African Journal of Biotechnology Vol. 10(71), pp. 15998-16005, 14 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1046
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

The antimicrobial effect of Origanum compactum essential oil, nisin and their combination against Escherichia coli in tryptic soy broth (TSB) and in sheep natural sausage casings during storage at 25 and 7°C

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Accepted 5 October, 2011

The aim of this study was to investigate the antimicrobial effect of oregano essential oil (EO) at 0.015, 0.03 or 0.06%, nisin at 800 or 1600 IU/g, and their combination on *Escherichia coli* in tryptic soy broth (TSB) and in sheep natural sausage casings. At 0.015%, EO presented a weak antibacterial activity against the pathogen in TSB. Consequently, only the levels of 0.03 and 0.06% were examined in casings. EO at 0.03 or 0.06% showed an inhibitory activity against *E. coli* O157:H7, *E. coli* ATCC 25922 and *E. coli* cocktail during storage at 25°C and at 7° C in casings. Treatment of casings or in TSB with nisin at 800 or 1600 IU/g did not show any antibacterial activity against *E. coli* strains. Synergy between EO and nisin was observed in both TSB and natural casings. The combination of EO at 0.03 or 0.06% and nisin at 800 or 1600 IU/g presented an additive effect against the pathogen, during storage at 25°C and at 7°C.

Key words: Oregano essential oil, nisin, natural casings, biopreservative, *Escherichia coli.*

INTRODUCTION

Natural casings are derived from the fibrous, connective tissue layer of the intestinal tract of animals (Andriessen, 1987). They are usually preserved by salting, curing and/or drying (Fischer and Schweflinghaus, 1988). The efficacy of common salt preservative methods against major zoonotic pathogens, such as *E. coli* O157:H7, *Salmonella* typhimurium, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium perfringens*, were confirmed by the studies of Houben (2005) and Wijnker et al. (2006). But in recent years, food companies have been redoubling efforts to reduce the use of salt or it substitution because of the adverse environmental impact of the large quantities of salt and water (for desalting) used by meat industry. Likely substances to achieve this effect are essential oils and bacteriocins.

In Morocco, the *Lamiaceae* family (more than 30 genera and 225 species of which more than 90 are endemics) represents a great ecological and economic interest, as it includes many medicinal, aromatic and honey-plant species (Benabid, 2000). Within this family, the genus *Origanum* is represented by five species of which three are endemic, including *Origanum compactum*. O. *compactum*, locally known as za'tar which is mainly used as a culinary condiment and largely employed in popular medicine for the treatment of ailments such as digestive and pulmonary disorders (Belakhdar, 1997; Benabid, 2000; Ennabili et al., 2000). O. *compactum* is also used as preservative for the melted butter (*smen*) and dryed fig (*Chriha*). Till date, different extracts of O. *compactum* plant have been tested for their biological activities. The essential oil extracted from this plant is mainly constituted of carvacrol, thymol, γ-terpinene and *p*-cymene (Bakkali et al., 2005; Mezzoug

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Abbreviations: EO, Essential oil; **TSB,** tryptic soy broth.

et al., 2007). Previous study reported that the anti-bacterial activity of these four monoterpene compounds is related to their ability to perturb membrane structure (Cristani et al., 2007). Oregano essential oil induces membrane damage (leakage of potassium and uptake of propidium iodide (PI) and bis-oxonol), ultrastructural alterations and the loss of cell viability (Bouhdid et al., 2008).

Nisin is the best known and studied bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*. It is the only bacteriocin that has been approved as a food additive in Europe and it is considered as safe bacteriocin in the United States (Delves-Broughton, 1990; Food and Drug Administration, 1998; Montville et al., 2001). Nisin has a known broad-spectrum inhibitory effect on Gram-positive bacteria (Hurst, 1981; Davies et al., 1999). It's able to kill bacteria via targeted pore formation after developing a stable complex with Lipid II, an essential intermediate in the bacterial cell-wall synthesis. Subsequent pore formation causes leakage of potassium ions and intracellular metabolites such as ATP (Breukink et al., 1999; Hsu et al., 2004). The affected bacteria die as a result of energy depletion and stagnation of intracellular biosynthetic processes (Ruhr and Sahl, 1985; Sahl and Bierbaum, 1998).

