

## Antimicrobial Activity of Olive Oil, Vinegar, and Various Beverages against Foodborne Pathogens

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### ABSTRACT

The survival of foodborne pathogens in aqueous extracts of olive oil, virgin olive oil, vinegar, and several beverages was evaluated. Vinegar and aqueous extracts of virgin olive oil showed the strongest bactericidal activity against all strains tested. Red and white wines also killed most strains after 5 min of contact, black and green tea extracts showed weak antimicrobial activity under these conditions, and no effect was observed for the remaining beverages (fruit juices, Coca-Cola, dairy products, coffee, and beer). The phenolic compound content of the aqueous olive oil and virgin olive oil extracts could explain their antibacterial activity, which was also confirmed in mayonnaises and salads used as food models. Virgin olive oil in mayonnaises and salads reduced the counts of inoculated *Salmonella* Enteritidis and *Listeria monocytogenes* by approximately 3 log CFU/g. Therefore, olive oil could be a hurdle component in certain processed foods and exert a protective effect against foodborne pathogens when contaminated foods are ingested.

Despite increased hygiene and advanced food production techniques, there is a serious problem concerning the growing number of foodborne illness outbreaks. While commercially produced foods are generally safe, home-made foods are more sensitive to microbial contamination. Virulent foodborne pathogens have recently been detected even in acidified products (17). At the same time, consumers are avoiding foods containing chemical preservatives. Therefore, the demand for natural biopreservatives and safer foods is increasing (5).

Plants, herbs, spices, and certain foods contain substances that possess antimicrobial activity. Among them, wine and tea are the two most reported foods with bactericidal activity. Survival of *Salmonella* Typhimurium, *Shigella sonnei*, and *Escherichia coli* in common beverages (cola, beer, milk, and wine) has been evaluated (26), and wine showed the highest antibacterial effect. Ethanol was ruled out as responsible for this activity when these pathogens were exposed to red and white wine and died after 20 min of contact (28). However, other researchers have attributed this activity to the ethanol content in wine, along with organic acids and low pH (18). Antilisterial activity has also been found in red grape juice free of ethanol and preservatives, which was related to its phenolic composition, in particular the polymeric phenolic fraction (22). Simple phenols in wine and grapes (e.g., gallic acid, flavanols, anthocyanins) have also exhibited antimicrobial activity (20, 29).

Tea extracts have been shown to have antibacterial activity against gram-positive and, to a lesser extent, gram-negative bacteria (27), and several studies have disclosed that purified catechin fractions from green and black tea

inhibited the growth of many bacterial species (9, 31). In addition, teaflavins from black tea showed strong antibacterial effects against *Bacillus cereus* (8). It seems, therefore, that the bactericidal activity of these teas could be due to the level of catechins and teaflavins.

Coffee extracts also exhibit strong antibacterial action against a broad spectrum of microorganisms (19, 27), and beer has been considered a safe product against pathogens for decades because of its low pH and ethanol content (14). Milk possesses a natural antimicrobial system, the lactoperoxidase system, that has been used on many occasions to preserve raw milk quality (24), and vinegar, because of its high content in acetic acid, was found to be markedly effective in preventing bacterial food poisoning (6, 30).

Olive oil is consumed directly on toast and in fresh salads, but it is also used in commercially processed foods (e.g., tuna, tomato, mayonnaise) and in many homemade dishes. Polyphenols present in the olive fruits, olive oil waste waters, and olive leaves possess antimicrobial activity against a broad spectrum of microorganisms (3, 7, 15). However, studies on the antimicrobial activity of olive oil polyphenols are few (11, 21) and are focused on simple phenols, which represent only a minor amount of the total content. Recently, we investigated the role of phenolic compounds on the bactericidal activity of many different olive oils (16). Four substances were identified as mainly responsible for this activity: hydroxytyrosol, tyrosol, and the dialdehydic form of decarboxymethyl ligstroside and oleuropein aglycons, with the last two having the strongest bactericidal activity.

The objective of this study was to compare the antimicrobial activity of aqueous extracts of olive oil and the above beverages against several foodborne pathogens. In addition, the antibacterial effect of virgin olive oil and olive oil on inoculated mayonnaises and salads was tested.

