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Effect of Mixed Antimicrobial Agents and Flavors in Active Packaging Films

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Active packaging is an emerging food technology to improve the quality and safety of food products. Many works have been developed to study the antimicrobial activity of essential oils. Essential oils have been traditionally used as flavorings in food, so they have an important odor impact but they have as well antimicrobial properties that could be used to protect the food. Recent developments in antimicrobial active packaging showed the efficiency of essential oils versus bread and bakery products among other applications. However, one of the main problems to face is the odor and taste they could provide to the packaged food. Using some aromas to mask the odor could be a good approach. That is why the main objective of this paper is to develop an antimicrobial packaging material based on the combination of the most active compounds of essential oils (hydrocinnamaldehyde, oregano essential oil, cinnamaldehyde, thymol, and carvacrol) together with some aromas commonly used in the food industry. A study of the concentration required to get the antimicrobial properties, the organoleptic compatibility with typical aroma present in many food systems (vanilla, banana, and strawberry), and the right combination of both systems has been carried out. Antimicrobial tests of both the mentioned aromas, the main components of some essential oils, and the combination of both groups were carried out against bacteria (*Enterococcus faecalis*, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella choleraesuis*, *Yersinia enterocolitica*, *Escherichia coli*), yeasts (*Candida albicans*, *Debaryomyces hansenii*, *Zygosaccharomyces rouxii*), and molds (*Botrytis cinerae*, *Aspergillus flavus*, *Penicillium roqueforti*, *Eurotium repens*, *Penicillium islandicum*, *Penicillium commune*, *Penicillium nalgiovensis*). The sensory properties of the combinations were evaluated with a triangular test and classification was by an order test; the odor threshold of the aroma compounds was also studied. The results reveal that none of the aromas had antimicrobial properties. The most antimicrobial compounds are thymol, carvacrol, and cinnamaldehyde, but none of them could be combined with banana aroma, whereas only thymol with strawberry aroma gave the right combined organoleptic profile. All of the antimicrobials under study could be combined with vanilla aroma, providing both antimicrobial property and the odor expected.

KEYWORDS: Aroma; antimicrobial; active packaging; compatibility; sensory analysis; food protection

INTRODUCTION

The new lifestyle in which food products are in the supermarket for long periods demands progressive increase in their shelf life. However, a longer shelf life should not imply the loss of properties, especially concerning foodstuffs in which two vital topics, food safety and food quality, can be emphasized. Microbial infections including the presence of molds in many food products are the most limiting reason why many products cannot sit for long periods. Also, the decrease of quality in terms of organoleptic properties such as aroma losses (*1*) affects the length of the shelf life. For these reasons, the development of new systems to guarantee both the safety and quality of packaged food is more and more important.

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Several techniques are currently applied to fight food deterioration such as modified atmosphere, vacuum packaging, sterilization processes, and freezing. However, emerging technologies such as active packaging provide a promising solution in which the shelf life of the packaged product can be considerably extended without affecting either the food or the process, as the solution is in the packaging material itself. This way, the food can be healthier, more natural, and free of preservatives while maintaining all of the good properties, including food safety, as the preservatives remain in the packaging, which is not eaten. One of the key points in active packaging is the active agent to be used, and this depends on the objective of the new packaging material, which means if antimicrobial or antioxidant properties are required or even the specific aromas for a kind of food. This paper deals with a study carried out with active packaging that

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Table 1. Odor Threshold (12 Panelists; Samples Compared against Control)

aroma	sample	correct answer	significant difference (p = 0.05)?
vanilla	0.01%	3	no
vanilla	0.1%	10	yes
vanilla	1%	8	yes
vanilla	0.1 vs 1%	5	no
vanilla	0.01 vs 0.1%	8	yes
strawberry	0.1%	5	no
strawberry	1%	10	yes
strawberry	5%	12	yes
banana	0.1%	8	yes
banana	1%	11	yes
banana	5%	12	yes

incorporates an aroma together with a natural active agent with antimicrobial properties. All of them have been accepted as food additives; therefore, the food additives would be incorporated into the packaging material instead of being added to the food. This represents a great advantage versus the current system of adding preservatives to the food to extend its shelf life.

Working with natural compounds, it is well-known that essential oils from plants and spices behave as excellent antimicrobials or antioxidants (*2−16*), and most of them additionally provide a characteristic aroma. Also, some papers emphasize that thymol, carvacrol, and cinnamaldehyde are the major components and mainly responsible for the antimicrobial properties of most of the essential oils (*17−24*), although it is true that other compounds also present at lower concentrations in the essential oils have also antimicrobial performance. However, although there is a clear interest in this area, as recent research demonstrates (*25−28*), only a few of active packaging materials are commercially available (*16*, *21*, *23*).

