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Phenolic compounds contained in little-known wild fruits as antiadhesive agents against beverage-spoiling bacteria *Asaia* spp.

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Abstract: The aim of the study was to evaluate antioxidant activity and total phenolic content of three fruit juices: elderberry (*Sambucus nigra*), lingonberry (*Vaccinium vitis-idaea*) and cornelian cherry (*Cornus mas*), and their action against adhesion of bacterial strains of *Asaia lannensis* and *Asaia bogorensis* isolated from spoiled soft drinks. The antioxidant profiles were determined by total antioxidant capacity (DPPH), and ferric-reducing antioxidant power (FRAP). Additionally polyphenol content (TPC) was investigated. Chemical compositions of juices were tested by chromatographic techniques HPLC and LC-MS. Adhesion properties of *Asaia* spp. cells to various abiotic materials were evaluated by luminometry, plate count and fluorescence microscopy. Antioxidant activity of fruit juices expressed as inhibitory concentration (IC_{50}) ranged from 0.042±0.001 (cornelian cherry) to 0.021±0.001 g/mL (elderberry). TPC ranged from 8.02±0.027 (elderberry) to 2.33±0.013 mg/mL (cornelian cherry). Cyanidin-3-sambubioside-5-glucoside, cyanidin-3-glucoside, cyanidin-3-sambubioside were detected as the major anthocyanins and caffeic, cinnamic, gallic, protocatechuic, and p-coumaric as the major phenolic acids. The significant linear correlation was noted between TPC and antioxidant capacity. In the presence of fruit juices the significant decrease of bacterial adhesion from 74% (elderberry) to 67% (lingonberry) was observed. The high phenolic content indicated that these compounds may contribute to the reduction of *Asaia* spp. adhesion.

Keywords: *Asaia* spp.; Fruit juices; Berry juices; Polyphenols; Antiadhesion

1. Introduction

The presence of spoilage microorganisms in the production lines increases the risk of cross-contamination of products and, in the case of certain groups of microorganisms, it can have a significant effect on the risks for the public health [1]. Representative microorganisms, increasingly isolated from functional drinks, are Gram-negative, acetic acid bacteria *Asaia* spp. Numerous studies on *Asaia* spp. and their presence in non-alcoholic beverages are described in the literature [2-3]. It was noted that the growth of these bacteria causes significant changes in organoleptic qualities of final products, such as turbidity and flock formation. Furthermore, *Asaia* strains are characterized by strong adhesive abilities on food contact materials. Consequently, the adhesion and proliferation of these bacteria on solid surfaces leads to the formation of biofilms which can be potential sources of product contamination [2]. What is more *Asaia* spp. are considered to be opportunistic pathogens. They can cause infections in people with immunodeficiency, such as pediatric patients, children and patients with a history of intravenous drug abuse. Moreover, *Asaia* strains are characterized by high resistance to chemical preservatives commonly used to improve the microbiological stability of food,

such as benzoates, sorbates and dimethyldicabonate. In general, in the case of microbial cells forming biofilms, increased resistance to commonly used disinfectants such as quaternary ammonium compounds, peracetic acid and hydrogen peroxide is observed [4-5]. Therefore, there is a growing demand for effective alternatives to the chemicals used against these bacteria [6].

Medicinal plants have historically proven values as natural sources of molecules with therapeutic potential. However, in the past decades, pharmaceutical industry has focused mainly on synthetic compounds as drug discovery source [7]. On the other hand, social interest in old medicinal plants is growing, and the global market of herbal medicines stands at over $ 60 billion annually and generates increasing interest and publicity. The importance of plants results from the fact that bioactive compounds found in the wild cannot be reproduced in the laboratory. As a result, it is estimated that about 60% of antimicrobial drugs discovered in the past few decades are of natural origin [8]. The use of plants with antimicrobial activities is particularly important in the food industry. Many research projects aim to identify and characterize natural products by the combined and synergistic use of computational techniques, ethnopharmacological knowledge, chemistry, and a broad range of cell-based models [9].

Among the plant origin sources of bioactive compounds characterized by antimicrobial and antiadhesive activities, berries are one of the most prominent. Recent reviews showed that phenolic compounds from berries show antimicrobial activity against fungi, viruses, and bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) strains [10]. *In vitro* studies showed that particular berries, like cranberry (*Vaccinium macrocarpon*), cloudberries (*Rubus chamaemorus*), bilberries (*Vaccinium myrtillus*) and strawberry are characterized by antimicrobial action against pathogens belonging to *Escherichia*, *Salmonella*, *Staphylococcus*, *Helicobacter*, *Clostridium* and *Campylobacter* genera or their inflammatory agent such as endotoxin lipopolysaccharide (LPS) [11-13]. Effective activity of fruit juices was also noted against yeasts and molds such as *Candida krusei*, *Candida albicans*, *Trichophyton tonsurans*, and *Aspergillus fumigatus* [14]. These fruits are rich source of anthocyanins, phenolic acids, flavanols, flavonols and tannins with health promoting actions, and have been used for centuries in folk medicine as natural remedies for many diseases [15]. What is more, these compounds show high antioxidant capacity and anti-ulcer, anti-inflammatory, anti-cancer and anti-microbial properties [16-17]. Additionally fruit juices can be used as natural food colorings and aromas in the food industry, which excludes the need to use synthetic equivalents.

While some fruits are common on supermarket shelves around the world, there are many other wild-growing fruit that are not so well-known. These include elderberry (*Sambucus nigra*), lingonberry (*Vaccinium vitis-idaea*) as well as cornelian cherry (*Cornus mas*). Their common feature is that they grow as perennial wild plant in Europe and they are known from traditional folk medicine. *Sambucus nigra* fruits and flowers have been used in traditional medicine internally for treatment of disorders of the respiratory and gastrointestinal tracts, mouth, skin, and for viral infections, fever, colds, and influenza [18]. *V. vitis-idaea* has a long history of use as a antihemorrhagic, antiseptic and anti-urogenital agent. *Cornus mas* is considered as the least known in Europe but these uncommon fruits are rich in vitamin C, and can be used to fight cold and flu [19]. Early studies of these wild-growing fruits focused mainly on their direct antimicrobial potential against pathogenic bacteria [20]. However, much less attention has been paid to activities against spoilage microflora of production lines in the beverage industry. Therefore, the aim of our study was to characterize bioactive components of these three juices, evaluate their antioxidant activity *in vitro*, as well as their action against adhesion of bacteria *Asaia* spp. isolated from spoiled soft drinks.

2. Results and Discussion

2.1. Carbohydrates content

Each fruit juice was analyzed for two of the most abundant dietary sugars: glucose and fructose by spectrophotometric method using enzymatic assay kits specific for these carbohydrates. This method is characterized by high sensitivity, with lower limit of detection equaling 0.332 mg/L. Sugar content varied depending on the type of fruit. All tested fruit juices contained fructose, but the highest content of this monosaccharide was found in the juice of cornelian cherry (5.56±0.061 g/100 mL) (Table 1). However, this juice contained the lowest glucose content equaling 2.97±0.046 g/100 mL. The lowest fructose content (3.29±0.015 g/100 mL) was noted for elderberry juice, and this value was similar to the elderberry glucose content (3.19±0.022 g/100 mL). The results of our research are in line with the available literature. Carbohydrate content in elderberry is 18%, 11.5% of which are monosaccharides. Over 95% of these are fructose and glucose present in similar amounts [18]. According to Veberic et al. (2009), fresh elderberry fruits contain fructose and glucose, and their amounts range from 3.39±0.093 to 5.25±0.143 and from 3.333±0.067 to 5.23±0.053 g/100 g, respectively [21]. The reducing sugars content in cornelian cherry range from 5.2 to 12.00 g per 100 g of fresh weight, with an average value of 8.1±1.6 g/100 g. Similarly to *S. nigra* and *C. mas*, the main sugars in lingonberry juice are glucose and fructose, which contents are 4.3 g/100 mL and 4.4 g/100 mL, respectively [22]. The differences in sugars contents can be attributed primarily to the genotype of the plants, as well as geographical location and prevailing climatic conditions [23].

