feteasca Neagra, Milestii Mici, must of 2021) was contaminated with *B. bruxellensis* (5.9%). Even though the infection load was 9.4×10^2 cfu/ cm^3, corresponding to the Cq 28.7, the wine from this must at a later stage had no detectable *B. bruxellensis*, resulting in no contamination of mature wine of the 2021 vintage with *B. bruxellensis*. In the 2022 vintage, none of the musts had detectable amounts

of *B. bruxellensis*; however, two out of the analyzed twenty-one wines (wine Feteasca Alba, Straseni and wine Feteasca Regala of 2022) were infected with *B. bruxellensis* (Feteasca Alba, Straseni—2.9 × 10^4 cfu/cm^3, Cq value 23.85; Feteasca Regala—3.6 × 10^3 cfu/cm^3, Cq value 27.1). In 2023, two out of the twenty-six musts (Rkatsiteli and Merlot), constituting 7.7% of the samples, had a detectable amount of *B. bruxellensis* (2.5 × 10^2 cfu/cm^3 in the case of Rkatsiteli and 3.7 × 10^3 cfu/cm^3 in the case of Merlot, Milestii Mici). As for wines of the 2023 vintage, nineteen out of twenty-two wines (86.3%) had a detectable amount of *B. bruxellensis* (Cq < 40), and nine out of twenty-two wines (50%) had a significant amount (Cq < 35). Therefore, the two-year trend (2022 and 2023) shows a prevalence of *Brettanomyces* proliferation in wines compared to musts. This is in concordance with previously published data [58], which stated that *Brettanomyces* is a bigger concern at later stages of wine-making. Moreover, wine contamination with *Brettanomyces* increased in 2023 compared to 2021 and 2022. This growing trend of wine contamination with *Brettanomyces* is very worrying. One possible explanation is that *Brettanomyces*, once introduced with grape berries to the micro-winery, persist, proliferate and are established on the equipment. This is consistent with previous findings [59]. Therefore, the barrels, presses, hoses, pumps, vessels, filters, etc., can serve as a contamination source themselves.

In only one of the two musts (Merlot, Milestii Mici) infected with *B. bruxellensis* in 2023, the infection persisted at the wine stage and even increased (3.7 × 10^3 cfu/cm^3, 3.7 × 10^3 cfu/cm^3 at the must stage and 4.1 × 10^4 cfu/cm^3 at the wine stage). The highest amount of *B. bruxellensis* was detected in 2023 in Cabernet Sauvignon (2.0 × 10^6 cfu/cm^3). The results are shown in Table 7.

Table 7. The level of *B. bruxellensis* infection in musts and wines.

Year	Variety	Region PGI	Stage	Concentration, cfu/cm^3
2021	Feteasca Neagra	Codru, Milestii Mici	Must	974 ± 141
2022	Feteasca Alba	Codru, Straseni	Wine	29,805 ± 642
2022	Feteasca Regala	Codru	Wine	3611 ± 225
2023	Rkatsiteli	Codru	Must	253 ± 286
2023	Merlot	Codru	Must	3708 ± 294
2023	Feteasca Neagra	Codru, Stauceni	Wine	53,328 ± 911
2023	Feteasca Neagra	Valul lui Traian, Cantemir	Wine	1692 ± 579
2023	Ametist	Codru	Wine	7696 ± 824
2023	Feteasca Alba	Codru, Cricova	Wine	37,119 ± 3863
2023	Augustina	Codru	Wine	971 ± 225
2023	Malena	Codru	Wine	266 ± 220
2023	Rara Neagra	Stefan Voda	Wine	803 ± 204
2023	Merlot	Codru	Wine	41,479 ± 2628
2023	Cabernet	Codru	Wine	1,980,379 ± 227,165

Moreover, no significant difference in cfu/cm^3 for samples of red and white wines was elucidated. This is consistent with the observations that even though *Brettanomyces* is more of a problem in red wines [60], it can also be present in white wines [4]. This also speaks in favor of the possibility that the infection came at a later stage of wine-making, since the difference in wine-making techniques between white and red wines, as well as later harvest dates of red grapes did not seem to affect *Brettanomyces* concentration.