Together with antimicrobial packaging, incorporation in the food packaging of specific aromas for food is also challenging. The use of ethanol as an antimicrobial is a common practice, for example, in the bakery industry, and to mask the ethanol, vanilla aroma is often used. Instead of ethanol or an ethanol releaser, essential oils could be incoroporated in the packaging, but in this case it is important to know the compatibility of the odor with the typical aromas used in the food indsutry. On the other hand, the antimicrobial properties of the aromas commonly used in food were never before studied. The taste and odor provided by the essential oils are not always acceptable, and new combinations are required to get both antimicrobial and acceptable organoleptic properties. For these reasons, it is important to find the right combination of the properties of essential oils and the aromas that would provide the required active packaging material. Sensory studies together with antimicrobial tests can supply the information required for getting the best new active material for use in products with basic vanillin, strawberry, or banana aroma. This paper describes the study carried out in this area, in which the sensory studies allowed the selection of the right combination of an aroma and an essential oil that supply the aroma profile and antimicrobial properties, respectively.

MATERIALS AND METHODS

Active Films. The films containing the aromas were produced in the laboratory by incorporating a known concentration (w/w) of aroma and/or antimicrobial compounds in films of polypropylene (PP), suitable for use in food packaging, via a manufacturing process protected by European Patent EP1657181 held by the company ARTIBAL S.A. Briefly, the active material was prepared by applying to the material a formulation containing the aromas, the essential oils, or both.

Table 2. Comparison of Threshold Values for Different Plastic Size Containing Vanilla (12 Panelists)

aroma	sample	correct answer	significant difference (p = 0.05)?
vanilla	0.25−1%	8	yes
vanilla	1−2%	5	no

The aromas used were strawberry flavor, supplied by Jumel Alimentación (Murcia, España); banana (1-butanol, 3-methyl acetate; CAS 123-92-2); and vanilla (benzaldehyde, 4-hydroxy-3-methoxy; CAS 121-33-5). The antimicrobials used were thymol (CAS 89-83-8), carvacrol (CAS 499-75-2), cinnamaldehyde (CAS 14371-10-9), hydrocinnamaldehyde (CAS 104-53-0), and oregano essential oil, supplied by Sigma Aldrich.

Microbial Cultures. The following foodborne microbial strains were selected for use in the tests: Gram-positive bacteria *Enterococcus faecalis* (29212 ATCC, American Type Culture Collection (ATCC)), *Listeria monocytogenes* (7644 CECT, Colección Española de Cultivos Tipo (CECT)), *Bacillus cereus* (495 CECT), and *Staphylococcus aureus* (29213 ATCC); Gram-negative bacteria *Salmonella choleraesuis* (4000 CECT), *Yersinia enterocolitica* (4315 CECT), and *Escherichia coli* (29252 ATCC); yeasts *Candida albicans* (ATCC 64550), *Debaryomyces hansenii* (10353 CECT), *Zygosaccharomyces rouxii* (11928 CECT), and *Botrytis cinerae*; and molds *Aspergillus flavus* (CECT 2687), *Penicillium roqueforti* (21319 IBT; Culture Collection of Fungi (Lyngby, Denmark)), *Eurotium repens* (1800 IBT), *Penicillium islandicum* (2762 NT CECT), *Penicillium commune* (21314 IBT), and *Penicillium nalgiovensis* (12105 IBT). The strains were stored at −18 °C in sterilized skimmed milk and subcultured as follows. Bacteria were subcultured in Mueller−Hinton agar supplied by Sumalsa (Zaragoza, Spain) at 30 °C for 48 h (Gram-positive) or 24 h (Gram-negative). Fungi were subcultured on Sabouraud cloramphenicol agar either at 30 °C for 48 h (yeast) or at 36.5 °C for 7 days (molds).

Antimicrobial Activity Tests. Inhibition tests were prepared in triplicate by inoculating plates of the appropriate solidified agar medium in Petri dishes with 100 μL of a physiological saline solution containing 10^8 colony-forming units (CFU) per milliliter of the organism. Then, each antimicrobial film was placed over the top of a Petri dish, with no direct contact between it and the microorganism. After the incubation period, the number of colonies that had formed on each plate was counted for bacteria and yeast, and the result for molds was given by the percentage of growth in the diameter of the Petri dish between the blank and the sample. Controls without sample films and blanks with PP films without active compounds were included. In the case of the mold *B. cinerae*, the results are included in the plot of yeasts instead of in the molds because the culture and the measurement as CFU were similar to that applied to the yeasts.

Sensory Panel. The test panel that carried out the sensory experiments was composed of 12 trained individuals belonging to the laboratory staff. Sensory evaluations were performed in a tasting room. The average age of the panelists was 36 years old, and 70% were female.

