Inactivation of *Listeria monocytogenes* in Raw Fruits by Enterocin AS-48

ANTONIO COBO MOLINOS,1 HIKIMATE ABRIQUEL,2 NABIL BEN OMAR,1 ROSARIO LUCAS,1 EVA VALDIVIA,2 AND ANTONIO GÁLVEZ1*

1Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071–Jaén, Spain; and 2Departamento de Microbiología, and 3Instituto de Biotecnología, Facultad de Ciencias, Universidad de Granada, 18071–Granada, Spain

ABSTRACT

The purpose of this study was to determine the effect of enterocin AS-48 on *Listeria monocytogenes* CECT 4032 in fruits and fruit juice. Fruits were contaminated with a *L. monocytogenes* cell suspension, washed with enterocin AS-48 (25 μg/ml) or with sterile distilled water as control, and stored at different temperatures (−20, 6, 15, 22°C). Washing treatments significantly inhibited or completely inactivated *L. monocytogenes* in strawberries, raspberries, and blackberries stored at 15 and 22°C for up to 2 days and in blackberries and strawberries at 6°C for up to 7 days. Washing treatments with enterocin AS-48 also reduced viable counts in sliced melon, watermelon, pear, and kiwi but did not avoid proliferation of survivors during storage at 15 and 22°C. Added enterocin (25 μg/ml) completely inactivated *L. monocytogenes* in watermelon juice within 24 h. To enhance the antilisterial activity of treatments, enterocin AS-48 was tested in combination with other antimicrobial substances on sliced melon stored at 22°C. The combinations of enterocin AS-48 and trisodium trimetaphosphate, sodium lactate, lactic acid, polyphosphoric acid, carvacrol, hydrocinnamic acid, p-hydroxybenzoic acid, n-propyl p-hydroxybenzoate, or 2-nitropropanol showed increased antilisterial activities compared with each antimicrobial tested separately. Washing treatments with enterocin AS-48 in combination with 12 mM carvacrol, as well as with 100 mM n-propyl p-hydroxybenzoate, avoided regrowth of *Listeria* during storage at 22°C. Results from this study indicate that enterocin AS-48 alone or in combination with other preservatives could serve as an additional hurdle against *L. monocytogenes* in fruits and fruit juices.

Consumer demands for minimally processed, ready-to-eat fruits have increased considerably in the last decades (4). Preparation steps such as peeling, cutting, or slicing remove or damage the protective fruit surfaces and allow microbial infiltration of the tissues. Spread of microorganisms from fruit surfaces during processing might compromise the safety of fresh-cut fruits (18, 52, 56). Furthermore, cutters and slicers can be potent sources of contamination in that they usually provide inaccessible sites, which harbor bacteria. Many reported foodborne outbreaks have been associated with fruit or fruit products, including fresh-cut melon and watermelon and their juices (7, 12, 13, 38, 40, 54, 57), unpasteurized orange juice (5, 10, 17), unpasteurized apple juice (8), apple cider (39, 43), raspberries (28), frozen strawberries (9, 44), frozen raspberries (48), fresh-cut fruit (29), and mangoes (11). Several reports have determined the incidence, survival, and growth of bacterial pathogens on watermelon and melon slices (18–20, 22, 47). In particular, melon and watermelon products are regarded as potentially hazardous foods by the U.S. Food and Drug Administration (39) because they could favor the growth of pathogenic microorganisms because of their low acidity (pH 5.2 to 6.7) and high water activity (0.97 to 0.99). *Listeria monocytogenes* is a foodborne pathogen causing listeriosis (33). Growth of *L. monocytogenes* has been demonstrated in a number of vegetables under refrigerated and ambient conditions (27, 47), in nonacidic fruits (54), and on the outer surface of acidic fruits such as tomatoes (6) and peeled Hamlin oranges (45) stored above 20°C. *L. monocytogenes* has also been isolated from strawberries obtained from retail markets (30) and from dried fruits (37). Recalls of fresh-cut melons (58) and fresh-cut fruit salad because of possible contamination with *L. monocytogenes* (31) have been documented.