However, *Brettanomyces* concentrations varied in different samples of the same year. For example, the red wine Rara Neagra (2023, Stefan Voda) was infected to 8 × 10^2 cfu/cm^3, while the red wine Cabernet Sauvignon (2023, Codru) had 2 × 10^6 cfu/cm^3. Similarly, the white wine Rkatsiteli (2023, Codru) had 2.5 × 10^2 cfu/cm^3, while Feteasca Alba (2023,

Codru) had 3.7×10^4 cfu/cm^3 (Table 7). A possible explanation could be that a certain wine matrix favors the propagation of *Brettanomyces* to a greater extent than others, resulting in high *Brettanomyces* content in corresponding wines [61]. This is consistent with our data, that must infection with *Brettanomyces* did not correlate with the infection of wines made from these musts. Since the grapes come to the winery from different regions, the population of *Brettanomyces* is heterogeneous and apparently can be represented by different genotypes. This assumption is partially supported by the data presented in the next section. The concentration of vinyl phenols and ethyl phenols produced by *Brettanomyces* depends not only *on Brettanomyces* concentration but also on the *Brettanomyces* strain [62] and wine matrix [25].

Another possibility is the higher prevalence of *Brettanomyces* in the 2023 vintage. This hypothesis conforms with previous research [63] showing very different levels of *Brettanomyces* infection of wine in two consecutive vintages.

A small number of infected samples in 2021 and 2022 did not allow for statistical evaluation of the effect of the year on *B. bruxellensis* infection. However, the average infection load in wines in 2022 and 2023 did not differ significantly.

Most of the wine samples were obtained from vineyards of the Codru and Stefan Voda PGI regions, and no statistical difference was identified in *Brettanomyces* propagation in wines obtained from grapes cultivated in the mentioned regions. Therefore, it could be stated that climatic, edaphic and biotic factors, as well as agrotechnical practices of grapevine cultivation in two different regions of Moldova, do not play a major role in *Brettanomyces bruxellensis* propagation in mature wines. In addition, the data imply that this species under favorable conditions propagates equally on both white and red wines, not manifesting specific media preference for its growth and development. However, depending on the grape variety, the *Brettanomyces* concentration may have a very different impact on the quality of the final product. Since greater potential of volatile phenol production arises from higher levels of precursors (ferulic and *p*-coumaric acids) [64], and the amount of these contents differs depending on grape variety, with white wines containing a concentration of *p*-coumaric acid 5–25% compared to red wines, probably due to the lack of the maceration stage [65], the quality of red wines is more likely to be impaired by *Brettanomyces* infection.

To our knowledge, this is the first study of *Brettanomlyces* dynamics in a Moldavian winery testing local Moldavian–Romanian varieties and local Moldavian new selection varieties. This has potential implications for Moldavian and Romanian wine-makers since the wine matrix is known to be important for wine spoilage by *Brettanomyces*.

3.4. Genetic Sequencing

An important issue for wine-makers is the source of *B. bruxellensis* in musts and wines. Because of the growing dynamics of wine infection with *Brettanomyces*, it was important to find out the source of contamination in the micro-winery. We assumed that if the *B. bruxellensis* isolated from different wines of the winery were genetically similar, the contamination must have occurred in preceding years and the cells survived the sanitation practices of the winery. Otherwise, in case genetic divergence is detected in different wines, the *B. bruxellensis* cells most likely are introduced with the grape berries. However, this does not exclude the possibility that the contamination occurred during several years, and each year new's genotype of *Brettanomyces* was introduced in the micro-winery, resulting in a non-homogeneous population. In order to clarify if the *B. bruxellensis* contamination of different wines in the micro-winery had a common origin, *B. bruxellensis* from different wines of 2023 was isolated and a genomic fragment flanked by ITS1 and ITS4 primers was amplified and sequenced using ITS1 primer. Sequences were submitted to the NCBI database with

the following accession numbers—BrettS2: PQ219467 [66]; BrettS4: PQ219468 [67]; BrettS10: PQ219469 [68]; BrettS12: PQ219470 [69]; BrettS13: PQ219471 [70]. Figure 4a shows the alignment of the obtained sequences of different isolates.