Sample Preparation. The films were prepared as explained above. In the triangular test there were different concentrations of aroma compounds (vanilla, 0.01, 0.1, and 1%; banana, 0.1, 1, and 5%; and strawberry, 0.1, 1, and 5%) in the film. Then a 1 dm^2 piece of this active film was introduced into an opaque glass bottle of 75 cm^3 volume, so that the identification threshold was calculated. For the "classification by order" test, the samples were prepared in the same way, but now the samples contained as well the antimicrobial compounds according to the microbiological results. The combinations tested, according to the microbiological results, were 8% vanilla−2% thymol, 8% vanilla−2% carvacrol, 8% vanilla−2% cinnamaldehyde, 8% strawberry−2% thymol, 8% strawberry−2% carvacrol, 8% strawberry−2% cinnamaldehyde, 8% banana−2% thymol, 8% banana−2% carvacrol, and 8% banana−2% cinnamaldehyde.

These were the nominal concentrations of the active agents, but they did not correspond to the real concentrations in the headspace, which were between 1 ng/mL and 10 μg/mL of the compounds released by the active plastic depending on the compound (*13*, *14*, *21*, *23*).

Sensory Analysis. *Triangular Test (29, 30).* A triangular test between different concentrations of aroma compounds in the active and in the nonactive films was carried out to determine the lowest level that could be detected by the panelists. The panelists had three bottles containing the films, two of them with the same concentration of aroma compounds and

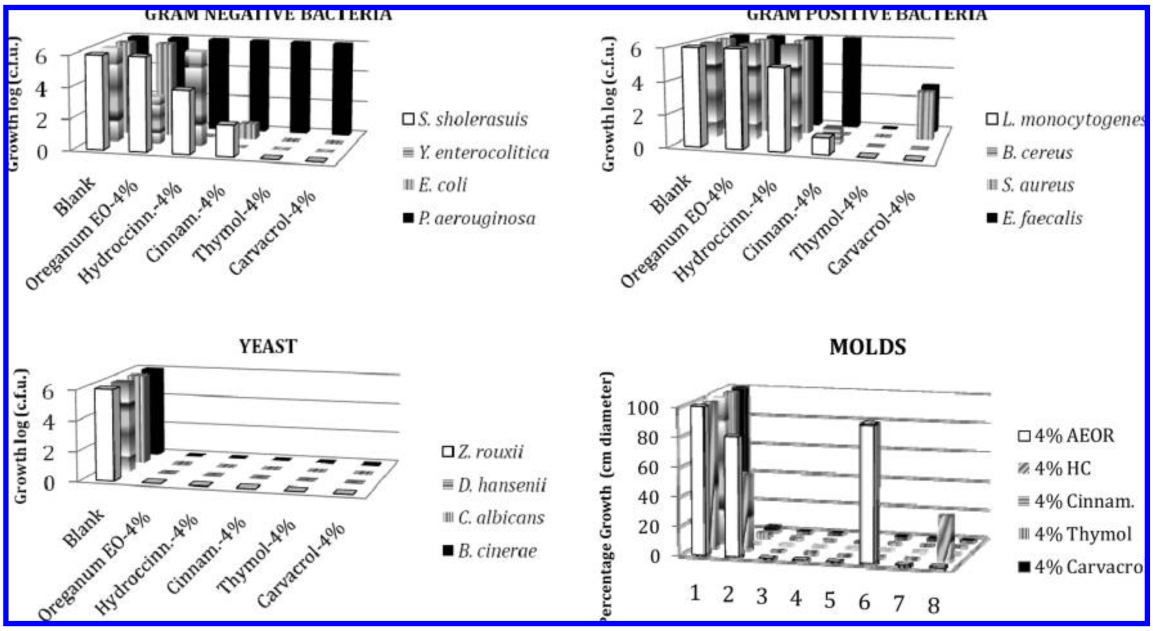

Figure 1. Microorganism growth in the presence of antimicrobial compounds (4%). Molds: 1, blank; 2, *Aspergillus flavus*; 3, *Eurotium repens*; 4, *Penicillium commune*; 5, *Penicillium expansum*; 6, *Penicillium nalgiovense*; 7, *Penicillium roqueforti*; 8, *Penicillium islandicum*.

