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MONITORING OF MICROBIAL CHANGES IN FRESH FRUIT BEVERAGES WITH THE ADDITION OF SELECTED MEDICINAL PLANTS

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ABSTRACT

In this study, we assessed the microbiological quality of fresh fruit smoothies made from three types of fruit, including versions with added medicinal plants. Our aim was to get closer to home conditions of smoothie preparation, so we did not use essential oils of medicinal plants but their whole parts. We quantified total microorganisms, coliform bacteria, yeasts, and filamentous fungi using the plate dilution method. Total microbial counts ranged from 5.12 to 6.67 log CFU/mL, with most samples showing a decrease after the addition of medicinal plants, particularly *Thymus serpyllum*. Coliform bacteria levels varied from 1.48 to 2.70 log CFU/mL, with the highest counts recorded in pear-based smoothies. A reduction in coliforms was observed only in samples containing apple and *Thymus serpyllum*, pear and *Thymus vulgaris*, and orange with *Mentha piperita*. Yeast counts ranged from 3.10 to 5.46 log CFU/mL, with medicinal plants influencing their abundance. Three samples showed no colonies and a low level of filamentous fungi contamination. . Morphological identification of microscopic fungi revealed four genera: *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*. *Cladosporium* was the most frequently isolated genus (50% of samples), while potentially toxinogenic *Aspergillus*, *Penicillium*, and *Alternaria* did not exceed 25% occurrence. Among yeasts, *Geotrichum candidum* was dominant. MALDI-TOF MS analysis identified three genera and six species of coliform bacteria. *Klebsiella oxytoca* was the most common (48%), followed by *Enterobacter cloacae* (19%) and *Raoultella ornithinolytica* (14%). Our findings suggest that the addition of medicinal plants into smoothies may enhance microbiological quality by reducing microbial contamination, though further investigation is needed to determine their impact on coliform bacteria.

Keywords: smoothie, total microbial counts, coliform bacteria, filamentous fungi, yeast, MALDI-TOF MS Biotyper