The bacteriocin nisin is not generally active against Gram-negative bacteria, fungi and virus (Boziaris and Adams, 1999). In normal circumstance, Gram-negative bacteria are resistant to nisin mainly due to their impermeable outer membranes. However, nisin can be effective against Gram-negative bacteria if used in combination with agents destabilizing the outer membrane (Stevens et al., 1992). Recent studies showed that the inhibitory activity of nisin against Gram-negative food borne pathogens could be improved by a synergistic effect with other antimicrobial factors including chelators (Cutter and Siragusa, 1995; Murdock et al., 2007) or plant EO (Solomakos et al., 2008a, b).

Use of EO and nisin, natural preservatives, to inhibit growth of serious pathogens such as *E. coli* O157:H7 is of great interest to the meat industry. *E. coli* O157:H7 is recognized as an important cause of food borne disease. This pathogen, a Gram-negative, facultative anaerobe bacterium, has a low infection dose of 50–5 organisms (Betts, 2000). The three distinct virulence factors of this serotype of *E. coli* are the production of the cytoxic Shiga- like toxins (I and II), hemolysin synthesis, and the ability to adhere to and colonize intestinal surfaces. *E. coli* O157:H7 can cause hemorrhagic colitis, hemolytic uremic syndrome, and thrombocytopenic purpura, and can lead to death (Karmali, 1989). Since its first identification in 1982, *E. coli* O157:H7 has been isolated in numerous food borne outbreaks worldwide, with increasing frequency in the past decade (Betts, 2000; Vernozy-Rozand et al., 2002).

The aim of this work was to study the antimicrobial effect of oregano EO, nisin and their combination against *E. coli* O157:H7 and *E. coli* ATCC 25922 in sheep natural sausage casings without the use of salt, an ordinary preservative in natural casing industry.

MATERIALS AND METHODS

E. coli strain and inoculum preparation

E. coli CECT 4076 (serovar O157:H7) were obtained from the Faculty of Sciences Tétouan, Morocco, and *E. coli* ATCC 25922 was purchased from BIOBACT, Rabat Morocco. Bacteria were grown in brain heart infusion (BHI) broth (BIOKARD DIAGNOSTICS, Beauvais, France) for 24 h at 37°C, with two consecutive subcultures.

Extraction of oregano EO

O. compactum Bentham plant was collected from Dar Ben Karrich (Tetouan, North west of Morocco, Africa, 35° 30' 36" North, 5° 25' 12" West). The botanic identification of the plant material was made by Prof ENNABILI Abdeslam from National Institute of Medicinal and Aromatic Plants (University Sidi Mohamed Ben Abdellah, 30 000 Fes (Morocco). Voucher specimen (INP059) was deposited in the herbarium of that institute. Samples of 500 g of dried leaves of oregano were used for the preparation of EO as previously reported (Govaris et al., 2010).

Oregano EO chemical analysis

The essential oil chemical composition was analyzed using a gas chromatograph (GC) fitted to a mass spectrometer (MS). Operating in electron-impact EI (70 eV) mode. VB-5 (Methylpolysiloxane à 5% phenyl), column (30 m × 0.25 mm i. d., 0.25 µm thickness) was used (Centre National pour la Recherche Scientifique et Technique-CNRST, Rabat, Maroc). The chromatographic conditions were as follows: Injector and detector temperatures, 220 and 300°C, respectively; carrier gas, helium at flow rate of 1.4 ml/min; temperature program ramp from 40 to 300°C with gradient of 4°C min^{-1} (holding the initial and final temperature for 4 min).

