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Abstract: Fruit juices are an important part of the modern diet in many countries. However, few data are available concerning the microbiological quality of the fruit juices sold in Greece. Using standard microbiological procedures, we conducted a bacteriological survey of commercially sold, pasteurized shelf-stable fruit juices from retail markets. A total of 120 samples of fruit juices sold in various retail markets were examined for their bacteriological quality. The pH of the tested juices was 2.4 - 4.8. Bacteria were isolated from 51 samples (42.5%) and fungi from 78 samples (65%). Escherichia coli O157:H7 was detected in four of the analyzed samples (3.34%), and Staphylococcus aureus was detected in four different samples (3.34%). In 11 samples (9.1%), the total number of microorganisms detected was 125 colony-forming units (cfu). Acidophilic microorganisms were isolated from 26 samples (21.7%) and Blastomyces was detected in 46 samples (38.3%). All samples were negative for Lactobacillus, Clostridium perfrigens, Salmonella spp., Bacillus cereus, total coliforms, Escherichia coli, and Listeria monocytogenes.
Many of the microorganisms detected may cause disease in humans; thus, a number of the tested samples did not meet the Greek guidelines for the microbiological quality of juices. Use of a Hazard Analysis Critical Control Point (HACCP) system should be generally introduced into the food industry sector to improve the quality of fruit juices, as well as other manufactured foods.

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Dear Sir,

I send you attached our manuscript JFP-10-270 "Occurrence of microorganisms of public health and spoilage significance in fruit juices sold in retail markets of Greece", to be considered for publication to anaerobe.

Best Regards

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Running title: Microbiological monitoring of fruit juices

Occurrence of microorganisms of public health and spoilage significance in fruit juices sold in retail markets of Greece

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Key Words: Fruit juices, Microbiological quality, Greece, monitoring

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Abstract

Fruit juices are an important part of the modern diet in many countries. However, few data are available concerning the microbiological quality of the fruit juices sold in Greece. Using standard microbiological procedures, we conducted a bacteriological survey of commercially sold, pasteurized shelf-stable fruit juices from retail markets. A total of 120 samples of fruit juices sold in various retail markets were examined for their bacteriological quality. The pH of the tested juices was 2.4 - 4.8. Bacteria were isolated from 51 samples (42.5%) and fungi from 78 samples (65%). *Escherichia coli* O157:H7 was detected in four of the analyzed samples (3.34%), and *Staphylococcus aureus* was detected in four different samples (3.34%). In 11 samples (9.1%), the total number of microorganisms detected was 125 colony-forming units (cfu). Acidophilic microorganisms were isolated from 26 samples (21.7%) and *Blastomyces* was detected in 46 samples (38.3%). All samples were negative for *Lactobacillus*, *Clostridium perfrigens*, *Salmonella spp*., *Bacillus cereus*, total coliforms, *Escherichia coli*, *and Listeria monocytogenes.* Many of the microorganisms detected may cause disease in humans; thus, a number of the tested samples did not meet the Greek guidelines for the microbiological quality of juices. Use of a Hazard Analysis Critical Control Point (HACCP) system should be generally introduced into the food industry sector to improve the quality of fruit juices, as well as other manufactured foods.

Juices, like most acidic foods, frequently become spoiled as a result of contamination with aerobic acid-tolerant bacteria, together with yeasts and molds. Fruits and vegetables contain the nutrients necessary to support the rapid growth of foodborne pathogens, yet outbreaks of illness caused by consumption of fruits and vegetables are less frequent than outbreaks involving other foods. This higher level of protection is due in part to proper pasteurization and to the presence of external barriers such as the peels and rind of fruits, which prevent microorganisms from entering and subsequently growing in the interior of the fruit or vegetables [1, 5]. However, in some cases, such as when fruits have become wounded or slices have been freshly cut, this external barrier is broken, creating an opportunity for bacterial colonization of the interior of the fruit. The occasional presence of foodborne pathogens has been recognized for many years. Thus, pathogens as well as spoilage microorganisms can contaminate fruits and vegetables via several different routes and at several points throughout the pre-harvest and post-harvest process [6].