INTRODUCTION

Fruits are an essential part of the human diet, providing important macronutrients, micronutrients, dietary fibres, vitamins, minerals, and a wide range of bioactive compounds **(Hussein *et al.*, 2018)**. Their rich nutritional profile has been strongly associated with numerous health benefits, leading to widespread recommendations for including fruits as a key component of a healthy diet. Regular fruit consumption has been linked to the prevention of various diseases, including high blood pressure, cancer, diabetes, cardiovascular diseases, digestive disorders, bone problems, immune system disorders, and even ageing-related effects **(Famiani *et al.*, 2020; Aleksandrova *et al.*, 2021; Kurowska *et al.*, 2023)**. The health-promoting effects of fruits are largely attributed to their phytochemicals, particularly (poly)phenols, due to their anti-inflammatory and antioxidant properties **(Fratta Pasini & Cominacini, 2023; Hong & Qin, 2023; Nani *et al.*, 2021)**.
Medicinal plants are a rich source of bioactive compounds with significant pharmacological properties **(Albahri *et al.*, 2023)**. Over 1340 plants have been identified with antimicrobial activity, and more than 30000 antimicrobial compounds have been isolated from plants **(Abdallah *et al.*, 2023)**. Approximately 14–28% of higher plant species are classified as medicinal, with 74% of bioactive plant-derived compounds discovered based on their traditional medicinal use **(Conly & Johnston, 2005)**. The antimicrobial compounds derived from medicinal plants exhibit diverse mechanisms of action distinct from conventional antimicrobials, making them valuable for combating resistant microbial strains **(Truelove *et al.*, 2020)**. Due to their chemically complex nature, these compounds have significant therapeutic potential, with fewer side effects and a lower likelihood of resistance development compared to synthetic drugs **(Prabhu & Singh, 2019)**. Key phytochemicals such as spermidine, rutin, quercetin, tocopherol, and carotenoids contribute to their antimicrobial, antioxidative, anti-inflammatory, and antiviral properties **(Khwaif *et al.*, 2010; Rather *et al.*, 2017)**. These bioactive compounds primarily target microbial cell membranes, affecting their structure, integrity, permeability, or functionality **(Karp & Engberg, 2004; Cabello, 2006)**.
Fresh fruit beverages are highly nutritious but also perishable, serving as an ideal medium for the growth and multiplication of microorganisms **(Mengistu *et al.*, 2022; Aneja *et al.*, 2014)**. Despite their beneficial nutritional properties, including high potassium content, low sodium levels, and natural vitamin C essential for collagen formation and iron absorption **(Aneja *et al.*, 2014)**, fruit juices naturally harbour a microbiota originating from fruit surfaces during harvest, transport, storage, and processing **(Tournas *et al.*, 2006)**. This microbiota includes acid-tolerant bacteria and fungi such as yeasts (*Candida, Dekkera, Hanseniaspora, Pichia, Saccharomyces, Zygosaccharomyces*) and filamentous fungi (*Penicillium, Byssochlamys, Aspergillus, Paecilomyces, Mucor, Cladosporium, Fusarium, Botrytis, Talaromyces*). Additionally, lactic acid bacteria (*Lactobacillus, Leuconostoc*) and acetic acid bacteria (*Acetobacter, Gluconobacter*) are frequently isolated from fruit juices **(ICMSF, 2005)**.
Microbial spoilage in fruit beverages manifests as cloud loss, off-flavours, gas production, and alterations in colour, texture, and appearance, ultimately leading to product degradation **(Sperber & Doyle, 2009; Sospedra *et al.*, 2012)**. Although fruit juices have an acidic pH (<4.5), which serves as a natural barrier to microbial growth, foodborne pathogens such as *Escherichia coli* and *Salmonella* can survive in these environments due to their acid stress response **(Malik *et al.*, 2020)**. This highlights the potential risk of contamination due to unsanitary conditions, poor hygienic practices, and low-quality water sources used during production. Consequently, several foodborne outbreaks related to unpasteurised fruit juices have been reported worldwide in the past two decades **(Raybaudi-Massilia *et al.*, 2009; Ghenghesh *et al.*, 2005)**. Moreover, the presence of pathogenic bacteria, including *Escherichia coli, Listeria monocytogenes*, and *Salmonella* spp., further underscores the need for proper microbiological safety measures in fruit-based beverages **(Silva & Evelyn, 2023)**.
The objective of our study was to quantitatively determine the total microbial counts, coliforms, yeasts, and microscopic filamentous fungi in fresh fruit beverages and beverages supplemented with medicinal plants. Additionally, we aimed to identify microscopic fungi morphologically, and to identify coliform bacteria using the MALDI-TOF MS.

MATERIAL AND METHODS

Plant materials and smoothing

Three types of fruit were selected, as detailed in Table 1. Fresh fruits were purchased from supermarket chains in Nitra, Slovakia. They were washed under running drinking water, peeled, and cut into smaller pieces. For smoothie preparation, 300 g of chopped fruit was blended using a smoothie blender. The resulting mixture was transferred into a sterile flask and mixed with 100 mL of drinking water to simulate domestic conditions. The smoothie was enriched with selected medicinal plants to evaluate their individual effects. Each smoothie sample was prepared separately with either fresh mint (*Mentha piperita*), wild thyme (*Thymus serpyllum*), or thyme (*Thymus vulgaris*), all obtained from home gardens. Specifically, the mint-enriched smoothies contained 7 leaves of *Mentha piperita*, the wild thyme-enriched smoothies contained 20 leaves of *Thymus serpyllum*, and the thyme-enriched smoothies contained 20 leaves of *Thymus vulgaris*. The prepared samples were placed in a laboratory shaker (Orbital Shaker Incubator ES-20, Biosan, Latvia) and shaken for 15 minutes to facilitate the release of antimicrobial compounds.