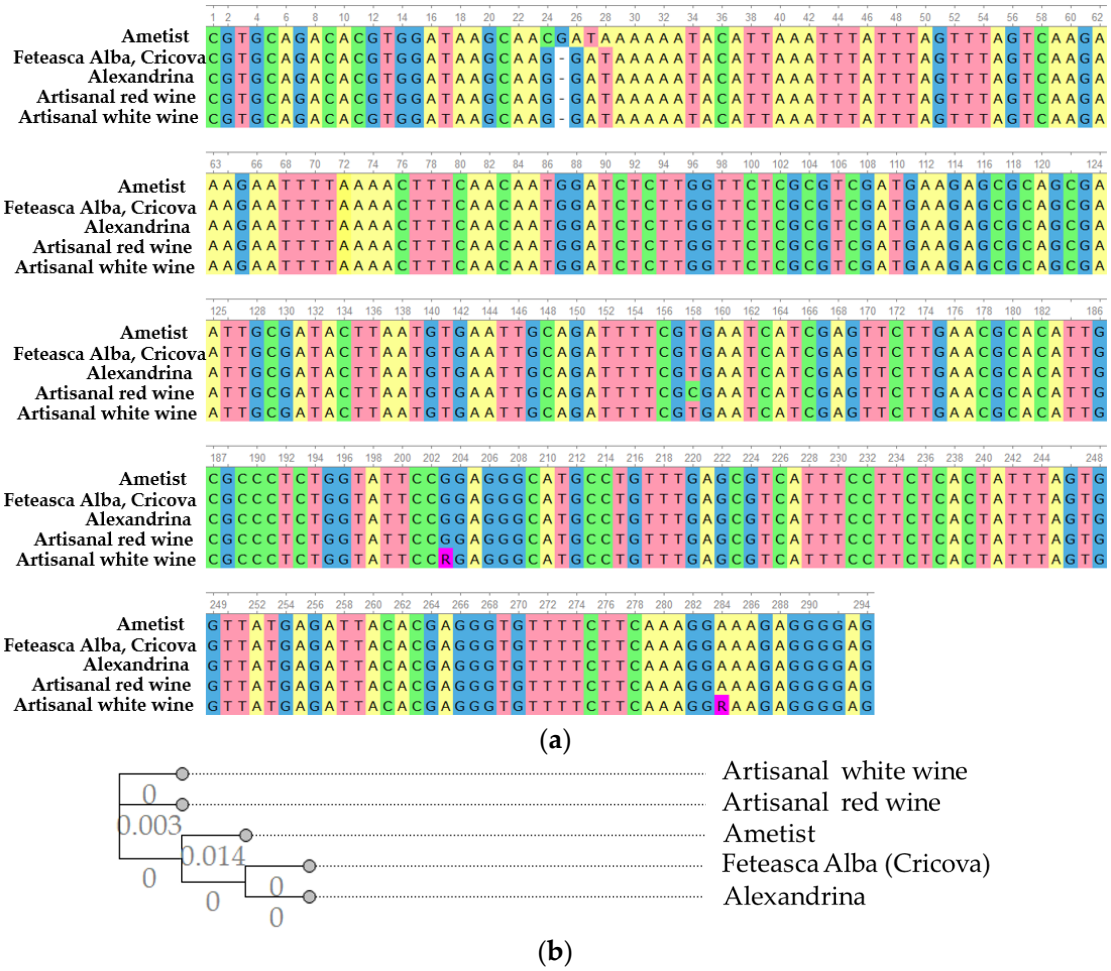

Figure 4. ITS1-ITS4 nucleotide sequences from individual *B. bruxellensis* isolates from artisanal wines and wines from micro-winery. (**a**) Alignment; (**b**) dendrogram of similarities. Different colours indicate different nucleotide bases.

Since there were no available data of *B. bruxellensis* sequences isolated form wines of Moldova, two artisanal wines, white and red, were also used as a control.

Interestingly, *B. bruxellensis* isolated from three different wines made in the micro-winery had a polymorphism in the sequenced fragment, with Ametist, infected with *B. bruxellensis* isolate, previously described as an isolate of South African and Lebanon origin, while Feteasca Alba, Cricova and Alexandrina, contaminated with a different *B. bruxellensis* isolate, were previously found in the Mediterranean wine-making region (Table 8).

