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Research Article

Microbiological Quality and Prebiotic Activity of Table Olives on Pathogenic Microorganisms and Lactic Acid Bacteria

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Abstract

Table olive is one of the most important fermented vegetables in the world and its principal production zone is the Mediterranean area. The aim of this work was to proceed to the microbiological characterization and antimicrobial action of olives and its brines for verify selective effects on microbiota and prebiotic effects. Four olive varieties were studied (Sevillana, Arbequina, Marfil and Empeltre). To the microbiological quality were researched lactic acid bacteria (LAB) and several pathogens microorganisms. To the antimicrobial activity the effects from olives were analyzed using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for bacteria, and disc diffusion method for mould and yeasts. Variability in microbiological quality was observed between pulp and brine and between olives studied. Sevillana olives can stimulate the growth of LAB. This may be due to the type of treatment used or the olives would be acting as prebiotic, stimulating the growth of Lactobacillus spp. For the antimicrobial activity, gram-positive and gram-negative bacteria have the same sensitivity to the tested olives. For mould and yeasts, Arbequina olives had the higher antimicrobial activity.

ABBREVIATIONS

LAB: Lacticacid Bacteria; MIC: Mínimum Inhibitory Concentration; MBC: Mínimum Bactericidal Concentration; INT: p-Iodonitrotetrazolium Violet Salt; CFU: Colony Forming Units; TSA: Trypticasein Soy Agar; MK: Mac Conkey Agar; BP: Baird Parker Agar Base;MRS: MRS Agar;SPS: SPS Agar; TSN: Tryptone Sulfite Neomycin Agar; S: Sabouraud Dextrose Agar + Chloramphenicol; X: XLT4 Agar; Mono: Rapid´*L.Mono* Chromogenic Medium

INTRODUCTION

Table olive is one of the most important fermented vegetables and its principal production zone is the Mediterranean area, although its production and consumption are spread all over the world [1]. In European Union the producer countries are Spain, Italy, Greece, Portugal and France [2]. Olive fruit is a drupe that consists of a bitter component (a glucoside called oleuropein), a low concentration of sugar (2.6–6.0%) and a high oil content (12–30%), although these values can change according of maturity and the olive variety [3]. Such characteristics prevent olives from being consumed directly from the tree and have been promoted a series of processes to make them eatable .The benefits of table olives in nutrition are associated besides the presence of monounsaturated fat acids, the minor components, such as phenolic (oleuropein, tyrosol and hydroxytirosol) and triterpenic acids (maslinic, oleanolic and ursolic) [4,5].

Table olives can be produced as Spanish-style green olives in brine (about 60% of the production), as Greek-style naturally black olives in brine, and asripe olives by alkaline oxidation (Californian-style), according to well-established processes [6,7]. Olives can be picked at different stages of maturity, and they are processed to eliminate the characteristic bitterness caused by their oleuropein glucoside, and thus to make them suitable for human consumption [8].The fermentation of Spanish-style treated olives is due to lactic acid bacteria (LAB), while in Greek and Californian processed black olives the organisms responsible for fermentation are yeasts, and LAB represent a small proportion of the total microflora [9,10].

Lactobacillus plantarum and *Lactobacillus pentosus* are the main LAB involved in table olive fermentation [3,11] [12,13]. Randazzo et al. [14] highlighted the dominance of *L. plantarum*, its high versatility and adaptation in olive brine. Several authors in the recent years investigated the effects of phenolic fractions; in particular, oleuropein and its hydrolysis products, oleuropeinaglycon, elenolic acid and hydroxytyrosol, have been pointed out as responsible for the inhibition of LAB growth

in olive brines [15-17]. The microbial composition in brine is influenced by (a) the indigenous microbiota present in olives, (b) intrinsic factors of the fruit such as pH, water activity, diffusion of nutrients from the drupe (depending on the structure of the olive skin) and levels of antimicrobial compounds such as oleuropein, and (c) extrinsic factors such as temperature, oxygen availability and salt concentration in brine [18]. Traditionally, table olive industries let the process develop spontaneously without monitoring the microbiota [19]. No official microbiological criteria for table olives are available. However, the Standard 66-1981 (Rev. 1-1987) of the Codex Alimentarius(1987) [20] prescribed the minimum requirements related to hygiene for table olives. And the final product must be free from microorganisms and parasites in amounts which may represent a hazard to health and shall not contain any substance originating from microorganisms in amounts which may represent a hazard to health.

