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Microbiota of Fresh and Canned Green Table Olives and Antibiotic Resistance of Foodborne Pathogens

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Abstract

Olives are the main part of the healthy, largely vegetarian, Mediterranean diet known to have aquintessential role in prolonging lifespan. All parts of the olive plant - leaves, roots, flowers and fruits are rich in beneficial bacteria. Often, during olive processing and storage foodborne bacteria grow and spoil the product. The aim of this study was dual - to investigate the microbiota in fresh and canned green table olives, and to test foodborne bacteria isolated from olives for their antibiotic resistance. The presence of pathogens as *clostridia*, *Staphylococci*, fecal enterococci, yeasts, *Candida* spp. and *Escherichia coli* and coliforms was assessed using selective, differential and chromogenic media. Randomly picked colonies were further subcultured and tested for antibiotic resistance towards 17 antibiotics and six antifungals. Our results showed that fresh olives contained total number of microorganisms (CFU) - $3x10^2$, fecal enterococci - $2.4x10^4$, *Candida* – $1.5x10^2$ CFU/g and total number of yeasts and molds - $2.3x10^3$. Staphylococci, clostridia and *E. coli* and coliforms were not detected in fresh olives. In contrast, no bacteria, yeasts and molds, *Candida*, *Staphylococci*, *clostridia* and *E. coli* and coliforms were observed in canned green olives. Two isolates from fresh olives were further analyzed. They were identified as *Enterococcus faecium* and *Candida krusei*. The antibiotic resistance analysis demonstrated resistance of *E. faecium* 3391 to nine out of 17 antibiotics, including Linezolid, an antibiotic used for treatment of severe infections. *C. krusei* 3389 showed resistance to two out of six antifungals – to Itraconazole and Fluconazole, belonging to the class of triazole compounds.

Keywords: food microbiology, green olives, fecal enterococci, *Candida*, antibiotic resistance

Резюме

Маслините са основна част от здравословната средиземноморска диета, основана главно на растителни продукти. Тя е от ключово значение за удължаване продължителността на живота. Всички части на маслиновото дърво – листа, корени, цветове и плодове са богати на полезни бактерии. Често обаче, по време на обработването и съхранението на готовите вече маслини се развиват патогенни микроорганизми. Целта на нашето изследване е двукомпонентно - на първо място, да се изследва микробиологичното съдържание на свежи и стерилизирани зелени маслини и на второ място – да се култивират изолати от пробите, които да бъдат изследвани за антибиотична резистентност. Наличието на патогенните клостридии, стафилококи, фекални етерококи, дрожди и плесени, кандиди и *Escherichia coli* и колиформи е изучено чрез използването на селективни, диференциални и хромогенни хранителни среди. Случайно подбрани колонии са култивирани и изследвани за резистентност към 17 антибиотици и 6 противогъбични препарата. Резултатите от проведеното изследване показаха, че свежите маслини съдържат общ брой микроорганизми (КФЕ) - $3x10^2$, фекални ентерококи - $2.4x10^4$, кандиди – $1.5x10^2$ КФЕ/г, а общият брой дрожди и плесени беше $2.3x10^3$ КФЕ/г. Стафилококи, клостридии и *E. coli* и колиформи не бяха регистрирани в свежите маслини. За разлика от свежите маслини, в стерилизираните маслини не беше установена нито една от изследваните микроорганизмови групи. Бяха култивирани два изолата от свежите маслини, които бяха иденти-

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фицирани като *Enterococcus faecium* и *Candida krusei*. *E. faecium* 3391 показа резистентнот към 9 от 17 изследвани антибиотици, единият от които Линезолид, използван при лечението на тежки инфекции. Вторият изолат – *C. krusei* 3389 показа резистентност към 2 от 6 изследвани противогъбични препарата – итраконазол и флуконазол, и двата от групата на триазоловите съединения.

