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preliminary communication

Biological Activity of Sour Cherry Fruit on the Bacterial Flora of Human Saliva *in vitro*

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Summary

This study is the first report on the antibacterial effect of Hungarian sour cherry cultivars. Biological activity of sour cherry juices prepared from fruits Érdi jubileum, Érdi bőtermő, Maliga emléke and Kántorjánosi 3 harvested at different maturity stages was investigated on bacteria present in human saliva. The influence of sour cherry on a mixed bacterial flora of human saliva of 10 volunteers was determined by different experimental approaches. Bactericidal effects were evaluated by minimum inhibitory concentration (MIC) using agar diffusion methods and by minimum bactericidal dilution (MBD) assays counting the number of surviving bacterial cells in the diluted juices. Time-dependent antibacterial effects were also determined by monitoring the decrease in bacterial cell numbers after the treatment with undiluted juices. The investigated sour cherry juices displayed an impressive bactericidal effect against human saliva bacteria (10–100× reduction of cell numbers) within a short time frame (10–40 min). Érdi jubileum was more effective (100 000× reduction of cell number after 270 min) than the other studied cultivars. Bactericidal effect was influenced by ripening of samples of Érdi jubileum obtained at different harvesting dates. Biologically active components were effective against a large spectrum of opportunistic bacterial pathogens such as *Pseudomonas*, *Klebsiella*, *Pantoea* spp. and *Escherichia coli*, including the antibiotic-resistant *Pseudomonas aeruginosa* but they were ineffective against beneficial probiotic *Lactobacillus* spp. Results confirmed the antibacterial potential of all the investigated sour cherry fruits, therefore the consumption of the fruit or its juice for positive influence on oral hygiene is highly recommended.

Key words: Prunus cerasus, Érdi jubileum, polyphenols, anthocyanins, minimum inhibitory concentration, minimum bactericidal dilution, time-kill assay

Introduction

Consumption of fruit has a great influence on nutrition because we can control the intake of micronutrients and biologically active molecules. Furthermore, several studies have pointed out that many of these compounds show biological properties of interest, mostly in relation to their antibacterial effects (*1*). The antioxidant activities of fruits and vegetables have also been thoroughly studied (*2*,*3*).

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Human saliva consists of numerous bacterial cells of around 10^{10} colony-forming units (CFU) per mL of different species which are part of the normal flora, but others are pathogenic (*4*). Salivary bacteria may induce endogenous diseases in cases of weak immune system. The physiological state of the inner environment depends on fluctuations in the outside environment and the influence of stress-induced diseases (*5*), which hardly depends on nutrition (*6*). Saliva can be a clinical thermometer signalling the weakened physiological state of individuals. Many diseases like diabetes and obesity can also change the ratio of bacterial populations in the mouth. Affected individuals have different species of oral bacteria than healthy people (*7*). Among the most frequently occurring bacteria in human saliva, there are *Streptococcus* and *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* (*7*). Bioactive compounds of fruits most likely exert their antibacterial effects through the balance of redox homeostasis of the human body and by their cumulative action on free radicals and stimulative actions on antioxidant–related defences (*1–3*). In our previous work we found a high anthocyanin (ACY) and total polyphenol content (TPC) in numerous Hungarian sour cherry cultivars (*8,9*) which confirmed the results of other research groups (*10–12*). However, these biologically active components in sour cherry fruits and their bactericidal effects on human saliva have not been examined in depth until now.

The aim of our investigations is to detect the bactericidal effects of ACY and TPC present in Hungarian sour cherry cultivars on opportunistic and pathogenic bacteria found in human saliva and the beneficial *Lactobacillus* spp.

Materials and Methods

Preparation of fruit samples

Samples Érdi jubileum, Érdi bőtermő, Maliga emléke and Kántorjánosi were harvested at the optimal time of ripening, and the extremely ripe Érdi jubileum were harvested 5 days later. Samples were homogenized in a blender and the juices were stored in deep freeze (–25 °C for 2 months). Before use they were filtered through a filter paper (Sartorius Stedim, Aubagne, France). Total phenolic content (TPC) was determined according to the method of Singleton and Rossi Jr. (*13*) at 765 nm with a U–2800A Hitachi double beam spectrophotometer (Hitachi, Tokyo, Japan). Quantification was done on the basis of a standard curve of gallic acid and expressed as gallic acid equivalents (GAE). Determination of total anthocyanin content (ACY) was carried out according to the method of Füleki and Francis (*14*). Absorbance was measured at 530 nm.