Decontamination of fruit surfaces during processing is not always possible nor fully effective. One of the approaches tested to reduce bacterial pathogens on fruit surfaces is the application of bacteriocins (21). Nisin reduced *L. monocytogenes* populations on honeydew melon slices and apple slices, especially when used in combination with a phage mixture (32). In combination with sodium lactate, potassium sorbate, or both, application of nisin as sanitizer treatment on whole and fresh-cut cantaloupe gave significant reductions of *Salmonella* (55). Decontamination of whole cantaloupe and honeydew melon surfaces with a combination of hydrogen peroxide, nisin, sodium lactate, and citric acid as a sanitizer prevented further transfer of the inoculated *L. monocytogenes* and *Escherichia coli* to fresh-cut pieces (53).

Enterocin AS-48 is a broad-spectrum cyclic antimicrobial peptide (reviewed in Maqueda et al. (35)) that is now being tested against foodborne bacterial pathogens (2, 3, 16, 23–26, 34, 41, 42). In a previous study, enterocin AS-48 was tested for decontamination of *L. monocytogenes* in-
oculated in green asparagus, alfalfa, and soybean sprouts (16). Application of washing treatments containing enterocin AS-48 (25 μg/ml) reduced viable cell counts of L. monocytogenes below detection limits in refrigerated samples but failed to inhibit regrowth at 22°C. Application of washing treatments containing AS-48 in combination with several other antimicrobials afforded complete inactivation of the listeria in sprouts during storage at 22°C (16). On the basis of this previous knowledge (and considering that processed fruits are a completely different food system compared with sprouts), the purpose of this study was to determine the efficacy of enterocin AS-48 alone and in combination with chemical preservatives against L. monocytogenes inoculated on whole as well as sliced fruits under different storage conditions.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. L. monocytogenes CECT 4032 was provided by the Spanish Type Culture Collection (CECT) and was isolated in Colindale, United Kingdom, from a patient with meningitis associated with eating contaminated cheese. Enterococcus faecalis A-48-32 (36) was used for production of enterocin AS-48, and E. faecalis S-47 from our collection was used for standard determination of bacteriocin activity. All strains were cultivated with the use of a 1% inoculum on brain-heart infusion (Scharlab, Barcelona, Spain) broth at 37°C and stored at 4°C.

Preparation of enterocin AS-48 and chemical preservatives. Enterocin AS-48 was recovered from cultured broths of the producer strain E. faecalis A-48-32 in CMG medium by cation exchange chromatography as described elsewhere (1). Bacteriocin concentrates were filtered through 0.22-μm-pore-size low-protein binding filters (Milllex GV, Millipore Corp., Belford, Mass.) under sterile conditions and tested for bacteriocin activity against the indicator strain E. faecalis S-47 by the agar well diffusion method using stainless steel cylinders of 8-mm (outer) diameter. Immersion solutions were prepared by diluting bacteriocin concentrates (500 μg/ml) in sterile distilled water or in aqueous solutions of the chemical preservatives to be tested in the case of combined treatments.

Trisodium trimetaphosphate, sodium lactate, lactic acid, polyphosphoric acid, p-hydroxybenzoic acid, n-propyl p-hydroxybenzoate, and 2-nitropropanol were purchased from Sigma (Madrid, Spain). Carvacrol and hydrocinnamic acid were from Fluka (Madrid, Spain). The commercial solutions or concentrated stock solutions prepared by dissolving the solid compounds in sterile distilled water or in ethanol were diluted at least 20-fold in sterile distilled water to prepare the immersion solutions for use as washing treatments. All solutions were prepared fresh before use.