Table 1. Sugar content and antioxidant properties of investigated fruit juices

Berry juice	Sugar content [g/100 mL]		TPC [mg GAE/mL]	Antioxidant activity	
	Fructose	**Glucose**		**DPPH IC_{50} [g/mL]**	**FRAP IC_{50} [g/mL]**
Cornelian cherry (*Cornus mas*)	5.56±0.061	2.97±0.046	2.33±0.013	0.045±0.001	0.042±0.001
Lingonberry (*Vaccinium vitis-idaea*)	3.89±0.043^{c}	4.54±0.071^{c}	4.87±0.044^{c}	0.054±0.002^{c}	0.030±0.002^{c}
Elderberry (*Sambucus nigra*)	3.29±0.015^{c}	3.19±0.022^{b}	8.02±0.027^{c}	0.072±0.001^{c}	0.021±0.001^{c}

GAE – gallic acid equivalents; Values are means of three determinations ± standard deviation. Values in the same column with the different superscript lowercase letters are statistically different ($p < 0.05$). ^{a} – $p \geq 0.05$; ^{b} – 0.05÷0.005; ^{c} – $p < 0.005$; The results were compared to those received for *C. mas*.

2.2. Antioxidant capacity and total phenolic content

Generally, several assays have been frequently used to estimate antioxidant capacity in fruits and their products, such as 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP). What is more, in the antioxidant capacity assays, peroxyl (ORAC), hydroxyl (HORAC), superoxide anion (SORAC), peroxynitrite (NORAC), and singlet oxygen (SOAC) radicals/oxidants are used [24]. However, FRAP and DPPH which are based generally on a single electron transfer (SET) mechanisms and measure the ability of an antioxidant to transfer one electron to reduce compound are still the most commonly used. DPPH test measures the ability to scavenge free radicals, while the FRAP assay quantifies the total concentration of redox-active compounds. Both tests are simple, relatively rapid, reproducible and do not require specialized equipment, thus can be used for assessing antioxidant activity in foods and plant extracts.

The results of DPPH test obtained in our study indicate that cornelian cherry juice showed the strongest antioxidant properties (IC_{50} = 0.045±0.001 g/mL), while the lowest capacity was obtained for elderberry juice (IC_{50} = 0.072±0.001 g/mL). It has been documented that the water extract of elderberry had lower DPPH radical scavenging capacity than bilberry or chokeberry, but

129 comparable to raspberry fruit extract [25]. On the other hand, in the study of Jakobek et al. (2007)
130 elderberry juice showed stronger antioxidant activity than black currant, red raspberry, sour cherry,
131 sweet cherry, as well as strawberry juices [26]. It is worth noting that wild growing *C. mas* shows
132 stronger DPPH radical scavenging ability than the cultivated forms [27]. According to Georgieva
133 and co-workers (2016), lingonberry showed lower DPPH capacity than strawberry, raspberry and
134 bilberry, while Tarko et al. (2015) noted that beverages supplemented with 2% lingonberry juice
135 exhibited lower antioxidant activity than products enriched with 2% cornelian cherry juice [28-29].

136 The FRAP test results obtained in our study indicate that the elderberry juice (IC_{50} = 0.021±0.001
137 g/mL) was characterized by the strongest activity, followed by lingonberry juice (IC_{50} = 0.030±0.002
138 g/mL). The results of DDPH and FRAP assays obtained in our study strongly correlate with the
139 results of TPC tests. For FRAP method R coefficient amounted to 0.9896 (y= -0.0037x+0.0496) while
140 for DPPH this value was 0.9917 (y= 0.0048x+0.0327). TPC was the highest in elderberry juice, lower
141 in lingonberry juice, while the amount of polyphenols was the lowest in cornelian cherry juice (Table
142 1). Due to the highest TPC elderberry juice was characterized by the strongest antioxidant activity
143 measured using FRAP assay. According to Tarko and co-workers (2015) TPC in lingonberry juice is
144 comparable to cornelian cherry and accounts 51±1.1 and 51±1.0 mg catechin/100 mL, respectively
145 [29]. In the work of Šamec and Piljac-Žegarac (2011) cornelian juice was characterized by high
146 content of phenols reaching the level of 501.58±10.11 mg GAE/100 g, thereby providing a richer
147 source of phenolic compounds than red (147.39±2.42 mg GAE/100 g) and white (60.12±3.05 mg
148 GAE/100 g) grapes [30]. These results agree with those obtained by Moldovan and co-workers
149 (2016), who noted that the TPC for fresh *C. mas* fruits extract was 489.94±17.88 mg GAE/100 g [31].
150 However, the *C. mas* juice tested in our study (recalculating 233±1.00 mg GAE/100 mL) was
151 characterized by lower polyphenols content than those presented by the other authors. Again, these
152 differences may arise not only from geographic determinants, but primarily from plant genotypes.

153 According to a review of the literature conducted by Nile and Park (2014) elderberry fruits are one of
154 the richest sources of phenolic compounds among berries, with TPC 104 mg GAE/g, while Sidor and
155 Gramza-Michałowska (2015) have reported that this value ranges from 3.6 mg GAE/g to 19.5 mg
156 GAE/g [18, 32]. On the other hand, Silva et al. (2017) have shown that elderberry contains
157 approximately 1.19 g GAE/100 g which is slightly higher than the values obtained in our study [33].
158 Differences in the obtained values may be due to many factors. For example, climate conditions, fruit
159 variety as well as processing methods (heating, filtration, crushing), and storage conditions (air,
160 temperature) can cause changes in the composition of (poly)phenols [30]. It is also worth to note that
161 the stability of anthocyanins from elderberry is higher in the presence of glucose, but significantly
162 lower in the presence of fructose [18]. Furthermore, antioxidant capacity of biomolecules is
163 significantly influenced by the structure. The activity may be attributed to the enhanced stabilization
164 of the radical state during electron transfer when assayed relative to compounds that lacked the
165 orthodiphenolic structure. The addition of a glycoside residue, such as glucose, at position 3 on the
166 C-ring or methylation of the 3′ and/or 5′ hydroxyl group on the anthocyanidin B-ring, has been
167 shown to reduce the antioxidant capacity and radical scavenging activity [35].

168 *2.3. Phenolic profiles*

169 The tested fruit juices are rich source of phenolic acids (PAs) as well as flavonols, flavanols and
170 anthocyanins (Table 2). The presence of these groups of compounds was detected by HPLC and
171 confirmed by LC-MS, therefore additional compounds were also reported (Table 3). In the cornelian
172 cherry the highest content among phenolic acids was obtained for gallic acid (2.025±0.314 μg/mL),
173 while caffeic acid was the major acid for elderberry juice (2.603±0.313 μg/mL). However, despite the
174 greatest variety of compounds in lingonberry juice, the extract contained the lowest amount of
175 phenolic acids.