Introduction

Olives are known to be rich in valuable nutrients and bioactive components of medicinal interest (Gnanbari *et al*., 2012; Essafi *et al*., 2019). Olive fruits contain appreciable concentrations of over 30 different phenolic compounds. Phenolic substances, both hydrophilic and lipophilic, are minor components in olives but have generated great interest due to their beneficial effect on human health. Hydrophilic phenol derivatives are phenolic acids, phenolic alcohols, flavonoids and secoiridoids. The lipophilic phenolic fraction possesses a broad range of biological activities such as antimicrobial, anti-inflammatory, antidislipidemic, antihypertensive, antioxidant, anticarcinogenic, antihypertensive, cardiotonic, laxative and antiplatelet (Gnanbari *et al*., 2012). The main beneficial effect of olives and olive oil is their anticancer effect and protection from cardiovascular disease (Oven *et al*., 2004; Gnanbari *et al*., 2012; Essafi *et al*., 2019). Recently, Francisco *et al*. (2019), reported another positive effect of olives and olive oil on human health, associated with the prevention of rheumatic diseases. Peyrol *et al*. (2017) described the prevention of MetS (Metabolic Syndrome) by olive products. The main phenolic component in olives responsible for their health beneficial effect is hydroxytyrosol (Rafehi *et al*., 2012), a superior antioxidant and radical scavenger (Fig. 1A), which induces apoptosis and arrests the cell cycle in cancer cells. The hydroxytyrosol demonstrated antimicrobial activity (Tuck *et al*., 2002) and is renally excreted via many metabolites as glucuronide conjugate, sulfate conjugate, homovalliic acid and 3,4 – dihydrophenylacetaldehide. Along with hydroxytyrosol, other phenolic compounds as tyrosol (Fig. 1B), the bitter olive glycoside – oleuropein (Fig. 1C), oleocanthal (Fig. 1D), and oleacein (Fig. 1E) are known to exert antiatherogenic, neuroprotective and endocrine effect (Karkovich *et al*., 2019).

Although olives and olive oil are abundant in antimicrobial substances, contamination with pathogens of animal or human sources is possible during their growth, harvest, transportation and storage. Contamination can emerge via treatment of soil with organic fertilizers, manure, sewage water, irrigation water or by the pathogens persisting in vegetables (Hamilton *et al*., 2006). The aim of our study was to compare microbiota in samples of fresh and canned green table olives and to study olive foodborne pathogens for antibiotic resistance.

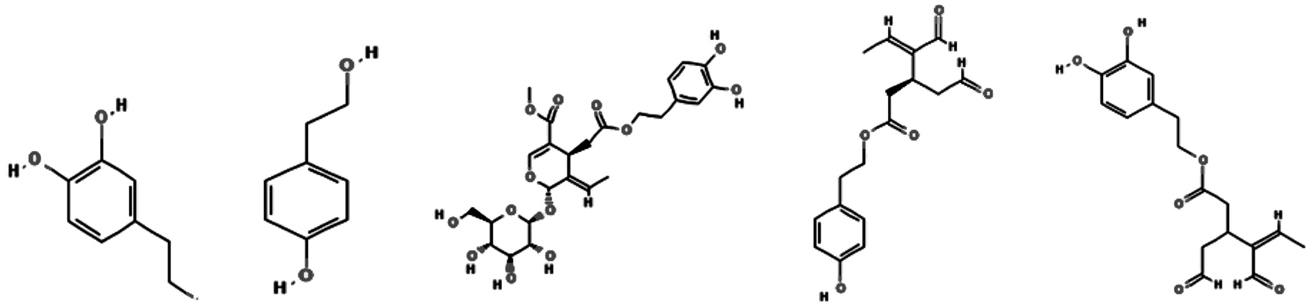
Materials and Methods

Sampling and sample preparation

Samples of fresh and canned green table olives were purchased in 2019 in big grocery stores in Sofia, Bulgaria, and were comparatively investigated for presence of viable microbiota. Ten grams of each sample were aseptically smashed and added to 90 ml Ringer’s solution. The purpose of using Ringer's solution was to completely dissolve the fats in the products. Starting sample solutions were diluted decimally (10^{-1} to 10^{-7}) further 0.1 ml of each was spread on agar plates, a routine method of Koch for counting microorganisms.

Total number of viable bacteria

CFU of viable bacteria was examined on nutrient agar (HiMedia, India and Oxoid, England). The agar was inoculated with 0.1 ml of each dilution. Cultivation was carried out at 30°C for 48h. Enumeration was performed using the method of Koch.



A B C D E

Fig. 1. Structural formula of hydroxytyrosol (A), tyrosol (B), oleuropein (C), oleocanthal (D), oleacein (E) (Pubchem)

Number of fecal enterococci

KEAA (Kanamycin-Esculin-Azide agar, Merck, Germany) was applied for detection of fecal enterococci. Metabolizing esculin in the medium, enterococci turn the white-colored agar black and are visible as transparent to white-colored colonies. Being a component of the medium, kanamycin inhibited the growth of other Gram (+) bacteria. The agar was inoculated with 0.1 ml of each dilution. Incubation was carried out for 48h at 30°C. Enterococcal counts were assessed using the method of Koch.

