

# Smoothie Drinks as a Possible Source of Resistant and Biofilm-forming Bacteria

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## Abstract

Smoothie drinks are currently very popular drinks sold especially in fast food establishments. However, smoothies are significant source of microorganisms with high level of antibiotic resistance. The aim of the study was to evaluate the microbiological quality of smoothies purchased in Eastern Bohemia. A higher prevalence of mesophilic aerobic bacteria (5.4–7.2 log CFU/mL), yeast (4.4–5.9 log CFU/mL), and coliform bacteria (3.1–6.0 log CFU/mL) was observed in vegetable smoothies, in which even the occurrence of enterococci (1.6–3.3 log CFU/mL) was observed. However, the occurrence of *S. aureus*, *Salmonella* spp. and *Listeria* spp. was not observed in any sample. Nevertheless, a significant occurrence of resistant microbial strains was observed in all samples. The highest level of resistance was found in isolates from smoothie drinks with a dominant vegetable content (green-smoothie drinks). Considerable resistance was observed in Gram-negative rods, especially to amoxicillin (82.2%) and amoxicillin with clavulanic acid (55.6%). Among enterococci, only one vancomycin-resistant strain was detected. The vast majority of isolated strains were able to form biofilms at a significant level, which increases the clinical importance of these microorganisms and their resistance. The highest biofilm production was found in *Pseudomonas aeruginosa*, *Kocuria kristinae* and *Klebsiella pneumoniae*. Overall, significant biofilm production was also noted among isolates of *Candida* spp. **Keywords:** Antibiotic resistance, resistant bacteria, biofilm, smoothie drink.

## 1. Introduction

Fresh fruit and vegetable products are often not subjected to such technological interventions that would ensure the inactivation of pathogenic microorganisms or their effective removal before consumption [Li et al., 2021]. From fresh drinks it is possible to isolate, in particular, types of microorganisms that are adapted to a highly acidic environment (yeasts, fungi and lactic acid bacteria). A potential risk is also the presence of pathogenic species, especially psychrotrophic ones (e.g. *Listeria*

*monocytogenes*). In particular, there may be a risk of microbiological contamination at street sales points. Several studies have already looked into the microbiological quality of smoothie drinks [Krahulcová et al., 2021; Salamandane et al., 2021].

Microorganism resistance has been increasing considerably in recent years, and is becoming a global problem. According to WHO reports from 2014, resistance to antimicrobial substances is one of the main global threats in the spectrum of infectious diseases. Biofilm formation is a key virulence factor for many microorganisms. Biofilms are up to 1000 times more resistant to inhibitory effects compared to their planktonic forms [Koo et al., 2013; Koo et al., 2017].

The aim of the study was to evaluate the microbiological quality of ready-to-eat smoothie drinks prepared in various fresh bars in Eastern Bohemia region. In addition, all isolates were evaluated for their level of resistance to antimicrobials used in clinical practice and their biofilm-forming ability.

## 2. Material and Methods

### 2.1. Analysed samples

Samples of smoothie drinks were purchased in five Fresh bars in different cities of the Czech Republic (Pardubice, Hradec Králové). For the purposes of the study, the samples were marked with the letter **F** for a drink with a predominant component of fruit and **V** for a drink with a predominant component of vegetables (Table 1). The samples were transported to the laboratory in sterile containers immediately after their preparation and kept refrigerated during transport. Subsequently, the samples were immediately analysed.

### 2.2. Microbiological testing

According to preliminary experiments, a dilution series from 10<sup>-1</sup> to 10<sup>-5</sup> in physiological saline with peptone was prepared from each sample.