In USA, Canada, New Zealand, and several EU countries, public health organizations have run campaigns recommending the daily consumption of at least five daily servings of fruit and vegetables. In Greece, freshly pasteurized juices are consumed throughout the year [*2*]. In the past these beverages were not regarded as potentially hazardous [3,12]. However, in recent decades, juices have become frequent vehicle for transmitting pathogens, such as enteroha emorrhagic *Escherichia coli* O157:H7 [7], *Salmonella* [*9*] and *Cryptosporidium* [*8*]. Thus, the belief that high-acid foods are of minimal concern with regard to pathogenic bacteria has been challenged (*11*). Nevertheless, only a few studies have reported the incidence of bacteria in juices, mainly in non-pasteurized ones *(13, 21)*. Although they cannot grow at low pH, some strains of Enterobacteriaceae, including certain strains of *E. coli*, *Shigella,* and *Salmonella*, may survive for several days or even weeks in acidic foods (*15*). Also, the extreme acid tolerance of *E. coli* O157:H7 has raised further doubts about the safety of acidic foods (*29*). Some fruits, including oranges, are susceptible to attack by pathogenic fungi because of their low pH, higher moisture content, and nutrient composition *(17)*. Molds and yeasts tolerate conditions of high osmotic pressure and low pH and can grow at refrigerator temperatures (two factors that generally prohibit the growth of competitive bacteria) and can therefore cause spoilage in the processed product (*25, 26*). Typical yeast species found in citrus juices are *Candida parapsilosis, Candida stellata, Saccharomyces cerevisiae, Torulaspora delbrueckii, and Zygosaccharomyces rouxii,* although species from the genera *Rhodotorula*, *Pichia*, *Hanseniaspora,* and *Metschnikowia* are also common (Tournas et al, 2006). Despite the economic importance of juices, there are only a few reports investigating the presence of yeasts in them (*22, 23*).

The objective of the present study was to evaluate the microbiological quality of domestic and imported pasteurized shelf-stable fruit juices sold in retail stores in Greece. The potential presence of *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus* and *epidermis*, *Bacillus cereus*, *Escherichia coli O157:H7*, *Clostridium perfrigens*, *Lactobacillus*, *Escherichia coli*, acidophilic bacteria, fecal streptococci, *Blastomyces*, fungi, and yeasts were evaluated. Also total microbial counts and total coliforms counts were obtained.

MATERIALS AND METHODS

All fruit juice samples were purchased in local retail shops in Greece by experienced laboratory personnel. The number of samples as well as the juice type tested is shown in Table 1. It should be noted that fruit and vegetable juices containing dairy products (e.g., smoothies), freshly squeezed juices in sealed packages, and mixed juices with more than three ingredients were excluded from the sampling program.

In the present study, 120 samples of shelf-stable fruit juices were purchased over a period of one year from six different retail shops: 63 (52,5%) juices were imported , and the rest 57 (47,5%) were of domestic origin. The fruit juice samples were produced by five foreign-owned and seven Greek companies. Samples (500 ml) were transported to the laboratory in coolboxes (4-8^{o}C, in order not to change its microbiological flora) and were analyzed immediately. After homogenization of the content by manual shaking, the caps were disinfected with 70% alcohol, punctured with sterile tweezers, and 100-ml portions of juice were transferred into different sterile flasks. One flask was used to measure the pH of the juice (without dilution) and to calculate the amount of 1 N NaOH needed for neutralization. The appropriate amount of 1 N NaOH was added to the juice in the second flask. The third flask was used for analysis of non-neutralized juice. Once opened, the pH was directly measured.

Juice samples were assessed for the presence of *Lactobacillus*, *Clostridium perfrigens*, *Salmonella spp*., *Bacillus cereus*, *Staphylococcus aureus, Staphylococcus epidermis*, *E.coli O157:H7, generic E.coli*, *Blastomyces,* fecal streptococci, acidophilic microorganisms, total coliforms, total number of aerobic microorganisms, molds and yeasts.

