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Combined antimicrobial effect of bacteriocins of LAB isolated from a traditional brine table olives and essential oils against foodborne pathogens.

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ABSTRACT

The study aimed to determine the antimicrobial activity of bacteriocinogenic lactic acid bacteria isolated from olive brine and three essential oils (Thyme, Rosemary and Oregano Eos) alone and in combination against the Gram-positive *L. monocytogenes* and the Gram-negative *E. coli* O157: H7. The antimicrobial activity was determined by evaluation of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the tested antimicrobial agents. The evaluation demonstrates that the two CFSs of lactic strains and the purified bacteriocins inhibit the growth of both *L. monocytogenes* and *E. coli*. The results showed that thyme EO is most effective followed by rosemary and Oregano Eos. Thus, all combination executed against *L. monocytogenes* using the checkerboard technique showed an additive effect unless the CFSs of LAB in combination with Thyme EO which showed no interactive effect. Whereas the combination against *E. coli*, only two bacteriocins in combination with Thyme EO and ELBC02 bacteriocin in combination with Rosemary EO that showed an additive effect. The results obtained in these studies coupled with the right knowledge opens the way to the combination as a new field of application whose goal is confined to conservation and the safety of food.

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Keywords: Bacteriocin(s), Lactic acid bacteria, Antimicrobial(s), Essential oil, combination, Food preservation.

1. Introduction

Today, the health safety of food has become a discipline in its own right according to the Codex Alimentarius Commission (CAC) (Motarjemi, 2014). However, it is also generally recognized that biological hazards pose the greatest immediate threat to food safety in matters of public health, for example, the ability of food poisoning bacteria to cause severe acute disease outbreaks that still presents a threat in the food supply chain (Motarjemi and Lelieveld, 2014). Although he's better known the role of lactic acid bacteria (LAB) in the control of the security and the reduction of hygienic and toxicological risks (Stiles and Holzapfel, 1997), by their ability to produce more natural antimicrobial compounds and reduce the pH of foods a desired level. these bacteria have several potential applications as functional bacteria in the field of food processing especially in the preparation of dairy products, as participate in biochemical events that occur during cheese ripening (proteolysis lipolysis) and also contribute to pleasant sensory profile (Halm et al., 1993) the final product by the improvement of the nutritional value of vegetables fish, meats and prolong their service life why not also by the control mold growth by the action of antifungal properties of LAB (Hassan Gourama, 1995). The accumulation of studies conducted in recent years clearly indicate that the application of bacteriocins from lactic acid bacteria in food preservation may offer several advantages (And and Hoover, 2003; Naidu, 2010; Vijay Simha and al., 2012) ; they have relatively broad antimicrobial spectrum against numerous bacteria and pathogens of foodborne, show a mode of bactericidal action and bacteriostatic which will extend the life span of the food and thus reduce the economic losses due to the deterioration of food. We therefore remain convinced that the bacteriocins can be exploited in foods like natural substance in a large variety of industrial applications especially in the field of food preservation such as bio-preservation agents. However their antibacterial activity remains limited due to resistant strains as Gram negative bacteria. Another solution proposed to master microbiological hazards and avoid this problem is the development of processes called as combined technologies Barriers, each of the microbial development mastery of factors can be considered as a barrier. The bacteriocins-LAB can be also be added in combination with another antibacterial agent such as essential oils which have been found to exert antibacterial activity against pathogenic bacteria (Solomakos et al., 2008a, 2008b; Ruiz et al., 2009), with traditional preservation methods such as heat treatment moderates or emerging alternatives such that the electric field pulse or the high hydrostatic pressures (Burt, 2004; Ait-Ouazzou A, 2012; Lamia CHERRAT, 2013) The purpose of these combined processes would be to set up a series of obstacles that microorganism would not be able to overcome, reaching the desired level of inactivation while reducing the intensity of each process. The aim of this study was to evaluate the antimicrobial efficiency of bacteriocins (ELBC02 and ELBB08 produced by *Enterococcus faecium*) and EOs (Thyme, Rosemary and Oregano) alone and in combination against *L. monocytogenes*, and *E. coli* O157:H7.