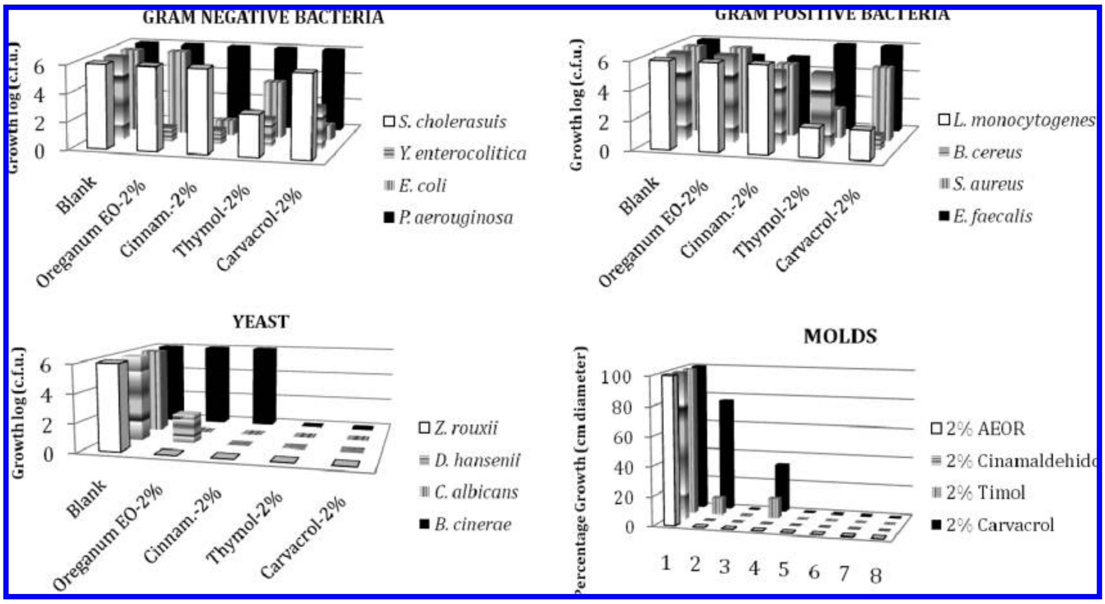

Figure 2. Microorganism growth in the presence of antimicrobial compounds (2%). Molds: 1, blank; 2, *Aspergillus flavus*; 3, *Eurotium repens*; 4, *Penicillium commune*; 5, *Penicillium expansum*; 6, *Penicillium nalgiovense*; 7, *Penicillium roqueforti*; 8, *Penicillium islandicum*.

the third one with a different concentration. They had to determine the different one. The aroma was then released by the active plastic sample to the headspace of the bottle in a similar way as would occur in the real packaged food, where the aroma is perceived in the headspace. The size of the plastic sample was calculated according to the headspace in a packaged bakery product. Thus, it was assumed that the perception of aroma in the bottle would simulate the real perception of the consumer when opening the packaged food.

Classification by Order Test. This test was carried out by 6 trained individuals. In this test each panelist had four samples and these samples must be ordered according to their intensity. Samples were prepared containing only the aroma compounds, and the aroma plus different antimicrobial compounds. The objective was to know which antimicrobial compound provided the best match with the aroma compounds (*31*).

Statistical Analysis. Organoleptic results were analyzed using the Fisher and Friedman tests. The Friedman test is a nonparametric test (distribution-free) used to compare observations repeated on the same subjects. This is also called a nonparametric randomized block analysis of variance and explains significant differences between the samples tested in the classification by order test (*31*). According to the Friedman test the following equation was applied to compare the observations made by the panelists:

$$F = \frac{12}{JP}\frac{X}{(P+1)}$$

where $X = \Sigma R_j^2 - \frac{1}{J(P+1)}$ J is the number of panelists, P is the number of freedom degrees, and R is the score of the sum of the order for each option tested.

RESULTS AND DISCUSSION

This paper has three main objectives: (a) determination of odor thresholds of selected food aromas to get information about the necessary aroma amounts that should be used; (b) study of the