Fruit preparation and bacteriocin treatments. Raw fruits were purchased at local supermarkets and kept under refrigeration for not longer than 24 h until use. Fruits were used either as whole pieces (raspberries), sliced (blackberries and strawberries), or peeled and sliced (pear, kiwi, melon, and watermelon). Slices were ca. 1.0 by 1.0 by 1.0 cm in size. Juice from watermelon was prepared from peeled watermelon with a Moulinex Frutti Pro (Moulinex, France) fruit juice extractor. All manipulations were carried out under aseptic conditions. Fruits and fruit slices (ca. 3 g) were artificially contaminated (10 μl per piece) with a sterile saline solution suspension of L. monocytogenes CECT 4032 (1.0 × 10⁶ CFU/ml) previously grown overnight in brain heart infusion broth at 37°C. After inoculation, fruits were allowed to dry for 1 h at room temperature and then were treated for immersion for 5 min at room temperature in 5 ml of sterile distilled water (controls) or distilled water containing enterocin AS-48 (25 μg/ml). After immersion treatments, excess immersion solution was drained on sterile filter paper, and samples were stored in sterile capped 50-ml polypropylene test tubes placed in a freezer or in refrigerated incubation chambers (Memmert, Schwabach, Germany) at desired incubation temperatures (−20, 6, 15, 22°C) for different periods of time. At each step, duplicate samples (3 g each) were mixed with 5 ml of sterile saline solution (0.85% NaCl) and pumped for 3 min in a Stomacher 80 (Biomaster, Seward Limited, Worthing, UK) before they were serially diluted in sterile saline solution and spread in triplicate on plates of PALCAM agar with added Listeria supplement (Merck & Co. Inc., Whitehouse Station, N.J.). Plates were incubated at 37°C for 48 h, and the number of colonies showing features typical of Listeria was determined to calculate viable cell counts. Confirmation of L. monocytogenes was done by PCR amplification of the hlyA gene with primers DG69 (GTGCCGCACAAGAAAAGGTTA) and DG74 (CGCCACACTGGATAC) as described by Choi and Hong (15). The expected 636-bp amplicon was visualized after agarose gel electrophoresis. Before being artificially contaminated, a control sample of the raw material was tested for the presence of L. monocytogenes as described above, and positive samples were discarded.

Juice from watermelon was inoculated (1%, vol/vol) with a L. monocytogenes cell suspension (5.0 × 10⁶ CFU/ml) prepared as described above. Bacteriocin treatment of juice was carried out by adding enterocin AS-48 to a final concentration of 25 μg/ml. Storage and sample analyses were carried out essentially as described above.

Combined treatments of enterocin AS-48 and chemical preservatives were carried out on L. monocytogenes artificially contaminated melon slices essentially as described above by using immersion solutions containing enterocin AS-48 (25 μg/ml final concentration), the corresponding chemical compounds, or both. Viable counts of Listeria were determined as described above after immersion treatment (time 0) and after 24 h of incubation at 22°C.

Statistical analysis. The average data from duplicate trials and standard deviations were calculated with the Excel program (Microsoft Corp., Redmond, Wash.). To determine the statistical significance of the data, a t test was performed at the 95% confidence interval with Statgraphics Plus version 5.1 (Statistical Graphics Corp., Herndon, Va.). The significance of combined treatments was determined by comparison of data from the same incubation time.

RESULTS

Effect of enterocin AS-48 on whole fruits, sliced fruits, and fruit juice stored at different temperatures. Whole raspberries as well as sliced blackberries and strawberries inoculated with L. monocytogenes were treated with the bacteriocin solution. In the raspberries, enterocin AS-48 reduced the concentration of Listeria by 1 to 1.3 log units after treatment, but no additional reduction was observed during storage of treated samples at −20°C (Fig. 1A and 1B). However, in the samples stored at 6°C, the concentration of Listeria was reduced below detection limits (30 CFU/g) at least during days 1, 3, and 7 (Fig. 1A). Significant reductions (P < 0.05) were also obtained in sam-
FIGURE 1. Effect of washing treatments with enterocin AS-48 (25 μg/ml) on L. monocytogenes CECT 4032 inoculated on whole raspberries (A, B), sliced blackberries (C, D), and sliced strawberries (E, F). After treatment, fruits were stored at −20°C (squares), 6°C (rhombs) (A, C, E), 15°C (circles), and 22°C (triangles) (B, D, F). Open symbols, controls; closed symbols, samples treated with enterocin AS-48. Data represent the mean ± SD (error bars) of two independent experiments.

samples stored at 15 and 22°C, although slight growth was observed in both cases after 2 days of incubation (Fig. 1B).

In sliced blackberries stored at −20 and 6°C, treatment with bacteriocin reduced the counts of viable Listeria below the detection limits after the first 24 h (Fig. 1C). Treatment with bacteriocin also reduced the counts of Listeria below detection limits after 8 h at 15°C and 24 h at 22°C (Fig. 1D).

In sliced strawberries, Listeria showed a lower survival and growth capacity, especially in the samples stored at 6°C (Fig. 1E). For most of the samples, no viable Listeria was detected after bacteriocin treatment and storage for all temperatures tested (Fig. 1E and 1F).