Determination of coliform bacteria and *Escherichia coli* was performed using the selective and differentially chromogenic medium Chromocult Coliform Agar (Merck, Germany; 37 °C for 24 h). For the purposes of this study, the determination of the total number of mesophilic aerobic bacteria on Plate Count Agar (Sigma-Aldrich, USA; 30 °C for 24 h), the determination of the total number of yeasts and fungi on Dichloran Rose Bengal Chloramphenicol Agar (Merck, Germany; 25 °C for 5 days), determination of the numbers of enterococci on Slanetz–Bartley agar (Oxoid Ltd., UK; 37 °C for 24 h), and determination of coagulase-positive staphylococci using Baird–Parker agar medium (Sigma-Aldrich, USA); 37 °C for 24 h) were also performed. For the detection of *Listeria monocytogenes*, primary multiplication was carried out in half broth according to Fraser (Sigma-Aldrich, USA; 30 °C for 24 h). After primary enrichment, 0.1 mL of the culture was transferred to 10 mL of secondary enrichment Fraser medium (Sigma-Aldrich, USA; 37 °C for 24 h) with subsequent inoculation of both primary and secondary multiplication on ALOA and PALCAM agar (Merck, Germany; 37 °C for 48 h). Detection of *Salmonella* spp. involved primary non-selective propagation in buffered peptone water (37 °C for 24 h). After incubation, the obtained culture was inoculated (secondary multiplication) into RVS broth (0.1 mL; 41.5 °C for 24 h) and MKTTn broth (1 mL; 37 °C for 24 h). Subsequently, the culture was inoculated onto selective agar media XLD and RAMBACH agar (Merck, Germany; 37 °C for 24 h). All analyses were performed in duplicate and repeated twice independently. The resulting values are the mean with the expressed standard deviation (SD). All strains were sub-cultured on Mueller-Hinton agar with subsequent identification using MALDI-TOF MS (Bruker Daltonics GmbH, Germany) with MBT Compass Software (MBT Compass Library Revision H 2021).

### 2.3. Monitoring of antibiotic resistance level

Susceptibility of isolates to amoxicillin (AMO, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), ampicillin (AMP, 2 µg); cefepime (CPM, 30 µg), cefotaxime (CTX, 5 µg), clarithromycin (CLA, 15 µg), clindamycin (CLI, 2 µg), clotrimazole (CLO, 10 µg), colistin (COL, 10 µg), cotrimoxazole (COT, 25 µg), cefpodoxime (CPD, 10 µg), cefuroxime (CRX, 30 µg), ciprofloxacin (CIP, 5 µg), doxycycline (DOX, 30 µg), econazole (ECO, 10 µg), linezolid (LIN, 10 µg), natamycin (NAT, 50 µg), nystatine (NYS, 100 µg), ofloxacin (OFL, 5 µg), oxacillin (OXA, 30 µg), penicillin (PNC, 1 µg), ticarcillin/clavulanic acid (TIM, 85 µg) and vancomycin (VAN, 5 µg) were tested by a previously described disk diffusion method [EUCAST, 2019] using antimicrobial disks purchased from Oxoid Ltd. (Basingstoke, UK), Bioanalyse Ltd. (Ankara, Turkey), and ITEST plus s.r.o. (Hradec Králové, Czech Republic).

Antibiograms and minimal inhibitory concentrations (MICs) were evaluated using a BACMED 6iG2 automated reader and analyser (Aspiag, Litomyšl, Czech Republic).

### 2.4. Monitoring of biofilm formation ability

Biofilm formation of isolated strains was monitored in flat-bottomed microtiter plates (SPL Life Sciences Co., Ltd., Pocheon-si, South Korea) as previously described [Silha et al., 2020]. There were 8 wells in each experiment, and the experiments were independently repeated 3 times. The level of biofilm formation was categorized as non-adherent ( $OD \leq OD_C$ ) or biofilm-forming strains ( $OD > OD_C$ ), where  $OD_C$  (*cut-off* OD) is defined as three standard deviations above the mean OD of the negative control (blank value). *Cut-off* OD ( $OD_C$ ) was defined as three standard deviations above the mean OD of the negative control. The measured and calculated  $OD/OD_C$  (0.111/0.120) values were the same for all measurements.