176

177 Table 2. Phenolic profiles of investigated berry juices

Proposed molecule	Concentration [μg/mL]		
	Cornus mas	*Vaccinium vitis-idaea*	*Sambucus nigra*
Caffeic acid	nd	nd	2.603±0.313[d]
Cinnamic acid	0.143±0.011	0.191±0.014[b]	nd
Gallic acid	2.025±0.314	0.071±0.009[c]	0.286±0.082[c]
Protocatechuic acid	0.379±0.271	0.497±0.087[a]	0.550±0.057[a]
p-coumaric acid	0.108±0.048	0.179±0.052[a]	nd
Rosmarinic acid	0.128±0.062	0.128±0.037[a]	0.128±0.019[a]
4-hydroxybenzoic acid	nd	0.150±0.074[a]	0.265±0.096[a]
Catechin	nd	0.662±0.121[a]	0.918±0.107[a]
Epicatechin	nd	0.304±0.082[d]	nd
Rutin	nd	nd	1.321±0.307[d]
Delphinidin-3-glucoside	nd	nd	2.057±0.371[d]
Cyanidin-3-sambubioside-5-glucoside	nd	nd	2.260±0.219[d]
Cyanidin-3-glucoside	0.280±0.039	0.605±0.054[c]	3.738±0.147[c]
Cyanidin-3-sambubioside	nd	nd	3.143±0.262[d]
Cyanidin-3-robinobioside	0.321±0.041	nd	nd
Petunidin-3-galactoside	nd	0.320±0.057[d]	nd
Petunidin-3-glucoside	nd	0.528±0.052[d]	nd
Pelargonidin-3-glucoside	0.380±0.052	0.359±0.063[a]	nd
Pelargonidin-3-rutinoside	nd	0.344±0.074[d]	nd
Pelargonidin-3-robinobioside	0.302±0.022	nd	nd

178 Values are means of three determinations ± standard deviation. Values in the same row with the
179 different superscript lowercase letters are statistically different ($p < 0.05$). [a] – $p \geq 0.05$; [b] – 0.05÷0.005;
180 [c] – $p < 0.005$; [d] – not compared; The results were compared to those received for *C. mas*. Anthocyanin
181 contents were expressed as μg of cyanidin-3-glucoside per one mL. nd – not detected

182 Our results for cornelian cherry agreed with this obtain by Moldovan et al. (2016) who found that
183 these fruits contain mainly chlorogenic and caffeic acids [31]. However, in their article the authors
184 pointed that the main phenolic acid in the fruit is ellagic acid. Deng et al. (2013) have noted the
185 presence of chlorogenic and gallic acids in *Cornus mas* fruits [36]. On the other hand Radovanović et
186 al. (2013) found gallic, p-coumaric and caffeic acids as major phenolic acids in these fruits [37]. The
187 lingonberry juice analysed in our research contained protocatechuic acid as a major phenolic acid,
188 with the concentration of 0.497±0.087 μg/mL. Our results on the PAs in the lingonberry juice are in
189 line with these in available literature. In the work of Häkkinen (1999), the most abundant phenolic
190 acids in lingonberry were p-coumaric acid (19.9%), ferulic acid (7.0%), caffeic acid (2.6%),
191 hydroxy-benzoic acid (2.1%) and ellagic acid (1.1%) [38]. On the other hand, Mattila and co-workers
192 (2006) found that main PAs were protocatechuic, vanillic, cinnamic, and gallic acids [39].
193 Subsequently, results of chemical analysis of elderberry juice showed that caffeic acid is the major
194 acid from this group of phenolic compounds. The study conducted by Tarko et al. (2017) noted the
195 presence of caffeic acid in elderberry while Lee and Finn (2007) found cinnamic and chlorogenic as
196 the main PAs in *S .nigra* [40-41]. Furthermore, Mikulic-Petkovsek et al. (2015) described that different
197 genotypes of elderberry contained different derivatives of coumaric, caffeic and cinnamic acids [42].

198 Although we did not detect cinnamic acid in our studies using HPLC nor LC-MS techniques, the
199 second of these methods showed that the juice of elderberry contains chlorogenic acid.

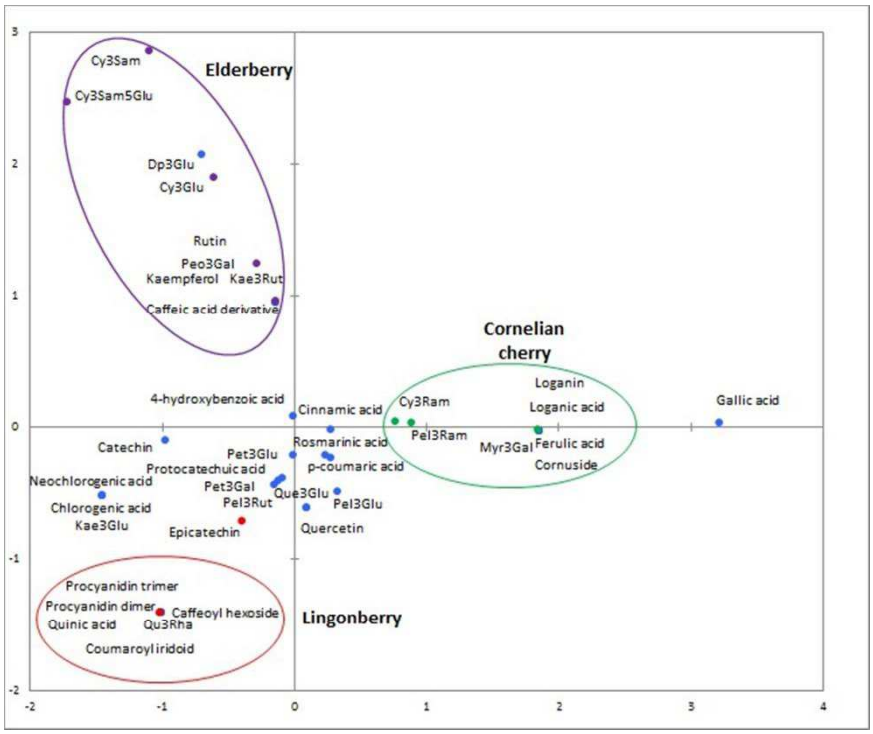
200 Table 3. Major polyphenolic compounds present in the tested juices, using LC-MS method.

Aglycone class	Proposed molecule	λ_{max} (nm)	$[M-H]^-$	MS^2	*Cornus mas*	*Vaccinium vitis-idaea*	*Sambucus nigra*
Phenolic acids	Caffeic	279	179	135	-	-	+
	Caffeic acid derivative	234, 279	341	177, 195	-	-	+
	Caffeoyl hexoside	231, 282	341	179	-	+	-
	Chlorogenic	295, 323	353	191	-	+	+
	Neochlorogenic	323	353	179, 191	-	+	+
	Ferulic	237, 323	193	149, 173	+	-	-
	Gallic	237, 276	205	111, 125, 173	+	+	+
	Quinic	235, 284	191	111, 173	-	+	-
Flavonols	Kaempferol	239, 279, 325	285	213, 257	-	-	+
	Kaempferol-3-glucoside	263, 344	447	255, 284, 327	-	+	+
	Kaempferol-3-rutinoside	265, 342	593	285	-	-	+
	Quercetin	235, 279, 341	301	229, 255	+	+	+
	Quercetin-3-glucoside	257, 353	463	301	+	+	+
	Quercetin-3-rhamnoside	257, 349	447	301	-	+	-
	Quercetin-3-O-rutinoside	256, 350	609	301	-	-	+
	Myricetin-3-galactoside	238, 278	491	317	+	-	-
Anthocyanins	Delphinidin-3-glucoside	276	463	301	-	+	+
	Cyanidin-3-glucoside	282	449	287	+	+	+
	Petunidin-3-glucoside	236, 269	479	317	+	+	-
	Peonidin-3-galactoside	235, 280	465	301	-	-	+
	Pelargonidin-3-robinobioside	271	577	431, 269	+	-	-
	Cyanidin-3-samburoside	279	581	449, 287	-	-	+
	Cyanidin-3-robinobioside	280	593	447, 285	+	-	-
Flavanols	Catechin	233, 280	289	205, 245	-	+	-
	Epicatechin	231, 281	289	205, 245	-	+	-
Proantho-cyanidins	Procyanidin dimer	281	575	425, 407	-	+	-
	Procyanidin trimer	277	863	575	-	+	-