In sliced pear samples, washing treatment with bacteriocin reduced the initial counts of Listeria by 3.44 log units up to levels below detection limits (Fig. 2A and 2B). However, viable Listeria were detected at concentrations between 0.69 and 1.67 log units during storage of the treated samples at −20 and 6°C, although these values always were significantly lower (P < 0.05) than controls. For the treated samples stored at 15 and 22°C, a greater recovery of Listeria population was observed during storage, although the counts obtained were significantly lower (P < 0.05) in both cases after 48 h compared with the control samples.

In sliced kiwi, bacteriocin treatment caused a reduction near 2.9 log units (Fig. 2C and 2D). No viable Listeria was detected from days 1 to 7 in the treated samples stored at −20°C, whereas in the samples stored at 6°C the counts oscillated between 1 and 1.92 log units, being significantly lower (P < 0.05) than controls (Fig. 2C). The samples stored at 15 and 22°C showed very similar results, with an increase in the concentration of viable Listeria up to ca. 2 log units during storage (Fig. 2D).

The results obtained in sliced melon and in watermelon were very similar, with reductions of viable cell counts from 0.63 to 1 log unit after treatments (Fig. 3). In treated samples stored at −20 or 6°C, Listeria counts remained stable at least until day 5, with a slight increase of the population by day 7 of storage at 6°C (Fig. 3A and 3C). Viable cell counts of treated samples stored at 6°C were significantly lower (P < 0.05) than controls, in which Listeria was able to grow up to 5.7 log units (Fig. 3A and 3C). Bacteriocin treatment did not prevent growth of Listeria in samples stored at 15 or 22°C (Fig. 3B and 3D).

Juice from watermelon was inoculated with an initial concentration of Listeria of ca. 4.5 log units and supplemented or not with AS-48 (25 μg/ml final concentration). In the samples treated with enterocin AS-48, the concentration of Listeria decreased gradually depending on time and storage temperature (Fig. 3E and 3F). Thus, in the sam-
FIGURE 2. Effect of washing treatments with enterocin AS-48 (25 µg/ml) on L. monocytogenes CECT 4032 inoculated on sliced pear (A, B) and sliced kiwi (C, D). After treatment, fruits were stored at −20°C (squares), 6°C (rhombus) (A, C), 15°C (circles), and 22°C (triangles) (B, D). Open symbols, controls; closed symbols, samples treated with enterocin AS-48. Data represent the mean ± SD (error bars) of two independent experiments.

FIGURE 3. Effect of enterocin AS-48 (25 µg/ml) on L. monocytogenes CECT 4032 after application of washing treatments on sliced melon (A, B) and sliced watermelon (C, D) or bacteriocin addition in juice from watermelon (E, F). After treatment, fruit and fruit juice samples were stored at −20°C (squares), 6°C (rhombus) (A, C), 15°C (circles), and 22°C (triangles) (B, D). Open symbols, controls; closed symbols, samples treated with enterocin AS-48. Data represent the mean ± SD (error bars) of two independent experiments.

Samples stored at −20 and 6°C, Listeria counts were significantly lower (P < 0.05) than control samples after 24 h and decreased below the detection limits after 3 days of storage (Fig. 3E). The loss of viability was more pronounced in samples stored at 15 and 22°C, in which viable counts were significantly lower (P < 0.05) than controls after 8 h of incubation, without detectable Listeria after 24 h (Fig. 3F).
FIGURE 4. Effect of combined washing treatments on L. monocytogenes CECT 4032 inoculated on sliced melon stored at 22°C. The chemical compounds tested were trisodium trimetaphosphate (TSTMP) at 1% (A) and 2% (B), sodium lactate at 1% (C) and 2% (D), 0.5% lactic acid (E), and 0.1% polyphosphoric acid (F). Samples were treated with the antimicrobial compounds alone (●) or in combination with AS-48 at 25 μg/ml (▲). Untreated controls (○). Sample treated with AS-48 alone (◇). Data represent the mean ± SD (error bars) of two independent experiments.