**Table 1.** Smoothie drinks samples.

Fresh Bar A	
FA	Strawberry, mint, apple, lime
VA1	Cucumber, spinach, mint, apple, pineapple
VA2	Spinach, orange, mango, banana
Fresh Bar B	
FB	Banana, strawberries, orange
VB	Avocado, mango, spinach, apple
Fresh Bar C	
FC	Banana, kiwi, pear, pineapple
VC	Spinach, chia seeds, kiwi, mango, apple, dates, water
Fresh Bar D	
FD	Apple, orange, banana, strawberries, carrot, honey
VD	Spinach, celeriac, lemon, apple, mango
Fresh Bar E	
VE	Cucumber, spinach, mint, apple, pineapple

FX—Fruit\* smoothie drink; VX—Vegetable\* smoothie drink; \* — Categorization by vendor based on predominant smoothie ingredient; <sup>1</sup> – Pardubice; <sup>2</sup> – Hradec Králové.

## 3. Results and Discussion

### 3.1. Microbial quality of smoothies

In fruit smoothie drinks, the total number of mesophilic aerobic bacteria was determined in the range of 4.4–5.3 log CFU/mL, and the occurrence of yeast was in the range of 4.3–6.1 log CFU/mL. The occurrence of coliform bacteria was also observed in 3 samples (75 %) of fruit smoothies. The occurrence of coliform bacteria was at a relatively low level (1.5–2.1 log CFU/mL) in our study compared to values of 2.0–4.2 log CFU/mL according to an earlier study focused on the quality of smoothie drinks in Slovakia [Krahulcová et al., 2021]. Only one sample (FC) did not contain any coliform bacteria or enterococci. Enterococci, *S. aureus* and fungi were not determined in any fruit smoothie sample. Pathogenic bacteria *Salmonella* spp. and *Listeria monocytogenes* were also not detected.

In the case of vegetable samples, no listeria, salmonella or *S. aureus* were observed. The total number of mesophilic aerobic bacteria and the number of yeasts ranged from 5.4

to 7.2 log CFU/mL and 4.4–5.9 log CFU/mL, respectively. The presence of indicator microorganisms, namely coliform bacteria (3.1–6.0 log CFU/mL) and/or enterococci (1.6–3.3 log CFU/mL), was detected in all samples. Overall, the lowest microbiological quality was found in the sample labelled VA1, in which the presence of coliform bacteria, enterococci and yeasts and fungi was detected, at levels of  $6.0 \pm 0.05$ ,  $3.3 \pm 0.09$ , and  $8.0 \pm 0.08$  log CFU/mL, respectively, while the highest numbers of mesophilic aerobic bacteria (7.2 log CFU/mL) were also observed in this sample.

Analysis of ten smoothie samples purchased in the Czech Republic revealed the presence of coliform bacteria in the range of 1.5–6.0 log CFU/mL. *Enterobacter bugandensis*, *Klebsiella variicola* and *Klebsiella pneumoniae* were among the most frequently detected representatives of coliform bacteria. The presence of the indicator bacteria *E. coli* was not detected in any of the smoothie drink samples.

In a recent study dealing with the microbiological quality of smoothie drinks in Slovakia, the presence of coliform bacteria at the level of 2.0–4.2 log CFU/mL was confirmed, and is in agreement with the conclusions of our study for all monitored samples of smoothie drinks. At the same time, however, the presence of enterococci was also observed in 90 % of the samples, at a level of 1.6–2.9 log CFU/mL [Krahulcová et al., 2021]. In our experiments, enterococci were determined in only 30 % of smoothie drink samples (green smoothie drinks VA1, VA2 and VB). Most often it was *Enterococcus mundtii* and in one case it was *Enterococcus casseliflavus*.