Microbiological analysis requires sterile materials and a relatively long storage life. Therefore the physical and biological stability of juices was examined after boiling them in a water bath (100 °C/3 min) and deep freezing (–25 °C) for a month.

Bacteria

Saliva samples were collected from 10 healthy young and middle-aged volunteers, members of the Department of Pomology, Faculty of Horticultural Science, Corvinus University of Budapest, Budapest, Hungary. The saliva samples were mixed and 100 μL were spread out on King B medium (*15*). The mixed bacterial flora of 10 individuals was developed after a 48-hour incubation at 30 °C. All types of bacterial colonies grown on plates were mixed and considered as single isolates during experiments. To study the spectrum of bactericidal effects of sour cherry juices, a number of strains of opportunistic and pathogenic species and two strains of a beneficial bacterial species were tested. Reference bacteria were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Hungary. The majority of these species are also registered in international collections as well: Japan Collection of Microorganisms (JCM), Czech Collection of Microorganisms (CCM), American Type Culture Collection (ATCC), or Collection of Marine Microorganisms (CMM). The above mentioned test organisms were the following: *Escherichia coli* B 01728 (NCAIM), *E. coli* 5172 (CCM), *Klebsiella pneumoniae* ssp. *pneumoniae* 132 (NCAIM), *K. pneumoniae* ssp. *pneumoniae* B 01686 (NCAIM), *K. pneumoniae* ssp. *pneumoniae* 10031 (ATCC), *Lactobacillus fermentum* B 01146 (NCAIM), *Lactobacillus plantarum* B 02142 (NCAIM), *Pantoea agglomerans* 83873/1 (NPHMOS), *P. agglomerans* B 02248 (NCAIM), *P. agglomerans* 123 (JCM), *Pseudomonas aeruginosa* B 011687 (NCAIM), *P. aeruginosa* 9027 (ATCC) and *Staphylococcus aureus* B 01065 (NCAIM). *Pantoea agglomerans* C–1 was obtained from the laboratory of Virginia O. Stochvell, Oregon State University, Corvallis, OR, USA.

Agar diffusion method

Antibacterial activity of juice preparations was determined by agar diffusion method, which has been used in a number of studies (*16,17*), with only slight modifications: 1-mL suspensions of 24-hour-old salivary bacteria (10^7 CFU/mL) adjusted with spectrophotometer at 560 nm were mixed with 3 mL of melted (45 °C) nutrient agar (1 %) and poured into Petri dishes containing nutrient agar (2 %) layer. Wells were punched by cork borer (10 mm diameter) containing 125-μL aliquots of test samples. Inhibition zones were measured in mm after 24 h of incubation. Juices were sterilized (100 °C/3 min) and 125 μL were pipetted into each well. Dishes were kept for 5 h at 4 °C (to force diffusion), followed by 24 h of incubation at 30 °C. Clear, transparent zones of growth inhibition around the wells on the turbid bacterial lawn, representing antibacterial activity, were measured in mm. As a negative control, sterile distilled water was used in one well (Fig. 1).

Physical stability of juices regarding their biological activity was studied and was also expressed by inhibition zones around wells using salivary bacteria as indicators. Heat treatment was made in water bath at 100 °C for 3 min; frozen juice samples were kept at –25 °C for 2 months. Fresh juice was used as control.

The antibacterial spectrum of juices was compared with the above mentioned method using characterized test bacterial species obtained from the international culture collections. The agar diffusion method was slightly modified in favour of *Lactobacillus* spp., where the upper agar layer (1 %) consisted of indicator bacterial cells coated with a superior agar layer to assure optimal conditions for the development of aerotolerant bacteria.