Others	Coumaroyl iridoid	238, 282	366	309	-	+	-
	Cornuside	242, 274	541	169, 347	+	-	-
	Loganic	239, 279	375	213, 169	+	-	-
	Loganic acid	239, 279	375	213, 169	+	-	-

Another groups of phenolic compounds occurring in fruits are anthocyanins, flavonols and flavanols. They are responsible for the attractive red, orange, blue, purple and even black colour of fruit. Our results of HPLC analysis showed that only catechin, epicatechin and rutin are present among flavonols and flavanols. On the other hand, the results indicate the presence of delphinidin, cyanidin, petunidin and pelargonidin derivatives. Elderberry was characterized by a variety of anthocyanins and their high concentration. The highest content was noted for cyanidin-3-glucoside, cyanidin-3-sambubioside and cyanidin-3-sambubioside-5-glucoside, these were: 3.738±0.147 μg/mL, 3.143±0.262 μg/mL and 2.260±0.219 μg/mL, respectively. It is worth noting that cyanidin-3-sambubioside and cyanidin-3-sambubioside-5-glucoside were identified only in *S. nigra* juice (Figure 1).

Figure 1. Principal components analysis (PCA) of chemical components identified using HPLC and LC-MS methods. The compounds characteristic for elderberry are marked in purple, lingonberry in red; cornelian cherry in green. Blue markers correspond to compounds that are common to tested juices.



These results are in accordance with those obtained by other researchers. For example, Silva et al. (2017) have noted that three main anthocyanidins in elderberry were cyanidin-3-glucoside, cyanidin-3-sambubioside and cyanidin 3-sambubioside-5-glucoside [33]. However, in their studies the concentrations of these compounds reached higher levels amounting to 4.27±0.52 g/100 g, 5.59±0.63 g/100 g and 1.79±0.45 g/100 g of dried weight. Comparable data were described in the study conducted by Lee and Finn (2007) [41].

In our study, lingonberry juice was noted as a source of glucosides, rutinoside of cyanidin, petunidin and pelargonidin. The most abundant anthocyanins detected in this juice was cyanidin-3-glucoside

with the concentration of 0.605±0.054 μg/mL. Other polyphenols in lingonberry juice were detected by LC-MS. These were mainly flavonols (kaempferol-3-glucoside, quercetin, quercetin-3-glucoside, quercetin-3-rhamnoside) as well as catechin, epicatechin, procyanidin dimer and procyanidin trimer. It is noteworthy that out of all the tested juices only the lingonberry contained proanthocyanidins. Reports have shown that proanthocyanidins in lingonberry and American cranberry are responsible for health promoting and antimicrobial activity of the juices [12].

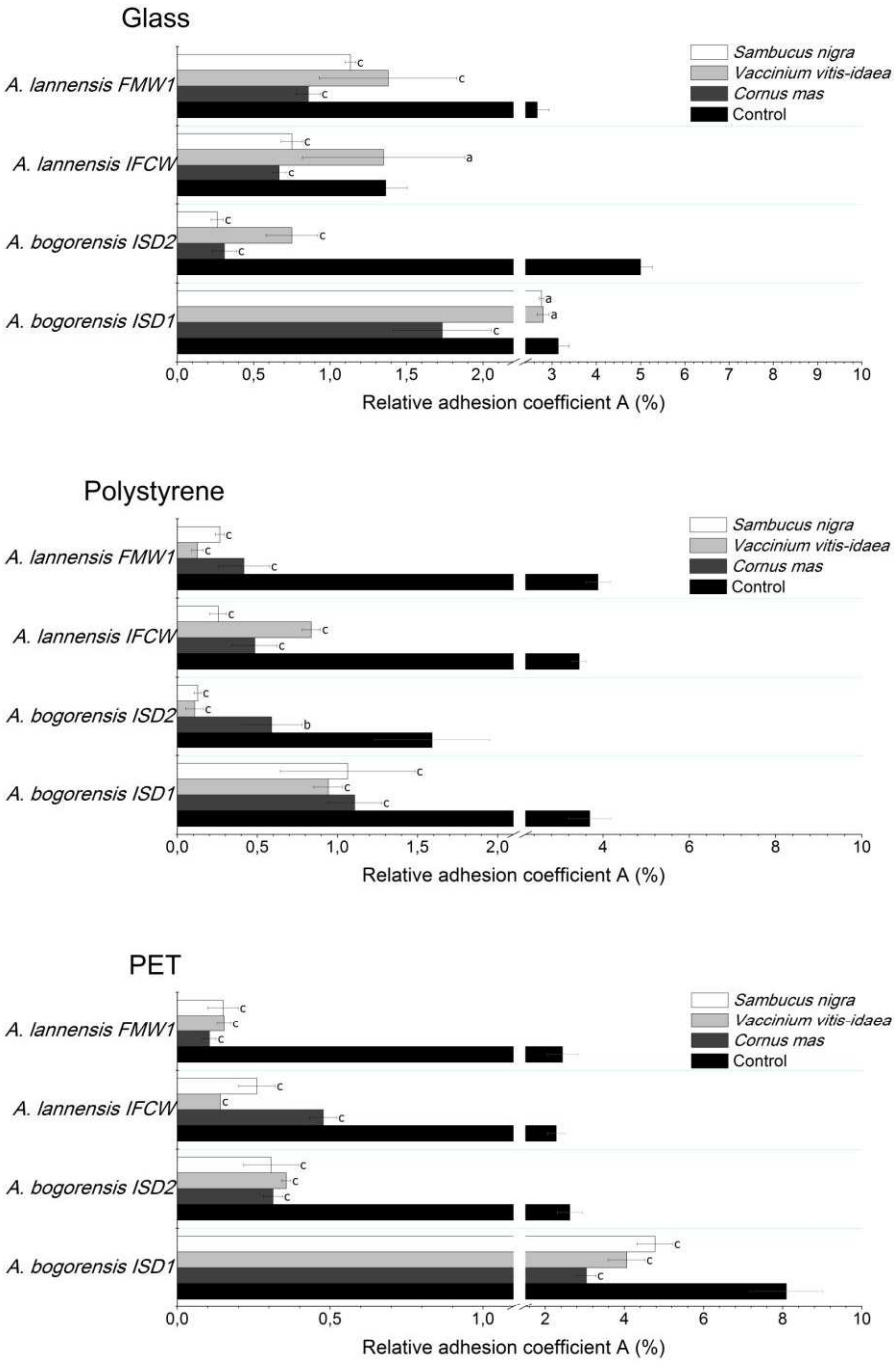
The least varied composition of anthocyanins and other phenolic compounds detected by HPLC and LC-MS techniques was in the *Cornus mas* juice. Among anthocyanidins cyanidin-3-glucoside (0.280±0.039 μg/mL), petunidin-3-glucoside (0.380±0.052 μg/mL), cyanidin-3-robinobioside (0.321±0.041 μg/mL) and pelargonidin-3-robinobioside (0.302±0.022 μg/mL) were noted. Flavonoids identified in this juice with the use of liquid chromatography mass spectrometry included quercetin, quercetin-3-glucoside and myricetin-3-galactoside. It has been found that cornelian cherry contained quercetin, kaempferol, as well as cyanidin-3-galactoside, pelargonidin-3-glucoside, pelargonidin-3-rutinoside. In the cornelian cherry fruits, cultivars of Bosnia and Herzegovina, the major component is peonidin-3-glucoside followed by cyanidin-3-galactoside [19]. Milenković-Andjelković (2015) described that the main anthocyanidin in cornelian cherry was pelargonidin-3-glucoside, which agrees with our results [43]. Taking into account the results of the above studies and results obtained in our case it can be stated that differences in fruit composition may occur due to genetic conditions of plants as well as climatic conditions in a given region. It has been described that genetic factors of the plant, as well as sun exposure, temperature, humidity, availability of nutrients and overall soil properties can influence the levels of particular anthocyanins and the content of polyphenols in general [23].