Number of Candida spp.

Both *Candida* conventional (HiMedia, India) and Differential chromogenic agars (HiMedia, India) were used for detecting *Candida* spp. Colonies of *Candida* grew black-colored on the white agar when conventional agar was used, and white-to-pink when grown on a differential chromogenic agar. While conventional agar has to be autoclaved, chromogenic agar should be prepared only by boiling according to the manufacturer's instructions. The agar was inoculated with 0.1 ml of each dilution. Incubation lasted 7 days at 28°C and the enumeration was performed according to Koch's method.

Total number of yeasts and molds

The total number of yeasts and molds was analyzed on YGC agar (Yeast peptone-Glucose-Chloramphenicol agar). Petri dishes were inoculated with 0.1 ml of each dilution. Incubation lasted 7 days at 28°C.Yeasts and moldswere enumerated according to the method of Koch.

The enumeration of *Clostridium perfringens* was carried out on SPS agar (Sulphite-Polymyxin-Sulphadiazine Agar, HiMedia, India). One ml of inoculum of each dilution was poured out in the petri dish bottom and covered with 20 ml of SPS agar for deep seeding and creating anaerobic conditions. Incubation lasted 48h at 37°C and the enumeration was performed via the Koch method.

Number of Staphylococci

MSA (Manitol-Salt agar, 6.5% NaCl, Merck, Germany) was used to study the presence of members of the *Staphylococcus* genus. On the orange-pink agar, staphylococcal colonies grew gold-colored surrounded by a visible well-shaped yellow halo. Petri dishes were inoculated with 0.1 ml of the dilutions, further incubated for 48h at 30°C and enumerated by the method of Koch.

Number of E. coli and coliforms

On VRBA agar (Violet Red Bile Agar) or DCLA (Deoxycholate Lactose Agar) was spread 0.1 ml of each dilution was spread on VRBA agar (Violet Red Bile Agar) or DCLA (Deoxycholate Lactose Agar). Incubation lasted 48h at 30°C and the number of *E. coli* and coliforms was assessed using the method of Koch.

Isolation and characterization of bacterial strains from green table olives

Randomly selected colonies were picked up from each plate for further cultivation and identification. Among the grown colonies, two isolates were chosen for further analysis. Isolates were sub-cultured on different media such as Columbia agar with 5% sheep blood, Brucella agar with 5% horse blood (BD, USA), MacConkey agar (BD, USA), Sabouraud dextrose agar and BBL CHROMagar *Candida* (BD, USA). Their identification was carried out using Crystal (BD, USA) system; VITEK 2 System (bioMerieux, France) and API 20 C AUX *Candida* (bioMerieux, France). Strains were stored at -20°C at New Bulgarian University Microbial Culture Collection (NBU-MCC).

Antibiotic and antifungal susceptibility tests of the microorganisms isolated from green table olives was performed using the standard disk agar diffusion method. The bacterial and fungal isolates were spread at concentration/turbidity equal to 0.5 McFarland on blood agar and *Candida* agar, respectively. Seventeen antibiotics currently used in clinics (BD, USA) – Cefoxitin (30 µg), Amikacin (30 µg), Rifamycin (5 µg), Erythromycin (15 µg), Clindamycin (2 µg), Gentamicin (30 µg), Gentamicin (120 µg), Ciprofloxacin (5 µg), Amoxicillin/Clavulanic acid (20/10 µg), Ceftriaxone (30 µg), Vancomicin (5 µg), Teicoplanin (30 µg), Levofloxacin (5 µg), Linezolid (30 µg), Sulfmethoxasole/Trimethoprim (25 µg), Tigecicline (15 µg), Ampicillin (2 µg), and six antifungals (HiMedia, India) – Clotrimazole (10 µg), Voriconazole (1 µg), Itraconazole (10 µg), Nystatin (50 µg), Ketoconazole (10 µg), Fluconazole (25 µg) were applied. The results obtained were evaluated according to the EUCAST system, version 7.0-2019 (www. eucast.org).

Results

Total number of viable bacteria

Our investigation showed presence of total number of bacteria in fresh olives at the amount of $3x10^2$ CFU/g. The recorded microbial counts did not exceed the standard limits (Enikova, 2010). The canned green olives were free from all seven microbial groups tested (Table 1).