For the determination of *Lactobacillus,* a protocol described by Cheriguene and colleagues was performed *(10)*. *Clostridium perfrigens, acidophiles and total plate counts were conducted as described in chapters 32,19 and 7, respectively, of the Compendium* (*4)*.

For the analysis of *B.cereus*, Salmonella spp., *L.monocytogenes*, *S.aureus* and *epidermis*, fecal streptococci, *E. coli* O157:H7, the following protocol was performed. 50 ml of neutralized juice was centrifuged (Sigma 3K30) at 10000xg for 15 min in order to condense the sample. The supernatant was discarded, and the pellet was resuspended in 1 ml of PBS. The resulting aliquot was treated according to methods described in chapters 32, 37, 36, 39, 8, and 35 (*4*). Plates containing 25–250 colonies were selected and counted, and the average number of colony-forming units (CFU) per ml was calculated. The results were expressed as CFU/ml of sample.

For the analysis of fungi, only non-neutralized samples were tested. An aliquot (2 ml) of each dilution of non-neutralized sample was plated in duplicate onto potato dextrose acidified agar (PDA) and dichloran-glycerol agar (DG-18). Plates were incubated at 25±1^{o}C for 72±3 h, and those containing 25–100 colonies were selected. The colonies were counted, and the average number of CFU/ml was calculated according to ch.20 and 21 (*4)*.

All the media used in the analyses were from Oxoid (Basingstoke, Hampshire, England).

RESULTS

The pH of the tested juices ranged from 2.4 (lemon juice) to 4.8 (cocktail-type juice). The average pH of all the juices was 3.47 (Table 1). Sixty-three (52.5%) imported juices as well as fifty-seven (47.5%) domestic juices were analyzed. The total number of contaminated samples (bacterial and/or fungal) according to juice type is depicted in Figure 1.

None of the tested samples was found to be positive for *L. monocytogenes*, *Lactobacillus*, *Salmonella* spp., *S. aureus*, *B. cereus*, *C. perfrigens*, total coliforms or *E. coli*. Fifty-one (42.5%) of the samples presented bacterial contamination. In two samples, *S. epidermis* was detected at levels >100 CFU/ml and four samples had levels between 1 and 100 CFU/ml. Also, in one sample, the total number of microorganisms was >100 CFU/ml. Coagulase-positive staphylococci, streptococci, and *E. coli* O157:H7, were of <10 CFU/ml . Of the 120 samples, 26 (21.7%) samples were positive for acidophilic microorganisms (>0 cfu/ml). In four of the samples, acidophilic microorganisms were detected at levels >100CFU/ml, and in another four, the levels were between 10 and 100 CFU/ml. Two juice samples (orange) had levels of acidophilic microorganisms>1000 CFU/ml, and two other samples (pineapple and mixed) had levels between >100 and 1000 CFU/ml. The positive samples for bacterial contamination are broken down according to microorganism and juice type in Table 2.

No firm correlation can be made between the microbiological quality and brand type or bacteriological quality and juice type as there were insufficient numbers of samples representing some brands or juice types. Brand 3 showed no positive results for any type of juice where as brand 9 showed all its products to be found positive in a microorganism (Figure 2). . In the fungal analysis, 46 samples were positive for *Blastomyces*. In the 120 total samples, 9 types of fungi were detected (Table 3).

DISCUSSION

The objective of the present study was to evaluate the microbiological quality of commercially sold juices in Greece. It is commonly accepted that products derived from fresh vegetables and fruits contain microorganisms, and some of these microorganisms are potentially hazardous to public health (*11*). As was shown in the present study, some juices may contain bacteria or fungi that, if not treated properly, which may cause serious illness in consumers or food spoilage. Although this is not the case for the majority of products, there is still a risk for consumers of fruit juices. Very few studies concerning the microbiological quality of fruit juices in Greece from the public health view in the literature.(22,26, 27)

Fruit juices are popular beverages with an important role in human nutrition (*2*). Fruit juices and purees are defined as fermentable, but unfermented products obtained by mechanical processing of fresh fruits. The presence of undesired metabolites derived from microbial growth can arise from the use of rotten fruits or from defects in the production line or subsequent contamination (*29*).