Table 1 Fruits information

Fruit name	Variety	Part used	Source
Apple	Golden Delicious	Pulp	Poland
Pear	Conference	Pulp	Belgium
Orange	Navelina	Pulp	Greece

Total microbial count

The undiluted smoothie sample was labelled as 10^0. From this, a series of tenfold dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) was prepared. To initiate the dilution process, 5 mL of smoothie sample was mixed with 45 mL of distilled water. The sample was then homogenised using a horizontal shaker for 30 minutes, resulting in a 10^{-1} dilution. Subsequent dilutions were prepared by transferring 1 mL of the previous dilution into 9 mL of sterile distilled water, following the same procedure for each step. The total colony counts of the bacteria were determined using the pour plate method on plate count agar (PCA, Conda, Spain). For each dilution triplicate plates were prepared in accordance with Slovak Technical Standard **STN EN ISO 4833**. The plates were incubated aerobically at 30°C for 72 hours in an inverted position. After incubation, bacterial colonies were counted to assess microbial load. For enumeration, only Petri dishes containing between 10 and 300 colonies in two consecutive dilutions were considered.

Coliform count

The quantification of coliform bacteria followed a procedure similar to that used for total microbial count determination. However, in this case, medium with crystal violet, neutral red, bile salts, and lactose (VRBA, Himedia, India) was used. Inoculation was performed using 0.1 mL of the undiluted sample, as well as dilutions from 10^{-1} to 10^{-3}, applying the surface plating method. The plates were incubated at 37°C ± 1°C for 24 ± 2 hours in an inverted position. For enumeration, only Petri dishes containing between 10 and 150 colonies in two consecutive dilutions were considered. Slovak Technical Standards **STN EN ISO 4832** were used.

Yeasts and filamentous microscopic fungi count

The quantification of yeasts and filamentous microscopic fungi followed a similar procedure to the previous microbial determinations, with the key difference being the use of Dichloran Rose Bengal Chloramphenicol (DRBC, Himedia, India) agar as the selective medium. A 0.1 mL aliquot of both undiluted and diluted samples (10^{-1} to 10^{-3}) was inoculated onto the surface of the solidified medium in triplicate. The plates were then incubated at 25°C ± 1°C for 5 to 7 days. As with coliform bacteria, only Petri dishes containing between 10 and 150 colonies in two consecutive dilutions were considered valid for analysis. Slovak Technical Standard **STN EN ISO 21527-2** was used.

Morphological identification of microscopic fungi

The taxonomic identification of all isolates was performed through macroscopic and microscopic examination, following the methodology described by **Pitt & Hocking (2009)** and **(Samson *et al.*, 2010)**.

Microbial identification of coliform bacteria with the MALDI-TOF MS Biotyper

Distilled water (300 µL) was pipetted into Eppendorf tubes. The biological material was carefully removed from Petri plates and transferred into the tubes containing water, then vortexed thoroughly. Subsequently, 900 µL of ethanol was added, and the tubes were vortexed again. The samples were centrifuged at 14000 rpm for two minutes, the supernatant was poured off, and centrifugation was repeated. The remaining ethanol was removed, and the pellet was allowed to dry for a few minutes. Formic acid (70%, 50 µL) was added to the pellet, mixed thoroughly by pipetting and vortexing, followed by the addition of 50 µL of acetonitrile. After mixing thoroughly, the tubes were centrifuged again. A 1-µL aliquot of the supernatant was pipetted onto a MALDI-TOF plate and allowed to dry. After drying, the sample was overlaid with 1 µL of MALDI matrix solution (Sigma-Aldrich, Germany). Once dried, the plate was placed into the ionisation chamber of the mass spectrometer for microbial identification. An identification score of 2.00–3.00 indicates a high-confidence identification at the species level, while a score of 1.70–1.99 corresponds to a low-confidence identification at the genus level. Scores below 1.70 (0–1.69) indicate unsuccessful strain identification.

