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# Enhancing food preservation and safety: Synergistic effects of *Allium*-derived organosulfur compounds and outer membrane permeabilization peptide L-11

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ABSTRACT

Ready-to-eat (RTE) foods, characterized by the lack of a terminal heating step prior to consumption, offer enhanced organoleptic qualities compared to their cooked counterparts, as they retain more of their natural flavors, textures, and nutritional content. However, these untreated products pose a risk for foodborne illnesses if not promptly consumed and/or adequately stored under refrigeration or in a frozen state. This investigation delves into the synergistic antimicrobial effects of a novel human-mimetic peptide, L-11, in conjunction with organosulfur compounds derived from *Allium* species—namely allicin, diallyl sulfide (DAS), dipropyl sulfide (DPS), propyl propane thiosulfinate (PTS), and propyl propane thiosulfonate (PTSO)—on the inhibition and control of Gram-negative foodborne pathogens across various food matrices. Notably, despite the minimal individual antimicrobial activity of peptide L-11 and the evaluated organosulfur compounds against target bacteria (*Escherichia coli*, *Salmonella enterica*, *Yersinia enterocolitica*, and *Shigella sonnei*), their combinations exhibited remarkable synergistic effects *in vitro* with until 32-fold reduction for some combinations. Specifically, combinations including PTS and PTSO demonstrated up to a 99% reduction in bacterial proliferation within RTE food models such as salmorejo, aioli, pumpkin cream, and spinach cream. These findings highlight the potential applications of these synergistic combinations in extending the shelf-life and enhancing the safety of RTE foods. To the best of our knowledge, this represents the inaugural report of such synergistic interactions between organosulfur compounds and peptides in extending the shelf-life of RTE foods.

## 1. Introduction

The World Health Organization (WHO) has highlighted the grave impact of unsafe food consumption, attributing over 600 million cases of foodborne diseases annually worldwide, with around 420,000 resultant fatalities. Particularly alarming is the toll on children under five, who account for approximately 125,000 of these deaths. The economic implications are also significant, with an estimated $110 billion lost annually due to decreased productivity and medical expenses (WHO, 2015). Addressing the needs of a growing global population for safe, nutritious, and healthy food is a substantial challenge for the food industry, national governments, and international bodies. The adoption of the "One Health" approach, recognizing the interconnectedness of human, animal, and environmental health, is increasingly seen as vital for the comprehensive management of food safety and security issues (Garcia et al., 2020). Furthermore, the rise of antibiotic-resistant pathogens within the food chain has heightened vigilance across food safety sectors. This trend underscores the urgent need for ongoing surveillance, research, and the development of new strategies to curb resistance spread. The movement of resistant bacteria through the food system not

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only complicates the management of foodborne illnesses but also poses a significant threat to global public health. A coordinated, multidisciplinary response involving all stakeholders in food production, regulation, and disease prevention is essential to address this challenge ((BIOHAZ) Hazards et al., 2021; Kim & Ahn, 2022; Larsson & Flach, 2022; Verraes et al., 2013).