Fruit juices are often infested by bacterial and yeast species that can survive different storage conditions. The ability of several bacteria such as *E.coli* O157:H7 to survive in acidified vegetable products is of concern because of previously documented outbreaks associated with fruit juices (*12, 19, 29*). Moreover, the increased resistance of several bacteria such as *Salmonella* typhimurium and *Salmonella* Sentfenberg in low pH (or extreme pH conditions) or heat has been established by several researchers (*18, 24)*. The enhanced acid resistance found could enable them to survive for prolonged time periods in the gastrointestinal tract, increasing the risk of illness. Furthermore, it should be taken into account that microbial growth in acidified media also induces a cross-protection response against heat that should also be considered for the design of pasteurization processes for acid foods (*3*). *Alicyclobacillus acidoterrestris*, a thermoacidophilic spore-forming bacterium, has caused spoilage of fruit juices which had been treated with thermal processes. It has been shown that in the range of pH 2.8 to 4.0, heat resistance was observed at lower temperatures, but not at the higher temperatures (*20*). In our study, the presence of *E.coli* O157:H7, *S.aureus*, *S.epidermis, fecal streptococci* and acidophilic microorganisms was documented even in very low counts..

Fruit juices are often infested by yeast species that can survive different storage conditions. The most common yeasts identified in freshly squeezed juices (orange, lemon, grapefruit, apple) included *Candida species, Trichosporon mucoides, Kloeckera spp.,* yeast-like fungus *Cryptococcus neoformans (29)*. *Candida guillermondii* prevailed in 55.95 % of all samples (*29*). In our study, Blastomyces as well as C.Hellenica were the most prevalent as it is shown in Table 3. Also the presence of Saccharomyces and Zygosaccharomyces

Pasteurization of juices can be done for temporary preservation (pre-pasteurization) and in this case this operation is carried out with continuous equipment (heat exchangers etc.). Pasteurization conditions are usually at 75°C in continuous stream. Pasteurization of bottled juice is then carried out just before delivery to the market; this is performed in water baths at 75° C until the point where the juice reaches 68° C. In cases when the final pasteurization is done without pre-pasteurization and temporary storage, modern methods use a rapid pasteurization followed by aseptic filling in receptacles. Rapid pasteurization conditions are as follows: temperature about 80° C, over 10-60 sec followed by cooling. The presence of bacteria in pasteurized juices should be probably due to the use of rotten fruit and/or from defects in the production line or subsequent contamination in the production line. However, some heat-resistant fungi such as Blastomyces are capable of surviving pasteurization and causing spoilage to the fruit juices. *Blastomyces* survives in soil that contains organic debris (rotting wood, animal droppings, plant material).

Minimally processed produce is exposed to a range of conditions during production and distribution, and this may increase the potential for microbial contamination, highlighting the need of applying good hygiene practices from farm to fork to prevent contamination and/or bacterial growth. The application of hazard analysis and critical control points (HACCP) procedures by food operators may contribute to the protection of public health (*16*). There is currently no law in Greece requiring that the microbiological quality of juices be assessed before these products are made available in the market. There need to be at least some guidelines to help prevent potential food poisoning from juices that contain hazardous bacteria or fungi. Such guidelines exist for water and even other foods, but for the moment there are none concerning juices. In order to protect consumers from accidental illness, the juice-producing companies should be monitored via a set of tests that include the microbiological examination of all equipment used in the process, as well as the final product.