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## MATERIALS AND METHODS

**Beverages.** Peach juice (pH 4.0), pineapple juice (pH 4.0), orange juice (pH 4.0), cow's milk (pH 7.0), yogurt drink (pH 4.6), Coca-Cola (pH 2.7), beer with 5% alcohol (pH 4.6), alcohol-free beer (pH 4.3), red wine (pH 3.7, 13% alcohol), white wine (pH 3.3, 11.5% alcohol), vinegar (pH 2.9, 5% acetic acid), coffee, tea, virgin olive oil, and olive oil were purchased from local department stores. One and a half grams of coffee powder or decaffeinated coffee powder was mixed in 160 ml of tap water for 5 min at boiling temperature to obtain coffee extracts. The pH values of caffeinated and decaffeinated coffee suspensions were 5.3 and 5.7, respectively. Green and black tea infusions were made by steeping 2 g of leaves in 160 ml of boiling water for 5 min. The pH values of green and black tea suspensions were 7.0 and 6.0, respectively. Virgin olive oil and olive oil extracts were obtained by mixing 5 g of oil and 5 ml of saline (0.85% NaCl) for 1 min at room temperature. The mixtures were centrifuged, and the aqueous phase was collected with a Pasteur pipette. The pH values of the virgin olive oil and olive oil extracts were 3.8 and 4.1, respectively.

**Bacterial strains and culture conditions.** Two strains each of *Salmonella enterica* serovar Enteritidis (CECT 4300 and CECT 4156), *Listeria monocytogenes* (CECT 4031 and CECT 4032), *Staphylococcus aureus* (CECT 86 and CECT 239), and *E. coli* O157:H7 (CECT 4267 and CECT 5947) were obtained from the Spanish Type Culture Collection (CECT) at Burjassot, Valencia, Spain. One strain of *Yersinia* sp. 5057655 and *S. sonnei* JCP were a gift from Dr. José-Carlos Palomares (Valme University Hospital, Seville, Spain). *L. monocytogenes* and *Yersinia* sp. were grown in brain heart infusion broth (Oxoid, Basingstoke, UK). *S. aureus*, *Salmonella* Enteritidis, *S. sonnei*, and *E. coli* were cultured in nutrient broth containing, per liter, 5 g of "Lab-lemco" powder (Oxoid), 10 g of meat peptone (Pronadisa, Laboratorios Conda, Madrid, Spain), and 5 g of NaCl (Panreac, Barcelona, Spain).

**Bactericidal effect of beverages.** One hundred microliters of diluted inoculum (in saline) from the overnight broth cultures was added to 4 ml of each product and left for 5 min at room temperature with occasional vortexing. A preliminary screening revealed that most of the products tested (fruit juices, dairy products, beers, Coca-Cola and coffee, teas, and olive oil extracts) showed low antibacterial activity. As a consequence, an initial population of  $\sim 10^5$  CFU/ml was chosen to test the bactericidal effect of these beverages, and an initial population of  $\sim 10^6$  CFU/ml was used for virgin olive oil extract, vinegar, and wines.

After 5 min of contact, survivors were determined by plating these mixtures on the appropriate nonselective agar media, both spreading 0.1 ml on the surface (limit of detection was 10 CFU/ml for all liquid products) and plating the  $10^{-1}$  dilution (0.1% peptone water) with a spiral plater (model WASP 2, Don Whitley Sci. Ltd., Shipley, UK). Colonies were enumerated with an automated counter (Counterstat, IUL Instruments, Barcelona, Spain). Each experiment was replicated twice, and duplicates were always included.

**Mayonnaise preparation and experimental design.** Milk mayonnaises were prepared by adding 300 ml of oil (sunflower oil, olive oil, or virgin olive oil), 150 ml of cow's milk, 11 ml of fresh lemon juice, and 1 ml of a diluted *L. monocytogenes* culture to a vessel to obtain an initial inoculum of  $\sim 2 \times 10^3$  CFU/ml. The components were mixed with a Braun MR 5000 M hand blender (Braun Española S.A., Barcelona, Spain) until the oil emulsified. The pH mean values were 4.9, 5.4, and 4.5 for sunflower oil, olive oil, and virgin olive oil milk mayonnaises, res-

spectively. After 10 and 30 min, 0.1-g samples were spread onto brain heart infusion agar plates and incubated for up to 48 h at 37°C; colonies were then counted (limit of detection, 10 CFU/g). Milk mayonnaise (instead of egg) is prepared in many restaurants in Spain to prevent *Salmonella* contamination.