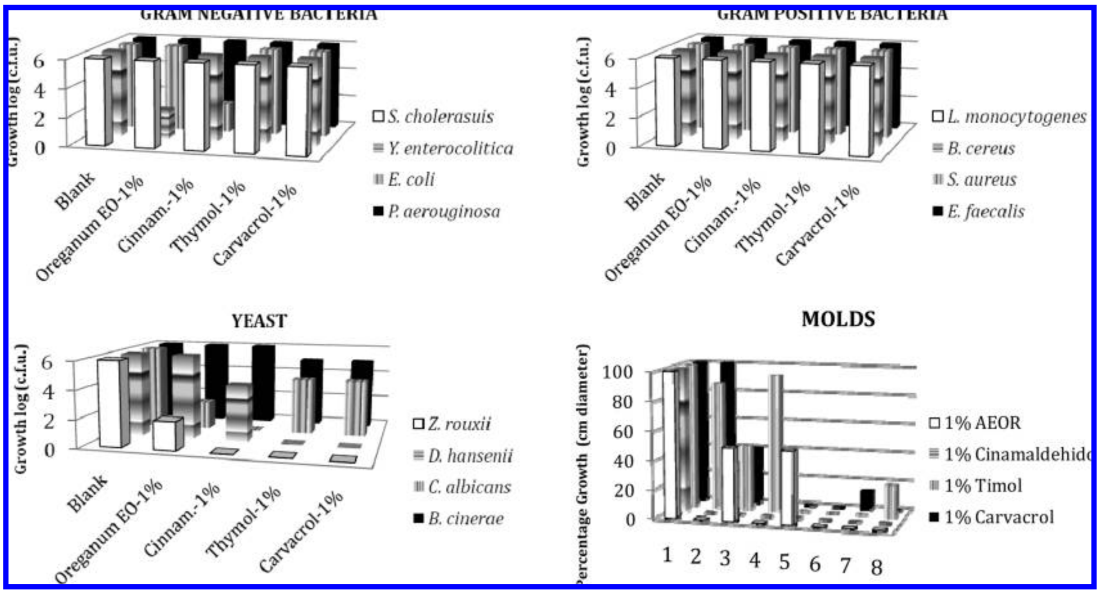

Figure 3. Microorganism growth in the presence of antimicrobial compounds (1%). Molds: 1, blank; 2, *Aspergillus flavus*; 3, *Eurotium repens*; 4, *Penicillium commune*; 5, *Penicillium expansum*; 6, *Penicillium nalgiovense*; 7, *Penicillium roquefort*i; 8, *Penicillium islandicum*.

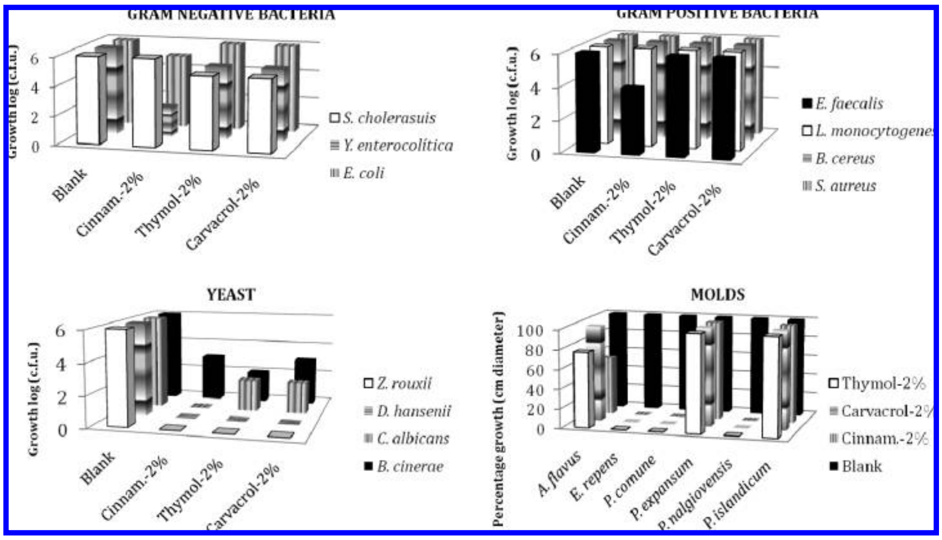

Figure 4. Microorganism growth in the presence of of antimicrobial compounds (2%) + vanilla flavor (8%).

antimicrobial properties of different natural antimicrobial agents and selection of the most efficient ones; and (c) compatibility and sensory study of the combination of aromas and antimicrobial agents.

The results obtained are described in the corresponding sections.

Odor Threshold. Twelve panelists participated in the odor threshold evaluation, and the results were analyzed using a binomial law table settled for one-third probability.

As can be seen in **Table 1**, the lowest concentration of vanilla detected by the panelists was 0.1%, as only three panelists detected the vanilla at 0.01%, and also there were no significant differences between this concentration (0.1%) and 1%. This means that a greater amount of vanilla in the sample film is not perceived as a stronger flavor by the panelists. Thus, when the vanilla flavor is desirable, it is only necessary to reach the threshold in the headspace; additional amounts of vanilla do not produce a stronger perception.

When the concentration of strawberry was 1%, the panelists identified the samples as being different from the control, but they could not identify the aroma. However, when the concentration of strawberry was 5%, the panelists described the aroma as strawberry-like. Therefore, 1% was established as the detection threshold and 5% as the identification threshold.

According to **Table 1**, for the aroma of banana, 0.1% was enough to say that the samples were different, but it was not enough to identify the banana aroma. A 5% concentration level was needed to have the identification threshold, which means that the characteristic compounds of banana aroma were not released at very low concentration level.

Additional tests were carried out modifying the size of the active film, and instead of 1 dm^2, 0.25 and 2 dm^2 were studied with vanilla. The main objective of this test was to check if the threshold of 0.1% was the same independent of the sample film size. Using the triangular test the thresholds of different sample film sizes, 1 versus 0.25 dm^2, were compared, as the small value simulates bigger packaging, because the volume-tp-surface ratio would be higher in the case of 0.25 dm^2 than in the case of 1 dm^2, or 1 versus 2 dm^2, which simulates the smaller packaging.

