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Microbiological Safety of Commercial Pasteurized Apple and Orange Juices

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Abstract

In this study we describe the assessment of the microbiological quality of 12 commercial pasteurized apple and orange juices. Orange juice is the most widely consumed fruit juice in the world and together with apple juice are an essential part of human diet. Although pasteurization is the preferable method for bacterial elimination, some heat-resistant microorganisms and their spores could survive the heat treatment and cause spoilage hence triggering serious outbreaks. Our study revealed that the microbiological quality of all 12 investigated pasteurized juices (6 apple juices and 6 orange juices) meet the national, European and international standards. The results obtained indicated that the juices were free of aerobic mesophilic bacteria and facultative anaerobes, yeasts, molds or of diarrheagenic *E. coli* and coliform bacteria described in the standards. No heat-resistant spore-forming bacteria had grown during the enrichment experiment. Based on our results, we can conclude that all tested juices are safe for consumption.

Keywords: safety of fruit juices, apple, orange, microbiology, *E. coli*.

Резюме

В това изследване е проведен анализ на микробиологичното качество на 12 плодови сока - пастьоризирани ябълков и портокалов сок, налични в магазинната мрежа. Портокаловият сок е най-консумираният в световен мащаб плодов сок, който заедно с ябълковия сок заемат водещи позиции в диетата на човека. Въпреки, че пастьоризацията е най-предпочитаният и използван метод за предотвратяване развитието на микроорганизми в соковете, някои резистентни към висока температура микроорганизми и техните спори биха могли да я преживеят и да доведат до развала на соковете, а оттам и до нежелани за здравето на човека последствия. Нашето изследване показа, че всички 12 изследвани ябълкови и портокалови сокове са микробиологично чисти и отговарят на националните, европейските и интернационалните стандарти. В изследваните сокове не бяха открити аеробни мезофилни бактерии и факултативни анаероби, дрожди и плесени, както и диарогенни *Escherichia coli* и колиформи, които да надхвърлят стойностите, наложени в стандартите. Също така, не бяха установени и температурно-устойчиви микроорганизми и техни спори по време на експериментие с набогатяване. Въз основа на получените резултати, може да се заключи, че всички изследвани сокове са безопасни за консумация от потребителите.

Introduction

Fruits and fruit juices represent an important part of human diet supplying the body with carbohydrates, minerals, vitamins, antioxidants, phenolic compounds, fibers and organic acids. The vast number of organic acids is responsible for the acidic pH values of juices. Due to their rich chemical content, juices play a significant role in the prevention of cardiovascular diseases, diabetes and cancer, and have a pronounced immunomodulatory effect (Slavin and Lloyd, 2012; Liu, 2013). Fruits and fruit juices represent one of the mandatory food groups that any individual should take daily along with vegetables, grains, dairy products, meat, fish, oils, nuts and spices. The high sugar and water contents of fruit juices are favorable for the proliferation of a cohort of bacteria (Caballero *et al*., 2003; Tribst *et al*., 2009; Juneja and Sofos, 2010; Montville *et al*., 2012; Leff and Feirer, 2013). The prevention of microbial growth in fruit juices

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is achieved through pasteurization - the method most commonly applied in industry. Usually, pasteurization for commercial juices is carried out at 82°C for 15 sec (Kemal *et al*., 2014; Peruttzi, 2017). It is considered that during this process the vegetative microbial cells and spores of molds and spore-forming bacteria are killed but at the same time the method possesses the disadvantage of destroying certain enzymes, particularly phenol oxidases. Pasteurization may also inactivate important substances as phenolic antioxidants (Sun, 2009). Besides common microorganisms, another target of pasteurization are the heat-resistant fungi and acid-tolerant spore-forming bacteria like *Clostridium pasteurianum*, *Bacillus coagulans* and *Alicyclobacillus acidoterrestris* (Walker and Phillips, 2008; Smit *et al*., 2011; Osopale *et al*., 2016). It is also known that many of the pathogenic species survive under refrigeration conditions and can be harmful to humans.

Generally, fruit juices are spoiled by three main groups of microorganisms – bacteria, molds and yeasts that are present in the raw material. Tolerating low pH and high sugar content, yeasts spoil juices by producing CO_2 and alcohol, enhancing the juice turbidity, causing flocculation and phase separation – all due to the action of microbial enzymes on pectin (Caballero *et al*., 2003).