2.4. Bacterial adhesion

The determination of bacterial attachment and biofilm formation was conducted in the culture medium suitable for growth and adhesion of *Asaia* spp. strains isolated from commercial soft drinks [2-3]. As the parameter determining the affinity of bacterial cells to the surface, the relative adhesion coefficient A(%) was calculated. The intensity of biofilm formation was assessed by luminometric measurement and expressed in relative light units (RLUs). Bacterial cells and biofilm structures were observed using fluorescence microscopy with LIVE/DEAD BacLight Bacterial kit. The results of coefficient A(%) for adhesion of *Asaia lannensis* and *A. bogorensis* strains to glass (G), polystyrene (PS) and polyethylene terephthalate (PET) are presented in Figure 2, while Figure 3 shows the results of luminometric measurements. All of the tested *Asaia* strains showed strongest adhesion to polyethylene terephthalate material in minimal medium with the average value of 3.86±0.46%, slightly weaker effect was observed in the case of polystyrene (3.15±0.32%) and glass (3.04±0.23%). Results of adhesion coefficient A(%) were confirmed by the luminometric method in which relative lights units reached 13923±1360 RLU/cm^2 (PET), 12692±855 RLU/cm^2 (PS) and 3556±241 RLU/cm^2 (G) respectively. Comparison of results and obtained p values showed that these differences are statistically significant. Based on this information it can be stated that the adhesion of *Asaia* spp. are characterized by stronger adhesion to plastic materials.

The comparative results were obtained in previous studies conducted by Kręgiel (2013) [2]. It has been documented that the surface's roughness and hydrophobicity significantly affects bacterial attachment and biofilm development. For instance, low surface energy promotes the adhesion of microorganisms. Polystyrene and polyethylene terephthalate used in our study are generally characterized by lower surface energy than glass. However, the key factor affecting the adhesion is the environment. It has been noted that adhesion of *Asaia* spp. in media containing sucrose as the only carbon source is enhanced in comparison to environments with glucose or fructose content [3]. What is more, it is believed that modification of media composition through the use of antimicrobial substances, that are safe to consumers health, is the best strategy to prevent biofouling in soft drinks

271 technology.



272

273 Figure 2. Adhesion of *Asaia* strains to glass, polystyrene and PET in minimal medium with addition
274 of 10% elderberry, lingonberry and cornelian cherry. Results are expressed as relative adhesion
275 coefficient A(%). Values are means of three determinations ± standard deviation. Values with the
276 different letters are statistically different ($p < 0.05$). a – $p \geq 0.05$; b – 0.05÷0.005; c – $p < 0.005$; The
277 results were compared to those received for control medium.

278

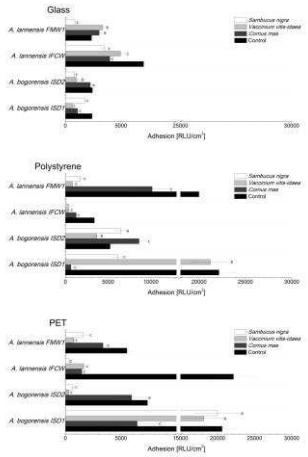


Figure 3. Adhesion of *Asaia* strains to glass, polystyrene and PET in minimal medium with addition of 10% elderberry, lingonberry and cornelian cherry. Results are expressed in RLU/cm^2. Values are means of three determinations ± standard deviation. Values with the different letters are statistically different ($p < 0.05$). a – $p \geq 0.05$; b – 0.05÷0.005; c – $p < 0.005$; The results were compared to those received for control medium.

Application of 10% lingonberry, cornelian cherry and elderberry juices as a supplements to the minimal medium resulted in a significant decrease in the relative adhesion coefficient A(%) and

luminometric measurements results. Slight decrease of A(%) in the medium containing lingonberry was observed only in the case of *Asaia lannensis* IFCW strain. The strongest anti-adhesive properties were noted for elderberry juice, which inhibited the adhesion of *Asaia* spp. to the polystyrene carrier by 87% on average. Slightly weaker properties were noted for lingonberry juice (85%), and cornelian cherry (77%). Considering the species of tested bacteria, tested juices were characterized by a stronger anti-adhesive activity in relation to *A. lannensis*, inhibiting the attachment of the cells by 75%. The strongest inhibition was noted for *A. lannensis* FMW1 in the medium with lingonberry, from 3.88% to 0.12% (97%). Generally, elderberry and cornelian cherry juices showed comparable antiadhesive properties against tested strains. Average results of the reduction of relative adhesion coefficient A(%) in these juices for all of the carriers was 74% and 73%, while in the case of lingonberry it was 67%. Luminometric results (RLU/cm^2) confirmed significant reduction of *Asaia* spp. adhesion in the majority of tested juices. The most pronounced decrease in the results of luminometric measurements was noted for adhesion of *A. lannensis* IFCW in the medium with elderberry juice: from 22153 RLU/cm^2 to 339 RLU/cm^2 (98%). Again, the used juices showed stronger activity against the *A. lannensis* causing a decrease in adhesion, by 60% on average. In general, *S. nigra* juice was characterized by the strongest anti-adhesive properties. The average reduction of adhesion (measured by the luminometric method) was 59%, while for *V. vitis-idaea* and *C. mas* it was 52% and 37%, respectively.

Obviously, the applied techniques of adhesion analysis involve measurement errors, but neither method is perfect. The techniques were selected by taking into account two important criteria: (1) complementary description of the effect of fruit juices on population and vitality of *Asaia* spp. cells attached to the surfaces; (2) industrial applicability, where the bacteria produce biofilms on industrial lines, contaminate products and cause significant financial losses [3]. Generally, the plate count method allows to determinate culturable microorganisms, while the luminometric technique enables to estimate total biological material on the abiotic surfaces. This method is based on ATP quantification and can be used to evaluate the total number of adhered cells, but also bacteria that are unable to grow, extracellular polymeric substances containing small amount of ATP, as well as organic material from culture media. What is more, luminometric measurements may be influenced by bioactive compounds, such as (poly)phenolics, contained in the environment. Due to the mechanism of the luminometry measurement which is based on the enzymatic reaction of luciferin oxidation to oxyluciferin, the presence of antioxidants can influence the final results and may cause differences [44].