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Table 1: Types of shelf-stable juices tested and pH range

Juice type	**No. of samples analyzed**	**pH range**	**mean pH**
Cocktail	29	2.6-4.8	3.5
Strawberry	3	3.2-4.3	3.6
Peach	2	3.2-3.7	3.5
Pineapple	13	3.2-4.0	3.5
Apricot	3	3.1-3.7	3.4
Watermelon	4	3.1-3.9	3.5
Lemon	3	2.4-2.9	2.7
Nectar	19	2.7-3.9	3.5
Orange	34	2.7-4.5	3.6
Banana	3	3.6-3.8	3.7
Cherry	3	3.1-3.6	3.3
Grapefruit	4	2.7-3.3	3.0
Total	**120**	**2.4-4.8**	**3.47**

Figure 1: Number of total and contaminated (bacterial and fungal) samples according to juice type (n=120)

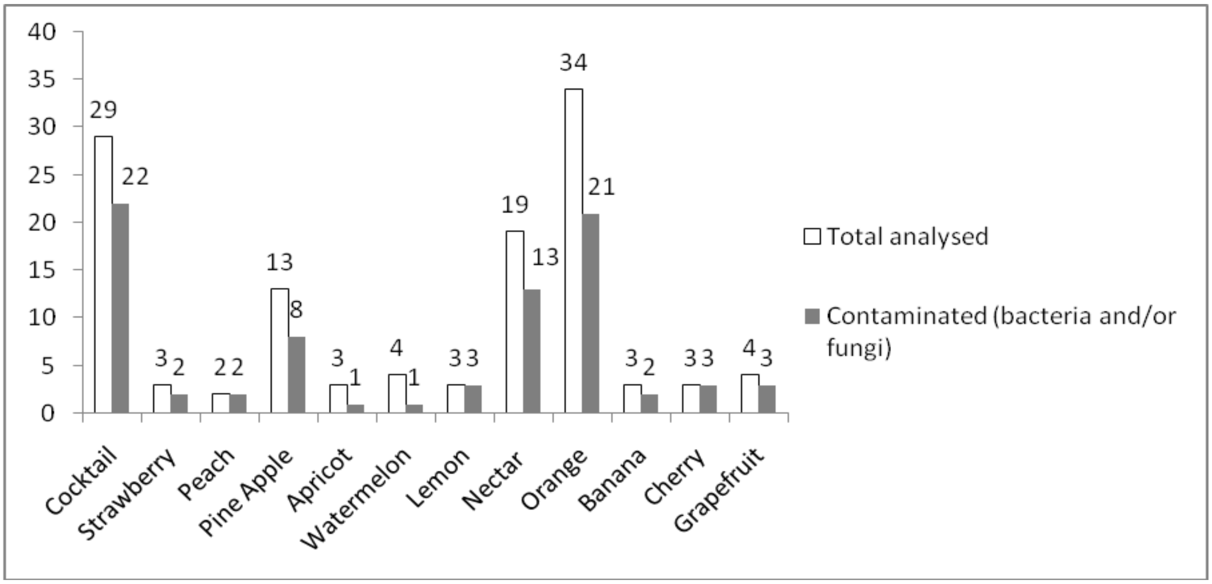
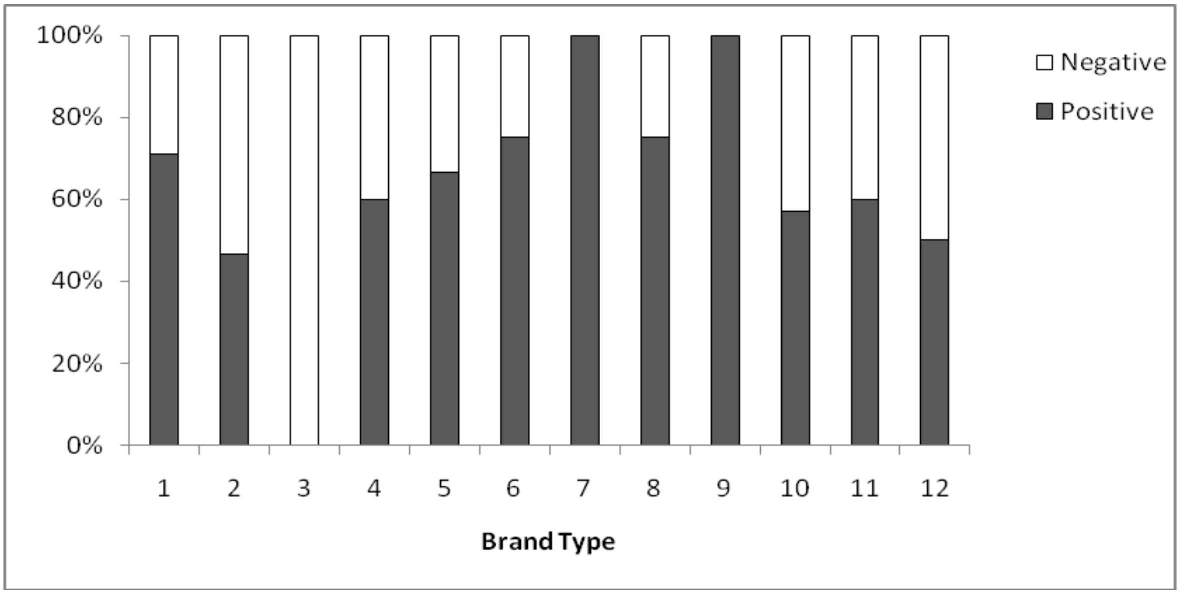