Fruit juices of apples, peaches, strawberries and grapes harbor a vast bacterial population dominated by taxa belonging to the *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla as reported by Leff and Feirer (2013). Other important human pathogens associated with juices belong to the genera *Erwinia*, *Pseudomonas*, *Salmonella* and the species *L. monocytogenes* and *E. coli* These bacteria can cause serious outbreaks. In addition to the bacteria mentioned above, lactic acid bacteria are also considered as common juice spoilers. In the past decade, growing attention has been given to the species belonging to genus *Alicyclobacillus*, described to be prevalent in fruit-based products due to their ability to survive the acidic juice environment (Walker and Phillips, 2008). *Alicyclobacillus* is a genus of thermo-acidophilic, endospore-forming bacteria. Furthermore, *Alicyclobacillus* endospores are resistant to the low pH of fruit juices and the pasteurization treatment (Smit *et al*., 2011; Osopale *et al*., 2016). The predominant metabolite synthesized by *Alicyclobacillus* responsible for juice spoilage is 2-methoxyphenol (guaiacol) (Wall, 2000). Witthuhn *et al*. (2016) reported that *Alicyclobacillus* was detected in 26 out of 225 juice samples investigated, i.e. more than 11% of juices studied were contaminated with this bacterium.

Being mostly aerobic, molds spoil juices via changing the odor and forming mycelial mat on the juice surface (Caballero *et al*., 2003). The heat-liable asexual spores (conidia) of the most common genera such as *Cladosporium*, *Penicuillium*, *Botritis*, *Aspergillus*, *Mucor*, *Rhizopus*, and *Fusarium* could be easily killed by heating at 60°C for 5-10 min (Peruttzi, 2017). In contrast, the heat-resistant species of genus *Byssochlamys*, *Neosartorya* and *Talaromyces* survive heat treatment owing to their thick wall. They have been reported in canned blueberries and tomato juice (Kotzekidou, 2009) and have become a serious industrial problem. Extremely harmful for the human health are a few mycotoxins such as byssochlamic acid (isolated from *Byssochlamus fulva*) (Weinedborner, 2001) (Fig. 1.), Bissotoxin A (isolated from *B. fulva*) (Kramer *et al*., 1976) and Patulin (Fig. 2.) (isolated from *B. nivea*) (Rice *et al*., 1977). All these toxins have been detected in strains colonizing fruit juices.

One of most infectious bacterium colonizing fruits and invading fruit juices is the diarrheagenic *E. coli* O157: H7. First detected in apple cider

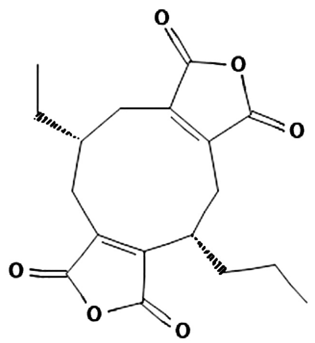

Fig. 1. Structural formula of Byssochlamic acid (PubChem)

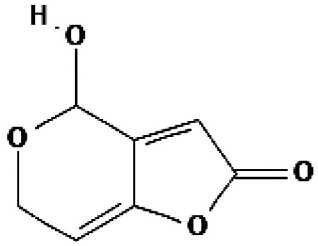

Fig. 2. Structural formula of Patulin (PubChem)

in Massachusetts, USA, in 1991, it is reported to cause hemorrhagic diarrhea and hemolytic uremic syndrome (HUS). The complicated etiology of these illnesses combined with the very low infectious dose (100 cells) identify this bacterium as one of most dangerous food-borne bacterial pathogens (Montville *et al*., 2012).

The first object of our investigation, apple juice, is produced from the most frequently consumed apple fruit. Apple contains a broad variety of vitamins - A, B_1, B_2, B_3, B_6, B_9, C, E, H, P, K (highest values are Vit. C and Vit. K); minerals – Fe, K, Mg, Na, S, P, I, Cl, Al, B, V, Co, Mn, Cu, Mo, Ni, Pb, F, Cr, Zn (the highest concentration is K); water, proteins, saturated, monounsaturated and polyunsaturated fatty acids, organic acids (malic-, quinic- and citromalic acid), amino acids including 10 essential amino acids, mono-, oligo- and polysaccharides (primarily glucose and fructose, cellulose, starch; the total CH content is 10.3 g/100g apple), tannins (polyphenols), amides and other nitrogenous compounds, soluble pectin and a diverse range of esters e.g. ethyl and methyl-iso-valerate esters. Esters have been reported to be essential for the typical apple aroma. Potassium is the most abundant mineral in apple juice at 1229 mg/l, and among the amino acids asparagine is the prevailing one (Caballero *et al*., 2003; Liu, 2013).