The relative amount of individual components of the total oil was expressed as a percentage peak area relative to total peak area. Library search was carried out using the combination of NKS library with 75 000 spectra and a personnel aromatic library.

Nisin preparation

Nisin from *L. lactis* (subsp. *lactis)* was obtained from Sigma-Aldrich (N5764). Nisin was solubilised in 0.02 M HCl at a concentration of 10 mg/ml with heating (60 to 70°C) to aid solubilisation. The solution was sterilized by filtration through 0.22 µm membrane filters (Millex, Millipore) prior to use.

Antimicrobial activity in broth

Oregano EO, nisin and their combination were added to 40 ml sterile TSB in 100 ml glass bottles. The EO was added at concentrations of 0, 0.015, 0.03 and 0.06% while nisin at concentrations of 0, 800 and 1600 IU/ml in TSB. In the combined addition, EO and nisin were also mixed in TSB at the combination of [EO 0,015% + nisin 800 IU], or [EO 0.015% + nisin 1600 IU], [EO 0.03% + nisin 800 IU] or [EO 0.03% + nisin 1600 IU], and [EO 0.06% + nisin 800 IU] or [EO 0.06% + nisin 1600 IU]. Following

inoculation with the pathogen, broth samples of all examined treatments were incubated at 37°C for 32 h. Sampling for microbiological analysis of inoculated broth was performed after 0, 4, 8, 23, 27 and 32 h of incubation.

Treatment of sheep natural sausage casings

Dry salted Australian sheep casings (AA 20 to 22) were obtained from BOA Company (Boyauderie d'Atlas Tangier Morocco). These casings were previously cleaned, scarped, selected and salted according to the Company Standard Operating Procedures. For the experimental treatment, eight hanks were transferred to the microbiology laboratory. Casings were first desalinated for 30 min with flowing lukewarm tap water (temperature 25 ± 2°C). This reduces the residual salt level of dry-salted casings to less than 3 wt% (Lee et al., 1994). Thereafter casings were drained overnight at 3°C to reduce water level. Prior to casings, inoculation with pathogen and the addition of EO and/ or nisin, casing was also examined for any contamination by bacteria or the tested pathogen, as described in microbiological analysis.

Casing samples (100 g) were placed in stomacher plastic bags (Whirl-Paks, Paris, France) and inoculated with inocula of *E. coli* O157:H7, *E. coli* ATCC 25922 and their mixture (ca 10^6cfu/g). The inoculated samples were homogenized in a stomacher (Stomacher/Lab Blender 400) for 1 min at room temperature to ensure proper distribution of the pathogen. Following homogenization, casings were placed in sterilized glass bottles under a biohazard hood; 100 ml of sterilized water were added.

Each of the inoculated samples was treated separately with different EO, nisin and their combination. The EO was added at concentration of 0, 0.03 and 0.06%, while nisin at concentrations of 800 and 1600 UI/ml in the combined addition, EO and nisin were also mixed at the combination of [EO 0.03% + nisin 800 IU] or [EO 0.03% + nisin 1600 IU], and [EO 0.06% + nisin 800 IU] or [EO 0.06% + nisin 1600 IU].

To make uniform distribution of the added compounds, treated casing samples were further homogenized by shaking each bottle manually. All glass bottles with samples from all treatments were stored under aerobic conditions at 7 or 25°C for 25 days.

Microbiological analysis

10 g of casing for each treatment were transferred into individual sterile plastic bags (Whirl-Paks, Paris, France) and mixed with 90 ml of tryptone salt broth (TSB) (BIOKARD, Beauvais, France) using a Stomacher (Stomacher/Lab Blender 400) for 2 min. Appropriate serial decimal dilutions were made in tryptone salt broth. TSB samples were also serially diluted (1:10) in tryptone salt broth. Sample dilutions (0.1 ml) of either TSB or casing were spread plated on appropriate media in duplicate. Populations of *E. coli* strains were determined on selective McConkey agar (BIOKARD, Beauvais, France) at 37°C for 48 h.