Statistical analysis

Basic descriptive statistical methods, including mean and standard deviation, were used to evaluate the results. Additionally, the statistical techniques of paired t-test, Analysis of Variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test were employed to compare the results and determine statistically significant differences between the data groups. Statistical analyses were conducted using XLSTAT software (Addinsoft, Pris, France). Significance was determined at a level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of the quantitative determination of the total microbial counts, coliform bacteria, yeasts, and microscopic filamentous fungi are presented in Table 2. This table provides data on the quantity of individual microbial groups in fresh fruit smoothies without the addition of medicinal plants (control sample) and in smoothies enriched with medicinal plants. In the smoothie samples, total microbial counts (TMC) ranged from 5.12 to 6.67 log CFU/mL, with the highest values recorded in the pear-based beverage. However, TMC slightly increased after adding *Mentha piperita* leaves to the apple smoothie and *Thymus vulgaris* leaves to the orange smoothie. In most of the other samples, a reduction in total microbial counts was observed. *Thymus serpyllum* led to a decrease in microbial counts in all samples, with the most significant reduction observed in the apple smoothie. *Mentha piperita* demonstrated the most pronounced antimicrobial effect when combined with the orange smoothie, resulting in the most significant reduction of the microbial population. On the other hand, *Thymus vulgaris* showed the strongest influence on the total microbial count (TMC) in apple and pear beverages. However, a shaking time of just 15 minutes is not sufficient to conclusively determine an antibacterial effect of the plant extracts on the microorganisms. The number of TMV in pear samples, including treated variants, were significantly the higher ($p \leq 0.05$) in comparison with the apple and orange groups. The Orange + MP sample showed the lowest TMC, being significantly different ($p \leq 0.05$) from all other groups.

Table 2 Quantitative representation of microbial groups in smoothie samples

Sample	TMC	CB	Yeast	FF
	log CFU/mL			
Apple	5.57 ±0.03^{a}	1.64 ±0.03^{a}	5.46 ±0.03^{a}	1.00 ±0.03 ^{a}
Apple + TS	5.39 ±0.02^{b}	1.48 ±0.03^{b}	4.02 ±0.03^{b}	1.48 ±0.03 ^{b}
Apple + MP	5.64 ±0.02^{a}	1.89 ±0.03^{c}	5.00 ±0.03^{ad}	1.48 ±0.05 ^{b}
Apple + TV	5.50 ±0.03	2.05 ±0.02^{d}	4.11 ±0.03^{be}	1.00 ±0.01 ^{a}
Pear	6.67 ±0.03^{c}	2.64 ±0.03^{e}	3.99 ±0.05^{b}	1.30 ±0.03 ^{c}
Pear + TS	6.61 ±0.03 ^{c}	2.67 ±0.02^{e}	3.10 ±0.04^{c}	1.00 ±0.02 ^{a}
Pear + MP	6.67 ±0.03 ^{c}	2.70 ±0.04^{e}	3.73 ±0.05^{bc}	1.61 ±0.03^{d}
Pear + TV	6.60 ±0.03 ^{c}	2.43 ±0.03^{f}	3.13 ±0.03^{c}	1.48 ±0.02 ^{b}
Orange	5.51 ±0.01^{a}	2.16 ±0.02^{g}	4.99 ±0.03^{ad}	< 1^{e}
Orange + TS	5.45 ±0.03^{b}	2.41 ±0.04^{f}	4.88 ±0.03^{d}	< 1 ^{e}
Orange + MP	5.12 ±0.02^{d}	2.00 ±0.04^{dg}	4.21 ±0.04^{e}	1.61 ±0.02 ^{d}
Orange + TV	5.56 ±0.03^{a}	2.40 ±0.02^{f}	4.29 ±0.03^{e}	< 1 ^{e}

Legend: TS - *Thymus serpyllum*, MP - *Mentha piperita*, TV - *Thymus vulgaris*, , TMC - total microbial counts, CB - coliform bacteria, FF - filamentous fungi. The obtained results present the average values and standard deviations (SD) of the plate counts, with each measurement performed in triplicate. Values within the same column marked with different letters indicate statistically significant differences as determined by Tukey’s Honest Significant Difference (HSD) test ($p \leq 0.05$).