Table 1. Number of bacteria and fungi in fresh and canned green table olives

Microorganisms/ Sample	Total number bacteria /MPA/	Fecal enterococci /KEAA/	Fungi /*Candida* agar/	Total number Yeast&Molds /YGC/	Staphylococci /MSA/	*C. perfringens* /SPS agar/	*E. coli* and coliforms /VRBA/
1. Fresh green olives	$3x10^2$	$2.4x10^4$	$1,5x\ 10^2$	$2.3x10^3$	0	0	0
2. Canned green olives	0	0	0	0	0	0	0

Number of fecal enterococci

As seen in Table 1, fecal enterococci grew in fresh olives at a concentration of $2.4x10^2$ CFU/g in contrast to canned olives, where no enterococcal colony was observed. Enterococci are not included in the standards as target microorganisms to be monitored in foods and no limits are described. However, recently, due to the broad spectrum of diseases caused in humans by these bacteria, their number in food is in focus in many reports.

Number of fungi (Candida spp.)

During the enumeration period of 7 days, no colonies grew from either fresh or canned olives. On the 7-10th day, a few black colonies were observed in each petri dish with standard *Candida* agar from fresh olives. The colonies were further transferred on *Candida* CHROMagar, where they grew pink to light purple in color, hence were proven to belong to *Candida*.

Total number of molds and yeasts

Our study demonstrated presence of yeasts and molds only in fresh olives ($2.3x10^3$ CFU/g). No molds and yeasts grew from the canned olive sample (Table 1).

Number of Staphylococci

As shown in Table 1, both fresh and canned green table olives were negative for *Staphylococci*.

Number of C. perfringens

The investigated samples of fresh and canned olives contained no *clostridia*.

Number of E. coli and coliforms

Our analysis proved that the studied fresh and canned olives were not contaminated with *E. coli* or coliforms, and no colonies of these bacteria were cultivated on VRBA or on DCLA agar.

Isolation and identification of bacterial strains from green table olives

Randomly selected colonies grown from the fresh olives sample, were selected and further analyzed. Of the colonies formed, two isolates, one cultivated on Candida CHROMagar and the second on KEAA agar, were selected for identification and for studying their antibiotic resistance. Using Crystal, VITEK 2 and API 20 C AUX Candida, these strains were identified as *Candida krusei* and *Enterococcus faecium*, respectively. Both strains were stored at New Bulgarian University Microbial Culture Collection (NBU-MCC) under the following numbers: *C. krusei* 3389 and *E. faecium* 3391.

Antibiotic and antifungal susceptibility of the isolates from table green olives was tested by the standard disk diffusion method.

As seen in Fig. 2, the antibiotic susceptibility of *E. faecium* 3391 is diverse. The strain demonstrated sensitivity to eight (47%) and resistance to nine antibiotics (53%) (see Table 2).

Fig. 2. Agar diffusion susceptibility test of *E. faecium* 3391 towards 17 antibiotics

Table 2 demonstrates that the isolate *E. faecium* 3391 is sensitive to Rifamicin, Gentamicin 120, Ceftriaxone, Vancomicin, Teicoplanin, Levofloxacin, Sulfmethoxasole/Trimethoprim and Tigecicline. The strain showed resistance to a greater number of antibiotics: Cefoxitin, Amikacin, Erythromycin, Clindamycin, Gentamicin 30, Ciprofloxacin, Amoxicillin/Clavulanic acid, Linezolid and Ampicillin. An important finding is the resistance of *E. faecium* 3391 to the antibiotic Linezolid, which is used in fighting severe infections.

Figure 3 presents the antifungal susceptibility test of the strain *C. krusei* 3389.

This pathogenic fungus showed sensitivity to four (67%) and resistance to two antifungals (33 %) (See Table 3).