Egg mayonnaises were similarly prepared, except that two eggs were used instead of milk, and *Salmonella* Enteritidis was the target microorganism. Additionally, egg mayonnaises without lemon juice were studied. The pH mean values were 4.7, 4.9, and 4.6 for sunflower oil, olive oil, and virgin olive oil egg mayonnaises, respectively, and 7.8, 7.4, and 7.2 for the same mayonnaises without lemon juice. These mayonnaise experiments were replicated twice.

**Salad preparation and experimental design.** To prevent interference from background microbiota, inner leaves of commercial iceberg lettuce were first washed with tap water and then immersed in a chlorine solution (230 ppm of sodium hypochlorite) at 30 to 35°C for 15 min. Finally, lettuce leaves were washed with tap water to leach chlorine. Fifty grams of lettuce was aseptically cut with a scissors and mixed with 27.5 ml of oil (sunflower oil, olive oil, or virgin olive oil), 25 ml of saline water (0.1% NaCl) containing the inoculum, and 2.5 ml of fresh lemon juice, vinegar, or water as the control. The salads were inoculated with *L. monocytogenes* at a level of  $2 \times 10^3$  CFU/g. All the ingredients were thoroughly hand mixed with autoclaved forks. After 30 min of occasional blending, salads were transferred to a stomacher bag together with 100 ml of 0.85% NaCl and blended for 60 s at high speed in a Seward 400 stomacher (Seward Medical Ltd., London, UK). Surviving cells were enumerated by spiral plating 50- and 100- $\mu$ l aliquots on brain heart infusion plates, which were incubated at 37°C for up to 3 days (limit of detection, 20 CFU/ml).

**Analysis of polyphenols.** Phenolic extracts of olive oils were obtained following the procedure described elsewhere (2). Briefly, 0.6 g of olive oil was extracted with  $3 \times 0.6$  ml of *N,N*-dimethylformamide; the extract was then washed with hexane, and  $N_2$  was bubbled into the *N,N*-dimethylformamide extract to eliminate residual hexane. Finally, the extract was filtered through a 0.45- $\mu$ m-pore-size membrane and injected into the chromatograph.

Analysis of polyphenols in the aqueous olive oil extracts was made by directly injecting the solution into the chromatograph after filtration through a 0.45- $\mu$ m-pore-size filter.

The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters column heater module (Waters, Milford, Mass.). A Sherisorb ODS-2 (5- $\mu$ m; inner diameter, 25 cm by 4.6 mm; Waters) column was used. Separation was achieved with an elution gradient (2) having an initial composition of 90% water (pH adjusted to 3.0 with phosphoric acid) and 10% methanol. A flow rate of 1 ml/min and a temperature of 35°C were used in all experiments. A Waters 996 diode array detector and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) were connected in series, and quantification of compounds was done as described elsewhere (16). Phenolic extracts were analyzed by liquid chromatography-mass spectrometry with a ZMD4 mass spectrometer (Waters) equipped with an electrospray (ESI) probe, working in the negative-ion mode. Cone voltage fragmentation was 20 V, capillary voltage was 3 kV, desolvation temperature was 250°C, source temperature was 120°C, and extractor voltage was 12 V. A constant flow rate of 1 ml/min was used for each analysis with a split ratio of approximately 5:1 (UV detector:mass spectrometry).

## RESULTS AND DISCUSSION

All tested pathogens survived in the fruit juices studied after 5 min of contact; none of these juices produced a cell