Table 2 shows the results obtained. From the results we can conclude that a very low concentration of the aroma is required to

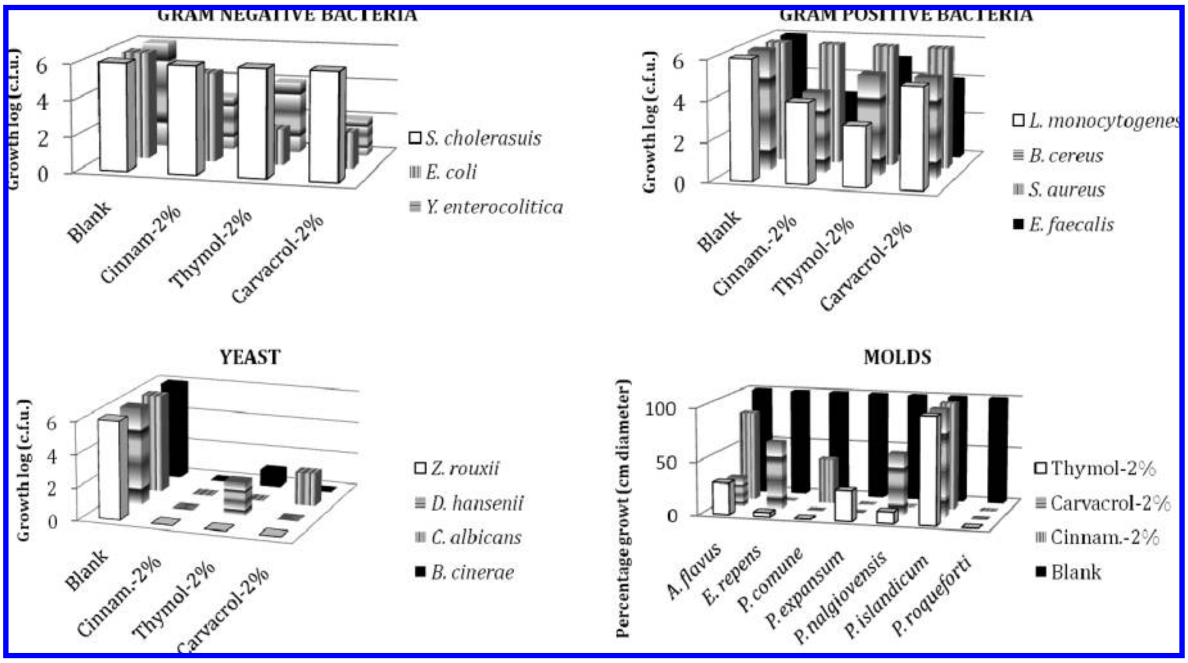

Figure 5. Microorganism growth in the presence of antimicrobial compounds (2%) + strawberry flavor (8%).

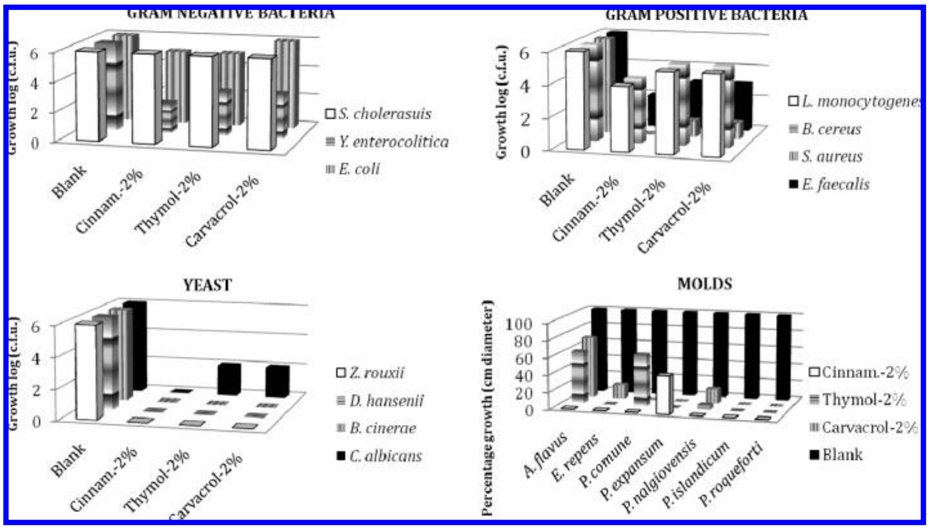

Figure 6. Microorganism growth in the presence of antimicrobial compounds (2%) + banana flavor (8%).

perceive the vanilla odor. Such a concentration is generated by 1 dm^2 of PP containing a concentration of 0.1% of vanilla in 75 cm^3 of air volume. When the concentration of vanilla increases, the perception does not increase. This means that the threshold of 0.1% vanilla can be used for smaller packages but bigger packages require a higher concentration of vanilla to be perceived by the consumer. In other words, the threshold of aroma should be optimized according to the size of the packaging, as it is a function of both the concentration of the aroma in the film and the film size involved in the package.