2. Materials and methods

2.1. Bacterial strains and media.

Lactic acid bacteria considered in this study were isolated from traditional brine table olives and have been identified as *Enterococcus faecium* (Gaamouche et al., 2014). The strains were stored at −20 °C in MRS broth (DE MAN, ROGOSA, SHARPE) (Biokar Diagnostics, France) with 15% glycerol. The indicator strains chosen for their pathogenic effect were *Listreria monocytogenes* (CECT 4031), has been obtained from Spanish Collection of Type Cultures CECT and *Escherichia coli* O157: H7 from strain collection. The strains were stored at −20 °C in Mueller–Hinton broth medium with 15% glycerol. Prior to each experiment, stock cultures were grown through two consecutive 24 h growth cycles in Mueller–Hinton and MRS broth medium at 37 °C.

2.2. Preparation of essential oils

Three different essential oils (Thyme, Rosemary and Oregano Eos) were purchased from Herbalist's shop. These Eos were chosen based on their potential in preservation of food products against food pathogens. Each essential oil was dissolved in 0.5% (v/v) of Tween 80 (Sigma-Aldrich). Series of dilutions of each EO over the range of 0.5 to 56 µL / mL were prepared in Muller Hinton Broth (Liofilchem) (MHB). The mixture was then stirred.

2.3. Preparation of bacteriocin

The Enterococcus faecium LBB08 and LBC02 isolated from a traditional brine table olives were cultured in MRS broth at 37°C/24h. The supernatant containing the bacteriocins was collected after centrifugation for 15 min at 10,000 x g. The obtained supernatant was then heated for 10 min at 70 °C and filter-sterilized through a 0.2 µm pore-size filter (Turgis et al., 2012). The bacteriocins ELBB08 and ELBC02 of the two lactic strains were purified by the method of Yang (Gaamouche et al., 2014), and the proteinaceous activity and nature of the bacteriocin was confirmed against *L. monocytogenes*. Twofold dilutions of the supernatants and purified bacteriocins were assayed at serial concentrations of 1000; 555; 278; 222; 139; 83.3; 55.5 and 28 ppm which were then used in the assay for determining the minimum inhibitory concentration (MIC) mentioned below.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC)

The MIC values of Eos were determined by microbroth dilution assay using resazurin as indicator of bacterial growth. Three EOs were diluted to obtain serial concentrations of 0.5 to 56 µL/mL. The serial concentrations of EOs were added to each well in a 96-well microtiter plate with a volume of 180 µL. Each well was then inoculated with 20 µL of a pathogenic suspension, adjusted to reach 10^6 CFU mL−1, was added to each well. The microplate was incubated aerobically for 24 h at 37 °C (Turgis et al., 2012). For MIC determination, 5 µL of the resazurin (Sigma-Aldrich) 0.01% (w/v) was added to each well, and MIC was defined by recording the lowest EO concentration of the wells that did not change color. To determine the MBC values, 10 µL of broth from the uncolored wells was inoculated in MHA and incubated for 18–24 h at 37 °C, and MBC was defined as the lowest recorded EO concentration of the wells in which bacteria failed to grow in MHA. (Cherrat et al., 2014). The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the LAB CFSs and bacteriocins. A volume of 180 µL of each dilution of purified bacteriocin and CFS were filled into 96-wells microplate, and each well was then inoculated with 20 µL of a pathogenic strain at a concentration of 10^6 CFU/ml. Plates were incubated at 37°C for 24 h. After incubation, bacterial growth was evaluated by the presence of turbidity and a pellet on the well bottom. According to the Clinical and Laboratory Standards Institute guidelines (CLSI 2006) broth microdilution, the MIC value was defined as the lowest concentration of the antibacterial agent that completely inhibited the growth of the organism as detected with the naked eye (no macroscopically visible growth) (Ben Slama et al., 2013). The MBC was calculated by performing viable cell counts in MHA after 24 h of incubation at 37 °C. A number of wells were reserved in each plate to test for sterility control (no inoculum added), and the inocula viability (no antibacterial agent added).

