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MULTIDRUG-RESISTANT BACTERIA ASSOCIATED TO STRAWBERRIES AND GOOSEBERRIES AT VARIOUS STAGES OF RIPENESS

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Abstract

The lack of understanding about proper food manufacturing, transport, storage, and marketing methods has resulted in a high level of contamination with pathogenic bacteria that cause gastrointestinal disorders and induce drug resistance, which is a serious issue for the food sector. Due to their popularity as a fruit in the province of Imbabura, Ecuador, this issue has sparked a lot of interest in evaluating the quality and microbiological safety of strawberries and cape gooseberries at different ripening stages. According to bacteriological analyses, the microbiota of strawberries was dominated by Escherichia coli (1.61 x 10^3 CFU/g) at ripeness stages 4 (three-quarters of the fruit has turned red) and 6 (red colour, they feel slightly soft when squeezed, ready to eat), while the microbiota of gooseberries was predominated by Enterobacter spp. (1.11 x 10^2 CFU/g) at ripe stage 6 (intense yellow, ready to eat). The differences in the physiochemical characteristics (pH, total solids, acidity) at the time of harvest results in a difference in some pathogenic bacteria's adaptation. The selected clones per each ripe stage showed multi-antibiotic resistance (MAR). The calculated MAR index varies from 0.17 to 1.00 and was ripe stage-, and fruit dependent. The most resistant clones were E. coli for strawberries and Enterobacter spp. for gooseberries. Thus, it is crucial to set up efficient control measures and develop coordinated strategies to guarantee the microbiological quality of these foods.

Key words: *Strawberry, gooseberry, food safety, pathogens, antibiotic resistance.*

INTRODUCTION

The promotion of healthier lifestyles increases the consumption of fresh products such as tropical fruits. They offer bioactive phytochemicals such as flavonoids and phenolic compounds, which have been linked to several health advantages (Septembre-Malaterre et al., 2018). Worldwide, health organizations launched campaigns encouraging people to eat at least "5 a day" portions of fruit and vegetables every day (Lafarga et al., 2019). In fact, consuming fruit on a regular basis appears to help avoid a wide range of chronic disorders (Samtiya et al., 2021). Strawberry (*Fragaria x ananassa*) and cape gooseberry or uvilla (*Physalis peruviana*) are the most consumed fruits valued for their flavour, aroma, and nutritional quality. Eating fresh fruits is a healthy habit that should be adopted by everyone. Contrarily, fruits do not go through any stage of processing to guarantee the effective eradication or inactivation of pathogenic bacteria, so the customer is exposed to a higher risk of illness (Drobek et al., 2021). Fruit-associated pathogens pose safety concerns as they may carry antibiotic-resistance genes (ARG). Recent studies indicated that strawberries' ARG diversity and abundance decreased when they were fertilized with aerobic and anaerobic composting manure (Zhang et al., 2022). In addition, despite growing interest in plant microbiomes, perennial fruit trees' associated microbial communities continue to be underappreciated (Sangiorgio et al., 2022). Compared to fruits off the ground, strawberry plants crawl on the ground, making their fruits more susceptible to soil ARG contamination (Zhang et al., 2022). Thus, due to their production in the external environment, there is an increased risk of contamination with various pathogenic microorganisms, some of which cause foodborne illnesses (Pangahl et al., 2022). Postharvest losses of between 20 and 45% have been recorded for gooseberries. Up to 50% of strawberries can be lost due to the

Botrytis cinerea fungus's influence (Azam et al., 2019). The fruits start to degrade after harvest because of mechanical damage, increased rates of respiration and transpiration, and mold infection. Early, we prospect the quality of strawberries and gooseberries purchased from a local retail market and a local farm in Ecuador (Tenea et al., 2023). The results indicated the presence of a high number of microorganisms in fruits independently of their origin. However, the composition of the raw materials and their physicochemical characteristics determine the degree of contamination and the existence of a specific indicator. We also assessed the selected clones' antibiotic resistance profiles, which showed strong resistance to several drugs. This could be explained by the fact that the fruits came from a field where the farmers used animal waste as fertilizer. In this context, to understand how the microorganisms might spread from farm to table, the present study presents a comparative bacteriological analysis and antibiotic resistance patterns of microorganisms isolated from both gooseberries and strawberries collected from fields at the early ripeness stage of 2, 4, and 6 (ready-to-eat) fruits.

