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Effect of sodium benzoate and potassium sorbate on *Burkholderia cepacia, Paraburkholderia tropica* and *Paraburkholderia fungorum* in flavored noncarbonated water

Wirkung von Natriumbenzoat und Kaliumsorbat auf Burkholderia cepacia, Paraburkholderia tropica und Paraburkholderia fungorum in aromatisiertem Wasser ohne Kohlensäure

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Summary

Potassium sorbate and sodium benzoate are commonly used preservatives in the food industry. This work was conducted with the aim of verifying the effectiveness of these two preservatives on the bacteria *Burkholderia cepacia, Paraburkholderia tropica* and *Paraburkholderia fungorum,* which were isolated from the environment of nonalcoholic noncarbonated beverages. The sensitivity of bacteria to preservatives was tested in commercially available flavored noncarbonated water. It has been shown that potassium sorbate and its combination with sodium benzoate can limit the growth of the bacteria studied but not completely suppress it. On the other hand, sodium benzoate was the least effective preservative ($p < 0.05$). Moreover, in this work it was shown that the bacteria *B. cepacia, P. tropica* and *P. fungorum* can use sodium benzoate as a source of carbon for their growth; therefore, diauxic growth was observed in flavored still water with the addition of sodium benzoate for all bacteria studied.

Keywords: growth curve, preservatives, diauxic growth, acid tolerance, benzoate utilization

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Introduction

Flavored mineral and spring waters are sorted into the food category "Beverages" and the subcategory "Flavored drinks" according to EU legislation (Regulation (EC) No 1333/2008). Flavorings of this type of beverage are regulated by Regulation (EC) No 1334/2008. Bottled flavored waters commonly contain natural or artificial flavors, as well as sugar or sweeteners, acidity regulators, natural or artificial colors and preservatives (Tireki, 2021). They can also be fortified with other functional components, such as herbal extracts, vitamins, and minerals (Ashurst, 2016; Kregiel, 2015; Tireki, 2021).

Due to their high-water activity and composition, soft drinks, and thus flavored waters, are a good growth environment for microorganisms (Curutiu et al., 2019). On the other hand, they are characterized by a low pH, which is achieved with the addition of acidity regulators, and only a narrow group of microorganisms can survive and multiply under these conditions (Lawlor 2009; Silva et al., 2022; Tribst et al., 2009).

Therefore, acid-tolerant microorganisms represent the main microbial risk (Curutiu et al., 2019; Kregiel, 2015; Silva et al., 2022). Bacteria frequently isolated from noncarbonated beverages are those of the genera *Acetobacter, Gluconobacter* and other acetic acid bacteria, such as the genus *Asaia,* which is associated with flavored bottled waters and iced teas (Kregiel et al. 2018; Silva et al., 2022). The products of the metabolism of these bacteria are ethanol, acetate, succinate, and formate, which cause off-odors and off-flavors in beverages (Bintsis 2018; Kregiel 2015).

The Burkholderia cepacia complex (Bcc) is a group of more than twenty-five phenotypically similar Gram-negative bacteria (De Volder et al., 2020; Depoorter et al., 2020; Martina et al. 2018). These bacteria are ubiquitous, opportunistic pathogens of humans (De Volder et al., 2020; DeSmet et al., 2013; Jimenez et al., 2015; Lupo et al., 2015; Peterson et al., 2013). Many Bcc bacteria are naturally resistant to a wide range of industrial biocides used in food and beverage preservation (Song et al. 2018). Notably, their ability to form biofilms helps them survive in adverse, nutrient-poor environments (Cuzzi et al., 2014; De Volder et al., 2020). The genus *Paraburkholderia* is remarkably close to *Burkholderia,* with the primary difference being a lower genomic G+C content in *Paraburkholderia* (Vandamme et al., 2017; Vanwijnsberghe et al., 2021). Currently, the genus *Paraburkholderia* includes over seventy species, which occur mainly in the soil and occasionally in the aquatic environment (Vanwijnsberghe et al., 2021). However, some species of the genus *Paraburkholderia,* including *P. fungorum* and *P. tropica,* can contribute to infections in humans and animals (Deris et al., 2010; Gerrits et al., 2005; Vanwijnsberghe et al., 2021).