Statistical analysis

Analysis of the variance of the data was performed using the ANOVA-MANOVA using statistical software STATISTICA 6.0 (1997 edition). Significant differences ($p < 0.05$) between mean values of triplicate sample were determined.

RESULTS AND DISCUSSION

GC-MS analysis of the oregano EO showed that the carvacrol was the most abundant individual component (36.31%), followed by thymol (25.25%), γ-terpinene (16.88%), *p*-cymene (9.21%). Other compounds as α-terpinene (2.33%), isocaryophyllene (2.01%), and linalool (1.35%) were found in minor percent. These results are in agreement with those reported in previous studies. The aromatic monoterpenes mainly presented in the genus *Origanum* are: *p*-Cymene, thymol, carvacrol and their precursor, γ -terpinene (Skoula et al., 1999). The analysis of essential oils of *O. compactum* from various Moroccan regions revealed the presence of three main components with variable rates; thymol (0 to 43.4%), carvacrol (3.8 to 71%) and *p*-cymene (0 to 25.4%) *(*Van Den Broucke and Lemli, 1980*)*. Similar results were obtained by other authors *(*Bouchra et al., 2003; Lahlou and Berrada, 2001*).*

The antimicrobial activity of EO at 0.015, 0.03 and 0.06%, nisin at 800 or 1600 IU/g and theirs combinations, on *E. coli* O157:H7 in TSB broth are presented in The inhibitory effect of EO at 0.015% in broth against *E. coli* strains was relatively low. The addition of EO at supplementation levels of 0.03 and 0.06% in broth, showed a higher inhibitory effect as compared to the addition at 0.015 and 0.06% levels which showed no growth of viable cells of the pathogen on plates from 32 h (Table 1). These results show that the antimicrobial proprieties of oregano essential oil are dose-dependent. Ozcan et al. (2003) observed that oregano EO at a concentration of 0.2% had no antibacterial activity against *E. coli* O157:H7, while at a concentration of 0.4% possessed a high activity. Of the major components of oregano EO studied here, carvacrol and thymol were found to possess antibacterial activity against *E. coli* O157: H7 in *in-vitro* experiments (Uyttendaele et al., 2004; Burt et al., 2005; Di Pasqua et al., 2006). Helander et al. (1998) investigated how oregano EO, carvacrol and thymol exert their antibacterial effects on *E. coli* O157: H7. These phenols disintegrated the membrane of the bacteria, leading to the release of membrane-associated material from the cells to the external medium. Gill and Holley (2006) found that carvacrol was capable of inhibiting the membrane bound ATPase activity of *E. coli*. However, the compounds present in the greatest proportions are not necessarily responsible for the total activity; the involvement of less abundant constituents should also be considered (Cimanga et al., 2002). Therefore, the activity could be ascribed to the presence of other components such as γ-terpinene, *p*-cymene, linalool and α-terpinene also known to possess an antibacterial activity. γ- terpinene present in our EO at level of 16.88% and *p*-cymene at 9.21%, showed no antibacterial activity against *E. coli* O157:H7 according to the findings of Burt et al. (2005) in *in-vitro* experiments. However, Kisko and Roller (2005) found that γ -terpinene possessed an inhibitory activity against *E. coli* O157:H7 in apple juice.

The addition of nisin at 800 or 1600 IU/ml in broth

Table 1. Effect of oregano EO, nisin and their combination on *E. coli* O157:H7 in TSB at 37°C for 32 h.