2.5. Determination of synergic effects between essential oils and bacteriocins by the checkerboard method

In this study the method of checkerboard described by (Davidson and Parish 1989) has been affected for the evaluation of the combination assays. The checkerboard method was performed using 96-well microplates to obtain the fractional inhibitory concentration (FIC) index of EO and bacteriocin combinations against each pathogenic bacterium. The microplate assay was arranged as follows: columns containing 25 µl of an EO (A) which was two-fold diluted in a model medium along the x axis. Rows of the same plates contained 25 µL of bacteriocin (B) which was also two-fold diluted

in the same medium along the y axis. Next, 90 µL of microbial suspensions, adjusted to reach 10^6 CFU mL−1, was added to each well. Plates were then incubated at 37 °C for 24 h (Turgis et al., 2012). The MIC of each EO compound alone or in combination with bacteriocin was taken as the lowest concentration that inhibited bacterial growth completely after 24 h according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2006) and resazurin test. Assays were performed in duplicate and then replicated. The fractional inhibitory concentration (FIC) index was calculated by adding the FIC values of antimicrobial compounds (a) and (b) (FICa+FICb). The FICa and FICb values represented as the lowest concentrations of EO and bacteriocin, respectively, that caused the inhibition of bacterial growth in the combination tests. Calculations were performed as follows:

- FICa = (MICacombined/MICaalone)
- FICb = (MICbcombined/MICbalone)
- FIC = FICa + FICb

3. Results

As shown in Tables 1, the results indicated that bacteriocins displayed remarkable antimicrobial activity, similar to the CFSs of LAB against bacterial indictors, with the lowest MIC values against the Gram-positive bacteria *L. monocytogenes* with a MIC of 83.3 ppm. The bacteriocins activity required to inhibit the growth or kill *E. coli* 139 ppm was unusual, with a difference observed between MICs and MBCs against *E. coli*. However regarding the bactericidal activity against *L. monocytogenes*, ELBB08 showed the highest activity. Differences in the MICs were observed depending on the Eos and the susceptibility of the tested species, the results showed that Gram-positive bacteria were more susceptible to the three EOs than Gram-negative ones. The best performing EOs were Thyme and Rosemary and the least effective was generally Oregano EO.

Table 1: Antimicrobial activity of the selected natural compounds by determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).

Test compound	Target bacterial strains			
	L. monocytogenes		*E. coli* O157: H7	
	MIC	MBC	MIC	MBC
LBB08[1]	83.3	278	139	555
LBC02[1]	83.3	278	139	555
ELBB08[1]	83.3	222	139	555
ELBC02[1]	83.3	278	139	555
Thyme EO[2]	14	21	28	>56,0
Rosemary EO[2]	21	21	56	>56,0
Oregano EO[2]	28	28	56	>56,0

[1]: Result in ppm; [2]: Result in µL/mL

The FIC indices values obtained from different combinations against pathogens bacteria are shown in Table 2.
The results show that the combined bacteriocins or CFSs LAB in combination with Rosemary EO displayed an additive activity against *L. monocytogenes* (CECT 4031) and caused an no interactive effect against *E. coli* O157: H7, except for enterocin LBC02, which was the only agent that generates an additive effect in the presence of Rosemary EO against *E. coli* O157: H7.

In combination with Thyme EO, the purified bacteriocins had an additive effect against *L. monocytogenes* and *E. coli* O157: H7, and induced no interactive effects in combination with CFSs LAB against the two bacterial pathogens. The Oregano EO had an additive effect against *L. monocytogenes* and indicated as no interactive effect against *E. coli* O157: H7 when it was combined with the purified bacteriocins or CFSs LAB.

Table 2: Fractional inhibitory concentration (FIC) indices of bacteriocins in combination with essential oils.

The combination	Bacterial strains			
	L. monocytogenes		*E. coli* O157: H7	
	FIC	Activitéa	FIC	Activitéa
Thyme EO +LBB08	1,3	I	2,2	I
Thyme EO +LBC02	1,3	I	2,2	I
Thyme EO +ELBB08	0,9	AD	0.6	AD
Thyme EO +ELBC02	0,9	AD	0,6	AD
Rosemary EO+LBB08	0,9	AD	1,4	I
Rosemary EO +LBC02	0,9	AD	1,4	I
Rosemary EO +ELBB08	0,9	AD	1,4	I
Rosemary EO +ELBC02	0,9	AD	0,9	AD
Oregano EO+LBB08	0,6	AD	2,0	I
Oregano EO +LBC02	0,6	AD	2,0	I
Oregano EO +ELBB08	0,6	AD	2,0	I
Oregano EO +ELBC02	0,6	AD	2,0	I

[a] FICa = (MICa combined/MICa alone) and FICb = (MICb combined/MICb alone). FIC=FICa+FICb. FIC≤0.5: synergic effect (S); 0.5bFIC≤1: additive effect (AD); 1bFIC≤4: no interactive effect (I); FIC>4: antagonistic effect (A).