Comparison of the biofilm structures in the control medium to these with berry juices is shown in Figure 4. Microscopic analysis of the effect of tested juices on the adhesion abilities of *Asaia* strains showed that cornelian cherries only slightly affect the structure of the developed biofilm, but reduce the viability of the cells in the structures. Bacterial viability kits used in our study are a mixture of SYTO® 9 (green-fluorescent nucleic acid) stain and propidium iodide (red-fluorescent nucleic acid) stain. Generally, SYTO® 9 used alone labels all bacteria in the population, those with damaged membranes as well as those with intact membranes. On the other hand, propidium iodide exhibits activity only in relation to bacteria with damaged membranes. At the same time it causes a reduction in the SYTO stain. As a result, undamaged cells present green fluorescence while cells with damaged membranes are red.

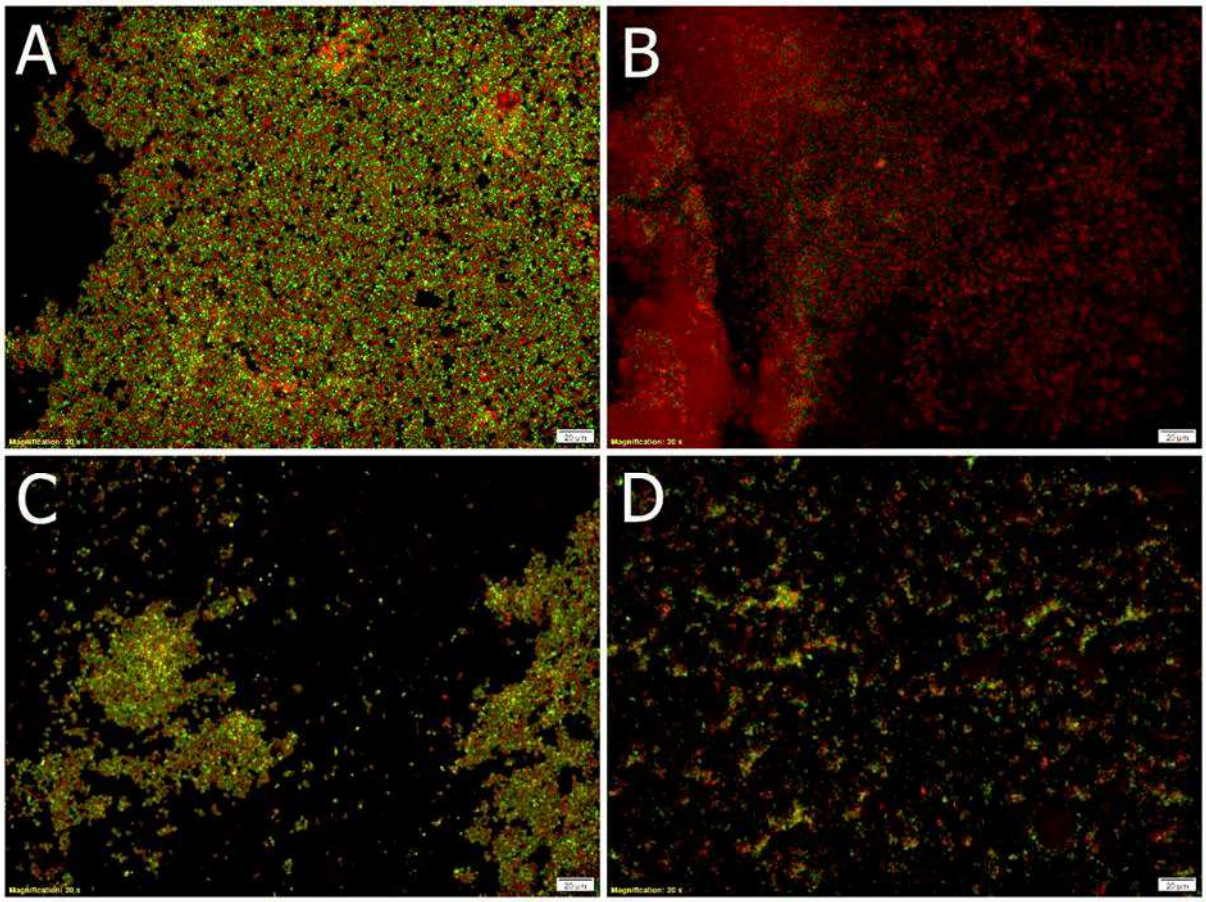


Figure 4. Microscopic observation of the biofilms formed in: (A) control (minimal medium); (B) medium with cornelian cherry juice; (C) medium with lingonberry juice; (D) medium with elderberry juice.

Comparing biofilm images obtained for control (Figure 4A) and culture conducted with cornelian cherry juice (Figure 4B) we noted the reduction of viability of *Asaia bogorensis* ISD1 bacterial cells. At the same time, in the case of cultures conducted with lingonberry (Figure 4C) and elderberry (Figure 4D), significant changes in the structure of the biofilm were noted. In the case of *A. bogorensis* biofilm in the medium supplemented with elderberry juice, micro-colonies were observed. A similar effect was noted for lingonberry juice. Presumably, both the *S. nigra* as well as *V. vitis-idaea* are characterized by anti-adhesive activities, preventing cell adhesion to the surface, and consequently preventing the development of biofilm.

According to the literature, proanthocyanidins in cranberry and lingonberry juice are characterized by strong antiadhesive activities against uropathogenic *Escherichia coli* strains. It was documented that these compounds show urinary tract infection-preventive effect [12]. What is more, flavonoids from *V. vitis-idaea* showed strong activity against oral pathogens. It has been noted that flavan-3-ols and procyanidins dimers were active against biofilm formation of *Streptococcus mutans* and *Fusobacterium nucleatum* [45]. It has been described that the 10% addition of the elderberry extract decreased the growth of *Streptococcus pyogenes* and *Branhamella catarrhalis* by 70% [46]. What is more, extract of elder fruits inhibits the growth of *Helicobacter pylori* by 20% [47]. On the other hand extracts from *Cornus mas* have been shown to possess strong antibacterial activity against both Gram-positive and Gram-negative bacteria: *Staphylococcus aureus* and *Pseudomonas aeruginosa* [48]. Epicatechin-(4β→8)-epicatechin-(4β→8,2β→O→7)-catechin also showed strong antimicrobial activity against *Porphyromonas gingivalis* and *Prevotella intermedia* [49]. Moreover, extracts containing tannins have been described as strong antibacterial against *Staphylococcus aureus*, *Helicobacter pylori*, *Clostridium perfringens*, *Bacillus cereus*, *Klebsiella* spp., and *Proteus* spp. [11]. It has been suggested that

355 the inhibitory effect on the bacterial growth and adhesion may not result from the activity of simple
356 phenolics compounds but trough the complex phenolic polymers. Generally, it is believed that
357 several mechanisms are responsible for the antimicrobial properties of phenolic compounds: (1)
358 destabilization and permeabilization of cytoplasmic membrane; (2) inhibition of extracellular
359 microbial enzymes; (3) direct actions on microbial metabolism, and deprivation of the substrates; (4)
360 blocking the microbial adhesins [32]. What is more, it has been noted that anthocyanins
361 (pelargonidin, delphinidin, cyanidin, as well as cyanidin-3-glucoside) are characterized by growth
362 inhibition of the DNA repair mutant strain of *E. coli*. Therefore, antibacterial activity of these
363 compounds can result from their reaction with DNA [11]. However, it is believed that antimicrobial
364 activity of a fruit extracts is a synergistic effect of (poly)phenolic compounds. Our results confirmed
365 this relationship. The average results of relative adhesion coefficient A(%) and luminometry
366 (RLU/cm^2) obtained in our study strongly correlate with the results of TPC tests. For A(%) method R
367 coefficient amounted to 0.9228, while for RLUs measurement R was equal to 0.9641. Thus, it can be
368 stated that the anti-adhesive properties of the tested juices depends on the content of polyphenols.