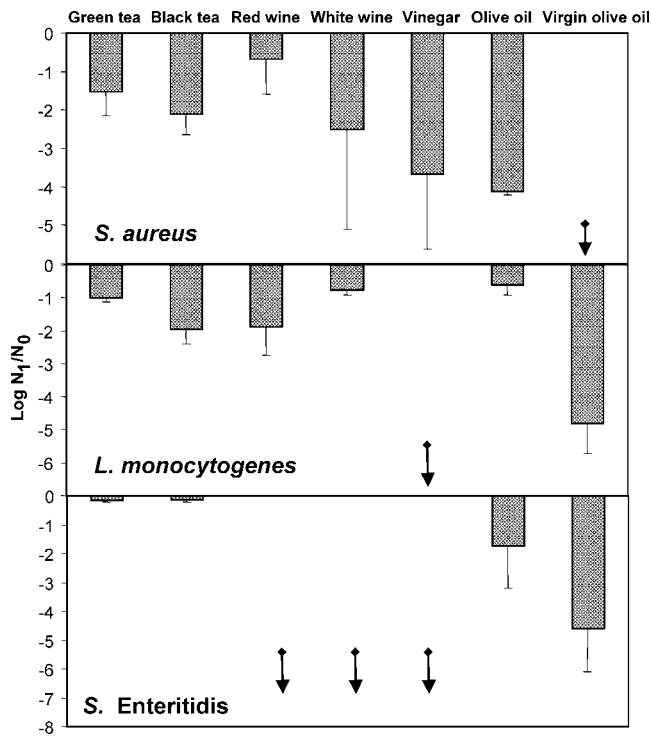


FIGURE 1. Bactericidal effect of wine, vinegar, and tea and olive oil extracts against several foodborne pathogens.  $N_0$  = CFU per milliliter inoculated,  $N_1$  = CFU per milliliter after 5 min of contact. Bars = the standard deviation of results from two strains of *S. aureus*, *L. monocytogenes*, and *Salmonella Enteritidis*. Arrows mean that the microorganism survival was below the detection limit.

reduction greater than 1 log (data not shown). To our knowledge, there are no articles dealing with the antimicrobial activity of fruit juices. However, the low pH ( $\sim 4.0$ ) and acidic environment could contribute to microbial control, with the survival and growth of *E. coli* in apple and orange juices now of great concern (17, 23). In this study, the microbial counts were determined after 5 min of contact to look for strong bactericidal effects, and it is obvious that the low pH was not sufficiently inhibitory to the foodborne pathogens tested.

Bacteria also survived for at least 5 min in dairy products and Coca-Cola. It could be predicted that these microorganisms would survive in milk at pH 7 and be affected by the pH 4.6 of the yogurt drink, but it was surprising that they survived even at a pH as low as 2.7, as in the case of Coca-Cola. In fact, these results confirmed those obtained by Sheth et al. (26), who observed the survival of foodborne pathogens in milk and cola for up to 48 h. It seems, therefore, that pH alone cannot significantly reduce the number of pathogens in these beverages. It has to be said that only yogurt drinks contained background microbiota (the lactic acid bacteria used for manufacture), which could be easily distinguished from the inoculated pathogens because of their different colony morphologies and growth rates, but neither of the other liquid products nor the mayonnaises or salads contained background microbiota that could interfere with the counts of the inoculated pathogens.

It is assumed that beer is currently free of pathogenic

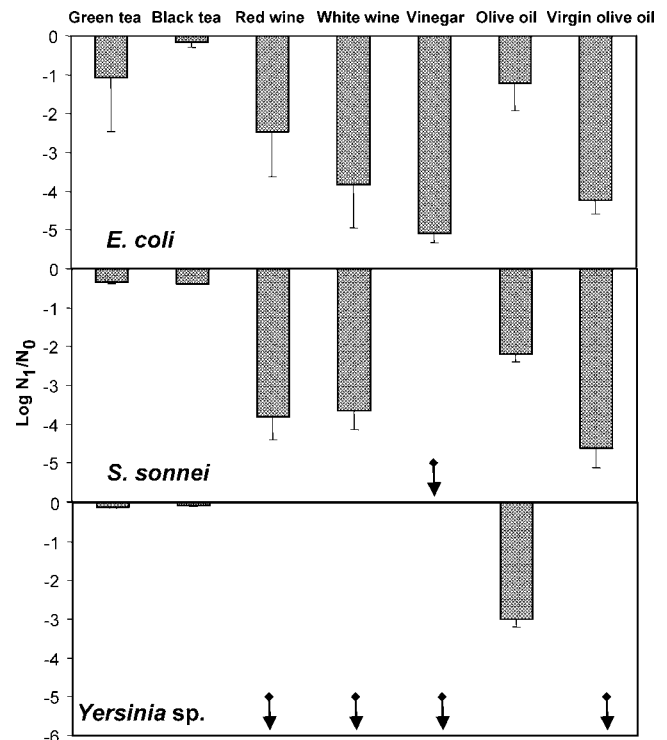


FIGURE 2. Bactericidal effect of wine, vinegar, and tea and olive oil extracts against several foodborne pathogens.  $N_0$  = CFU per milliliter inoculated,  $N_1$  = CFU per milliliter after 5 min of contact. Bars = the standard deviation of results from two strains of *E. coli* and duplicate runs for a single strain of *S. sonnei* and *Yersinia sp.* Arrows mean that the microorganism survival was below the detection limit.