Table 2. Antibiotic susceptibility test of *E. faecium* 3391

Antibiotic/Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
E. faecium 3391	R	R	S	R	R	R	S	R	R	S	S	S	S	R	S	S	R

R- Resistant, S- Sensitive
Cefoxitin (1), Amikacin (2), Rifamycin (3), Erythromycin (4), Clindamycin (5), Gentamicin 30 (6), Gentamicin 120 (7), Ciprofloxacin (8), Amoxicillin/Clavulanic acid (9), Ceftriaxone (10), Vancomicin (11), Teicoplanin (12), Levofloxacin (13), Linezolid (14), Sulfmethoxasole/Trimethoprim (15), Tigecicline (16), Ampicillin (17)

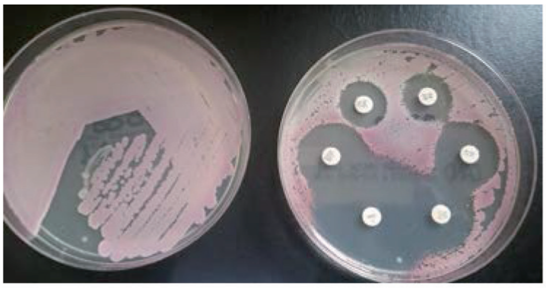


Fig. 3. Agar diffusion susceptibility test of *Candida krusei* 3389 towards 6 antifungals

Table 3. Antibiotic susceptibility test of *C. krusei* 3389

Antifungals/Strain	1	2	3	4	5	6
C. krusei 3389	S	S	R	S	S	R

R- Resistant, S- Sensitive
Clotrimazole (1), Voriconazole (2), Itraconazole (3), Nystatin (4), Ketoconazole (5), Fluconazole (6).

As seen in Table 3, *C. krusei* 3389 was sensitive to four (Clotrimazole, Voriconazole, Nystatin and Ketoconazole) and resistant to two antifungals (Itraconazole and Fluconazole).

Discussion

Both bacteria and yeasts are usually detected in olives during their harvesting or processing as described by Arroyo Lopez *et al*. (2008). Typically, the microbial community in olives is dominated by the yeasts *Saccharomyces cerevisiae* and *C. apicola*, as reported by Arroyo-Lopez *et al*. (2008), Medina *et al*. (2016), and Ciafardinni *et al*. (2018). The authors describe in their study 131 bacterial genera including over 350 taxonomic units detected in olives by pyrosequencing of 16S rDNA. The same research groups have found in olive samples members of the families *Enterobacteriaceae* and *Lactobacillaceae*, of the *Staphylococcus* genus and the most common spoilage bacteria, *Pseudomonas* and *Propionibacterium*.

Yeasts are present throughout the whole olive fermentation process, which is important as they produce compounds whose organoleptic properties improve the quality and flavor of olives (Arroyo-Lopez *et al*., 2008). Besides the yeasts *S. cerevisiae* and *C. apicola*, Medina *et al*. (2016) reported presence of *Pichia manshurica* and *P. galeiformis* in olives, and Mateus *et al*. (2016) of *C. boidinii, P. membranefaciens, Zygosaccharomyces mrakii, Priceomices carsonii* and *Wickerhampmyces anomalus*. Arroyo-Lopez *et al*. (2008) described other spoilage species as *C. boindii*, *Debaromyces hansenii, P. anomalia* and *Rhodothorula glutinis. C. apicola* is known as a highly osmotolerant ascomycete fungus, which produces sophorolipids (biosurfactants), membrane fatty acids and enzymes, such as reductases and proteases. This microscopic fungus is found naturally in wine and cachaça fermentation processes. Recently, *C. apicola* has been reported to secrete β-fructofuranosidases with fructosyltransferase activity, useful for prebiotic synthesis. The total number of yeasts detected during our analysiswas $2.3x10^3$ CFU/g (fresh olives) and 0 CFU/g (canned olives). No data about the presence of *C. krusei* in olives is described in the literature.

The main bacterial genus isolated from table olives is *Lactobacillus* with the predominant species – *Lactobacillus plantarum* and *L. pentosus* (Hurtado *et al*., 2012). Most often, the spoilage bacteria in olives belong to the family *Enterobacteriaceae*, family *Lactobacillaceae* and genera *Staphylococcus*, *Pseudomonas* and *Propionibacterium*. Recently, new bacteria were discussed by de Castro *et al*. (2018) responsible for the spoilage of green table olives in Spain – members of the family *Cardiobacteriaceae* and of the genus *Ruminococcus*. The genera *Alcalibacterium*, *Marinilactibacillus* and *Halolactibacillus* were discussed in the paper of Lucena-Padros and Luis Barba (2016). Our results coincide partially with the findings of the above authors, showing low presence of total number of bacteria at concentration $3x10^2$ CFU/g (fresh olives) and 0 CFU/g (canned olives) but differ as regards the presence of *E. coli* (*Enterobacteriaceae*), staphylococci and clostridia: both types of olive samples investigated were free of these bacteria. An interesting finding of our investigation was the high amount of fecal enterococci (2.4×10^4 CFU/g) in fresh olives. No results about the amount of enterococci in olives are described in the literature.