369 **3. Materials and Methods**

370 *3.1. Plant material*

371 Cornelian cherry (*Cornus mas*), lingonberry (*Vaccinium vitis-idaea*) and elderberry (*Sambucus nigra*)
372 fruits were collected fresh from local orchards and forests in central Poland in late July and early
373 August 2016. Following this, fruits were washed with water, slightly dried and frozen at – 20 °C. The
374 juice was obtained from defrosted fruits using a squeezer MES3000 (Bosch, Poland). Cloudy juice
375 was passed through a 20-µm-pore-size filter paper (Whatman, USA) once and then filtered and
376 sterilized simultaneously with 0.45-µm-pore-size membranes (Merck-Millipore, Germany).

377 *3.2. Bacterial cultures*

378 Four strains of bacteria *Asaia* spp., isolated from fruit-flavoured mineral waters and isotonic drinks
379 were used in the study – *Asaia bogorensis* ISD1 (GenBank KP234014), *A. bogorensis* ISD2 (GenBank
380 KP234015), *A. lannensis* IFCW (GenBank KP234011) and *A. lannensis* FMW1 (GenBank HQ917850).
381 These bacteria were identified using morphological, physiological and genetic methods and the
382 nucleotide sequences of 16S rRNA were deposited in the GenBank (NCBI) [50]. Bacterial strains
383 were deposited in the Pure Culture Collection of Industrial Microorganisms LOCK 105, at the
384 Institute of Fermentation Technology and Microbiology, Lodz University of Technology (Poland).

385 *3.3. Carriers*

386 Bacterial adhesion was evaluated to plastics and glass used as packaging materials for soft drinks.
387 For this purpose polystyrene (PS) (Coveris Rigid, Poland) and polyethylene terephthalate (PET)
388 (Coveris Rigid, Poland) were used. The rectangular slides measuring 76×26 mm were sterilized in
389 two stages: (1) carriers were kept in the 70% ethanol solution for 6 hours, and then (2) they were
390 placed in a laminar chamber (Telstar, Spain) and subjected to UV irradiation for 3 hours. White glass
391 slides (G) (Knittel Glass, Germany) were used as the reference material. Tested plastics are certified
392 by Polish National Institute of Public Health and intended for food contact.

393 *3.4. Chemical analysis of juices*

394 *3.4.1. Carbohydrates*

395 The monosaccharide profiles of the tested juices were determined enzymatically using a UV
396 spectrophotometer MULTISCAN GO (Thermo Scientific, USA) [51]. D-glucose and D-fructose
397 content was determined in accordance with the procedures of the manufacturer of K-FRUGL assay

kit (Megazyme, Ireland). The obtained results were calculated and expressed as grams of fructose or glucose per 100 mL of tested juice [g/100 mL].

3.4.2. Total phenolic content (TPC)

Total phenolic content (TPC) was determined in accordance with the modified Folin-Ciocalteu method using a 6405 UV/VIS spectrophotometer (Jenway, UK). 10 µL of ten-folded juice and 100 µL of 10% (v/v) the Folin-Ciocalteu's reagent were mixed and incubated for 4 minutes at room temperature. Subsequently 100 µL of 7% (w/v) sodium carbonate and 40 µL of distilled water were added. After the incubation of the mixture in darkness, at room temperature for 1.5 h, absorbance was measured at 765 nm. Simultaneously a standard curve of gallic acid was prepared using concentrations from 0 to 250 mg/L, and the correlation coefficient was 0.9998. Total phenolic content was calculated as mg of gallic acid equivalents (GAE) per mL of sample [mg GAE/mL].

3.4.3. Total antioxidant capacity (DPPH)

The total antioxidant capacity of juices was determined spectrophotometrically (Jenway, UK). DPPH was freshly prepared in 96% methanol. The stock solution was prepared by dissolving 24 mg of 1,1-diphenyl-2-picrylhydrazyl (DPPH) with 100 mL of methanol. The working solution was obtained by dilution of the stock solution with methanol to obtain an absorbance of approximately 1.00±0.05 at 515 nm. 150 µL of properly diluted (five-fold dilutions) sample juice was added to 2.85 mL of DPPH. The solution was incubated in darkness, at room temperature for 1 h. The results were expressed as IC_{50} [g/mL] – the concentration of the tested juice leading to 50% reduction of the initial DPPH concentration. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity [52].

3.4.3. Ferric-reducing antioxidant power (FRAP)

The ferric-reducing antioxidant power (FRAP) of the berries was tested following the assay of Oyaizu (1986) [53]. The sample of tested juice was diluted with sterile distilled water to obtain a series of five-fold dilutions. 0.5 mL of the proper dilution was added to 2.5 mL of 0.2 M phosphate buffer, pH 6.6 and 1% of potassium iron (III) hexacyanoferrate (II). In the control sample, distilled water was used instead of juice. Thereafter, the samples were incubated for 20 minutes at 50 °C, then immediately cooled and treated with 10% trichloroacetic acid (TCA). 2.5 mL of the supernatant was transferred to a new test tube with 2.5 mL of sterile distilled water and 1 mL of 0.1% (w/v) iron (III) chloride hexahydrate. The reducing power was determined spectrophotometrically (Jenway, UK) at 700 nm. The results were calculated and expressed as IC_{50} [g/mL].

3.4.4. Phenolic compounds

Before chromatographic analysis the juices were filtered with 0.45-µm-pore-size membranes (Merck-Millipore, Germany). The phenolic compounds contained in tested juices were characterized using HPLC with a diode array detector (DAD) (Finnigan Surveyor-PDA Plus detector) and a ChromQuest 5.0 chromatography software (Thermo Fisher Scientific, USA). Separation of anthocyanins using the HPLC method was achieved on a Lichrospher RP 18-5 (250 mm by 4.6 mm, 5 µm packing; Hichrom, UK). The elution conditions were as follows: flow rate of 0.8 mL/min; oven temperature of 25 °C; solvent A (5% (v/v) formic acid), and solvent B (95% (v/v) acetonitrile). Elution began with 3% solvent B for 2 min, then 3 to 15% solvent B for 13 min, 15 to 18% solvent B for 9 min, 18 to 25% solvent B for 31 min, and 25 to 30% solvent B for 5 min, followed by washing and re-equilibration of the column. The injection volume for all samples was 50 µL. Detection was conducted at 520 nm [3]. Individual anthocyanin contents were determined according to the linear calibration curve (R= 0.9947) and expressed as µg of cyanidin-3-glucoside per mL of sample.

Other phenolic compounds were also analysed using the HPLC method described by Mišan et al. (2011) [54]. This analysis was performed by using an Agilent 1200 series (Agilent, USA) liquid chromatograph equipped with a diode array detector (DAD), a binary pump, an online vacuum degasser, Chemstation Software (Agilent Technologies, USA), an autosampler and a column (4.6 mm by 50 mm, 1.8 μm packing, Agilent, Eclipse XDB-C18), at a flow rate of 1 mL/min. Solvent gradient was performed by varying the proportion of solvent A (methanol) to solvent B (1% (v/v) formic acid in water). The elution conditions were as follows: initial 10% solvent A (methanol); 0-10 min, 10-25% solvent A; 10-20 min, 25-60% solvent A; 20-30 min, 60-70% solvent A. The injection was done automatically using autosampler, and the volume of the tested sample and standards was 5 μL. The spectra were recorded within 60 min in the range 210-400 nm and chromatograms plotted at 280, 330 and 350 nm. The content of the phenolic compounds was determined according to its calibration curve and expressed as μg per mL of sample. Calibration curves were plotted on the basis of five calibration points and the correlation coefficients were calculated. For all investigated compounds correlation coefficient was higher than 0.9995.