Hour of incubation	Population (log cfu/ml)(mean values±SD)											
	Control	% oregano EO concentration (V/v)			Nisin (IU/ml)		EO % and nisin UI concentration					
		0.015	0.03	0.06	800	1600	0.015 + 800	0.015+1600	0.03 + 800	0.03 + 1600	0.06 + 800	0.06 + 1600
0	6.79±0.29	6.42±0.30	5.94±0.16	4.59±0.22	6.86±0.18	6.81±0.20	6.69±0.22	6.83±0.19	5.92±0.16	4.92±0.15	4.65±0.16	4.00±0.23
4	8.63±0.17	7.46±0.23	5.74±0.15	3.74±0.16	8.32±0.15	8.42±0.18	7.01±0.15	6.98±0.29	4.30±0.25	3.00±0.2	0.00±0	0.00±0
8	8.90±0.20	8.38±0.29	4.90±0.19	3.20±0.23	8.90±0.19	8.92±0.30	7.41±0.24	7.61±0.18	4.10±0.26	2.78±0.26	0.00±0	0.00±0
23	9.34±0.19	8.33±0.19	4.87±0.20	2.54±0.18	9.29±0.25	9.30±0.25	7.70±0.18	7.30±0.20	4.00±0.19	2.65±0.19	0.00±0	0.00±0
27	9.40±0.26	7.13±0.16	3.71±0.25	2.07±0.25	9.37±0.20	9.37±0.17	7.69±0.17	7.25±0.31	3.61±0.22	2.48±0.29	0.00±0	0.00±0
32	9.47±0.20	8.04±0.20	3.63±0.30	0.00±0.19	9.44±0.17	9.20±0.22	7,68±0.25	7.08±0.19	3.00±0.27	2.00±0.25	0.00±0	0.00±0

resulted in no significant difference ($P > 0.05$) from the control samples during incubation. These results are in agreement with previous findings that nisin is not active against Gram-negative bacteria, including *E. coli* O157:H7 (Cleveland et al., 2001; Olasupo et al., 2003). This might be due to the protective role of the outer membrane, which covers the cytoplasmic mem-brane and peptidoglycan layer of Gram-negative cells (Breukink and de Kruijff, 1999; Fang and Tsai, 2003; Helander and Mattila-Sandholm, 2000).

EO at 0.015% combined with nisin at 800 or 1600 IU/ml, presented an antimicrobial effect against the pathogen during incubation at 37°C for 32 h. EO at levels of 0.03% and nisin at 800 or

1600 IU/ml, resulted in an inhibitory effect against *E. coli* strains and combination of EO at 0.06% with nisin at 800 or 1600 IU/ml resulted no growth of viable cells of *E. coli* O157:H7 from 4 h. The additive effect of oregano EO with nisin against *E. coli* O157: H7 could be explained by the disinter-gration of the protective outer membrane caused by the oregano EO, which could lead to an induced sensitivity to nisin (Vaara, 1992; Helander and Mattila-Sandholm, 2000).

The inhibitory effects of *O. compactum* essential oil at 0.03 and 0.06%, nisin at 800 or 1600 IU/g, their combination on *E. coli* O157: H7 and *E. coli* ATCC 25922 and their mixture in natural sheep casings stored at 7 or 25°C for 25 days, are presented in Figures 1 and 2, respectively. During storage at 25°C, populations of the pathogen in the control samples and the samples treated with nisin at 800 or 1600 IU/g were similar ($P > 0.05$). However, the samples treated with EO at 0.03 and 0.06% presented populations of the pathogen significantly lower ($P < 0.05$) than the non-treated samples. In these samples, populations of *E. coli* strains were decreased to 0.64 and 0.91 $\log_{10}$ cfu/g, respectively on day 5 and kept approximately unchanged up to the end of storage. The samples treated with EO at 0.03 or 0.06% plus nisin at 800 or 1600 IU/g presented a great reduction of populations of the pathogen compared with the samples treated with EO alone and the samples treated with nisin.