In recent years it has increased the number of work focused on the analysis of the probiotic effect of lactic acid bacteria present in table olives [21,22]. However we did not find published articles on the prebiotic effect of the Spanish olive cultivars studied in this work. Thus,the aim of this work was to proceed the microbiological characterization of four Spanish olive cultivars and its packing brine and to verify their antimicrobial and prebiotic action.

MATERIALS AND METHODS

Samples

Four Spanish table olives cultivars were studied (Sevillana, Arbequina, Marfil and Empeltre). The Sevillana table olive was purchased in three different Barcelona´ markets. Arbequina, Marfil and Empeltre table olives were acquired with Spanish commercial or artisanal producers. All table olives were immersed in brines. These brines were also used in subsequent analysis.

Samples Preparation

From the olives samples was done their preparation on sterile conditions to isolate the aliquot in specific culture media and evaluated the microbiological quality. Olive pulp were cut into small pieces (25 g) and homogenized in a Stomacher bag for two minutes with 225 mL of sterile Ringer solution (10^{-1} dilution). Further 1 mL of 10^{-1} dilution was added to 9 mL of Ringer solution and then homogenized (10^{-2} dilution). A series of decimal dilutions until 10^{-5} dilution were made with the same solvent. For each sample of packing brine were made the same dilutions (10^{-1} to 10^{-5}).

In order to analyze the antimicrobial action of the olives samples in front of several microorganisms were made extracts of the pulp. For the extracts, each olive was dried with filter paper and weighted using a watch glass. The pulp was separated from olive stone and weighted (10 g). This pulp was homogenized with distilled water (5 mL) using Polytron (6 cycles of 30 seconds, 5-speed). During the washing tube was used 4 mL of water and added to homogenized pulp with a final volume of 9 mL. This extract was considered concentration 100%. Dilutions with sterile distilled water were made to obtained concentrations of 80%, 50%, 30% and 10%. The same dilutions were made with all olives packing brine.

Study of the microbiological quality of the olive pulps and packing brines

The microbiological quality analysis weres made on the olive pulp and on the packing brine.The different concentrations (10^{-1} to 10^{-5}) of the olive pulps and the packing brines was studied in the following plates: TSA (Trypticasein Soy Agar), MK (Mac Conkey Agar), BP (Baird Parker Agar Base), MRS (MRS Agar), SPS (SPS Agar), TSN (Tryptone Sulfite Neomycin Agar), S (Sabouraud Dextrose Agar + Chloramphenicol), X (XLT4 Agar), Mono (Rapid´*L.Mono* Chromogenic medium).

The TSA, MK, BP, X and mono plates were read in 24h, the MRS, SPS and TSN in 48h, and S in 96h.Three independent experiments were performed.

It were researched lactic acid bacteria, *Staphylococcus aureus*, coliforms and other enteric pathogens, molds and yeasts, *Clostridium perfringens*, *Salmonella* spp., *Listeria monocytogenes*, and a several pathogens microorganisms.

Microbiological parameters of antimicrobial activity of olives

The antimicrobial activity was performed using different concentrations (100% to 10%) of Arbequina, Empeltre and Marfilolive pulp extracts and packing brine.

To perform these analyses, MIC and MBC methods were used for bacteria and disc diffusion method for filamentous fungi and yeasts. These methods are described in items 2.5 and 2.6. Three independent experiments were performed.

The following microorganisms were used: lactic and pathogenic bacteria (*Lactobacillus brevis*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium)*, mould (*Aspergillus flavus*, *Penicillium rugulosum)*, and yeasts (*Candida albicans* and *Saccharomyces cerevisiae)*.

Study of the ability of microbial inhibition by dilution in microtiter plates using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The technique applied is a variation of that used by Eloff (1998) [21]. The assay was performed in 96-well plates. Different concentrations of pulp extracts and brine of each variety of olives were added in the wells (100 µL) in duplicate. Subsequently were added 50 µL of the microorganisms' suspension and the plates were incubated at 37°C for 24 h. Vancomycin and Neomycin were employed as positive controls against gram positive and gram negative bacteria, respectively.