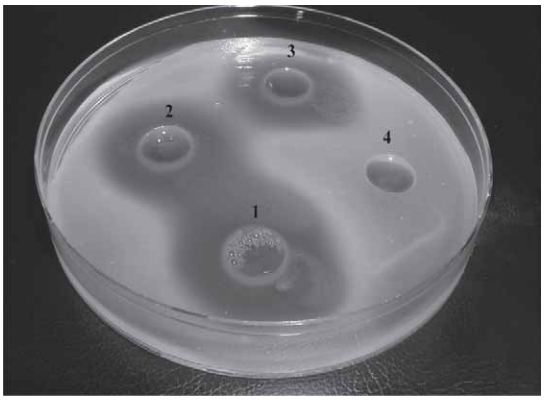

Fig. 1. Illustration of antibacterial effect measured by agar diffusion method after 24 h of incubation. Wells (*d*=10 mm) contained 125-μL aliquots of Érdi jubileum fruit juice: 1. undiluted, 2. 1:1 diluted juice, 3. 1:2 diluted juice, 4. control (distilled water)

Minimum inhibitory concentration (MIC) and minimum bactericidal dilution (MBD) assay

Antibacterial activity of juices was determined by two assays: MIC, a defined effectiveness of the lowest visible concentration of fruit juice in serial dilutions from 1:2 to 1:64 that produces a clear inhibition zone in agar diffusion wells within 24 h, and MBD, which considers the highest dilution that kills 99 % of the initial inoculum level. For MBD assay a serial dilution of the sterile juice was prepared, and then inoculated with indicator bacterial cells in a final concentration of 10^5 CFU/mL. Aliquots of samples (juice/bacterium 100:100, in μL) were poured into King agar B plates (Sigma-Aldrich, St. Louis, MO, USA) after 4 h. The number of viable cells was determined by colony growth on the plates after 48 h of incubation at 30 °C.

Time-kill assay

For determination of time-dependent antibacterial activity, only the biologically more active sour cherry samples (Érdi jubileum, extremely ripe Érdi jubileum and Maliga emléke) were used. Undiluted juices were inoculated to a final concentration of 2 to $2.5 \cdot 10^6$ CFU/mL. Samples were taken every 10 min in order to determine the viability of the cells after juice treatment, comparing them to control (sterile distilled water). Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were prepared from the samples, poured on the surface of King agar B plates and incubated at 30 °C for 2 days. For measurement, only those plates where colony numbers were about 30–300 per plate were used. Reduced colony numbers indicated the number of living bacterial cells for each time point.

Statistical analysis

To determine the time-kill assay of undiluted juices, five replicas were used. Statistical analysis was made by using SPSS software v. 14.0 (SPSS Inc., Woking, Surrey, UK). Univariate analysis was made using the Duncan's test to separate homogenous groups of sour cherry cultivars. To compare the mean differences among the homogenous groups, on the basis of the relative standard deviation (RSD), the value of the determination was 5 % (*N*=5).

Results

Sour cherry juices were stable after heat (boiling 100 °C/3 min) and cold (–25 °C) treatment for 2 months (Table 1). Only slight differences existed in the antibacterial activity among juices of cultivars measured by agar diffusion of MIC: Kántorjánosi produced an inhibition zone even at a 1:4 dilution, Maliga emléke at 1:5, Érdi jubileum and Érdi bőtermő at 1:7 and extremely ripe Érdi jubileum at 1:8 dilution. When their biological activity was determined by MBD, the juices showed more pronounced differences than measured by agar diffusion. A steady reduction in cell numbers was observed in all diluted juices (Table 2). There was a significant decrease in the initial cell numbers in each sample from $1.2 \cdot 10^5$ to $8.3 \cdot 10^2$ at all the dilutions used. Extremely ripe Érdi jubileum showed the highest activity at dilution 1:16, while the other samples were diluted to 1:5 to obtain the same bactericidal effect.