microorganisms because of its low pH and ethanol concentration, as well as the pasteurization treatment, but concern about bacteria in alcohol-free beer has increased (14). In this study, a microorganism reduction was not found in alcohol-free beer or in beer containing 5% alcohol, except in alcoholic beer inoculated with one of the two *Salmonella Enteritidis* strains, in which a 1.4-log reduction was observed. Hence, a relatively low pH (4 to 4.5) and ethanol concentration ( $\leq 5\%$ ) did not appear to rapidly kill foodborne pathogens inoculated in these beverages.

Bactericidal activity has also been found in aqueous coffee extracts, but data are contradictory, in particular about the growth inhibition of *E. coli* (19, 27). We did not detect any microbial reductions after 5 min of contact in any of the coffee extracts inoculated, which means that neither the concentration of caffeine in these extracts nor in the coffee polyphenols exerted rapid inhibitory action.

By contrast, wine, vinegar, tea, and olive oil extracts showed bactericidal activity against the foodborne pathogens tested (Figs. 1 and 2). Among them, vinegar showed the strongest bactericidal effect, followed by the aqueous extract of virgin olive oil, wines, and olive oil and tea extracts. Vinegar reduced the counts of inoculated *L. monocytogenes*, *Salmonella Enteritidis*, *S. sonnei*, and *Yersinia sp.* to levels below the detection limit and killed most of the *E. coli* and *S. aureus* cells. The strong bactericidal activity of vinegar is well known (6, 30) because of its high acetic acid content, and our data confirmed these previous



TABLE 1. Phenolic composition of the olive oil and virgin olive oil used in the experiments<sup>a</sup>

Compound	Initial oil		Aqueous extract	
	Virgin olive oil	Olive oil	Virgin olive oil	Olive oil
Hydroxytyrosol glycol	3.9 (0.0)	ND	1.6 (0.2)	ND
Hydroxytyrosol	52.8 (6.5)	14.1 (0.2)	36.9 (0.8)	13.5 (0.5)
Tyrosol	17.0 (0.3)	7.4 (0.2)	15.2 (0.3)	8.2 (0.2)
Hydroxytyrosol	ND	11.5 (0.1)	ND	4.0 (0.2)
Dialdehydic form of decarboxymethyl oleuropein aglycon	179.1 (11.9)	79.4 (5.1)	118.4 (6.0)	28.1 (0.7)
Dialdehydic form of decarboxymethyl ligstroside aglycon	78.5 (0.5)	35.5 (1.2)	26.7 (0.4)	5.8 (0.1)
L-Acetoxypinoresinol	16.3 (1.4)	21.0 (0.9)	1.8 (0.0)	3.7 (0.2)
Pinoresinol	56.9 (1.9)	37.7 (0.4)	5.4 (0.3)	3.5 (0.3)
Oleuropein aglycon	210.9 (12.0)	147.6 (0.7)	52.3 (3.5)	25.5 (1.3)
Ligstroside aglycon	65.6 (11.8)	62.7 (0.7)	5.0 (0.9)	1.8 (0.6)
Luteolin	3.2 (1.5)	2.1 (0.0)	ND	ND
Apigenin	0.4 (0.3)	0.6 (0.0)	ND	ND
Total	684.6 (48.1)	419.6 (9.5)	263.3 (12.4)	94.1 (4.1)

<sup>a</sup> Values are expressed as milligrams per kilogram with standard deviation in parentheses. ND, not detected.

results. Surprisingly, the aqueous extracts of virgin olive oil exerted an effect similar to that of vinegar, which is a very new and interesting finding. Neither *S. aureus* nor *Yersinia* sp. was detected after 5 min of contact with the aqueous extract of virgin olive oil, which means a >5-log reduction with reductions of >4 log for the remaining pathogens.