The current trend among consumers is a growing preference for minimally processed, fresh foods without artificial additives, prioritizing superior organoleptic qualities. Traditional food processing techniques, which typically use heat to reduce microbial contamination, can negatively impact the flavor, color, texture, and nutritional and bioactive properties of foods, particularly those sensitive to heat (Augusto, 2020). As a result, refrigerated ready-to-eat (RTE) plant-based foods, many of which avoid thermal preservation to retain sensory and functional attributes, are increasingly favored (Ferrari et al., 2019; Smigic et al., 2023; Tîrziu et al., 2020). However, these foods are vulnerable to microbiological risks, including foodborne bacteria, due in part to the necessity of human handling in their preparation. At the same time, there is a significant shift towards "clean label" preservation methods that avoid synthetic chemical preservatives (Gokoglu, 2019). In response, clean label antimicrobials from natural sources and with minimal processing are being explored. These include animal-derived compounds (e.g., lysozyme, lactoferrin, chitosan) microbial sources (e.g., bacteriocins, fermentates, protective cultures), and plant-origin bioactive compounds such as essential oils, phenolic extracts, and *Allium*-derived organosulfur compounds (AOSCs) (Carocho et al., 2015; Choi et al., 2024; Grant and Parveen, 2017; Lu et al., 2022). Among botanical preservatives, organosulfur compounds from *Allium* plants, such as garlic and onion, are particularly valued for their distinctive flavors and health benefits, including the prevention of various chronic diseases (Lu et al., 2022). These compounds exhibit a wide range of biological activities, such as antioxidant, anti-inflammatory, anticancer, immunomodulatory, and cardioprotective effects, and can modulate the gut microbiome and enhance immune function, offering a holistic approach to health and disease prevention (Guillamón et al., 2021; Lu et al., 2022). Traditionally utilized as flavoring agents, AOSCs are also recognized for their ability to inhibit the growth and toxin production of a wide array of microorganisms, including Gram-positive and Gram-negative bacteria, viruses, parasites, and fungi (Bhatwalkar et al., 2021; Falcón-Piñeiro et al., 2021; Krstin et al., 2018; Rouf et al., 2020; Sorlozano-Puerto et al., 2018, 2020). These properties highlight their potential as effective and natural food preservatives in the contemporary shift towards cleaner, safer, and more natural food preservation methods. The primary challenge in utilizing AOSCs as clean label antimicrobials in ready-to-eat (RTE) foods is their sensory impact. At concentrations sufficient for effective antimicrobial action, AOSCs can alter the sensory characteristics of food, potentially making them unappealing to consumers (Liu et al., 2022). Despite this limitation, interest in researching organosulfur-derived compounds and essential oils as antimicrobial agents has grown, driven by their broad-spectrum activity and GRAS (generally recognized as safe) status. An innovative approach to circumvent the organoleptic impact of AOSCs involves leveraging synergism in food preservation. This strategy combines multiple preservation methods, such as modified atmosphere, pH adjustment, salt/sugar concentration, antimicrobial peptides, essential oils, pulsed electric fields, cold plasma, irradiation, and high-pressure processing. These combinations aim to hinder microbial growth through various mechanisms, thereby enabling milder preservation techniques that do not compromise food's sensory attributes (Leistner, 1992; Liu et al., 2022). Particularly promising is the synergistic use of proteinaceous preservatives like bacteriocins and antimicrobial peptides with other preservation hurdles. This approach enhances the antimicrobial effect, thereby improving food safety and extending shelf life, while maintaining the attractiveness of food products. The bacteriocins low toxicity to eukaryotic cells, rapid action, and digestibility further underscore their suitability for food safety applications. Previous studies have demonstrated the effectiveness of combining these proteinaceous preservatives with essential oils to broaden the antimicrobial effect against both Gram-negative and Gram-positive bacteria (Liu et al., 2022; Tanhaeian et al., 2020). The L-11 peptide, composed of 11 amino acids, is derived from the human cathelicidin LL-37. While L-11 generally lacks the direct antimicrobial properties of its parent molecule, it is optimized for enhanced stability and efficacy. This peptide exhibits the ability to permeabilize the outer membrane of Gram-negative bacteria, disrupting their cell walls and making them more susceptible to hydrophobic antibiotics and other antimicrobial agents (Q. Li et al., 2021; Xia et al., 2021). L-11's ability to disrupt bacterial membranes and enhance the efficacy of other antimicrobial agents makes it a valuable tool in various applications, ranging from medical and clinical use to food safety (Cebrián et al., 2021; Q. Li et al., 2021; Xia et al., 2021). By incorporating L-11 into food preservation strategies, it is possible to enhance the antimicrobial effectiveness of natural compounds, such as organosulfur compounds from *Allium* species, thereby extending the shelf-life of ready-to-eat (RTE) foods and reducing the risk of foodborne illnesses.

In this study, we investigate the synergistic antimicrobial potential of three AOSCs derived from garlic—allicin, DAS, and DPS—alongside two compounds from onion—PPTS and PTSO. These AOSCs were combined with the Gram-negative outer-membrane disrupting peptide L-11, a human cathelicidin-derived undecapeptide known for its capability to permeabilize the outer membrane of gram-negative bacteria, thereby enhancing their susceptibility to antibiotics (Cebrián et al., 2021; Li et al., 2021; Xia et al., 2021). This study focuses on evaluating the effectiveness of these combinations against Gram-negative foodborne pathogens in various ready-to-eat (RTE) food models. The overarching goal is to develop an effective and natural antimicrobial strategy that enhances food safety and extends the shelf-life of RTE foods.