To prevent microbial growth in noncarbonated beverages, it is common to use preservatives like sodium benzoate and potassium sorbate (Musyoka et al., 2018). These are weak organic acid salts, whose antimicrobial effect is based on the accumulation of protons and anions inside the microbe. The accumulation leads to disruption of the normal function of cellular metabolism (Lopez-Malo et al., 2007; Musyoka et al., 2018). Benzoic acid and its salts as well as Sorbic acid and its salts reliably inhibit the growth of a wide range of yeasts, fungi. Moreover, sorbic acid is effective against bacteria except for lactic acid bacteria (Chipley, 2005; Stopforth et al., 2005).

This study was performed with the aim of verifying the effectiveness of commonly used preservatives, sodium benzoate and potassium sorbate, against the bacteria *Burkholderia cepacia, Paraburkholderia tropica* and *Paraburkholderia fungorum,* which were isolated from the environment of nonalcoholic noncarbonated beverages.

Material and Method

Bacterial cultures

The following bacterial species were used in this study: *Burkholderia cepacia, Paraburkholderia fungorum* and *Paraburkholderia tropica.* These bacteria were isolated from beverages that showed signs of spoilage – *B. cepacia* and *P. fungorum* from peach flavored green iced tea and *P. tropica* from lemon flavored green iced tea. One isolate per one culture was prepared and then externally identified using MALDI-TOF at the species level. These bacteria were stored on nutrient agar (Nutrient agar, Merck KGaA, Germany) in a thermostat at 4 °C for one week and subcultured immediately before use in the experiments. The purity of the bacterial cultures was checked using a Gram-stained microscopic slides.

Media preparation

The effectiveness of the selected preservatives was studied in commercially purchased flavored noncarbonated water. Composition of flavored unsaturated water provided on request from the manufacturer: water, sucrose 35 g/L, citric acid anhydrous 0,7 g/L, pH 3.00, natural flavor (raspberry, cranberry). The flavored water was filled into four 250 ml laboratory bottles GL 45 with a pouring ring and cap (SIMAX, Czech Republic). The first bottle of flavored water was left without the addition of preservatives, the second with the addition of 300 mg/L potassium sorbate (PS) (Sigma-Aldrich Chemie GmbH), the third with the addition of 150 mg/L sodium benzoate (SB) (Sigma-Aldrich Chemie GmbH) and the fourth with the addition of a combination of 250 mg/L potassium sorbate (PS) and 150 mg/L sodium benzoate (SB). The amount of preservative was chosen at the upper limit laid down in Regulation (EC) No 1333/2008 of the European Parliament and of the Council. The flavored still water solutions were sterilized in an autoclave at 121 °C for 15 minutes.

Sample preparation

Individual bacterial species were inoculated into tubes with sterile TSB (Tryptic Soy Broth, Merck KGaA, Germany) and incubated in the incubator at 30 °C for 48 hours. Post-incubation bacterial density was adjusted to an optical density of 4.00 McFarland using sterile TSB.

Ten milliliters of each prepared solution of flavored noncarbonated water were filled into sterile tubes (GAMA GROUP a.s., Czech Republic). For each bacterial species, two tubes of each solution were prepared. The prepared sets of solutions in tubes were inoculated with 100 µL of the bacterial suspension. The tubes were stored in the incubator at 30 °C throughout the experiment.

Monitoring of the effectiveness of preservatives on selected bacterial species

For the monitoring of bacterial growth, the densitometric method was chosen. The optical density was measured at 525 nm (DENSI-LA-METR II, Erba Lachema s.r.o., Czech Republic). As a blank, flavored, noncarbonated water without inoculation with any bacteria was measured. The growth of bacteria in flavored, noncarbonated water with and without additional preservatives was detected.

For the first 48 hours of the experiment, measurements were made twice a day for 12 hours. After that, the samples were measured at intervals of 2 days for 18 days. After the experiment contamination by molds and yeasts was checked by plating the samples on YGC agar (Yeast Extract Glucose Chloramphenicol Agar FIL-IDF, Merck KGaA, Germany).