There are a great number of articles dealing with the antimicrobial properties of tea (27, 31), and it was surprising that the black and green tea extracts produced only a slight bactericidal action against most pathogens. Tea extracts and their active principal components (catechins and teaflavins) have been described as growth inhibitors of both gram-positive and gram-negative bacteria (8, 9) but were inactive when they were tested in a food model (ground beef) (13), and our experiments showed reductions lower than 3 log for *S. aureus*, *L. monocytogenes*, and *E. coli* and a nonsignificant effect against *Salmonella* Enteritidis, *S. sonnei*, and *Yersinia* sp. (Figs. 1 and 2).

In contrast, both red and white wines were potent bacteria killers, in particular against *Salmonella* Enteritidis and *Yersinia* sp., whose cell counts were below the detection limit after 5 min of exposure. *S. aureus* and *L. monocytogenes* were the least sensitive pathogens to wines. These data are in agreement with previous findings (10, 26, 28) that relate the antimicrobial activity of wine to the synergistic action of organic acids, low pH, and ethanol (18) as well as phenolic compounds (20). This antimicrobial activity of wine has been known since antiquity, and researchers advise its consumption to protect against some food poisoning organisms (10), although the ingestion of ethanol must be taken into consideration.

The olive oil extracts exhibited weaker bactericidal action than virgin olive oil but stronger than tea extracts. Recently, we investigated the antimicrobial activity of virgin olive oil and olive oil extracts in vitro against a broad spectrum of bacteria (16), and we sought to compare these results with those of other well-documented bactericidal beverages. We also found that this activity was related to the phenolic content of these extracts, in particular the syner-

gistic action of hydroxytyrosol, tyrosol, and the dialdehydic form of decarboxymethyl ligstroside and oleuropein aglycons (16). Table 1 displays the phenolic composition of the oils and their aqueous extracts. As expected, virgin olive oil had a higher concentration of total and individual polyphenols than olive oil, and the same relationship can be observed for their aqueous extracts. Almost twice the amount of these substances was present in the virgin olive oil extracts than in the olive oil extracts, which can be related to the higher bactericidal effect of the former extracts. Since virgin olive oil is obtained directly from olive fruits and olive oil is a mixture of refined olive oil and virgin olive oil, the polyphenol content of olive oil comes from the initial content of the virgin olive oil added.

Olive oil may be consumed directly on bread and in fresh salads, but it is also used in many homemade dishes (e.g., mayonnaise, cakes) and processed foods (e.g., canned tuna, fried tomato, meat foods). Hence, the bactericidal activity of olive oil polyphenols may be exerted directly on these foods, but it may also act as a hurdle component in certain contaminated meals during their ingestion. Wine consumption was linked to a reduction in illness related to the spoilage of contaminated tuna sandwiches and raw oysters (1, 4), and it has been proposed as a digestive aid (28) to provide a protective effect against foodborne pathogens when contaminated foods are ingested. A similar suggestion, therefore, could be applied to olive oil on the basis of the results obtained in this study.

At the same time, mayonnaises and salads were used as food models to confirm the results obtained on the bactericidal effect of olive oil and virgin olive oil extracts. The survival of *Salmonella* Enteritidis inoculated in egg mayonnaises during their preparation and the effect of adding lemon juice were evaluated at 10 and 30 min. Results in Figure 3 confirmed the bactericidal action of the virgin olive oil and, to a lesser extent, of the olive oil in this food model. This effect was also time-dependent and seen only when lemon juice was added. Interestingly, viable counts of *Salmonella* Enteritidis were below the detectable limit in

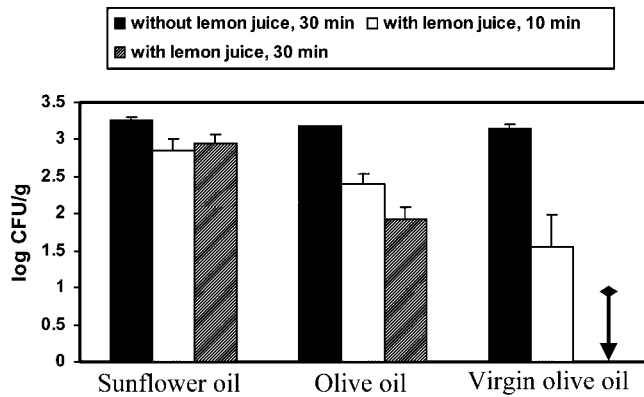


FIGURE 3. Survival of *Salmonella Enteritidis* in egg mayonnaises elaborated with different oils and initially inoculated with  $2 \times 10^3$  CFU/g. Bars = the standard deviation of two experiments, and the arrow means that the population was below the detection limit.