At the end of the incubation period 40 µL of 0.2 mg/mL INT (*p*-iodonitrotetrazolium violet salt) solution was added to each well. It was maintained at 37°C for 1h and analysis proceeded by color differences. The salt solution is an indicator of biological activity. Its components act as electron acceptors and are reduced, triggering color when there is activity of

microorganisms and being colorless if there is not. The lowest concentration indicating inhibition of growth was recorded as the MIC (Minimum Inhibitory Concentration). Each analysis was replicated 3 times.

Referring to the results of the MIC assay, the wells showing complete absence of growth were identified and 50 µL of each well were transferred to agar plates and incubated at indicated time and temperature for each microorganism. The complete absence of growth was considered as the minimum bactericidal concentration (MBC) [24,25].

Evaluation of the antimicrobial capacity of olives by disc diffusion method. Assay with filamentous fungi and yeasts

Antimicrobial activity of olive pulp extracts and packing brine was evaluated by a disc diffusion assay for filamentous fungi and yeasts. Mould and yeasts inoculum used are the order of 1-2 x 10^6CFU/mL. A petri plate containing the culture medium was inoculated with 100 µL of mould and yeasts suspensions, which was spread with a sterilized cotton bud. Approximately 50 µL of each olive pulp extract and packing brine in various concentrations was inoculated on a paper disc (6 mm of diameter), that were placed on a solid medium plate spread with mould and yeasts suspensions. After cultivating at 28°C for 96h, antimicrobial activity was compared by measuring the clear zone (including the 6 mm diameter of disc) generated around the paper disc. A following formula was applied:

Inhibition value = (Inhibition diameter in mm – Disc diameter (6 mm)) / 2

RESULTS AND DISCUSSION

Microbiological quality

The results of microbiological quality of Arbequina, Empeltre, Marfil and Sevillana olives and their brines are shown in Table 1. In general, variability was observed between pulp and brine and between olives studied, mainly between Sevillanaand the others three varieties of olives.

There was microorganism growth for Arbequina, Empeltre and Marfil olives (pulp and brine) only in TSA culture media.. Already Sevillana olives showed growth of microorganisms in the TSA, BP, S and MRS culture media.

With regard to microbiological quality, the growth of mesophilic aerobic bacteria, *Staphylococcus aureus*, mould and yeasts was expected in olives samples. However, mainly contamination by *Staphylococcus aureus*, should be avoidedbecause this pathogen can cause food-borne illness.

Staphylococcus aureus was detected in Sevillana olive pulp and its packing brine. These samples should be considered as unacceptable and improper for consumption. The occurrences of *S. aureus* in olive brines were reported by Pereira et al. [2] in four of thirty-five olives samples studied.

The growth of lactic acid bacteria in Sevillana olives or other olives may be due to theiracting as prebiotic, stimulating the growth of *Lactobacillus* spp. To be considered prebiotic, the substance fulfils the following aspects: resistance to digestion, fermentation by the large intestinal microbiota, and selective effect of the microbiota [28]. In our study, we analyzed this selective effect by comparing the antimicrobial activity of olives between pathogenic microorganisms and lactic acid bacteria. Resistance to digestion and fermentation by the large intestinal microbiota are still being evaluated in our laboratory.

The analysed Sevillana olive pulp and brine showed growth of mould (Table 1), unlike the other olives cultivars studied.. Probably, this was related with air surface of brine in tanks or barrels. Excessive mould growth origin a mouldy taste and can cause deterioration by consuming the acids produced during olive fermentation [2].

Regarding the yeasts growth, this work showed that Sevillana olive pulp and brine presented high growth (Table 1). Natural olives are fermented predominantly by yeasts [26,27], which would explain their detection levels as high as total mesophilic microorganisms.

Antimicrobial activity of olive extracts and packing brines

MIC and MBC analysis of bacteria: The results of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) analysis for bacteria studied are compiled in the Table 2.

The positive control had pinkish coloration after INT addition in all microorganisms, unlike the negative control that showed no color changed.