Table 1. Physical stability of sour cherry juices expressed as inhibition zones determined by agar diffusion method

Treatment	Cultivar			
	Maliga emléke	Kántor-jánosi	Érdi jubileum	Érdi jubileum+
	Inhibition zone/mm			
heating (100 °C/3 min)	26.1	26.5	27.5	24.6
freezing (–25 °C/2 months)	27.3	28	27.5	26
control (distilled water)	27.3	28	27.5	26

Érdi jubileum+=extremely ripe Érdi jubileum

Table 2. Correlation between dilution rate and antioxidant content of sour cherry juices

Cultivar	MBD	γ(TPC as GAE)	γ(ACY)	N(viable cells)/V
		mg/L	mg/L	mL^{-1}
Érdi jubileum	1:8	42.4	28.6	$15.0 \cdot 10^2$
Érdi jubileum+	1:16	24.2	20.0	$8.3 \cdot 10^2$
Érdi bőtermő	1:5	57.2	38.4	$6.0 \cdot 10^2$
Maliga emléke	1:5	56.5	34.6	$8.9 \cdot 10^2$
Kántorjánosi	1:5	36.6	17.0	$5.3 \cdot 10^2$

MBD=minimum bactericidal dilution, TPC=total polyphenolic content, GAE=gallic acid equivalent, ACY=anthocyanin content, Érdi jubileum+=extremely ripe Érdi jubileum
Initial cell number of each dilution was 10^5/mL

Experiments of the time-kill assay were also conducted with biologically more active sour cherry cultivars Maliga emléke and Érdi jubileum. Samples of extremely ripe Érdi jubileum were harvested 5 days later than Érdi jubileum, which provided a chance to compare the correlation between the effect of ripening, development of biological activity and bactericidal effects (Table 3). Bac-

Table 3. Time-kill assay of undiluted juices of sour cherry cultivars

t/min	Érdi jubileum	Érdi jubileum+	Maliga emléke	Control
0	$(2.2\cdot10^6\pm1.1\cdot10^3)^g$	$(2.4\cdot10^6\pm1.1\cdot10^3)^g$	$(2.3\cdot10^6\pm1.1\cdot10^3)^g$	$(2.2\cdot10^6\pm1.1\cdot10^3)^g$
10	$(1.9\cdot10^6\pm1.1\cdot10^3)^f$	$(1.3\cdot10^6\pm1\cdot10^3)^f$	$(1.7\cdot10^6\pm1.7\cdot10^2)^f$	$(2.1\cdot10^6\pm1.2\cdot10^3)^g$
20	$(5.8\cdot10^5\pm7.6\cdot10^2)^f$	$(2\cdot10^4\pm1.2\cdot10^2)^{ab}$	$(7.9\cdot10^4\pm7.6\cdot10^2)^{ab}$	$(2.2\cdot10^6\pm1.1\cdot10^3)^g$
40	$(2.1\cdot10^5\pm4\cdot10^2)^e$	$(9.3\cdot10^3\pm1.5\cdot10^2)^a$	$(3.9\cdot10^4\pm3\cdot10^2)^{ab}$	$(2.1\cdot10^6\pm0.8\cdot10^3)^g$
60	$(2\cdot10^5\pm7\cdot10^2)^e$	$(1.1\cdot10^4\pm1.5\cdot10^2)^a$	$(2.8\cdot10^4\pm1.1\cdot10^2)^{ab}$	$(2.2\cdot10^6\pm1.1\cdot10^3)^g$
80	$(1.9\cdot10^5\pm6\cdot10^2)^{cd}$	$(9.6\cdot10^3\pm1.65\cdot10^2)^a$	$(2.7\cdot10^4\pm1.5\cdot10^2)^{ab}$	$(2.0\cdot10^6\pm1.1\cdot10^2)^g$
100	$(1.8\cdot10^5\pm3\cdot10^2)^{cd}$	$(8.6\cdot10^3\pm2.3\cdot10^2)^a$	$(2.5\cdot10^4\pm1.5\cdot10^2)^{ab}$	$(2.1\cdot10^6\pm1.2\cdot10^3)^g$
120	$(1.7\cdot10^5\pm2\cdot10^2)^c$	$(7.5\cdot10^3\pm2\cdot10^2)^a$	$(2.6\cdot10^4\pm1.1\cdot10^2)^{ab}$	$(1.9\cdot10^6\pm1.1\cdot10^3)^g$
150	$(1.6\cdot10^5\pm3\cdot10^2)^c$	$(8.2\cdot10^3\pm3\cdot10^2)^a$	$(2.4\cdot10^4\pm1.1\cdot10^2)^{ab}$	$(2.2\cdot10^6\pm1.3\cdot10^3)^g$
180	$(1.4\cdot10^5\pm3\cdot10^2)^{bc}$	$(3.4\cdot10^3\pm1.5\cdot10^2)^a$	$(2.1\cdot10^3\pm1.2\cdot10^2)^{ab}$	$(2.3\cdot10^6\pm1.1\cdot10^3)^g$
210	$(1.1\cdot10^5\pm1.1\cdot10^2)^{ab}$	$(10^3\pm85)^a$	$(2.2\cdot10^3\pm3\cdot10^2)^{ab}$	$(2.2\cdot10^6\pm0.7\cdot10^3)^g$
240	$(9.5\cdot10^4\pm1.1\cdot10^2)^{ab}$	$(9.3\cdot10^2\pm115)^a$	$(1.8\cdot10^3\pm2\cdot10^2)^{ab}$	$(2.1\cdot10^6\pm0.9\cdot10^3)^g$
270	$(6.5\cdot10^4\pm1\cdot10^2)^{ab}$	$(3.4\cdot10^2\pm25)^a$	$(5.5\cdot10^3\pm10^2)^a$	$(2\cdot10^6\pm1.1\cdot10^3)^g$