## 2. Material and methods

### 2.1. Microorganisms and culture conditions

The bacterial strains *Escherichia coli* CECT 516, *Salmonella enterica* CECT 7159 and CECT 7160, *Shigella sonnei* CECT 547, and *Yersinia enterocolitica* CECT 754 were sourced from the CECT (Spanish Type Culture Collection) for this study. For the *in vitro* experiments, these strains were routinely cultured in Mueller Hinton broth (BD Difco, Leeuwarden, The Netherlands) at 37 °C with agitation at 200 rpm. When solid media were required, agarose was incorporated at a concentration of 1.5%. In food challenge tests, specific media were employed for the enumeration of each pathogen: ECC agar for *E. coli*, COMPASS *Salmonella* agar for *Salmonella* spp., SS Agar (Biokar Diagnostics, Allone, France) for *S. sonnei*, and Yersinia CIN agar (Condalab, Madrid, Spain) for *Y. enterocolitica*. These media selections facilitated the accurate detection and enumeration of the targeted foodborne pathogens within the food matrices under investigation.

### 2.2. Allium organosulfur-derived compounds and peptide

The organosulfur compounds allicin, DAS, DPS, PTS, and PTSO, all with a purity exceeding 95%, were procured from DOMCA SAU (Granada, Spain). These compounds were solubilized in polysorbate-80 to achieve the desired concentrations for the study. The L-11 peptide, featuring a purity greater than 95%, was synthesized by Pepscan (Lelystad, The Netherlands). Nisin Z was purchase from Handary (Brussels, Belgium). Unless otherwise noted, all additional reagents utilized in the experiments were sourced from Sigma-Aldrich Química S. L., (Madrid, Spain), ensuring high-quality and consistent materials for the research.

### 2.3. Minimal inhibitory concentration, minimal bactericidal concentration, and synergy test

The minimum inhibitory concentration (MIC) of each antimicrobial agent, was determined using the broth microdilution method, in line with the guidelines set forth by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2015). This involved the preparation of decreasing concentrations of AOSCs, peptide L-11, and nisin (used as positive control in synergism (Q. Li et al., 2021) from 256 to 0.25 μg/mL in 96-well plates which were then inoculated with the bacterial strains to achieve a final bacterial concentration of 5 $Log_{10}$ CFU/mL. To confirm the minimum bactericidal concentration (MBC), wells showing no bacterial growth, as indicated by the absence of absorbance at 620 nm, were subjected to further culturing on selective agar media. These cultures were then incubated at 37 °C for 24–48 h, allowing for the determination of the concentration at which bacterial growth was completely inhibited.

To assess synergistic effects, after MIC evaluations, the AOSCs MIC was evaluated in conjunction with a sub-MIC level of L-11 (4x, 8x, 16x, and 32x lower than the observed MIC for L-11 alone) and next, the fractional inhibitory concentration indices (FICI) were calculated using the previously described formula $FICI = FICa + FICb = MIC_{ac}/MIC_a + MIC_{bc}/MIC_b$ where: $MIC_a$ is the MIC for the AOSCs alone, $MIC_b$ for L-11 alone, $MIC_{ac}$ for the AOSCs in the presence of a $MIC_{bc}$ concetratio of L-11 peptide. The FICI was interpreted according to EUCAST as follows: synergistic, $FICI \leq 0.5$; additive, $0.5 < FICI \leq 1$; indifferent, $1 < FICI < 2$; antagonistic, $FICI \geq 2$. For the FICI calculations, twice the highest concentration tested was used in the cases where the MIC was not reached (Xia et al., 2021). For this procedure, the peptide was mixed with MHB at twice the target concentration, followed by the addition of 50 μL of this mixture into each well of a 96-well plate, except for the first column, which received 100 μL. The AOSCs or nisin, prepared at twice its final concentration, were introduced in the first column and then serially diluted across the plate from columns 1 to 11. Each well then received 50 μL of a bacterial suspension at $10^5$ CFU/mL. The assay, conducted in triplicate, was analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

### 2.4. Food trials

#### 2.4.1. Food preparation

Efficacy trials were performed to evaluate the antimicrobial properties of AOSCs, both individually and synergistically with the L-11 peptide, across a range of food models, including *salmorejo*, aioli, pumpkin cream, and spinach cream. The preparation processes for these food models are outlined as follows:

*Salmorejo* is a traditional ready-to-eat (RTE) dish from Spain, made with tomato, virgin olive oil, garlic, and bread. The preparation involved blending 5 kg of fresh tomatoes, which were then strained to remove seeds and skin. Subsequently, 1 kg of bread, 250 g of virgin olive oil, 10 g of salt, and 1 g of raw garlic, all sourced from a local vendor in Alhendín (Granada, Spain), were added to the tomato base. The mixture was then thoroughly blended until it achieved a creamy texture.