Polyphenols were characterized using a LC-MS method. Samples were injected onto an HPLC column coupled online to an LTQ Velos mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic separation was achieved with a Hypersil GOLD column (1.9 μm, 150 mm by 4.6 mm; Thermo Fisher Scientific, USA) operated at 45 °C. The mobile phase consisted of solvent A (1 mL of formic acid in 1 L of deionized water) and solvent B (95% (v/v) acetonitrile). The elution began with 96 to 85% solvent A for 8 min, then 85 to 82% solvent A for 12 min, 82 to 60% solvent A for 40 min, 60 to 50% solvent A for 4 min and then 3 min, and 50 to 96% solvent A for 2 min, followed by washing and re-equilibration of the column. Mass spectra were recorded within 60 min. The injection volume was 10 μL. The flow rate was set at 220 μL/min. Electrospray ionization (ESI) mass spectrometry was performed using the LTQ Velos MS (Thermo Fisher Scientific, USA) equipped with a heated electrospray ionization interface and controlled by Excalibur software (Chattanooga, USA). Mass spectra were acquired in negative mode over the *m*/*z* range of 120 to 1,000. The ionization spray voltage was 4 kV. The sheath gas flow rate was 25 mL/min, and auxiliary gas flow rate was 10 mL/min. The temperatures of source and desolvation were 350 °C and 280 °C, respectively [55].

3.5. Microbiological analysis

3.5.1. Culture media and growth conditions

Bacterial growth and adhesion were investigated in liquid minimal medium [2% sucrose (w/v), 0.3% $(NH_4)_2PO_4$ (w/v), 0.3% KH_2PO_4 (w/v), 0.3% $MgSO_4 \times 7H_2O$ (w/v), 0.05% (w/v) yeast extract, pH 5.8±0.05] [3]. 19.8 mL of sterile minimal medium were poured aseptically into 25-mL Erlenmeyer flasks, then sterile carriers – being surfaces for bacterial adhesion – were placed vertically into a liquid in such a way that half of the carrier was immersed in the medium, and the other part was above the liquid. Immediately after the preparation of fruit juices, they were added to the culture media to the final concentration of 10% (v/v). The addition of these juices caused the decrease in pH values. For minimal medium with *C. mas* juice the value was 4.30±0.05, while for *V. vitis-idaea* and *S. nigra* pH were 4.45±0.05 and 4.50±0.05, respectively. Culture media were inoculated with standardized bacterial suspensions in order to obtain bacterial cell concentration of $10^5 \div 10^6$ CFU/mL. Bacterial cultures were incubated for 6 days at 25 °C.

3.5.2. Bacterial adhesion

Determination of the number of bacteria in the liquid and those attached to the surface was conducted using the plate count method with CG agar medium [2% (w/v) glucose, 0.3% (w/v) peptone, 0.3% (w/v) yeast extract, 0.7% (w/v) $CaCO_3$, 2% (w/v) agar]. According to our previous studies, this medium is suitable for the growth of *Asaia* strains [6, 50]. In order to determine the level

of bacterial adhesion on abiotic surfaces, two methods – plate count and luminometry were used. For this purpose the carrier was removed from the medium, washed with sterile distilled water and then swabbed with ATP-free, HY-LiTE® sampling pens (Merck Millipore, Germany). The obtained results were converted to RLU per cm^2 of the carrier. In order to determine the number of viable bacterial cells attached to the tested surface, the carrier was removed from the medium, rinsed with sterile distilled water and then swabbed. Then, the swab was transferred into 0.85% (w/v) sodium chloride with 0.1% (v/v) Tween 80, vortexed vigorously, diluted and transferred onto GC agar medium. Inoculated plates were incubated at 25 °C for 96 h, and the characteristic pink-pale colonies of *Asaia* spp. were counted. The results were expressed as CFU per cm^2 of the carrier. The number of planktonic cells was also determined by plate count method and the results were presented as CFU per mL of culture medium. Based on the results obtained for both adhered and planktonic cells, the level of bacterial adhesion as the relative adhesion coefficient A(%) was calculated according to the formula described by Kręgiel (2013) [2].

3.5.3. Fluorescent microscopy

Visualization of bacterial biofilms was perform by fluorescence staining using a LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, USA). The kit contains two dyes: SYTO® 9 (green-fluorescent nucleic acid) stain and propidium iodide (red-fluorescent nucleic acid stain). Preparation of the staining solution was carried out in accordance with the manufacturers procedure, mixing the dyes in a ratio of 1:1. Biofilm was gently washed with PBS solution, and then the entire surface was covered with a staining solution. The sample was incubated in darkness for 20 min at 30 °C. Images of the cells were done using fluorescent microscope OLYMPUS BX53 equipped with filters with excitation wavelength ranged from 470 to 630 nm and a high-resolution digital colour camera (Soft Imaging System's Color View).

3.6. Statistics

Three independent experiments were performed, and from the obtained data, means with standard deviations were calculated. Statistical differences between the obtained adhesion results were compared using a one-way repeated measures analysis of variance (ANOVA; OriginPro 9.2.214, OriginLab Corp., USA). Statistical significance was set at the level of 5% ($P < 0.05$). In addition, correlation coefficients between results of DPPH, FRAP, TPC and those obtained for adhesion analysis were calculated. The chemical compositions of the tested fruit juices were compared using principal components analysis (PCA) using XLSTAT 2017 (Addinsoft, USA), complete statistical add-in for Microsoft Excel (Microsoft, USA). The outcomes from LC-MS were displayed as binary data (0 or 1) depending on whether a component was absent or present in the plant extract, while the outcomes from HPLC were displayed as a concentration of the specific component.

4. Conclusions

We have shown that some little-know, edible European fruits may present promising sources for the beverage industry, not only because of their strong antioxidant properties or high content of phenolic compounds. These juices may be a valuable supplement for functional beverages, inhibiting bacterial adhesion on abiotic surfaces. Therefore, multi-component fruit juices recognized by folk medicine may be used as components of modern functional drinks to improve their microbial stability. Further studies on the active components based on novel strategies by computational techniques, chemistry, and cell-based models are necessary. While the discovery and development of natural products represents a complex endeavor demanding a highly integrated interdisciplinary approach, the research trends clearly indicate that wild edible fruits may be among the most important sources of the new functional food in the future.

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Author Contributions: Hubert Antolak conceived and designed the experiments; Aleksandra Mišan performed the experiments on HPLC analysis; Hubert Antolak and Agata Czyżowska performed the analysis on HPLC (anthocyanidins) and LC-MS; Marijana Sakač and Olivera Đuragić contributed reagents/materials/analytical tools; Hubert Antolak and Marijana Sakač performed the TPC, DPPH and FRAP tests; Hubert Antolak performed the analysis of fructose and glucose content in tested juices; Hubert Antolak performed the microbiological analysis. Hubert Antolak and Dorota Kręgiel wrote the article.

Conflicts of Interest: The authors declare no conflict of interest

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