Érdi jubileum+=extremely ripe Érdi jubileum (harvested 5 days after Érdi jubileum)
Data are presented as mean values±standard deviation, $p<0.05$; $^{a–g}$ homogenous groups

tericidal effect evidently took place in both tested cultivars. From the early moments of treatment (0–20 min), a pronounced cell decay process started and continued. Reduction of viable cell numbers caused by Érdi jubileum juice resulted in the final bacterial concentration of $6.5\cdot10^4$, by Maliga emléke $5.5\cdot10^3$ and by extremely ripe Érdi jubileum $3.4\cdot10^2$ CFU/mL during the maximum incubation period of 270 min, in contrast to the control (distilled water) where cell numbers were stable at 2 to $2.2\cdot10^6$ CFU/mL (Fig. 2). Extremely ripe Érdi jubileum (harvested 5 days after Érdi jubileum) had higher total phenolic and anthocyanin contents than Érdi jubileum. Therefore, the observed bactericidal effect seemed to correlate well with the inner content, especially with ACY, and maturity stages of fruits (Table 2).

After analysing the antibacterial spectrum of sour cherry juices, it can be concluded that they displayed a wide-ranging effect against a number of opportunistic pathogens (Table 4). No such effect was exhibited against *Staphylococcus aureus* or *Lactobacillus* spp.

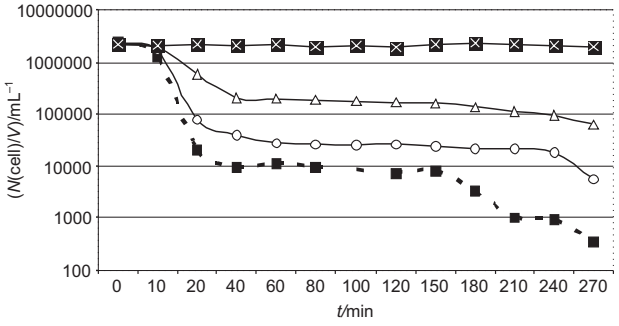

Fig. 2. Time-dependent decrease of bacterial cell number induced by undiluted sour cherry juices: —△— Érdi jubileum, —■— extremely ripe Érdi jubileum, —○— Maliga emléke, —⊠— control

Discussion

Previous works related antibacterial effects of different plant species to the presence of biologically active

Table 4. Antagonistic effect of sour cherry juices on bacteria frequently present in human saliva determined by agar diffusion method