FIGURE 5. Effect of combined washing treatments on L. monocytogenes CECT 4032 inoculated on sliced melon stored at 22°C. The chemical compounds tested were carvacrol at 6 mM (A) and 12 mM (B), hydrocinnamic acid (HC) at 5 mM (C), p-hydroxybenzoic acid (PHB) at 50 mM (D), n-propyl p-hydroxybenzoate (PPHB) at 50 mM (E) and 100 mM (F), and 2-nitropropanol at 0.1% (G) and 0.5% (H). Samples were treated with the antimicrobial compounds alone (●) or in combination with AS-48 at 25 μg/ml (▲). Untreated controls (○). Data represent the mean ± SD (error bars) of two independent experiments.

Application of enterocin AS-48 in combination with other antimicrobial agents in the washing treatments of sliced fruit. Because washing treatments with bacteriocin solutions exerted a very reduced protective effect against L. monocytogenes during storage in several of the sliced fruits tested, especially in those stored at higher temperatures, combined treatments of AS-48 (25 μg/ml) with chemical preservatives were assayed on sliced melon stored at 22°C as model. Growth of Listeria after washing with bacteriocin alone is shown in Figure 4A.

Although treatments with trisodium trimetaphosphate, sodium lactate, lactic acid, or polyphosphoric acid alone had no effect on Listeria viability, increasing bactericidal effects were observed in combination with enterocin AS-48 (Fig. 4). Reductions of viable cell counts for the combined treatments of trisodium trimetaphosphate as well as sodium lactate and AS-48 were proportional to the concentrations of preservative added (Fig. 4A through 4D). Combined treatments with lactic and polyphosphoric acids and enterocin AS-48 reduced viable cell counts below detection levels by at least 2.74 log units (Fig. 4E and 4F). During storage of the treated samples, growth of Listeria was markedly inhibited for the combined treatment with 2% lactate, but not for the other treatments (Fig. 4D). Nevertheless, viable cell counts for the combined treatments were significantly lower than controls in most of the samples (Fig. 4A through 4F).

Carvacrol had a concentration-dependent antilisterial effect (Fig. 5A and 5B). In the combined treatments with enterocin AS-48, viable cell counts of the treated samples were significantly lower (P < 0.05) than controls for 6 mM carvacrol (Fig. 5A) or were reduced below detection limits during the whole storage period for 12 mM carvacrol (Fig. 5B). Hydrocinnamic acid had no effect on the Listeria at 1 mM (data not shown), but it caused some growth inhibition at 5 mM when tested alone and reduced viable cell counts below detection levels within the first 8 h of incubation when tested in combination with enterocin AS-48 (Fig. 5C).

Washing with p-hydroxybenzoic acid at 20 mM in combination with enterocin AS-48 caused a significant re-
duction of 1.2 log units ($P < 0.05$) but did not prevent further proliferation of survivors (data not shown). At a higher concentration of 50 mM $p$-hydroxybenzoic acid, the combined treatment maintained the concentration of *Listeria* below 1.4 log units during storage (Fig. 5D). Treatment with 2-n-propyl $p$-hydroxybenzoate at 25 mM had no effect on *Listeria* viability (data not shown), but it did reduce viable cell counts when tested at 50 and 100 mM (Fig. 5E and 5F). For both concentrations, the combined treatment with AS-48 reduced the *Listeria* population below detection limits, although only the highest concentration of preservative prevented regrowth during storage (Fig. 5F).

In the combined treatments of 2-nitropropanol with enterocin AS-48, viable cell counts of *Listeria* were reduced significantly ($P < 0.05$) in proportion to the concentration of 2-nitropropanol used (Fig. 5G and 5H). Although regrowth was always observed during storage of samples, the counts of *Listeria* for the combined treatments were significantly lower ($P < 0.05$) compared with the controls (Fig. 5G and 5H).

**DISCUSSION**

Fruits and fruit juices can be the vehicle of transmission for foodborne pathogenic bacteria arriving from different sources such as the environment, manure, animal excreta, irrigation, and processing water, as well as harvesting and processing (27, 33). Because fruits (either as whole pieces or as ready-to-eat slices) are often stored under refrigeration, prolonged storage might facilitate proliferation of psychrotrophic bacteria such as *L. monocytogenes*. Food products that support growth of *L. monocytogenes* to levels in excess of 2 log CFU/g (14) are considered of higher risk. According to results from this study, *L. monocytogenes* CECT 4032 did not multiply in most of the fruits stored at 6°C, such as raspberries (pH 3.3), pear (pH 4.81), or kiwi (pH 3.49), but it proliferated on sliced melon (pH 4.82) and sliced watermelon (pH 5.74) to levels close to or exceeding 6 log CFU/g. It also showed a good capacity for survival in most of the fruits tested, except those with a lower pH such as blackberries (pH 2.50) and sliced strawberries (pH 2.70). Results from other authors have also reported variable decreases in the population of viable listeria in fruits and fruit juices depending on the fruit acidity and composition (6, 20, 45, 46). We should remark on the high degree of survival detected in samples stored at −20°C. Also, under temperature abuse conditions of 15 and 22°C, the population of *L. monocytogenes* increased in most of the fruits tested, except blackberries and strawberries. These results confirm the risk of *L. monocytogenes* contamination in fruits and the need to apply additional measures to prevent survival and proliferation of this pathogen.