Galovičová *et al.* (2021a) analyzed the metabolite composition of *Thymus serpyllum* essential oil using the MALDI-TOF MS Biotyper method. The main compounds identified were thymol (18.8%), carvacrol (17.4%), *o*-cymene (15.4%), and geraniol (10.7%). The highest antimicrobial activity was observed against *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and biofilm-forming bacteria such as *Enterococcus faecalis*. The major constituents of *Mentha piperita* essential oil comprising approximately 60% of the total oil are *d*-carvone, limonene, menthone, menthol, and pulegone, with content levels varying depending on the cultivation area **(Mahendran & Rahman, 2020)**. Among the polyphenolic compounds, luteolin, naringin, and rutin are present in significant amounts (**Gholamipourfard *et al.*, 2021**). The high antimicrobial activity of *M. piperita* is attributed especially to isomenthone, α-terpinene, pipertitinonoxide, trans-carveol, and β-caryophyllene. It exhibits inhibitory effects mainly against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (**Mehendran & Rahman, 2020**). **Galovičová *et al.* (2021b**) also evaluated the biological activity of *T. vulgaris* essential oil. The primary components reported were thymol (48.1%), *p*-cymene (11.7%), 1,8-cineole (6.7%), γ-terpinene (6.1%), and carvacrol (5.5%). This essential oil demonstrated natural efficacy against most Gram-positive and Gram-negative microorganisms, with strong inhibitory effects particularly against *E. coli* and *Staphylococcus aureus*.

Krahulcová *et al.* (2021) analysed the total aerobic bacteria in different types of fruit smoothies, reporting bacterial counts ranging from 2.9 to 7.3 log CFU/mL. These results correlate with bacterial population levels in fruits and vegetables, which vary between 3 and 7 log CFU/g depending on the type of ingredient **(Korir *et al.*, 2016; Abadias *et al.*, 2008)**. **Kačániová *et al.* (2021)** investigated the effect of plant extracts from *Calendula officinalis* L., *Ginkgo biloba*, *Thymus serpyllum*, *Matricaria recutita*, *Salvia officinalis* L., and *Mentha aquatica* var. *citrata* on microbial counts in grape juice. The juice sample without medicinal plant extracts contained 3.25 log CFU/mL, while the addition of *Thymus serpyllum* extract reduced the total microbial count to 1.72 log CFU/mL. Similarly, *Mentha aquatica* var. *citrata* extract had a comparable effect, lowering TMC to 1.62 log CFU/mL. In our study, we also observed a decrease in total microbial counts, despite using whole medicinal plant leaves instead of extracts. **Helal *et al.* (2006)** reported total bacterial counts of 4.87, 4.53, and 4.25 log CFU/mL in orange, guava, and banana juices, respectively. These bacteria belonged to: *Bacillus* sp., *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas* sp. Eight essential oils from local herbs were tested for antimicrobial activity against these bacterial strains, as well as filamentous fungi and yeasts. The antimicrobial activity of all tested oils was generally higher against bacteria than against fungi and yeasts. Compared to our samples, the effect of medicinal plant leaves on reducing microbial content in fruit smoothies was more pronounced for yeasts, followed by total microbial counts.

The coliform bacteria counts ranged from 1.48 to 2.70 log CFU/mL. The lowest values of CB were in the Apple + TS samples ($p \leq 0.05$), indicating a strong reducing effect of TS on the measured parameter in apple samples.

The highest count was recorded in pear beverage samples, while the lowest contamination was observed in apple-based samples. When medicinal plants were added, the bacterial count decreased in only 3 out of 9 cases. This suggests that the medicinal plants used were likely contaminated with coliform bacteria, leading to secondary contamination of the prepared fruit beverages. A reduction in coliform bacteria was observed only in beverages containing apple and *Thymus serpyllum*, pear and *Thymus vulgaris*, and orange and *Mentha piperita*.