Microbiological Results. Following the procedures described under Materials and Methods, a series of microbiological tests were carried out with the pure culture strains and the active films containing several mixtures of aroma and antimicrobial compounds. Three different concentrations, 1, 2, and 4%, of antimicrobial compounds were tested. The results obtained showed that most of the molds and yeast were inhibited with a concentration of 4% and also some of the bacteria tested, as shown in **Figure 1**. When the concentration decreased to the level of 2%, most of the molds and yeasts were inhibited, but the effect was not the same for the bacteria, as shown in **Figure 2**. A concentration of 1% was not enough even to prevent the growth of most of the yeasts, some of the molds, and any of the bacteria under study. For this reason, the selected concentration to study the odor impact was 2%, as molds (fungi) are usually the main problem of spoilage in packaged food.

As a result of this study, cinnamaldehyde, thymol, and carvacrol were selected to study the organoleptic compatibility with the aromas, as they were the most effective against the microorganisms, as can be seen in **Figures 1−3**. These results agree with those found in the literature (*13*, *14*, *21*, *24*).

Combination of Antimicrobials and Aromas and Antimicrobial Performance. The same experiment was carried out in Petri dishes covered by the active films containing the mixture of aroma and the selected antimicrobial compounds. **Figures 4− 6** show the plots of the antimicrobial behavior. As can be seen, the most active from an antimicrobial point of view is the mixture of 2% cinnamaldehyde and 8% strawberry for yeasts and molds.

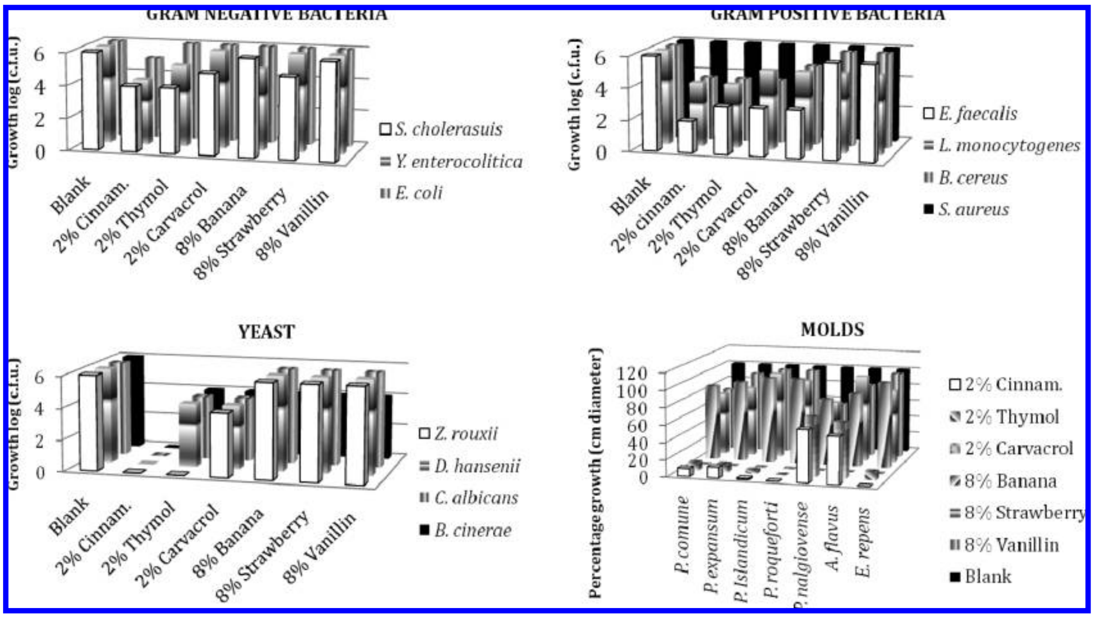

Figure 7. Microorganism growth in the presence of individual compounds.

Table 3. Organoleptic Compatibility

aroma	antimicrobial	score
banana	thymol	15
banana	carvacrol	11
banana	cinnamaldehyde	10
banana		24
strawberry	thymol	16
strawberry	carvacrol	11
strawberry	cinnamaldehyde	9
strawberry		24
vanilla	thymol	18
vanilla	carvacrol	12
vanilla	cinnamaldehyde	11
vanilla		19

The mixture with vanilla provided similar antimicrobial profile for molds with the three antimicrobial compounds, whereas the activity against bacteria was very low and again cinnamaldehyde was the best for yeasts. A similar behavior was found with the mixtures of banana and the antimicrobial compounds, although in this case the mixture was also efficient for *S. aureus* and 2% of cinnamaldehyde.