MATERIALS AND METHODS

Field fruit collection

Gooseberries (*Physalis peruviana,* variety Colombiana) at ripe stage 2 (green) and 4 (light yellow), and 6 (intense yellow, ready to eat) samples (20 fruits with calix/ field raw/ stage), and strawberries (*Fragaria* x *ananassa* variety Monterey) at the ripe stage 2 (3/4 white and 1/4 red color), 4 (three-quarters of the fruit has turned red) and 6 (red color, they feel slightly soft when squeezed, ready to eat) with no visible damages were used. This study was realized between October 2021-May 2023, regardless of the season, and the fruits were collected from the open fields of farmers from Imbabura Provence, Northern Ecuador. A total of 20 x 6 fields =120 fruits/ stage/ variety). The samples were translated to the laboratory and immediately proceeded to the bacteriological analysis. Three independent fruit harvests have been considered.

Bacteriological analysis

The microbiological quality was performed as previously described (Tenea et al., 2023). Briefly, the fruits (20 fruits/field raw/stage) were placed in a sterile zip bag containing buffered peptone water (0.1 %) and mixed vigorously by hand for 30s. The bags were placed at 37°C for 1.30 h and mixed gently at a 15 min interval, followed by centrifugation and recollection of the cells in 1 X PBS buffer. The presence of total coliforms, total aerobes, *E. coli*, *Salmonella* spp., *Shigella* spp., *Enterobacter* spp., yeasts, and molds was assessed in both Petrifilm (3M Science Applied to Life, MI, USA) and chromogenic media as described early (Tenea et al., 2023). CFU/(g) was used to express microbial counts. To test for antibiotic susceptibility, at least 10 colonies, stages, fruits, or biological markers (grouped by genus-level classification) were randomly selected, extracted, and purified.

Physicochemical analysis

Using a pH meter (Seven Compact S210, Mettler Toledo LCC, Columbus, OH, USA), 50 g of strawberry/gooseberry fruits were blended for 1 minute with 150 mL of distilled water to determine the pH. By titrating 10 mL of strawberry/gooseberry juice with 0.1 mol/L NaOH and using phenolphthalein as an indicator, titratable acidity (TA) was determined (AOAC, 1990). The tests were carried out three times. The data are given as a percentage of citric acid determined as previously described (Alharaty et al., 2020). A digital refractometer was used to calculate the fruit's total soluble solids (°Brix) and total sugar content (TSS).

Antibiotic susceptibility testing

A total of 145 and 142 random colonies from strawberries and gooseberries were selected and used for antibiotic susceptibility tests using commercial discs: Amoxicillin (AMX25: 25μg), Ampicillin (AN10: 10 μg), Gentamicin (CN10: 10 μg), Kanamycin (K30: 30 μg), Tetracycline (TE30: 30 μg), and Cefuroxime (CXM30: 30 μg). We employed the concentrations advised by the Scientific Committee on Animal Nutrition for the disk diffusion experiment (discs supplied by Merck, USA). According to the microbiological breakpoints specified by the FEEDAP guidelines, the Scan500 (Interscience, Fr) was used to automatically identify the inhibitory halos and grade them as susceptible, medium, or resistant (EFSA, 2012). As a reference, *E. coli* ATCC25922, *S. enterica* subsp. *enterica* ATCC51741, and *Enterobacter* spp. (lab strain) were used. The % of antibiotic resistance was determined as the number of total bacteria resistant / the number of total isolates tested. MAR index was calculated as the ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism is exposed to (Davis and Brown, 2016).

Hemolysis and catalase assay

The clones showing resistance to more than 3 antibiotics were evaluated for their hemolytic activity on Columbia agar containing 5% (w/v) human blood as described (Tenea et al., 2023). The isolates were divided into three groups based on the number of red blood cells that were lysed in the medium next to the colonies: alfa-hemolytic (green region near colony), beta-hemolytic (clear region near colony), and gamma-hemolytic (no region). The catalase assay was performed using hydrogen peroxide (H_2O_2) as substrate. An overnight culture of the selected colonies was obtained, and one drop was applied on a glass slide containing three drops of H_2O_2. The presence of bubbles indicates that the test is positive.

Statistical analysis

All experiments were run in triplicate and the results were reported as mean ± standard deviation. To identify significant differences between the means, the Fisher Least Significant Difference test was used (SPSS 13.0, Inc., Chicago, IL).