In all samples of smoothie drinks, the presence of yeast was detected at a density of 4.3–5.9 log CFU/mL. *Candida tropicalis*, *Candida lusitanae* and *Candida parapsilosis* were the most frequently isolated and identified yeasts from smoothie drinks based on MALDI-TOF MS.

Staphylococci were found in 60% of the samples, but none of the isolates were *S. aureus* or other coagulase-positive staphylococci. The most common representatives of staphylococci were *Staphylococcus epidermidis* and *Staphylococcus pasteurii*. Furthermore, the presence of *Pseudomonas aeruginosa* was also confirmed in two samples (FB, VA1).

### 3.2. Level of antibiotic resistance

Strains isolated from smoothie drinks were evaluated in terms of their sensitivity to selected basic antimicrobial sets for testing specified groups of microorganisms according to EUCAST and CLSI, as well as according to how the testing is set up as standard in clinical practice.

A total of 45 strains of Gram-negative rod-shaped bacteria were isolated from all samples. Resistance to amoxicillin was observed in 37 strains (82.2%). Only *Kosakonia cowanii* (1 strain), *Leclercia adecarboxylata* (1 strain), *Pantoea agglomerans* (5 strains) and *Pantoea stewartii* (1 strain) were evaluated as sensitive to amoxicillin. Amoxicillin is one of the most commonly used antibiotics in primary care. It is an aminopenicillin that was created due to increasing antimicrobial resistance. Among Gram-negative isolates, significant resistance to amoxicillin in combination with clavulanic acid was also observed in 25 (55.6 %) of isolates. *Acinetobacter* was isolated from two

samples, mainly vegetable smoothie drinks labeled VA1 and VD. *Acinetobacter* isolates were resistant to the greatest number of antibiotics of all Gram-negative rod isolates. *Acinetobacter baumannii* isolated from sample VA1 was even resistant to all the tested antibiotics from different groups (penicillins, cephalosporins, fluoroquinolones, aminoglycosides and sulfonamides). Over the past 30 years, *Acinetobacter baumannii* strains have acquired resistance to a wide range of newly synthesized antimicrobial agents and have become one of the most feared pathogens worldwide [Almasaudi et al., 2018].

Among the enterococci strains isolated from smoothie drinks, there was only one strain (25.0 %) with proven resistance to co-trimoxazole and vancomycin, *i.e.* Vancomycin-Resistant *Enterococcus* (VRE). In the 9 strains of staphylococci isolated, resistance was observed only to clarithromycin (55.6 %) and clindamycin (11.1 %). No resistance to any other antibiotics was observed.

Resistance to itraconazole was detected in 50.0 % of isolated *Candida* spp. and *Clavispora* spp. Itraconazole is an azole antifungal that is effective against a broad spectrum of clinically relevant fungi and is used as a first-line agent for the prevention and treatment of invasive superficial infections [Stepanovic et al., 2020]. In our study, *C. tropicalis* was confirmed in a total of 6 (60.0 %) smoothie drink samples. Of this number, only two strains were sensitive to all the tested antifungals (fluconazole, itraconazole, nystatin, clotrimazole, econazole and natamycin) and resistance to itraconazole was observed in the other strains (from samples FB, FC, FD, VA2 and VD).

Overall, it can be stated that most of the microbial strains were isolated from vegetable smoothie drinks, especially from those labelled VA1, VA2 and VC. Of the 15 isolates from sample VA1, 5 strains can be considered multi-resistant. Similarly, 3 and 6 strains were multi-resistant out of a total of 14 and 12 strains isolated from VA2 and VC samples, respectively.

### 3.3. Biofilm formation ability

The ability to form a biofilm was monitored in 84 strains isolated from smoothie drink samples, using the Christensen method in microtiter plates. The highest biofilm production was observed for *Pseudomonas aeruginosa* (sample FB; A=1.8075). One more strain of *Pseudomonas aeruginosa* with a significantly lower biofilm production was isolated within the study (sample VA1; A=0.3210). *P. aeruginosa* has already been recognized in the past as one of the most life-threatening bacteria [Thi et al., 2020].