4. Discussion

Because of the risk of security many efforts have been made to limit or restrict the use of chemicals for food preservation. The biological approach using microbial antagonists mainly bacteria and their metabolites to control the deterioration of food has become a subject of a thorough investigation (Sofos, 2014). Numerous studies have demonstrated that the production of bacteriocine-LAB can be successfully applied in foods to inhibit *L. monocytogenes* and other food pathogens (O'Sullivan et al., 2002; Rodgers, 2001). Based on our present results, LAB extract exhibited a good antibacterial effect against the tested strains, with low MICs values ranging from 83.3 to 139 ppm. (Alzubaidy et al., 2014) have also reported that the lactic bacteria supernatant rattled local dairy products have shown that the percent inhibition increases as the concentration of the supernatant increases. Other studies on the MICs (Lee et al., 2013) whose concentration is expressed in mg / mL, the comparison shows that the effectiveness of MIC represented by the two lactic

strains studied in our work reveal effective bacterial activity. Both supernatants of lactic strains of *E. faecium* and two bacteriocins purified have expressed a bacteriostatic and bactericidal activity against gram-positive strains and negative tested showing a strong sensitivity of *L. monocytogenes* (CECT 4031) compared to *E. coli O157*, thus allowing to quantitative information and more accurate than those demonstrated by the agar diffusion method (Gaamouche et al., 2014). As described before the bacteriocins had always shows efficacy against all strains of Listeria and a strong bacteriostatic and bactericidal activity, other studies on the MIC of various origins bacteriocins were performed specially against the strain of Listria (Ferchichi et al., 2001; Guyonnet et al., 2000; Sonsa-Ard et al., 2015). Gram positive bacteria are generally more sensitive to the bactericidal effect of bacteriocin-LAB, which can be explained by the presence in Gram-negative bacteria an additional outer membrane separated from the cell wall by the periplasmic space and which contains lipids (Gaamouche et al., 2014). The study conducted by (Casaus et al., 1997) indicates that the MIC values obtained with the different test organisms using , none of the bacteriocins were inhibitory to gram-negative bacteria in this assay namely *E. coli*. Also (Acuña et al., 2015) show that no activity was detected even at the high concentration of bacteriocin against *E. coli O157:H7*. Other (Woraprayote et al., 2015) showed that the MIC using the agar-diffusion method for both bateriocins produced by *Weissella hellenica* for *E. coli* ATCC 25922, whereas no activity was detected at high concentration of bacteriocin against *E. coli* O157 H7. The minimum inhibitory concentration and bactericidal are also variable according to the bacterial species and strains (Ennahar et al., 2000) and culture supernatant, purified or semi-purified preparation (Sebti, 2002; CHERGUI Achour, 2014). In this work the MBC values to target cells confirmed the bactericidal mode of action, in other study the bactericidal character is not ceding (Jordane JASNIEWSKI, 2008). The application of bacteriocins of LAB as antibacterial agent barriers of technology to master the deterioration of the quality of food as any process of conservation On the other hand The essential oils are widely used as antiseptic agents (Ait-Ouazzou et al., 2011; Baydar et al., 2004; Bendahou et al., 2008;, 200; Karaman et al., 2001). It has also been shown that numerous of essential oils possess a broad spectrum of activity against the pathogenic strains of food origin In our results all essential oils tested are active against strains used MIC and MBC from 14 µL / ml to 56 µL / mL. Essential oils can be a potential alternative to prevent the development and spread of micro-organisms responsible for the alteration of food meat (Burt, 2004), essential oils of cloves, Oregano and Thyme were effective against *L. monocytogenes* in meat products 5-10µL/g (Vrinda Menon and Garg, 2001), but the practical application of EO may be limited because of the flavor (Nowak et al., 2012). Among these oils that of Thyme is most active, this efficiency was observed in previous studies. (Rota et al., 2008) have postponed that this oil has expressed considerable antimicrobial activity against *L. monocytogenes* and *E. coli*. The antimicrobial activity of the essential oils is often correlated with their chemical constituents and their functional groups and potential synergistic interactions between the majority and minority compounds (Dorman and Deans, 2000; Burt, 2004; Ait-Ouazzou et al., 2011; Lamia CHERRAT, 2013). This antibacterial activity is often reduced to the activity of its controlling compounds or may be active, most compounds are already known for their antibacterial activity. Also for essential oils, Gram-negative bacteria studied in this work are generally less sensitive to essential oils as Gram positive (Lambert et al., 2001; Burt, 2004; Neve et al., 2004; Deba et al., 2008). However this has not been confirmed by other studies reporting that the susceptibility of bacteria is independent of Gram (Dorman and Deans, 2000). Essential oils are very complex mixtures of compounds majority and minority which often makes it difficult to explain their antioxidant and antimicrobial properties (Burt, 2004; Tepe et al., 2005) (Burt, 2004; Ait-Ouazzou et al., 2011). In particular despite the proven efficacy of essential oils in high concentrations to provide antimicrobial activity for the preservation of food in relation to an in vitro system, their application may be limited due to changes in the organoleptic quality, the texture of food or EOS interaction with food components (Turgis et al., 2012). In addition the presence of fat protein and carbohydrate strongly reduced the rate of antimicrobial activity of EO in foods (Nowak et al., 2012), for example the essential oil of peppermint was not