Orange juice, the second target of our study, is the most widely consumed fruit juice in the world, particularly appreciated by consumers for its organoleptic properties and its high content of potentially beneficial bioactive components (Liu, 2013). Among the citrus fruits, orange juice is the predominant fruit juice in the human diet. It contains abundance of vitamins - A, B_1, B_2, B_3, B_6, B_9, B_{12} C, D, E, K; minerals - K, Ca, I, Mg, P, Na, Zn; phenolic compounds, terpenes, tannins, saponins, flavonoids and alkaloids (Galaverna and Dall'Asta, 2014). Orange juice contains K concentrations that are even higher compared to apple juice – 1971 mg/l. Potassium is essential for human metabolism being responsible for the cardiac activity and together with Na regulates the water balance in the body. Potassium is crucial for the cognitive function and helps the evacuation of toxins from the body. Orange juice has been reported to prevent the formation of kidney stones because of the presence of citrates. The most abundant amino acid in orange juice is proline. The role of proline is important as it is known that this amino acid along with glycine and the proline derivate - hydroxyproline contributes to 57% of the total amino acids in the collagen (Liu, 2013; Li and Wu, 2018).

In this paper we describe the investigation of the microbiological quality of pasteurized commercial apple and orange juices, analyzing the total number of mesophilic microorganisms and facultative anaerobes, total number of molds and yeasts, and the number of diarrheagenic *E. coli* and coliform bacteria. The probability for heat-resistant spores to survive pasteurization was assessed as well.

Materials and Methods

Sampling

A total of 12 juice samples were investigated – three apple juice samples (1 liter Tetra pack), three orange juice samples (1 liter Tetra pack), three apple juice samples (200-250 ml Tetra pack) and three orange juice samples (200-250 ml Tetra pack). Juices were manufactured as follows: BBB juices by Gorna Bania Bottling Complany LtD, Happy day juices by RAUCH Fruchtsäfte GmbH & Co, and Florina juices by Best Fruits 17 Ltd. Samples were purchased from big retail stores in the spring of 2019.

A

B

Fig. 3. Apple and orange fruit juices. A) One liter Tetra pack apple andorange juices manufactured by BBB, Happy day and Florina Ltds; B) 200-250 ml Tetra pack apple and orange juices, commonly consumed by children (manufactured by BBB, Happy day and Florina Ltds).

Analysis of total number of microorganisms in one ml of juice

Aerobic mesophilic microorganisms and facultative anaerobes were evaluated using Nutrient agar (Hi-Media, India) and *via* the Koch's method, according to the methodology described in ISO 6887-1:2017. Nutrient agar Petri dishes were inoculated with 100 µl of each juice sample and cultivated for 72 h at 30°C. Analysis was performed in duplicate.

Analysis of total number of molds and yeasts in one ml of juice

Analysis of molds and yeasts was conducted on Sabouraud agar (Hi-Media, India) using Koch's method. 100 μl of each juice sample was spread on the agar and grown for 48 h (for fast growing molds and yeasts) to one week (for slowly growing molds) at 30°C. Analysis was performed via the horizontal method for enumeration of molds and yeasts, described in ISO 21527-1:2008. Analysis was done in duplicate.

Analysis of E. coli and coliform bacteria

Enumeration of *E. coli* and coliform bacteria was executed using Deoxycholate citratelactose agar (DCLA) (Scharlau, Spain). One L dH2O contains: meat extract - 10 g, lactose - 10 g, ferric citrate - 1g, sodium citrate - 20 g, sodium deoxycholate - 5 g, metal red - 0.02 g, agar-agar - 15 g. Petri dishes were inoculated with 100 μl of juice sample and cultivated for 24-48 h at 37°C. Enumeration was performed according to ISO 21528-2:2017. Analysis was carried out in duplicate.

DCLA is a selective and differential medium for gut pathogenic bacteria as *E. coli*, *Salmonella* and *Shigella*. It is rich in citrate and deoxycholate medium. Citrate and deoxycholate salts inhibit the growth of Gram (+) bacteria and other normal intestinal microorganisms allowing only the growth of the species of the family *Enterobacteriaceae*. Colonies of *E. coli* grow pink in color on DCLA, *Salmonella* spp. are colorless with or without black center, and *Shigella* spp. are visualized on the agar as colorless colonies.