This isolate is similar to the one infecting artisanal red wine (region Codru) and artisanal white wine (region Codru), except for one SNP (T to G substitution) in artisanal red wine and two ambiguous bases (possibly, a heterozygous) in artisanal white wine. *Brettanomyces* DNA fragments sequenced from both colonies from one wine sample had identical sequences.

DNA from two independent single colonies isolated from the wine Feteasca Regala were sequenced, resulting in chromatograms with overlapping peaks and alignment of the sequencing indicating the highest degree of similarity of 81% with *B. bruxellensis*. One

of the possibilities is that the *B. bruxellensis* isolate infecting this wine is heterozygous for an insertion or deletion. An alignment of the two sequences using BLAST algorithm showed 84% of matches, supporting the hypothesis that the two colonies could the same heterozygous isolate.

Table 8. Blast analysis of ITS1 sequences of *Brettanomyces bruxellensis*, isolated from different wines.

ID	Variety	Species	Similarity, %	Gene Bank Accession	Country of Origin	Source
BrettS2	Artisanal, red wine	*B. bruxellensis*	100	KY103313.1	France	Wine
BrettS4	Artisanal, white wine	*B. bruxellensis*	99.32	NR165974.1	Belgium	Beer
			99.32	MH393498.1	New Zealand	Combucha
			99.32	MH252564.1	France	Wine
BrettS10	Ametist	*B. bruxellensis*	100	KY103314.1	South Africa	Wine
			100	JQ327831.1	Lebanon	Wine tank
BrettS12	Alexandrina	*B. bruxellensis*	100	MT734879.1	Portugal	Wine
			100	MH252564.1	France	Wine
BrettS13	Feteasca Alba, Cricova	*B. bruxellensis*	100	MT734879.1	Portugal	Wine
			100	MH252564.1	France	Wine

Thus, based on these data, the introduction of different *Brettanomyces* isolates into the micro-winery with grapes seems to be a more likely hypothesis. However, it should be further confirmed by strain identification since, in this case, only primers for species identification were used. Nevertheless, even a relatively conservative fragment had some polymorphism in the *Brettanomyces* isolates that originated from different grapes.

4. Conclusions

The contamination levels of mature wines with respective species fluctuated in accordance with the wine-making season. The dynamics of the *Brettanomyces* infection of mature wines might speak in favor of the hypothesis that grape berries act as carriers for *B. bruxellensis* cells to enter the wine and the winery. Additionally, *Brettanomyces* concentrations varied in different samples of the same year, highlighting the importance of the wine matrix and wine-making techniques in the suppression of *Brettanomyces* growth.

To the best of our knowledge, this was the first sequencing of *Brettanomyces* isolated from local Moldovan wines produced in a micro-winery. The sequencing analysis revealed a polymorphism in the sequenced DNA fragment from *B.bruxellensis* isolates from different wines. This confirms the potential of sequencing analysis in tracing the contamination source.

The obtained results are applicable for any type of winery, micro-, small, medium and large; however, they are especially relevant for micro- and small wineries. Furthermore, they are of particular relevance for new wineries, possibly facing a similar issue of the growing *Brettanomyces* infection trend.

The management of wine spoilage microorganisms implies the prevention of contamination, detection of undesirable microorganisms and treatment of contaminated wines. This study is concentrated on detection and tracing the source of contamination; however, it would be interesting to assess the effect of different treatments on wine contamination with *Brettanomyces*, as well as the effect of different parameters on the accumulation of undesired *Brettanomyces* metabolites and their effect on the quality of the final product. Additionally, it is important to see how wine contamination with *Brettanomyces* can be reduced in the following years.

Author Contributions: Conceptualization D.Z. and I.M.; methodology, D.Z., I.M., V.M., C.G., S.R., R.S., E.B., F.I. and N.H.; writing—original draft preparation, D.Z., I.M., V.M., R.S. and A.B. writing—review and editing, D.Z., I.M., V.M., A.B., R.S., F.I. and N.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

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

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