From the smoothie samples, we identified three genera and six species of coliform bacteria using the MALDI-TOF MS Biotyper spectrometric method (Tab. 3). *Klebsiella oxytoca* (47.62%) and *Enterobacter cloacae* (19.05%) were the most frequently detected species in fruit smoothies. Additionally, *Klebsiella variicola* and *Raoultella ornithinolytica* were also present in the control sample.

Table 3 Isolated coliforms from smoothie samples

Sample	Isolated coliform bacteria species
Apple	*Klebsiella oxytoca, Enterobacter cloacea*
Apple + TS	*Klebsiella oxytoca, Enterobacter cloacea,*
Apple + MP	*Klebsiella oxytoca, Enterobacter cloacea, Raoultella ornithinolytica*
Apple + TV	*Klebsiella oxytoca, Enterobacter ludwigii, Raoultella ornithinolytica*
Pear	*Klebsiella variicola, Klebsiella oxytoca*
Pear + TS	*Klebsiella variicola, Klebsiella pneumoniae*
Pear + MP	*Klebsiella oxytoca*
Pear + TV	*Klebsiella oxytoca, Enterobacter cloacea*
Orange	*Raoultella ornithinolytica*
Orange + TS	*Klebsiella oxytoca*
Orange + MP	*Klebsiella oxytoca*
Orange + TV	*Klebsiella oxytoca*

Legend: TS - *Thymus serpyllum,* MP - *Mentha piperita,* TV - *Thymus vulgaris,* ND - not detected, TMC - total microbial counts, CB - coliform bacteria, FF - filamentous fungi

Malik *et al.* (2020) collected and analysed 80 fresh fruit juice samples from vendors in India. Contamination was found in 71 samples, and 105 microbial pathogens were isolated. Among the identified organisms, *Klebsiella* spp. (59.05%) was the most dominant, followed by *E. coli* (15.23%). Similarly, *Klebsiella* spp. (61.90%) was the most prevalent genus in our samples, followed by *Enterobacter* spp. (23.81%) and *Raoultella* spp. (14.29%). Another study on the microbial quality of fruit juices sold by street vendors in India reported a high microbial load of coliforms, faecal coliforms, *S. aureus*, and *Vibrio* spp. **(Mahale *et al.*, 2008)**. **Jesús *et al.* (2022)** analysed unpasteurised orange juice samples from various markets in Mexico and stated that a coliform bacteria count exceeding 2 log CFU/mL is considered an indicator of contamination. Such contamination can lead to rapid spoilage, poor sensory properties, and potential health risks for consumers. Up to 86% of the analysed beverage samples contained more than 2 log CFU/mL of coliform bacteria, and 85% tested positive for *E. coli* and *Salmonella* spp. In our study, this limit was exceeded in most samples, specifically in all pear and orange smoothies and in the apple smoothie with *Thymus vulgaris*. **Lee *et al.* (2021)** identified *Salmonella* and *E. coli* as problematic bacteria frequently isolated from fruit juices and beverages. They cited an example from the United States and Canada, where multiple individuals experienced health issues after consuming a fruit beverage contaminated with *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. **Kačániová *et al.* (2021)** investigated the microbiological quality of grape juice, reporting that coliform bacteria were able to grow only in the control sample. The addition of medicinal plants, particularly essential oil extracts of *Thymus serpyllum* and *Mentha aquatica*, effectively eliminated these bacteria. In contrast, complete inhibition of coliform bacteria did not occur in any of our smoothie samples. This is likely due to the fact that the medicinal plants used may have been contaminated, and essential oil extracts were not applied. **Ratajczak *et al.* (2023)** studied the effect of medicinal plant extracts on coliform bacteria in a single-component carrot beverage. The control sample, without medicinal plant extracts, contained 3.43 log CFU/mL of coliform bacteria. A reduction in bacterial count was observed after the addition of vanilla extract. However, the addition of peppermint (*Mentha piperita*) extract increased the coliform bacteria count to 4.1 log CFU/mL. Compared to their findings, the coliform bacteria counts in our study were lower. However, similar to their results, the addition of *Mentha piperita* also led to an increase in CFU/mL when combined with pear and apple smoothies.