effective in products with high fat levels (C. C. Tassou, 1995; Gill et al., 2002). Subsequently a combined effect of these OE with another antibacterial agent such as bacteriocins or LAB will reduce the individual concentrations of these compounds has an acceptable sensory level while maintaining the overall antimicrobial effect. In this regard the combinations of essential oils and protein substances could be a very promising natural alternative. Since these agents are known as great potential antimicrobial compounds.Despite the large flow of data however few studies have been conducted to study the interactions beween bacteriocins and other antimicrobial components. To enhance the antimicrobial activities of LAB bacteriocins against various bacterial foodborne have been used in combination with other antibacterial agents. This approach can also be used to reduce the appearance of resistant strains and the dose of antimicrobial agents (Yamazaki et al., 2004).The additive effects related to all bacteriocins acted against *L. monocytogenes* ($1<FIC\leq4$) in combination with the three essential oils (rosemary, thyme and oregano), also the case for the supernatant of two LAB in combination with essential oils of Rosemary and Oregano. These results are consistent with earlier work (Turgis et al., 2012) that showed that nisin in combination with Thyme and Oregano in combination with pediocin show additive effects against *L. monocytogenes*, while disclose only one synergistic activity was illustrated by nisin in combination with Origano against *L. monocytogenes*. In 2013 the first report on the combined effect enterocin A and Thyme oil against *L. monocytogenes* and *E. coli* O157: H7 has been studied in vitro by the enumeration of pathogenic bacteria populations by the determination of the minimum inhibitory concentration (Ghrairi and Hani, 2015). From the results obtained after the two bacteriocins showed an additive effect against *E. coli* in combination with Thyme, while ELBB08 bacteriocin in combination with Rosemary and two bacteriocins in combination with Oregano showed a non-interactive effect (Turgis et al., 2012). The exact mechanism of action of the essential oils and bacteriocins is unknown but some authors issued the hypothesis that these items result in the disruption of the cell membrane. According to (Ettayebi et al., 2000) the mechanism can be explained on the basis of destabilization induced by the essential oils of the bacterial membrane structure resulting in an increased permeability of nisin. In combination treatment therefore the concentration of intracellular nisin would be high to increase cell lysis. The use of OE and bacteriocin all play an important role in the formation of membrane pores which modify the permeability of the membrane proton motive power, efflux of amino acid and the pH gradient of bacteria. The sublethal Lesion gram-negative cells may facilitate the access of bacteriocins to the cytoplasmic membrane (Kalchayanand et al., 1992). Our data suggest that such combinations could be exploited as control strategies to fight against these pathogens, considering both the economic aspects and quality of food.

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