Presence of spores and spore-forming bacteria

In order to verify if the juices contain heat-resistant spore-forming or gut-colonizing bacteria, an indirect method was applied where juices were enriched in Peptone water. One liter of peptone water contained 10 g peptone from casein (HIMEDIA, India) and 5 g NaCl. The solution was adjusted to pH 7.2 ± 0.2. Erlenmayer flasks with 225 ml peptone water were inoculated with 25 ml juice samples (1:10) and further cultivated overnight for 24 h at 30°C. If present, the cultured bacteria should subsequently be isolated and studied for spore formation according to standard methods.

Results

Analysis of total number of microorganisms in one ml of juice

Our analysis showed no bacterial growth of aerobic mesophilic microorganisms in all six samples of one liter Tetra pack apple and orange juices (Table 1).

Analysis of total number of molds and yeasts in one ml of juice

The analysis for presence of molds and yeasts in one liter of apple and orange Tetra pack juices, did not establish growth of cells in either apple or orange juices produced by the two manufacturers - Florina and Happy Day. One colony per Petri dish was observed in both apple and orange juice manufactured by BBB Ltd. This result represents 5 CFU/ml juice. As the upper standard limit is 5-10 CFU/ml, this amount does not exceed the standard limits and cannot cause possible outbreaks (Fig. 4).

Analysis of E. coli and coliform bacteria

Figure 5 shows the analysis of *E. coli* and coliform bacteria presence in three orange and three apple juices. As seen from the figure, no single

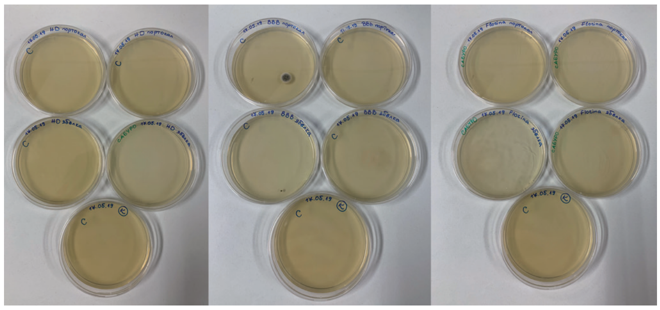

Fig. 4. Analysis of molds and yeasts in three apple and three orange juices (HD, BBB and Florina (1 liter Tetra pack)

colonies of *E. coli* colony or of *Salmonella* spp. or *Shigella* spp. were observed in either the apple or orange juices, 1 liter Tetra pack.

The lack of growth of *E. coli*, *Salmonella* spp. and *Shigella* spp. colonies on DCLA confirmed that the tested juices were free of pathogenic gut

Table 1. Presence of co-infection

Microrganisms/Juice type	HD apple juice	HD orange juice	BBB apple juice	BBB orange juice	Florina apple juice	Florina orange juice
Total number of aerobic mesophilic bacteria and FA	0	0	0	0	0	0
Total number of yeasts and molds	0	0	5	5	0	0
E. coli and coli-like bacteria	0	0	0	0	0	0

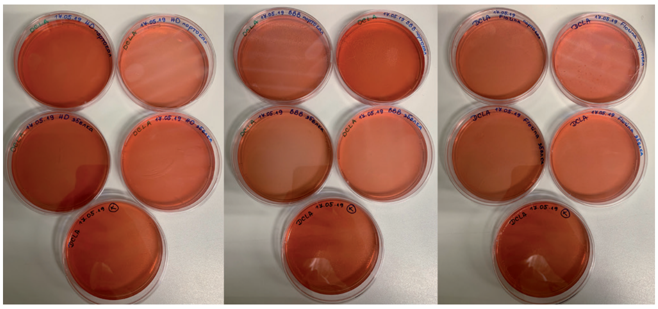

Fig. 5. Analysis of *E. coli* and coli-bacteria in three apple and three orange juices (HD, BBB and Florina (1 liter Tetra pack)

bacteria, especially of the lethal *E. coli* O157:H7, and entirely consistent with the ISO 21528-2:2017 standard. *E. coli*-tests are mandatory for the safety analyses of any kind of food. The amount of permissible colonies per g or per ml of product varies among different foods.