RESULTS AND DISCUSSIONS

A new trend in the food industry, rising consumer demand for natural products, contributed to an increase in the consumption of fresh foods over the past ten years (Melo and Quintas, 2023). Microbiological safety has raised significant concerns for the food industry and public health agencies despite being one of the most rapidly expanding sectors in recent years. Due to the lack of preceding microbiological lethal techniques, such food products may expose consumers to a risk of contracting a foodborne infection (Drobek et al., 2021). For example, enterococci can be found in the gastrointestinal tracts of both humans and animals as well as in foods that have either animal or plant origins. They have contributed to nosocomial infections, food poisoning, and the escalation of antibiotic resistance (Al-Kharousi et al., 2022). In this study, the bacteriological analysis indicated a comparable number of total aerobes in both fruits, which varies with the ripening stage (Figure 1).

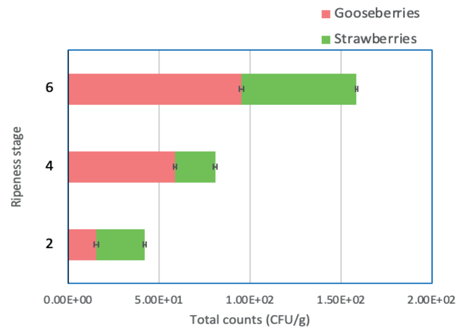
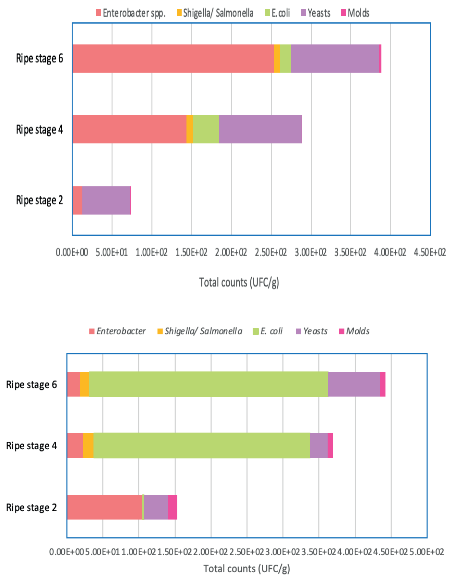


Figure 1. Total counts (UFC/g) enumerated in gooseberries and strawberries

Although gooseberries were bought with calix in this study, a greater number of indicators, *Enterobacter* spp., and *Shigella* spp., were detected at ripe stages 4 and 6 (Figure 2A). There were no *Salmonella* colonies detected, while a significant increase ($p < 0.05$) of *E. coli* and yeasts were detected at stage 4. The strong ability of some pathogenic bacteria to adapt and colonize the raw material to assure their metabolic activities may be related to the physiological characteristics at the maturity stage.



A).

B)

Figure 2. Total counts (UFC/g) of the indicator microorganisms detected in: A) - gooseberries and B) -strawberries at different ripe stages

Similarly, in strawberries, a high amount of indicator *E. coli* was detected at ripe 4 and 6, while *Enterobacter* was at stage 2 (Figure 2B). An increase in yeasts was observed at stage 6, which might have something to do with the fruit fermentation. According to Jensen et al. (2013), the microbiota found in healthy strawberries is diverse and contains potential plant pathogens, human pathogens, molds that produce mycotoxins, and biocontrol agents for plant diseases. The number of molds was higher in strawberries than in gooseberries at the sixth stage of ripeness. Although the fungus is usually present during the strawberry growing season and cannot be avoided, the amount of inoculum in each field can be reduced by removing dead leaves and ill fruit. After harvest, the fungus lives on tilled-in leaves and fruit as tiny, black, dormant sclerotia (Sengupta et al., 2020). The differences in the physiochemical characteristics (Table 1) at the time of harvest results in a difference in some pathogenic bacteria's adaptation. The proportion of Brix to titratable acidity (TSS/TA), which measures the amount of soluble solids and acidity in a substance, appears to be a good indicator of how sweet a fruit is and its overall acceptance (Martínez-Bolaños et al., 2008). However, in this study, the values were higher in strawberries than gooseberries indicating that the fruits were sweet. According to other studies, these values are cultivar-dependent and are impacted by the growing environment (Ikegaya et al., 2019).