During storage at 7°C, the initial populations of the pathogen in the control samples were significantly increased ($P < 0.05$). A similar ($P > 0.05$) population increase was also observed in the samples treated with nisin. However, the samples treated with EO at 0.03 and 0.06% presented populations of the pathogen significantly lower ($P < 0.05$) than the non-treated samples. A similar ($P > 0.05$) profile was exhibited by the samples treated with the combination of EO at 0.03 or 0.06% plus nisin at 800 or 1600 IU/g, which in turn were significantly lower ($P < 0.05$) than EO at 0.03 or 0.06%. In these samples, populations of *E. coli* decreased 3.59 and 3.83 $\log_{10}$ cfu/g respectively by the end of storage. It may be important to note that the non pathogenic *E. coli* ATCC 25922 and pathogenic *E. coli* O157: H7 strains or of the same strains presented no significant difference ($P > 0.05$) populations.

According to the present results, treatment of casings with EO at 0.03 and 0.06% had an inhibitory activity against *E. coli* O157: H7 and *E. coli* ATCC 25922 and their cocktail at 7°C, and also at 25°C. Previous studies had pointed out that a high incubation temperature enhanced the inhibitory activity of plant EO against food borne pathogens *in-vitro* (Smith-Palmer et al., 1998) or foods (Hao et al., 1998). As shown previously in TSB, nisin at 800 or 1600 IU/g possessed no antibacterial activity against *E. coli* strains in natural sausage casings during storage at 7 or 25°C. Fang and Tsai (2003) observed that nisin added at 10^2 IU/g in beef meat slurries was not active against *E. coli* O157: H7, during storage at 10 or 30°C. Solomakos et al. (2007) also showed that nisin at 500 or 1000 IU/g possessed no antibacterial activity against *E. coli* O157:H7