The data presented in Table 1 show optimal microbiological quality of the investigated juice samples. No aerobic mesophilic bacteria or facultative anaerobes were observed in the six investigated juices. According to the standard limits (ISO 6887-2017), the total number of microorganisms in juices should not exceed 50 CFU/ml. Five colonies of yeasts and molds in BBB orange and apple juice per ml does not exceed the Bulgarian, EU and USA standards as the standard limit (ISO 21527-1-2008) for yeast and mold spores is 5-10 CFU/ml. Samples were also free of *E. coli* and coliform bacteria. According to the standard (ISO 21528-2-2017), the upper limit for *E. coli* and coliform bacteria is 0.3-1 CFU/ml. The results displayed in Table 1 are convincing evidence that the studied six juices of 1 liter Tetra pack are microbiologically safe for consumption.

Similar assessment was conducted for the 200-250 ml orange and apple Tetra pack juices produced by the same manufacturers (Fig. 6). Due to the predominant consumption of juices in smaller boxes by children, the assessment of juice quality and safety is of significant importance.

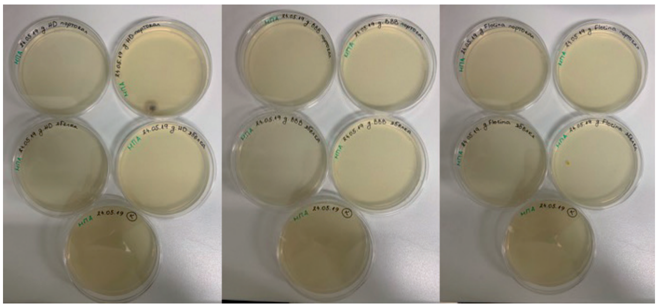

Fig. 6. Analysis of total number of microorganisms in three apple and three orange juices (HD, BBB and Florina (200-250 ml Tetra pack)

The results presented in Fig. 6 show no contamination with microorganisms (mesophylls and facultative anaerobes) of the juices consumed mainly by infants and children. Insignificant growth of a single colony per Petri dish was observed in two of the juice samples (HD orange and Florina apple). Petri dishes that showed no growth whatsoever demonstrated microbiological purity of children juices also regarding *E. coli* and coliforms (Table 2).

As seen from Table 2, no microbial growth was detected in all 200-250 ml Tetra pack orange and apple juices, except in the BBB apple juice sample (5 CFU/ml) and in Florina apple juice sample (5 CFU/ml). As the standard limit is 50 CFU/ml and the number of bacteria obtained during the experiment is below this value, all 6 apple and orange juices (200-250 ml Tetra pack) can be considered microbiologically safe for consumption.

Presence of spores and spore-forming bacteria

Our experiment showed that the peptone waterenriched apple juice samples (produced by BBB Ltd) – 1 liter and 200 ml Tetra packs were negative for bacterial growth, indicating that no heat-resistant spore-forming bacteria survived pasteurization. That is why, no further tests of bacteria and their spores were executed (Fig. 7).

The lack of bacterial growth in the enriched cultures demonstrated that no heat-resistant bacteria, for example *Alicyclobacillus acidoterrestris*, were

Table 2. Summarized results of microbiota in three apple and three orange juices (CFU/ml), 200-250 ml Tetra pack

Microrganisms/ Juice type	HD apple juice	HD orange juice	BBB apple juice	BBB orange juice	Florina apple juice	Florina orange juice
Total number of aerobic mesophilic bacteria and FA	0	5	0	0	5	0
Total number yeasts and molds	0	0	0	0	0	0
E. coli and coli-bacteria	0	0	0	0	0	0

Fig. 7. Peptone water-enriched samples of apple juice (1 liter and 250 ml Tetra pack) manufactured by BBB Ltd.

invading the apple fruits or further contaminating the apple juice. Enriched cultures were also free of heat-resistant spores.

Discussion

Juices are of significant importance for the human health. Detailed information about the beneficial role of fruits and vegetable in the diet was given by Liu (2013). Galaverna *et al*. (2014) reported the positive effect of antioxidants in orange juice on human health. Both apple and orange juice are known to prevent cardiovascular diseases, diabetes, cancer, and influence the immune system exerting a pronounced immunomodulatory effect. The high potassium concentration in both apple and orange juice helps the maintenance of normal blood pressure. Abundance of vitamins and minerals ensures optimal metabolism. Among vitamins, Vit. C, which is essential for the body, is present in high concentration in both juices (Caballero *et al*., 2003).

Summarizing the data from the literature overview, a number of papers have been continuously describing the enormous amount of bacteria developing in fruits and in fruit juices. Bacterial contamination of juices was reported by Leff and Feirer (2013), describing the main taxa colonizing the juices - *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* phyla. In contrast to freshly squeezed juices which are abundant in bacteria, pasteurized juices are considered to be free of bacteria (Caballero at al., 2003; Enikova, 2010). Usually, fruit juices are contaminated by microorganisms which colonize the fruit surface. Contamination occurs during harvesting and post-harvest periods. However, it is also possible that fruits and juice are invaded by microorganisms during transportation and storage (Kamal *et al*. 2014).