Bacteria	Cultivar				
	Érdi jubileum+	Érdi jubileum	Érdi botermo	Maliga emléke	Kántorjánosi
	Inhibition zone/mm				
Escherichia coli B 01728	30	30	30	30	30
Klebsiella pneumoniae ssp. *pneumoniae* 132	25	25	25	25	25
K. pneumoniae ssp. *pneumoniae* B 01686	25	24	24	24	24
Lactobacillus fermentum B 01146	0	0	0	0	0
Lactobacillus plantarum B 02142	0	0	0	0	0
Pantoea agglomerans C-1	25	25	25	25	24
Pantoea agglomerans 83873/1	25	25	25	25	24
Pantoea agglomerans B 02248	16	15	14	16	16
Pseudomonas aeruginosa B 011687	24	25	24	24	25
Staphylococcus aureus B 01065	0	0	0	0	0

Érdi jubileum+=extremely ripe Érdi jubileum

compounds (*18*). Experiments had been conducted with berry fruits (*19*,*20*), mango, orange, guava, black currant and pineapple juices (*21*), *Ribes* sp. (*22*), apples (*23*), pomegranate (*24*), wines (*25*), vegetables (*26*), medicinal plants (*7*,*16*,*27*) and different types of tea herbs (*28*,*29*) in similar ways but no data were available for sour cherries. Consequently, our study is the first report focusing on this fruit species and its beneficial actions on bacterial flora of human saliva. Our results are in contrast with another research group's work reporting that *Pseudomonas aeruginosa* survived in juices of blackcurrants, raspberries, mangos, pineapples, guavas and mixed fruits (*21*). Likewise, no inhibition of *Klebsiella pneumoniae* ssp. *pneumoniae* was observed by Lee *et al.* (*26*) using juices from vegetables and fruits. The juices from Hungarian sour cherry cultivars investigated in this study were able to limit the antibiotic (ciprofloxacin)-resistant strain of *P. aeruginosa* and also *K. pneumoniae.* Sour cherry juice showed bactericidal effect against all Gram(–) bacteria but not against the Gram(+) species tested. Hatano *et al.* (*30*) pointed to the instability of certain polyphenols, *e.g.* epigalocatechin gallate in a solution. We found that the bactericidal effect of sour cherry juices resisted extreme physical conditions since they were stable after boiling and freezing. Also, the harvesting time seems to have an influence on the increase of biologically active materials in fruits and on the antibacterial potential of Érdi jubileum. This cultivar seemed to be a promising model, because of its high ACY and TPC content determined previously by HPLC and spectrophotometric assays (*8*). Other researchers also found change of colour and ACY content of sour cherry ecotypes during fruit ripening and depending on geographical region (*31*). A correlation was established between the antioxidant content and antimicrobial properties at different ripening stages in the case of *Capsicum baccatum* L. var. *pendulum* fruits too (*32*). Parkar *et al.* (*33*) pointed out that *in vitro* antioxidant activity was assessed by determining the total reactive antioxidant potential which positively correlated with the amount of phenolics found in each sample. The mechanism of the bactericidal effect was not studied in this research, but a similar correlation between phenolic content and antioxidant potential of sour cherry fruits is a likely possibility. Didry *et al*. (*16*) suggested that the bactericidal activity of juices is a consequence of ACY and TPC. Although we found a lower ACY and TPC content in cultivar Maliga emléke than in extremely ripe Érdi jubileum, the former cultivar had a nearly similar bactericidal effect as extremely ripe Érdi jubileum. This could be either due to a different polyphenolic profile, the presence of other important biologically active substance(s), or the existence of a remarkable diversity in the chemical structure responsible for the biological activity which should be one of the foci of our future research.

Conclusion

The presented results show a high bactericidal effect of sour cherry fruits. The Hungarian sour cherry cultivars studied differ in their biological activity. Increase of anthocyanin concentration during ripening was evident and correlated with their bactericidal effect. Sour cherry fruit juices had a potential bactericidal effect on the bacteria which may affect oral health. They would be suitable for broader application in the food processing industry because of their resistance to deep freezing and boiling.

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