Application of enterocin AS-48 in washing treatments showed a higher efficacy against *L. monocytogenes* in fruits with a lower pH compared with those with a higher pH (such as sliced melon and watermelon), as well as in fruit samples stored under refrigeration at 6°C compared with those stored at 15 or 22°C. Washing treatments applied to ready-to-eat fruits that are stored under refrigeration could be useful to reduce the initial concentration of viable listeria, especially in those fruits with a higher pH in which the risk for proliferation of this bacterium is higher. In fruits, application of washing treatments is only recommended right before consumption to avoid damage caused by excess humidity (including mold proliferation). At that point, enterocin AS-48 could be included in washing treatments as an additional hurdle against *L. monocytogenes*.

To increase the efficacy of washing treatments on fruits with a higher pH, enterocin AS-48 was tested in combination with other antimicrobials in sliced melon stored at 22°C. The efficacy of treatments was enhanced clearly by all antimicrobials tested (trisodium trimetaphosphate, sodium lactate, lactic acid, polyphosphoric acid, carvacrol, hydrocinnamic acid, $p$-hydroxybenzoic acid, 2-n-propyl $p$-hydroxybenzoate, or 2-nitropropanol), with a concentration-dependent synergistic effect. In most cases, proliferation of the surviving fraction during storage of the treated samples was also inhibited, especially for the combinations of enterocin AS-48 and carvacrol or 2-n-propyl $p$-hydroxybenzoate. These results are of great relevance not only to avoid proliferation of *L. monocytogenes* in the treated fruits during temperature abuse conditions, but also in view of the general difficulties to apply disinfection treatments in this type of foodstuffs. As an alternative to sliced fruit treatment, Ukuku and Fett (54) applied washing treatments with chlorine solutions (1,000 ppm) or hydrogen peroxide (5%) against *L. monocytogenes* on whole cantaloupe to avoid contamination of the sliced fruit during processing. In other cases, treatment with ozone, chlorine dioxide, peroxycetic acid, and chlorinated trisodium phosphate has been reported to reduce the concentration of viable *L. monocytogenes* on strawberries and whole cantaloupe (49). Treatment of kiwifruit and honeydew melon with carvacrol has been found to delay spoilage without causing adverse organoleptic changes (50). Nisin has also been tested for fruit decontamination (32, 53, 55). In a previous study, it was shown that the antilisterial activity of enterocin AS-48 in washing treatments applied on sprouts was enhanced by sanitizers and other antimicrobial compounds (16). However, because of the peculiar characteristics of fruits and sliced fruit dishes (such as fruit salads) the types of decontamination treatments that could be used are much more limited compared with sprouts and other vegetables. Therefore, it is important to test the synergies of different compounds in order to decrease the concentrations added in the food to achieve the desired antimicrobial effects. Furthermore, this is the first report on the application of this bacteriocin for direct decontamination of fruits. Given the high susceptibility of whole and sliced fruits to organoleptic changes caused by treatment with disinfectants, application of enterocin AS-48 (an odorless and tasteless natural substance) alone or in combination with reduced concentrations of selected antimicrobials could provide a useful method to increase safety against *L. monocytogenes*, especially in fruits to be consumed by individuals with a higher susceptibility to this bacterium.

**ACKNOWLEDGMENTS**

This work was supported by the Spanish Ministry of Education (research project AGL2005-07665-C02-02/ALI). A. Cobo Molinos received
a fellowship from MAPFRE. We also acknowledge the Research Programme of the University of Jaén, and the Research Plan of the Junta de Andalucía (research group AGR230).

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