Statistics

The results are presented as an average ± standard deviation. The data obtained from the experiments were analyzed using Student's t-test for differences between groups at the significance level of $p < 0.05$. Statistical analyses were performed using Statistica 12.0 software (Statsoft Inc., Tulsa, OK, USA). All measurements were made in two parallels. The experiment was conducted twice to verify correctness.

Results

Selected bacterial species *Burkholderia cepacia*, *Paraburkholderia fungorum* and *Paraburkholderia tropica* were isolated from drinks that showed signs of spoilage. Their resistance to preservatives was tested in commercially purchased flavored noncarbonated water with an ingredient list free from sodium benzoate and potassium sorbate.

Figures No. 1–3 show the growth of the bacteria *P. fungorum, P. tropica* and *B. cepacia* in commercially purchased flavored noncarbonated water without the addition of preservatives, with the addition of PS, SB and their combination. In flavored water without the addition of preservatives, rapid exponential growth can be observed immediately after inoculation. In samples with the preservatives, there is a more gradual growth. It is also possible to distinguish the effectiveness of individual preservatives. The growth of *P. fungorum* and *P. tropica* differed significantly in each of the flavored noncarbonated water solutions with preservatives ($p < 0,05$). For *B. cepacia*, the efficacy of the use of PS and the use of a combination of PS and SB did not differ significantly ($p < 0.05$). In all bacteria, typical signs of diauxic growth can be observed in a solution with the addition of SB. In *P. fungorum,* the diauxic lag phase was terminated after the tenth day of cultivation, and the second exponential phase was entered. In the bacterium *B. cepacia,* this phenomenon occurred after the twelfth day of cultivation, and in the bacterium *P. tropica* after the fourteenth day of cultivation. Moreover, in the bacterium *P. tropica* this phenomenon occurs also in the control sample.

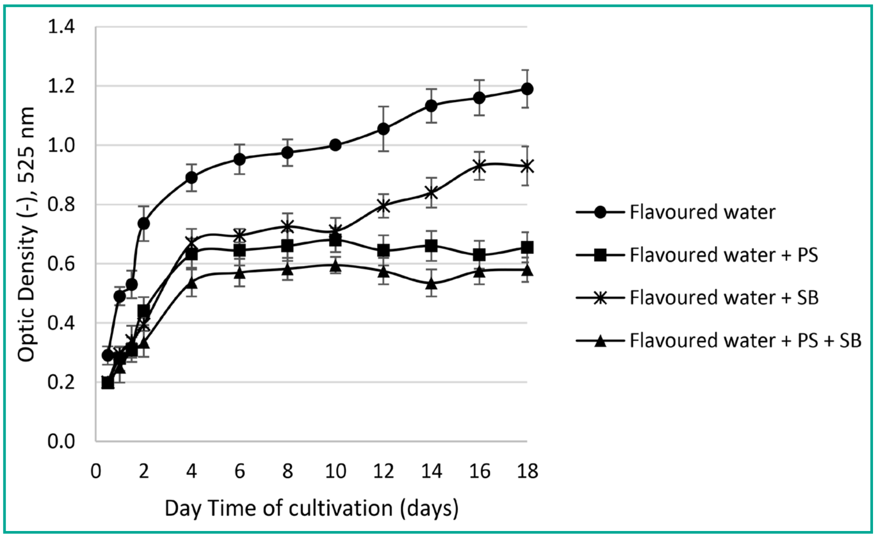

FIGURE 1: *Effect of preservatives in noncarbonated flavoured water on the growth of Paraburkholderia fungorum bacteria. (PS – potassium sorbate, SB – sodium benzoate, PS + SB sorbate + benzoate).*