During our investigation one bacterial isolate identified as *E. faecium*, was cultured and tested for its antibiotic resistance towards antibiotics tackling moderate or severe bacterial infections. Enterococci play a dual role being recognized both as probiotic but recently also as pathogenic bacteria with intrinsic or acquired resistance to almost all antibiotics currently in use (Tendolkar and Baghdayan, 2003). This Gram-positive bacterium which inhabits the gastro-intestinal tract of humans and animals can cause life–threatening infections in humans. Theresearch report of Franz and co-authors (Franz *et al*., 1996) described the strain *E. faecium* BFE 900 isolated from black olives. Apart from olives, enterococci are present in various Spanish foods. According to some authors (Ben Omar *et al*., 2004), the isolated enterococci were resistant to Erythromycin and Rifampin. These results coincide with the findings of our study – the isolate *E. faecium* 3391 also showed resistance to Rifampin. Rifampin is a macrocyclic antibiotic typically used in the treatment of tuberculosis and bacterial meningitis, inhibiting the DNA polymerase in *Mycobacterium tuberculosis*. *E. faecium* 3391 isolated during our investigation showed resistance to more than 53% of the antibiotics tested.

Tamang *et al*. (2016) described the following yeasts which colonize olives: *C. apicola*, *Pichia* sp., *P. manshurica/P. galeiformis, S. cerevisiae*. In the list of authors and in other reports, *C. krusei* is not included; hence no data about the antifungal activity of this microorganism is available and could not be discussed. Ciafardini *et al*. (2013) described *C. parapsilosis* in olive oil but not in olives. *C. krusei* 3389 isolated during our experiments was sensitive to four antifungals (Clotrimazole, Voriconazole, Nystatin and Ketoconazole) and resistant to two agents (Itraconazole and Fluconazole). The result obtained is alarming as Itraconazole is a broad-spectrum antimycotic triazole agent with a low toxicity profile used for treatment of a wide range of fungal infections such as vulvovaginal candidiasis, oral candidiasis, dermatophytoses e.g. tinea pedis, tinea cruris, tinea corporis, tinea manuum, *Pityriasis versicolor*, onychomycoses, systemic candidiasis, cryptococcal infections (including cryptococcal meningitis), histoplasmosis and aspergillosis. Fluconazole, the second antifungalsubstance the isolate *C. kruzei* 3389 was resistant to, also belongs to the class of triazole antifungals and is a remedy for a wide spectrum of candidiasis – oral, vaginal and of the blood, throat, esophagus and lungs. It is also applied to patients with bone marrow transplantation to prevent candidiasis. This antifungal is also a powerful cure for meningitis caused by the fungus *Cryptococcus* (Liu *et al*., 2016).

Coliforms were reported in olive oil (Zullo *et al*., 2018) but no data about their survival in olives were found in the literature. The samples investigated in our study were free of *E. coli* and coliforms.

As the investigated samples were free of *Staphylococci*, we have not discussed this bacterial genus here. .*Staphylococci*, which are sensitive to the phenolic compound oleuropein in olives, were likely to have been inhibited by this substance (Zanicelli *et al*., 2005).

Conclusions

Our analyses showed that fresh olives contained total number of microorganisms - 3×10^2 CFU/g, fecal enterococci - 2.4×10^4 CFU/g, *Candida* – 1.5×10^2 CFU/g and total number of yeasts and molds - 2.3×10^3; staphylococci, clostridia and *E. coli* and coliforms were not detected. While yeasts are typical of fresh olives, the presence of enterococci in high amounts is alarming. No bacteria, yeasts and molds, candida, staphylococci, clostridia and *E. coli* and coliforms were detected in canned green olives. Two isolates from fresh olives were sub-cultured. They were identified as *E. faecium* and *C. krusei*. Tests for antibiotic resistance demonstrated resistance of *E. faecium* 3391 to nine out of 17 antibiotics, including Linezolid, used for treatmentof severe infections. The widespread occurrence of antibiotic resistance of *E. faecium* isolated from fresh olives is meaningful and confirms that the food and foodborne bacteria contribute to the worldwide global antibiotic resistance.

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