Table 1: Microbiological quality of Arbequina, Empeltre, Marfil and Sevillana olives (pulp and brine) with reading time of 24h to TSA, MK, BP, X and mono plates, of 48h to MRS, SPS and TSN plates and of 96h to mould and yeasts.

Plates	Pulp	TSA	MK	BP	MRS	SPS	TSN	S(CFU/g)		X	Mono
Olives	Brine	(CFU/g)	(CFU/g)	(CFU/g)	(CFU/g)	(CFU/g)	(CFU/g)	Moulds	Yeasts		
Arbequina	Pulp	3.4 x 103	<10	<10	<10	<10	<10	<10	<10	N	N
	Brine	6,0 x 103	<10	<10	<10	<10	<10	<10	<10	N	N
Empeltre	Pulp	1.0 x 102	<10	<10	<10	<10	<10	<10	<10	N	N
	Brine	1.1 x 104	<10	<10	<10	<10	<10	<10	<10	N	N
Marfil	Pulp	<10	<10	<10	<10	<10	<10	<10	<10	N	N
	Brine	1.0 x 102	<10	<10	<10	<10	<10	<10	<10	N	N
Sevillana	Pulp	3.8 x 105	<10	5.3 x 102	6.7 x 106	<10	<10	1.0 x 103	7.0 x 103	N	N
	Brine	6.1 x 105	<10	7.0 x 102	8.1 x 106	<10	<10	2.6 x 104	8.9 x 104	N	N

The extract of Empeltre olive pulp is dark, almost black, hampering the differentiation of color after the addition of INT. So it was not possible to verify the MIC values for these samples. However, MBC was performed at all concentrations (10 to 100%). The results showed that even the highest concentration evaluated, Empeltre olive pulp has no antimicrobial effect on the microorganisms used in this study. According to these results and in the conditions studied, we could suggest that the Empeltre olive pulp would not be acting as prebiotic, for not having selective effects on microbiota, stimulating the growth of Lactobacillus and inhibiting pathogenic microorganisms.

Empeltreolive brine showed MIC values ranged from 30 to 50% after 24h of incubation. *L. plantarum* was the microorganism more resistant to this treatment. However, with the evaluation of the MBC, we found that the pathogenic microorganisms also showed resistance to higher concentrations of Empeltre olive brine.

We found that MIC treatment with Arbequina olive pulp, *Lactobacillus brevis* and *plantarum* were more sensitive than the pathogenic microorganisms. However, with MBC analysis we were able to verify that all bacteria were resistant to treatment. For the treatment with Arbequina packing brine, *Lactobacillus* showed more resistance to treatment than pathogenic microorganisms and MBC analysis showed that both were resistant. Thus, Arbequina olive packing brine presents a more suitable environment for the development of *Lactobacillus* than its own pulp.

It was not possible to determine the MIC values in the wells with *Lactobacillus* due to dark coloring of the Marfil olive pulp. There was a wide variation among pathogens microorganisms in relation to the MIC values. *Salmonella enteritidis* and *Salmonela typhimurium* did not show the same resistance to treatment than other pathogens. However, after evaluating the results of MBC we find that all microorganisms tested were resistant to treatments with a maximum of 80% pulp.

Marfil olive brine was inhibitorier than olive pulp with average MIC values ranged from 10 to 50%. For the MBC analysis these values increased for all microorganisms.

In general, when we analyze the MBC results of the olive pulps we can verify that the bacteria continued to grow regardless of the pulp concentration used, showing that the bactericidal concentration is higher than concentrations tested in our study. For the olive brines we found most of MBC results among those values that have been tested. So, olive brines had an antibacterial activity highest than olive pulp but also shows no selective effect of the microbiota.

Another result from this study was that gram-positive and gram-negative bacteria have practically the same sensitivity to the tested olive pulps and brines. Jabrane et al (2010) [25] verify that essential oils tested also do not differ in their antimicrobial activity against gram-positive and gram-negative bacteria.

Antimicrobial activity against mould and yeasts: In order to verify the antimicrobial activity of the three olive pulps and brines studied against mould and yeasts, the disc diffusion assay was conducted (Table 3).

Arbequina olive pulp extract resulted in the highest antimicrobial activity. The antimicrobial activity of Arbequina olive pulp extracts occurred in all concentrations evaluated of *Saccharomyces cerevisiae* and *Candida albicans*, with highest clear zones in 100% concentration. On the other hand, definitive clear zones were not seen in *Penicillium rugulosum* and *Aspergillus flavus*plates.