Table 1. Physicochemical characteristics of fruits

Sample	Ripe stage	TSS (°Brix)	pH	TA (citric acid %)	TSS/TA*
Gooseberries	2	8.41 ± 0.04	3.62 ± 0.04	1.89 ± 0.15	4.44 ± 0.05
	4	12.84 ± 0.02	3.46 ± 0.03	2.36 ± 0.12	5.50 ± 0.12
	6	13.65 ± 0.06	3.51 ± 0.07	2.06 ± 0.03	6.79 ± 0.03
Strawberries	2	6.87 ± 0.02	3.32 ± 0.02	0.50 ± 0.06	13.74 ± 0.06
	4	7.43 ± 0.08	3.36 ± 0.02	0.46 ± 0.21	16.15 ± 0.21
	6	7.36 ± 0.05	3.53 ± 0.02	0.25 ± 0.03	29.93 ± 0.03

*was calculated as the ratio between Brix and titratable acidity.

Raw fresh vegetables may include antimicrobial residues, antibiotic-resistant bacteria, and clinically significant antimicrobial resistance genes (Rahman et al., 2021). The development of antibiotic resistance associated with food-borne microbes has a harmful effect on human health (Krahulcová et al., 2021). According to Davis and Brown (2016), MAR index provides a reliable, efficient, and effective way to identify the origins of bacteria that are resistant to antibiotics. A MAR greater than 0.2 indicates a source of contamination with a significant risk (Davis and Brown, 2016). However, this study found that for gooseberries the MAR index was greater than 0.25 for more than 50% of the selected clones independently of their ripe stage (Figure 3). Instead, for strawberries at stage 2, more than 79% of clones showed a MAR index above 0.25. Interesting, 70% and 90% of the *E. coli* selected clones were resistant to tetracycline and kanamycin, respectively. The antibiotics from the tetracycline family are most frequently found in various types of manure.

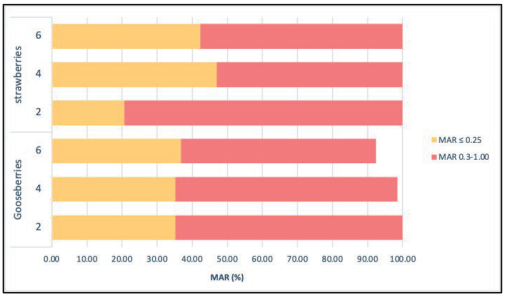


Figure 3. MAR index (%) calculated for a pool of indicator clones selected for gooseberries and strawberries.

According to previous research, the size of the farm and the concentrations of tetracycline found in the fertilized soils were directly connected with the use of tetracycline (Carballo et al., 2016). As a result, worries concerning the contribution of antibiotic residues to the antibiotic resistance reservoir are growing as they are released into the environment through the usage of animal wastes (Amador et al., 2019). Although in lower amounts, 8.33% *E. coli* and 11.11% of *Enterobacter* spp. clones at ripe stages 2 and 4, as well as 5.5% *E. coli* at ripe stage 6, were resistant to all antibiotics tested. Early studies indicate that despite being regarded as opportunistic pathogens, enterococci can act as a source of antibiotic resistance (McGowan et al., 2006). Additionally, strawberry fruits obtained from soil enriched with livestock manure, which is often used by farmers to increase productivity and fruit quality, could easily acquire antibiotic-resistance genes (Zhang et al., 2022). In addition, 84% of the selected *E. coli* and *Enterobacter* spp. clones from strawberries at stage 6 were beta-hemolytic, while *Enterobacter* spp, but not *E. coli* from gooseberries were catalase positive and beta-hemolytic regardless of their level of ripeness. Early research indicated that beta-hemolytic phenotypes of isolates from environmental conditions are considered as virulence-associated determinants and of clinical relevance (Mogrovejo-Arias et al., 2020).

CONCLUSIONS

To date, there are no studies evaluating the microbiota associated with gooseberries and strawberries at different ripeness stages and their antibiotic resistance patterns. The prevalence of microorganisms was higher at the maturity stage (ready-to-eat) in both fruits. Nonetheless, several AR microorganisms were detected in all clones independently of the ripe stage, suggesting that adequate handling and high cleanliness of fruits are required to reduce the possibility of AR bacterial propagation. These data may also have potential applications in the treatment of strawberry and gooseberry diseases, particularly those caused by economically significant infections which can affect fruit yield and quality postharvest.

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