Yeast counts ranged from 3.10 to 5.46 log CFU/mL, with the highest counts observed in apple smoothie samples. The addition of medicinal plants significantly influenced yeast abundance, with the greatest reduction recorded in the apple + medicinal plant combination. Number of yeast counts were significantly highest in the apple control sample and the pear + TS and pear + TV samples presented the lowest yeast levels ($p \leq 0.05$).

The presence of yeasts is attributed to their preference for sugar and low pH, which support their survival. **Staš *et al.* (2024)** describe yeasts as common contaminants of fruit juices and beverages, often negatively affecting their quality and shelf life. The most frequently detected yeast species in fruit beverages include *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces rouxii*. Yeasts are typically part of the natural microbiota of the fruit from which the beverage is made, however, contamination can also occur during production, packaging, storage, and distribution. A high level of contamination is particularly associated with fresh fruit beverages. Yeast counts can be reduced by using medicinal plants, which contain antimicrobial compounds. A series of experiments on plant-derived phenolic compounds confirmed the effectiveness of the modified broth microdilution method for assessing the anti-yeast activity of natural agents in fruit juices. Among the tested compounds, stilbenes - particularly pterostilbene - emerged as promising candidates for further investigation as beverage additives. Pterostilbene exhibited the strongest inhibitory activity against all three yeast strains, regardless of juice type. **Joshi *et al.* (2014)** examined the effect of peppermint (*Mentha piperita*) extract on yeast counts during the fermentation of an apple-based beverage. They reported a 5% reduction in active yeast cells after 48 hours of fermentation. For this reason, the use of peppermint is not suitable for fermented products, as a decrease in yeast count is undesirable in this context. Our results also confirm that peppermint contributes to the reduction of the yeast population.

The values of FF remained relatively low across most samples. Some treatments, particularly apple + MP, pear + MP, and orange + MP, showed increased FF counts, significantly higher ($p \leq 0.05$) than the apple and pear control samples.

Geotrichum candidum is an acid-tolerant, yeast-like fungus, often described as an intermediate between moulds and yeasts but classified as a yeast for more than 25 years. It is the most well-known species of the genus and is widely distributed, thriving on moist, nutrient-rich substrates. Apart from dairy products, *G. candidum* has been isolated from soil, water, air, maize, other cereals, rice grains, grapes, citrus fruits, bananas, tomatoes, cucumbers, frozen fruitcakes, fruit juices, bread, animals, and humans **(McSweeney & McNamara, 2022)**.

Geotrichum candidum formed numerous colonies on Petri dishes (Tab. 4), with counts ranging from 2.12 to 2.85 log CFU/mL. A decrease in CFU counts of this species was observed in 5 out of 9 samples, while an increase was recorded in pear beverages with *Thymus vulgaris* and orange beverages with *Thymus serpyllum* and *Thymus vulgaris.*

Table 4 Quantitative occurrence of *Geotrichum candidum* in smoothie samples

Sample	log CFU/mL
Apple	2.85
Apple + TS	2.73
Apple + MP	2.61
Apple + TV	2.85
Pear	2.36
Pear + TS	2.34
Pear + MP	2.16
Pear + TV	2.60
Orange	2.42
Orange + TS	2.47
Orange + MP	2.12
Orange + TV	2.54

Legend: TS - *Thymus serpyllum*, MP - *Mentha piperita*, TV - *Thymus vulgaris*, ND - not detected, TMC - total microbial counts, CB - coliform bacteria, FF - filamentous fungi

Foltinová *et al.* (2019) investigated the inhibitory effect of thyme essential oil on the growth of *Geotrichum candidum* isolates obtained from domestically sourced dairy products. They assessed the effect of the essential oil using the gaseous diffusion method and observed a 100% inhibitory effect, regardless of cultivation time. The antimicrobial effectiveness of essential oils depends on their chemical composition, with thyme oil being primarily composed of menthofuran and *p*-cymene. In our samples, the addition of thyme did not lead to a reduction of *G. candidum* CFU in any case.