To eliminate all pathogenic microorganisms and their spores from fruit juices already produced, industrial-scale pasteurization is used, described in details by Perutzzi *et al*. (2017). This process prevents contamination of juices and ensures their long shelf life, killing a plethora of bacteria such as *Salmonella*, *Shigella*, *E. coli*, *Acetobacter*, *Alycoclobacillus*, *Bacillus*, *Gluconobacter*, *Lactobacillus*, *Leuconostoc*, *Zymomonas*. It also kills yeasts such as *Candida*, *Pichia*, *Saccharomyces* and *Rhodotorula*. Pasteurization neutralizes the commonly encountered filamentous fungi such as *Penicillium*, *Byssochlamus*, *Fusarium*, *Botritis*, *Talaromyces*, *Aspergillus* and *Mucor*. During the heating process, many members of molds such as *Aspergillus*, *Eurotium*, *Cladosporium* and *Alternalia* are inactivated (Caballero *et al*., 2003; Trbst *et al*., 2009; Kemal *et al*., 2014)

Traditionally, pasteurization has been considered as a potential method for destruction of all microorganisms and their spores. Recently, a number of reports have been published describing the presence of the heat-resistant spore-forming bacterium *Alycyclobacillus* even in pasteurized juices (Smit *et al*., 2011; Osopale *et al*., 2016). Available data about the characteristics of this bacterium caught our attention and prompted us to conduct this investigation.

Target microbial groups in our investigation are mesophilic aerobic bacteria and facultative anaerobes, yeasts and molds, diarrheagenic *E. coli* and coliform bacteria as well as the possible heat-resistant spore-forming bacteria which survived the pasteurization. All these microorganisms have caused serious outbreaks round the world in the recent decade (Montville *et al*., 2012; Montville *et al*., 2017).

According to the international standards, very low amounts of microorganisms like bacteria, yeasts, molds, *E. coli* and coliform bacteria are permissible in juices. Being rich in carbohydrates, juices are a perfect source of fast growing bacteria (Sun, 2009).

As regards the total number of microorganisms (Table 1 and Table 2), our results meet the standard limits (ISO 6887-2017) max. Fifty CFU/ml juices of 1 liter tetra pack were free of microorganisms. Five colonies per ml were found in two 250 ml Tetra pack juices, which are insignificant and comply with the standard.

Molds and yeast total number in the investigated 12 juice samples also did not divert from the standard. All samples were free of molds and yeasts, except two samples which were positive, but still within the standard permissible values. ISO 21527-1-2008 prescribes the upper limit for yeast and mold spores ranging from 5 to 10 CFU/ml. The results obtained were 5 CFU/ml, indicating that both juices are safe products. The high toxicity of mycotoxins on human brain function has been well documented since the 1970s (Kramer *et al*., 1976; Rice *et al*., 1977) and has continued during the last two decades (Kotzekidou, 1997; Weinedborner, 2001), making the analysis performed an important one.

The presence of *E. coli* and coliform gut bacteria is permitted by the standard but at a very low concentration of 0.3-1 CFU/ml (ISO 21528-2-2017). All 12 samples were negative for *E. coli* presence. Serotype O157:H7 is one of the pathogenic *E. coli* which caused fatal outbreaks a few years ago in Germany. The body intoxication is due to the action of the highly toxic Vero- and Shiga toxins, synthesized by O157:H7 (Proft, 2013; Montville *et al*., 2017). As reported by Juneja and Sofos (2010), the very low infectious dose (100 cells) makes this strain one of the most potent pathogens transferred by fruits and vegetables.

Regarding the possible presence of heat-resistant bacteria and their spores in the enriched cultures of two apple juices, the lack of growth proved that the tested juices were of high microbiological quality, met the standards and could be considered microbiologically safe and suitable for use.

Conclusions

Our study revealed that all 12 investigated pasteurized juices (6 apple juices and 6 orange juices) meet the national, European and international standards in terms of their microbiological quality. Juices were free of aerobic mesophilic bacteria, facultative anaerobes, yeasts and molds as well as of the diarrheagenic *E. coli* and coliform bacteria. No heat-resistant spore-forming bacteria had grown via the enrichment experiment.

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