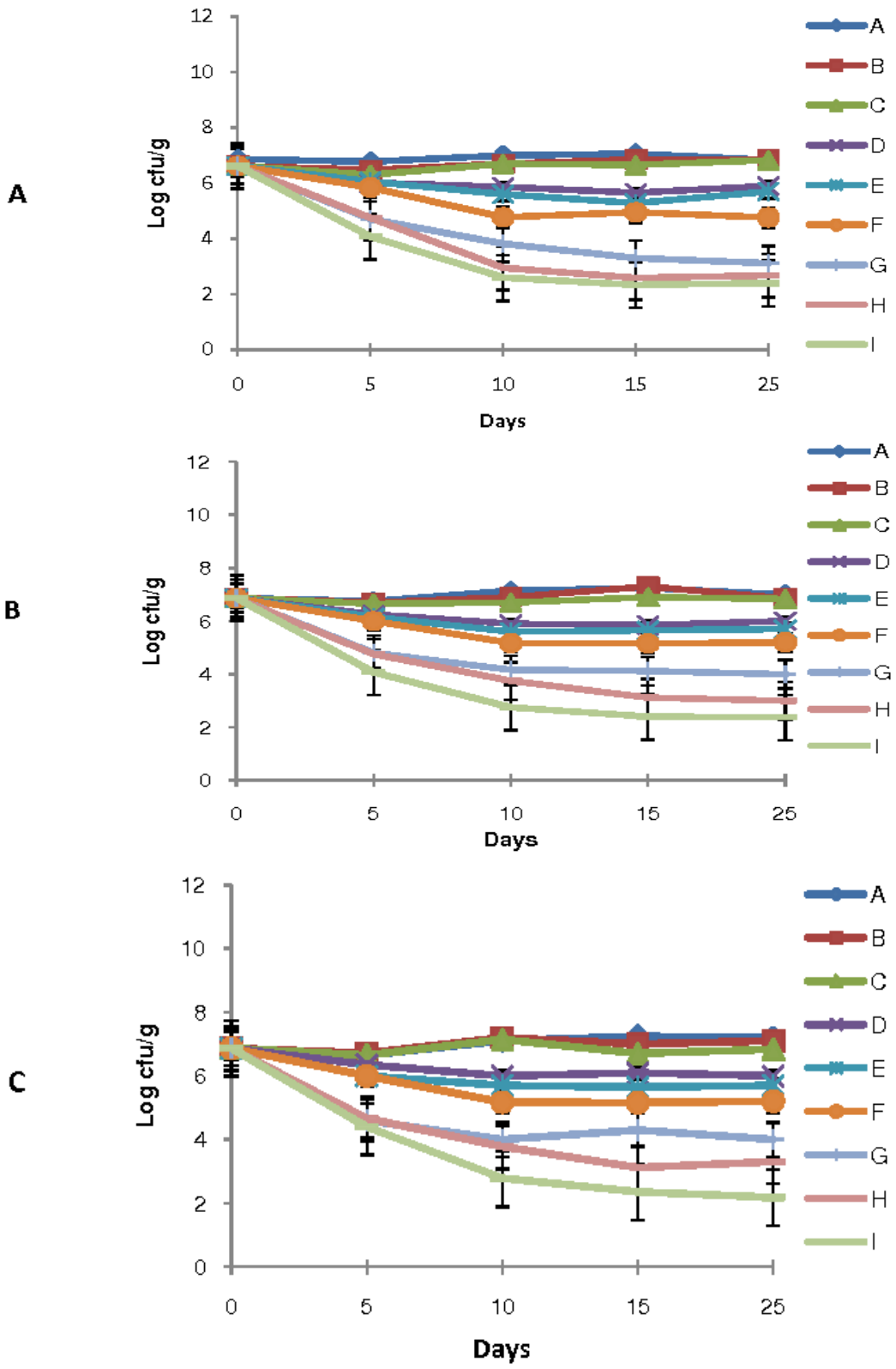

Figure 1. Effect of oregano EO, nisin and their combination on *E. coli* strains (A: *E. coli* O157, H7 strain; B: *E. coli* ATCC 25922 strains; C; cocktail strains) in sheep natural sausage casing during storage at 7℃ for 25 days. (-A-) Control, (-B-) nisin 800 IU/g, (-C-) nisin 1600 IU/g, (-D-) oregano 0.03%, (-E-) oregano 0.03% and nisin 800 IU/g, (-F-) oregano 0.03% and nisin 1600 IU/g, (-G-) oregano 0.06%, (-H) oregano 0.06% and nisin 800 IU/g, (-I-) oregano 0.06% and nisin 1600 IU/g.