In the fruit smoothie samples we prepared, contamination by microscopic filamentous fungi was low. In the orange smoothie samples, no colonies were detected, except in the sample with the addition of *Mentha piperita*. The presence of this medicinal plant also led to an increase in micromycete counts in the other samples. Among the microscopic filamentous fungi identified in our study, *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* were observed (Tab. 5). *Cladosporium* was the most frequently isolated genus, occurring in 50% of the samples. The potentially toxigenic genera *Aspergillus* and *Penicillium* were detected in 3 out of 12 samples (25% prevalence), while *Alternaria* was found in only two samples (17% occurrence).

Krisch *et al.* (2011) classify fruit beverages as high-risk foods due to the frequent presence of microscopic filamentous fungi and yeasts. The combination of low pH, low water activity, and relatively high sugar content creates favuorable conditions for the growth and reproduction of micromycetes. The addition of medicinal plants represents a natural and effective approach to reducing the number of microscopic filamentous fungi and inhibiting their mycotoxin production. This method is generally more acceptable to consumers compared to chemical preservatives. Fresh, unpasteurised fruit and vegetable juices from Korea were monitored by **Lee *et al.* (2021)**. Filamentous fungi were detected in 39 out of 52 samples, with an average contamination level of 3.47 log CFU/mL. **Lee *et al.* (2021)** also investigated seasonal variations in contamination levels. They reported the highest contamination in samples made from apples and pears purchased between August and October, while the lowest levels were observed in February and March. Our samples were prepared from fruit purchased in June and the contamination levels were significantly lower.

Table 5 Isolated micromycetes from smoothie samples

Sample	Isolated genera of microscopic filamentous fungi
Apple	*Penicillium*
Apple + TS	*Penicillium, Cladosporium*
Apple + MP	*Cladosporium*
Apple + TV	*Aspergillus*
Pear	*Aspergillus, Penicillium*
Pear + TS	*Cladosporium*
Pear + MP	*Cladosporium, Alternaria*
Pear + TV	*Cladosporium, Alternaria*
Orange	-
Orange + TS	-
Orange + MP	*Cladosporium, Aspergillus*
Orange + TV	-

Legend: TS - *Thymus serpyllum*, MP - *Mentha piperita*, TV - *Thymus vulgaris*, ND - not detected, TMC - total microbial counts, CB - coliform bacteria, FF - filamentous fungi

The mycobiota analysis of some Egyptian canned fruit juices and beverages conducted by **Abdel-Sater *et al.* (2001)** revealed a wide range of fungal species. The most prevalent fungi in all types of juices and beverages belonged to the genera *Aspergillus* and *Penicillium*. Analysis of five different fruit juices and beverages (five samples each) showed that all five apple beverage samples were contaminated with aflatoxin B_1 and G_1 at concentrations ranging from 20 to 30 mg/L, while 2 out of 5 guava juice samples were naturally contaminated with aflatoxin B_1 at 12 mg/L. All mango juice, grape, and peach beverage samples were free from naturally occurring mycotoxins. *Aspergillus* and *Penicillium* species were also detected in our samples, but only sporadically. The predominant genus was *Cladosporium*, which is not known to produce mycotoxins **(Pitt & Hocking, 2009)**.

CONCLUSION

Fruit-based beverages are becoming increasingly popular, mainly due to their hydrating effect and rapid energy supply. They are commonly prepared at home without heat treatment, which creates favourable conditions for microbial growth. This can reduce their quality and potentially pose a health risk to consumers. Medicinal plants contain numerous bioactive compounds with antimicrobial properties, which motivated us to use fruit smoothies as a matrix to test their effects. Our study demonstrated that adding medicinal plant leaves improved the microbiological parameters of the beverages compared to single-component fruit smoothies. The most significant effect was observed in yeast reduction, followed by a decrease in the total microbial count. Microscopic fungi were detected only sporadically, while no expected reduction was observed in coliform bacteria. Further research is needed to determine the effect of medicinal plants on coliform bacteria in fruit smoothies.

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