Table 2: Results of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Arbequina, Empeltre and Marfil olives pulp extracts and packing brines.

MICROORGANISMS	OLIVES	ARBEQUINA		EMPELTRE		MARFIL	
		MIC(%)	MBC(%)	MIC(%)	MBC(%)	MIC(%)	MBC(%)
L. brevis	Pulp	10	>100	/	>100	/	100
	Brine	>100	>100	30	50	30	53
L. Plantarum	Pulp	30	>100	/	>100	/	>100
	Brine	80	>100	50	80	23	97
B. sublitis	Pulp	100	>100	/	>100	100	>100
	Brine	50	>100	30	>100	43	>100
Pseudomonas	Pulp	>100	>100	/	>100	>100	>100
	Brine	30	87	30	77	50	93
E. coli	Pulp	93	>100	/	>100	87	>100
	Brine	30	87	43	50	30	70
Listeria	Pulp	100	>100	/	>100	93	100
	Brine	50	100	30	43	30	60
S. enteritidis	Pulp	50	>100	/	>100	37	>100
	Brine	30	80	30	80	10	70
S. typhimurium	Pulp	50	>100	/	>100	37	>100
	Brine	30	>100	30	67	10	60

Table 3: Inhibition values (in millimeters ± SD) observed in mould and yeast after treatment with different concentrations of Arbequina, Empeltre and Marfil olive pulp extracts and packing brines.

MICROORGANISMS		SACCHAROMYCES		CANDIDA		PENICILLIUM		ASPERGILLUS	
OLIVES		Pulp	Brine	Pulp	Brine	Pulp	Brine	Pulp	Brine
ARBEQUINA	PC	/	/	/	/	/	/	/	/
	10%	3.3 + 5.8	/	0.3 ± 0.2	/	/	/	/	/
	30%	2.3 ± 4.0	/	2.2 ± 2.9	/	/	/	/	/
	50%	4.0 ± 6.5	/	1.9 ± 2.0	/	/	/	/	/
	80%	2.7 ± 4.6	/	1.5 ± 2.6	/	/	/	/	/
	100%	6.7 ± 11.1	/	8.0 ± 7.7	/	/	/	/	/
EMPELTRE	PC	/	/	/	/	/	/	/	/
	10%	/	/	/	/	/	/	/	/
	30%	0.3 ± 0.6	/	0.3 ± 0.6	/	/	/	/	/
	50%	1.9 ± 3.3	/	0.8 ± 0.7	/	/	/	/	/
	80%	1.0 ± 1.7	/	0.5 ± 0.8	/	/	/	/	/
	100%	2.7 ± 4.6	/	1.75± 1.5	/	0.8±1.4	/	/	/
MARFIL	PC	/	/	/	/	/	/	/	/
	10%	0.3±0.4	/	/	/	/	/	/	/
	30%	0.5±0.5	/	/	/	/	/	/	/
	50%	0.6±0.5	/	/	/	/	/	/	/
	80%	0.3±0.4	/	0.2±0.2	/	/	/	/	/
	100%	1.7±0.9	/	/	/	/	/	0.5±0.5	/

Empeltre olive pulp extracts also showed antimicrobial activity against *Saccharomyces cerevisiae* and *Candida albicans* but with lower clear zones and did not show activity in the lower extract concentration (10%). This olive pulp had an antimicrobial activity against *Penicillium rugulosum* with concentration of 100%.

Marfil olive pulp extracts showed the lower antimicrobial action when compared with the others olives pulp extracts. This activity was detected in all concentrations to *Saccharomyces cerevisiae*, and only one concentration to *Candida albicans*(80%) and *Aspergillus flavus* (100%).

Olive brines have not antimicrobial activity. In three independent experiments grown mould and yeasts in all brine olives analyzed.

CONCLUSION

In general, the results obtained have shown that is necessary improve the quality and safety of olives using good practices in agriculture, hygiene and manufacturing.

Olive brines had an antibacterial activity highest than olive pulp extracts but, they have not antimicrobial activity against mould and yeasts.

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