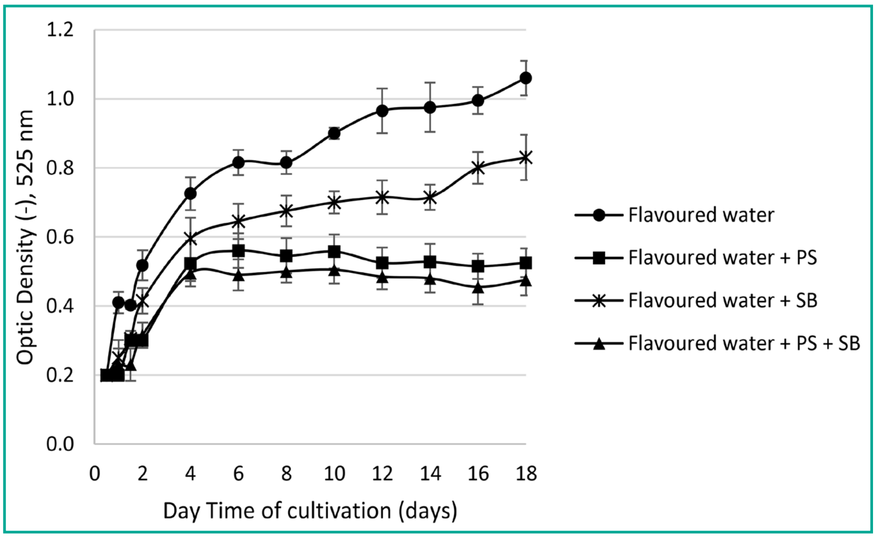

FIGURE 2: *Effect of preservatives in noncarbonated flavoured water on the growth of Paraburkholderia tropica. (PS – potassium sorbate, SB – sodium benzoate, PS + SB sorbate + benzoate).*

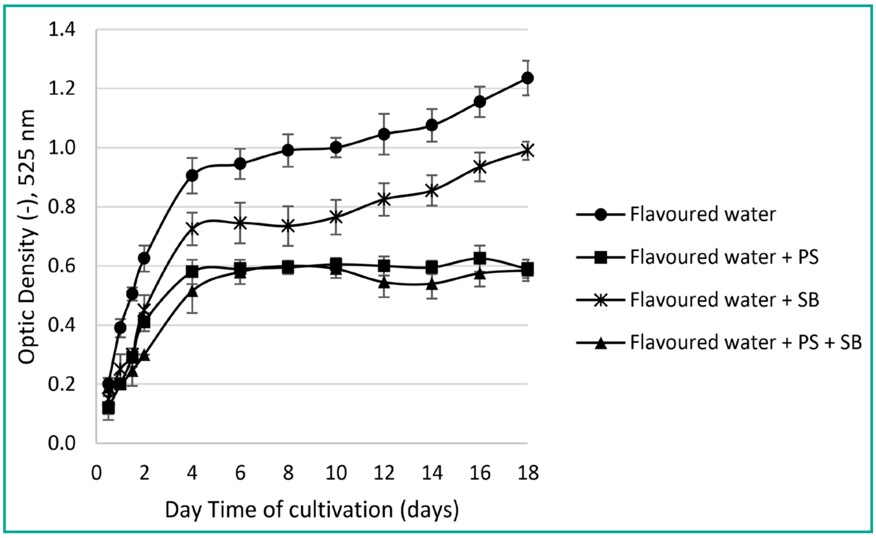

FIGURE 3: *Effect of preservatives in noncarbonated flavoured water on the growth of Burkholderia cepacia. (PS – potassium sorbate, SB – sodium benzoate, PS + SB sorbate + benzoate).*