Table 2: Number of samples containing bacteriological contamination according to microorganism and juice type.

Type of Juice	PC	AC	ST	SA	SE	EC1	Total
Cocktail	1	5	0	3	3	1	13
Strawberry	0	1	0	0	0	0	1
Peach	1	1	0	0	0	0	2
Pine Apple	1	2	0	0	0	0	3
Apricot	0	1	0	0	0	0	1
Watermelon	0	1	0	0	0	0	1
Lemon	0	0	0	0	1	0	1
Nectar	5	4	0	0	0	0	9
Orange	2	9	1	1	2	3	18
Banana	0	1	0	0	0	0	1
Cherry	0	0	0	0	0	0	0
Grapefruit	0	1	0	0	0	0	1
Total	**10**	**26**	**1**	**4**	**6**	**4**	**51**
% positive	**8.3**	**21.7**	**0.8**	**3.3**	**5.0**	**3.3**	**42.5**

PC: total number of microorganisms; AC: acidophilic microorganisms; ST: Fecal streptococci; SA: *S. aureus*; SE:*S. epidermis*; EC1:*E. coli* O157:H7.

Figure 2: % of positive samples for any microbiological contamination (>0 CFU/ml) according to brand type



Greek: 1, 3, 4, 5, 7, 8, 9 Imported: 2, 6, 10, 11, 12

Table 3: Samples positive for fungal contamination, according to juice type.

Juice type	TM	S	BL	CR	CH	CF	Z	CHOL	D	Total
Cocktail	1	0	13	1	5	0	1	1	0	**22**
Strawberry	0	0	1	0	0	0	0	0	0	**1**
Peach	0	0	1	0	0	0	0	0	0	**1**
Pine Apple	0	0	5	0	3	0	0	0	0	**8**
Apricot	0	0	0	0	0	0	0	0	0	**0**
Watermelon	0	0	1	0	0	0	0	0	0	**1**
Lemon	1	0	3	0	0	0	0	0	0	**4**
Nectar	1	0	5	1	4	0	0	0	1	**12**
Orange	1	1	13	0	5	1	0	0	0	**21**
Banana	1	0	1	0	0	0	0	0	0	**2**
Cherry	0	0	2	1	0	0	0	0	0	**3**
Grapefruit	0	0	1	0	1	1	0	0	0	**3**
Total	**5**	**1**	**46**	**3**	**18**	**2**	**1**	**1**	**1**	**78**
% positive										

TM, *Trichosporon mucoides*; S, *Saccharomyces kluyverii*; BL, *Blastomyces*; CR, *Cryptococcus albidus*; CH, *Candida hellenicus*; CF, *Candida famata*; Z, *Zygosaccharomyces spp*.; CHOL, *Candida holmii*, D, *Debarymyces polymorphus*.