To check if the presence of aromas in the active film produced some effect on the antimicrobial activity obtained with the mixtures, a new series of microbiological tests was carried out with active plastic films containing only 2% of the antimicrobial compounds, which means the same concentrations of thymol, carvacrol, and cinnamaldehyde each, and another series containing only 8% of each aroma in the sample film. **Figure 7** shows the results obtained. As expected, antimicrobial activity was found only with thymol, carvacrol, and cinnamaldehyde, confirming the previous references in this field (*2*, *13*, *21*). The same antimicrobial results as found previously with the mixtures were found here, confirming that the aroma compounds did not provide any antimicrobial activity. Only in the case of banana was a slight reduction in some of the microorganisms observed, but if the high concentration of aroma and the low logarithmic reduction of CFU obtained are taken into account, the antimicrobial activity could be considered negligible.

Table 4. Friedman Test

	vanilla	banana	strawberry
F	5	13.8	13.4

Sensory Study. Despite the important antimicrobial properties, the use of these compounds has been mainly limited due to the strong flavor that can be transferred to the food package as many studies have pointed out, but this point was never studied in detail (*32*−*34*). For this reason one of the main objectives of this study was to determine the compatibility of these antimicrobial compounds with three important and very common aromas used in the food industry (banana, vanilla, and strawberry). Using the combination of these antimicrobials and the aromas in the packaging, the aroma compounds usually added to the food could be likely reduced, although this is a subject outside the scope of the present paper.

Once the concentrations of both aromas and antimicrobial compounds were optimized, the organoleptic compatibility was studied. A ranking test in which the scores given by the panelists were listed was used to achieve this goal. The ranking test was performed by 12 trained panelists, and **Table 3** lists the points. From these values, the F was calculated.

The F values found for each option were 5 for vanilla, 13.8 for banana, and 13.4 for strawberry. According to the test, a value of $F > 7.6$ means that the samples are significantly different. From **Table 4** it was concluded that vanilla did not show significant differences for any of the selected options with a 99% of confidence level, whereas strawberry and banana gave significant differences.

As both banana and strawberry were significantly different, the Fisher test, which gives information on pair samples, was applied to them. The results obtained are shown in **Figure 8**. In all cases thymol was the antimicrobial agent that least modified the original aroma perception, as it had the second highest number of points.

Bars having the same letters in **Figure 8** indicate that there were not significant differences. As can be seen in the case of vanilla no significant differences were found in any pair of samples. However, in the case of banana, all of the samples with antimicrobial compounds were different from the blank, whereas in the

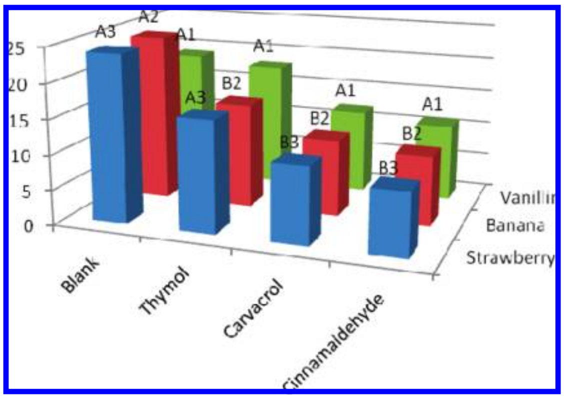

Figure 8. Fisher test applied to banana and strawberry samples.

strawberry samples the mixture with thymol did not show significant differences with the blank sample. These findings mean that the combination of banana aroma with any of the antimicrobials under study is not possible, as the banana flavor is significantly changed and, then, the organoleptic properties would not be those required for banana. In contrast, vanilla could be used in combination with any of the antimicrobials under study, as the vanilla flavor was not significantly modified. This is an interesting finding in terms of antimicrobial active packaging, especially for those applications to bread or bakery products in which vanilla is very commonly used as a flavoring compound.

From the study carried out several conclusions can be emphasized as follows:

(1) The combination of antimicrobial agents with each of the three aromas under study, vanilla, strawberry, and banana, influenced in different ways the aroma perception, that produced by cinnamaldehyde being stronger and more significant, followed by carvacrol and finally thymol.

(2) The vanilla perception was not modified by any of the antimicrobial agents, which means that vanillin could be mixed with any of them without affecting the aroma perception.

(3) Only thymol as antimicrobial agent did not show significant differences in the perception of strawberry. This means that only this antimicrobial agent could be used in an active plastic film combined with strawberry aroma or in a packaged product with strawberry aroma without modifying the original aroma.

(4) All of the antimicrobial agents under study significantly modified the aroma perception of banana. This implies that an active antimicrobial packaging with banana aroma is not possible using cinnamaldehyde, carvacrol, or thymol as antimicrobial agent.

(5) None of the aromas tested showed antimicrobial properties by themselves, but a slightly synergic effect was found in the case of the combinations of the antimicrobials under study and banana.

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