Discussion

The bacteria *B. cepacia, P. fungorum* and *P. tropica* were isolated from nonalcoholic, noncarbonated beverages which are characterized by a low pH. It has been experimentally confirmed that these bacteria can grow even in an acidic environment with a pH value of 3.0. In 2014, Stopnisek et al. addressed the acid tolerance of the genus *Burkholderia.* A total of thirty-one species of the genus *Burkholderia* were observed to grow in an environment with pH values of 3.5, 4.0 and 4.5. Only eight species were able to grow even at a pH of 3.5. This work coincides with the conclusion that within the genus *Burkholderia,* some species are acid tolerant and can grow at a very low pH. The genus *Paraburkholderia,* due to its considerable similarity with the genus *Burkholderia* (Vanwijnsberghe et al., 2021), can be assumed to have similar demands on conditions for growth. Lee and Jeon published a study in 2018 in which they described the newly discovered bacterium *Paraburkholderia aromaticivorans* sp. nov. Its growth was observed even at pH 3.0, with the optimum for growth being 5.0–6.0 (Lee and Jeon, 2018). Representatives of this genus are often isolated from acidic soils, where their acid tolerance gives them a competitive advantage over other soil bacteria (de Castro Pires et al. 2018; Paulitsch et al. 2019; Stopnisek et al. 2014). The results of this work, together with the above studies, suggest that the genus *Paraburkholderia,* similar to the genus *Burkholderia,* includes acid-tolerant species.

The hypothesis that the bacteria *B. cepacia, P. fungorum* and *P. tropica* are sensitive to the tested preservatives (PS, SB, and their combination) has not been fully confirmed. In flavored noncarbonated water with the addition of preservatives in the legally determined limit, there was a partial reduction in growth, but it was not completely suppressed. This phenomenon can be attributed to adaptive resistance, where exposure of a bacterial cell to stressful conditions leads to an increase in its resistance to the preservatives and biocides used (Fernández et al., 2011; Rushton et al., 2013). In a flavored noncarbonated water environment, SB is the least effective preservative for all bacteria. This is consistent with the findings of a 2013 study by Rushton et al., which studied the sensitivity of eighty-three bacterial strains of Burkholderia cepacia complex to eight preservatives (Rushton et a. 2013). Moreover, in a solution of flavored still water with the addition of SB, diauxic growth of all bacteria was observed. This phenomenon is typical when bacteria are cultivated in an environment with two sources of carbon that they can use for their growth (Monod, 1947). The most well-known example of diauxic is the growth of *Escherichia coli* on a mixture of glucose and lactose (Narang et al., 2007). In 2019, Morya et al. published a study investigating the ability of *Burkholderia* spp. to degrade various lignin-related compounds, including benzoic acid. The conclusion was that the bacteria of the genus *Burkholderia* spp. can use benzoic acid for their growth. For benzoic acid utilization, bacteria use the beta-ketoadipate pathway (Morya et al., 2019; Wang et al., 2018). Diauxic growth in the presence of SB can be explained by the utilization of benzoate even after the depletion of the primary carbon source.

In this experiment, the densitometric method was chosen to evaluate the effect of preservatives, as it is fast and can serve as an illustrative guideline for the effect of preservatives. In actual fact, however, it may not be completely correlated with the number of viable bacterial cells. Therefore, the results of this work can become the basis of a hypothesis for a more extensive and robust study focused on the resistance of bacteria of the genus *Burkholderia* and *Paraburkholderia* to various chemical and natural preservatives.

Conclusion

While potassium sorbate and sodium benzoate are legally permitted, they are not effective in suppressing the growth of *Burkholderia cepacia, Paraburkholderia tropica* and *Paraburkholderia fungorum.* In this study, it was shown that these acid-tolerant species can grow in an environment with a pH value of 3.0, which is characteristic of noncarbonated beverages. Thus, combined with their ability to form biofilms, they are a real risk in the beverage industry. The results of this study also showed that *B. cepacia, P. tropica* and *P. fungorum* can use sodium benzoate as a source of carbon for growth as well as some fungi and yeasts. The preservation of flavored noncarbonated waters is therefore insufficient for these bacteria. On the other hand, the use of potassium sorbate or its combination with sodium benzoate can be one of the degrees of obstruction effect in the production of this type of beverage and increase the bacterial safety of products that reach consumers.

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Credit | author statement

Gabriela Kuncová: Investigation, Writing - Original Draft, Visualization. Iveta Horsáková: Conceptualization, Methodology, Writing - Original Draft, Project administration. Ladislava Rýdlová: Validation, Formal analysis. Aleš Rajchl: Writing - Review & Editing, Supervision, Funding acquisition.

Conflict of Interest

All authors declare, that no conflict of interest exist.

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