Significant biofilm production was observed in the *Kocuria kristinae* strain (VE sample; A=1.0259). However, there are not many publications dealing with the formation of biofilms in this bacterium so far.

An increased ability to form a biofilm was also noted for some strains of coliform bacteria. The highest biofilm production was observed in the strain *Klebsiella pneumoniae* (sample FB; A=0.7581). Among strains of *Klebsiella* spp., strongly adherent strains were recorded, but also strains with relatively low biofilm activity (A=0.1810–0.7581). The lowest biofilm formation was

observed in the *Klebsiella oxytoca* strain (sample VA1; A=0.1336). *Enterobacter cloacae* strains with weak biofilm production were isolated from two samples within the study (sample VA1 and VB; A=0.1475 and 0.1747, respectively). *Citrobacter freundii* strains were also isolated from two samples (sample VA2 and VC; A=0.2473 and 0.2101, respectively) and were evaluated as moderately to weakly adherent strains from the point of view of biofilm formation. The occurrence of *E. coli* was not recorded in any of the samples, however, an *Escherichia hermannii* strain was isolated from the VD sample, with increased biofilm production (A=0.3802). Enterococci strains isolated from smoothie drinks were capable of biofilm formation, but in all cases at a relatively low level.

*Staphylococcus pasteurii* (FC sample; A=0.5714) exhibited the highest biofilm formation of all isolated staphylococci, and was categorized as strongly adherent. However, other staphylococcal isolates were rated as weakly adherent.

*Bacillus pumilus* strain (sample VE, A=0.5528) isolated in our study was evaluated as strongly adherent and with high biofilm production. *Bacillus pumilus* was also isolated from the FD sample, but with a low ability to form a biofilm (A=0.1426).

High levels of biofilm activity were observed among yeasts isolated from smoothie drinks. However, some non-albicans species are capable of higher biofilm production. Out of a total of 10 yeast strains, moderate biofilm formation was recorded in 6 strains of *Candida* spp. Strong biofilm production was even detected in 4 strains, for *Candida tropicalis* isolated from samples FB, VA2 and VD (A=0.6680, A=0.5998, and A=0.7105, respectively) and *Candida lusitanae* (A=0.6012).

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## 4. Conclusion

The presence of mesophilic aerobic bacteria in a higher cell density, as well as of coliform bacteria, enterococci, but also yeast were detected in the smoothie drinks. Even fungi were detected when analysing one of the smoothie drinks. As part of the identification of individual isolated colonies, the presence of Gram-negative rods, enterococci, staphylococci, pseudomonads, Gram-positive cocci, Gram-positive rods and bacilli was found. However, the presence of *S. aureus*, *Salmonella* spp. and *Listeria* spp. was not detected in any sample. The results of this study point to a significant risk of the presence of strains resistant to many antimicrobials. In some cases, they were even multi-resistant strains. A high resistance of Gram-negative rods was observed especially to amoxicillin, also in combination with clavulanic acid. The highest resistance was observed in the *Acinetobacter baumannii* strain, in which alarming resistance was detected to all monitored groups of antibiotics. Similarly, considerable resistance was also found for isolates of *Candida* spp. The formation of biofilms is one of the most important factors in the virulence of microorganisms, and this ability was described in basically all isolated strains of microorganisms in the study. Some strains of *Pseudomonas aeruginosa*, *Kocuria kristinae* and *Klebsiella pneumoniae* have been found to form a very strong biofilm, which drastically increases the clinical significance of strains isolated from smoothie drinks.

Not only on the basis of the results of this study, it is clearly necessary to pay attention to the proper observance of the level of hygiene and sanitation, and even more so for the preparation of food and drinks without preservative intervention or without the addition of antimicrobial substances.