mayonnaise made with virgin olive oil after 30 min, which means a reduction of 3 log CFU/g. Egg mayonnaise made with virgin olive oil (21) and inoculated after its preparation needed more than 48 h to reduce the number of microorganisms to an undetectable level. Investigators attribute the bactericidal action of olive oil in this assay to oil acidity and simple phenols. On the basis of our previous results (16), it is reasonable to assign the differences in bactericidal activity among oils to the different composition in phenolic compounds, in particular the four mentioned above.

Milk mayonnaises inoculated with *L. monocytogenes* during preparation were also used as food models. Again (Fig. 4), the use of virgin olive oil decreased bacterial populations below the detectable limit after 30 min, with this effect being weaker for olive oil because of its lower content in certain phenolic compounds (Table 1).

The survival of inoculated *L. monocytogenes* on lettuce seasoned with different types of oils, lemon juice, and vinegar after a 30-min treatment is reflected in Figure 5. The effectiveness of vinegar and lemon juice in reducing the counts of inoculated *Salmonella* Typhimurium on carrots and *Listeria innocua* on several fruits and vegetables has been reported (12, 25), and reductions of 1 to 3 log CFU/g were found. In our experiments, slight reductions of *L.*

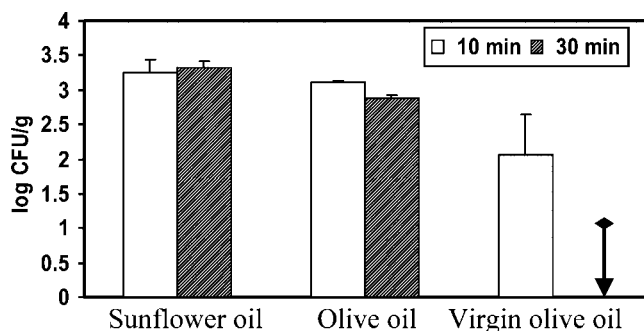


FIGURE 4. Survival of *L. monocytogenes* in milk mayonnaises elaborated with different oils and initially inoculated with  $2 \times 10^3$  CFU/g. Bars = the standard deviation of two experiments, and the arrow means that the population was below the detection limit.

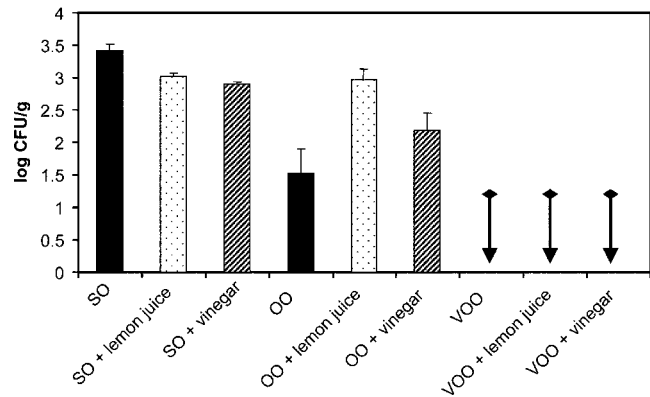


FIGURE 5. Survival of *L. monocytogenes* in lettuce salads elaborated with sunflower oil (SO), olive oil (OO), and virgin olive oil (VOO). Salads were dressed with lemon juice, vinegar, or neither. They were inoculated with  $2 \times 10^3$  CFU/g and analyzed 30 min after preparation. Bars = the standard deviation of two experiments, and arrows mean that the population was below the detection limit.

*monocytogenes* on lettuces seasoned with sunflower oil and vinegar or lemon juice alone were observed. By contrast, *L. monocytogenes* cells were killed after 30 min when virgin olive oil was used, regardless of the lemon juice or vinegar added. These results again confirmed the strong bactericidal activity of virgin olive oil and compounds diffused to the aqueous phase of the salad, such as certain phenolic compounds.

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