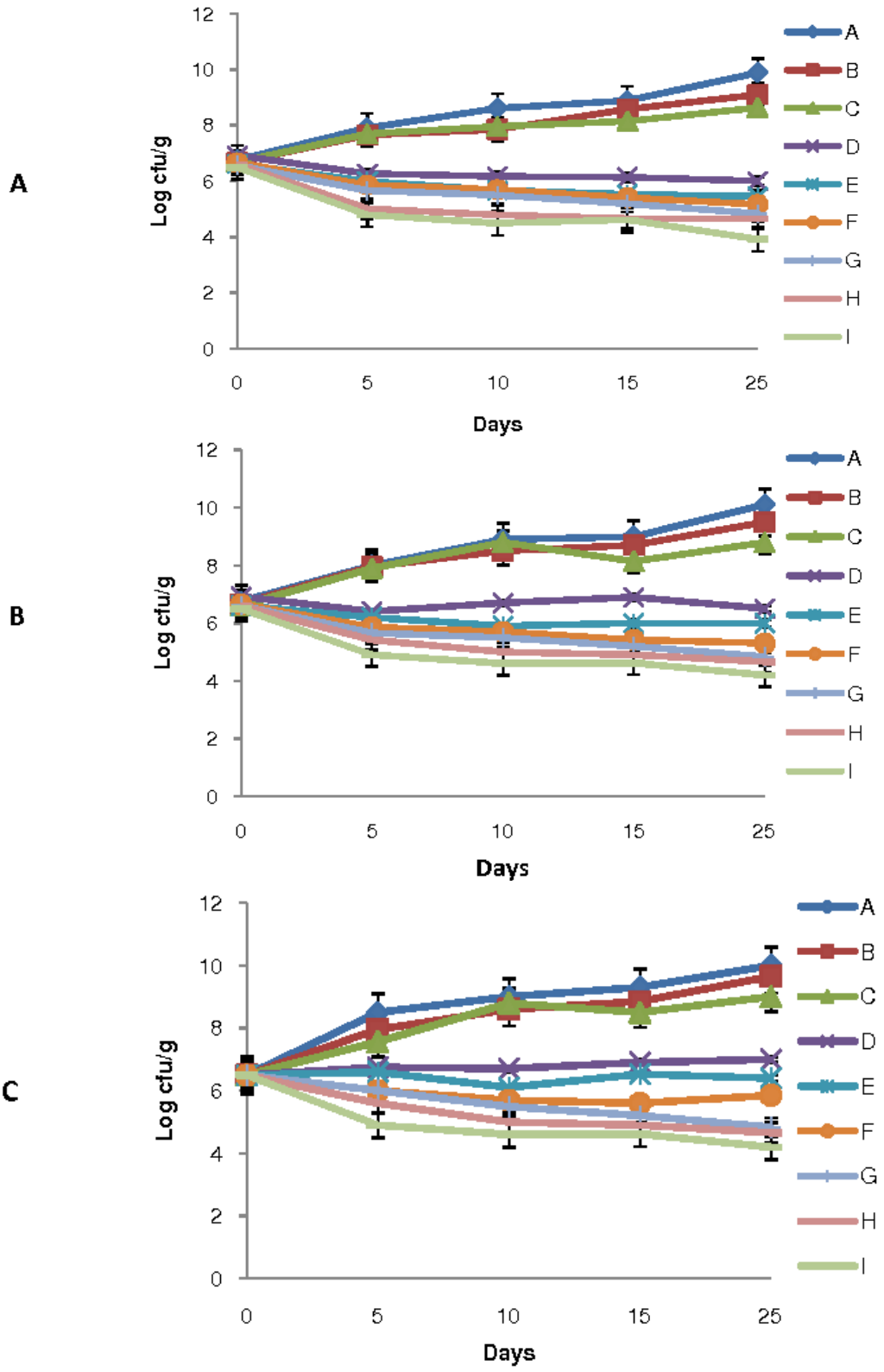

Figure 2. Effect of oregano EO, nisin and their combination on E. coli strains (A: *E. coli* O157:H7 strain; B: *E. coli* ATCC 25922 strains; C; cocktail strains) in sheep natural sausage casing during storage at 25°C for 25 days. (-A-) Control, (-B-) nisin 800 IU/g, (-C-) nisin 1600 IU/g, (-D-) oregano 0.03%, (-E-) oregano 0.03% and nisin 800 IU/g, (-F-) oregano 0.03% and nisin 1600 IU/g, (-G-) oregano 0.06%, (-H) oregano 0.06% and nisin 800 IU/g, (-I-) oregano 0.06% and nisin 1600 IU/g.

during storage at 4 or 10°C.

The combination of oregano EO at 0.03 and 0.06% and nisin at 800 or 1600 IU/g showed an additive effect against *E. coli* strains in natural sausage casings at refrigerated and not refrigerated temperature of storage.

Conclusion

In this study, it was demonstrated that the combination of *O. compactum* EO at 0.03 and 0.06% and nisin at 800 or 1600 IU/g showed an additive effect against the *E. coli* strains in natural sausage casings during storage at 7 and 25°C. This combination can augment the preservation of natural casings to inhibit the growth of this pathogen while replacing partially or completely the use of salt (ordinary preservative of natural casings).

ACKNOWLEDGEMENT

Authors are grateful to BOA group, Boyauderie de l'Atlas, Tangier Morocco for the financial support.

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