Aioli was created using 20 eggs, 800 g of extra virgin olive oil, 10 g of sliced raw garlic, and 5 g of salt, all procured from a local market in Alhendín (Granada, Spain). Garlic, salt, and eggs, which were previously brought to room temperature (20 °C) to aid in emulsification, were combined first. The mixture was then gradually emulsified with the oil using a mechanical mixer until the desired texture was achieved.

For both pumpkin cream and spinach cream, the process was identical, with the only variation being the use of either 4 kg of pumpkin or spinach. These vegetables, along with 2 L of milk, 500 ml of cream, 10 g of raw onion, and 10 g of salt (all sourced locally), were cooked and then blended together to form the cream. This method ensured a homogeneous mixture conducive to evaluating the antimicrobial effectiveness of the treatments in a consistent food matrix.

#### 2.4.2. Sensory analysis of foods

To mitigate the potent sensory impact of *Allium* compounds and select consumer-acceptable doses for food efficacy trials, a preliminary sensory evaluation was conducted (International Organization for Standardization., 2021). This assessment aimed to mirror consumer evaluations more objectively and realistically using aioli as the test food matrix. The sensory panel comprised 20 untrained participants, evenly split between ten women and ten men, ranging in age from 22 to 60 years, randomly chosen for diversity. The evaluation involved aioli samples with two different concentrations of AOSCs - 16 and 32 μg/g, along with a control batch without any added compound and another batch containing the peptide L-11 at a concentration of 12 μg/g. Each sample, weighing 10 g, was served in sterile plastic cups and brought to room temperature prior to assessment.

To ensure a clear palate for accurate sensory evaluation, panelists were provided with water and unsalted toast both at the start and between sampling different aiolis. They were asked to rate each sample on a 10-point scale, where a score of 0 represented the absence of an attribute and a score of 9 indicated an extremely intense presence of the attribute. The sensory characteristics evaluated included appearance (color, homogenization), odor (seasoning smell, garlic and onion smell), flavor (flavor intensity, garlic and onion taste, acidity, salting), and texture. This comprehensive sensory evaluation was conducted in a specially designed laboratory equipped with individual booths and white lighting to minimize external influences on the panelists' perceptions, thereby ensuring the integrity and reliability of the data collected.

#### 2.4.3. Challenge test

Following the selection of AOSCs doses that achieved sensory acceptance, antimicrobial efficacy trials, also known as challenge tests, were conducted based on the methodology described by (Ariza et al., 2021). In these tests, ready-to-eat (RTE) foods including *salmorejo*, aioli, pumpkin cream, and spinach cream were inoculated with *E. coli* CECT 516, *S. enterica* CECT 7160, *S. sonnei* CECT 457 and Y. enterocolitica CECT 745 respectively in sterile polypropylene containers to reach a final concentration of 2–3 $Log_{10}$ CFU/mL. Subsequently, the selected AOSCs (allicin, DAS, DPS at 16 μg/g, and PTS/PTSO at 8 or 16 μg/g) and the L-11 peptide (12 μg/g) were applied directly to the foods. Distilled water served as a negative control, while potassium sorbate at 1000 μg/g (Scharlab, Barcelona, Spain) was used as a positive control. All food samples were then stored at 4 °C, and two independent experiments were conducted for each food type.

Sample analysis involved collecting at intervals aligned with the shelf life of each RTE food. Food samples were diluted 1:10 with buffered peptone water (Biokar Diagnostics, Barcelona, Spain) and homogenized using a MASTICATOR mixer (IUL, Barcelona, Spain). The diluted samples were cultured on selective media plates and incubated at 37 °C for 24–48 h. The results were quantified as $Log_{10}$ CFU/mL over time. For instances where bacteria were not quantifiable due to being below the detection limit (<1 $Log_{10}$ CFU/g), further analysis was conducted through pre-enrichment in nutrient broth (Biokar Diagnostics, Barcelona, Spain), with findings reported as either the presence or absence of bacteria.

### 2.5. Statistical analysis

Statistical analysis of the data derived from two independent challenge test was conducted to evaluate the antimicrobial efficacy of the treatments. For each experiment, three independent samples per treatment were tested at each sampling time, resulting in a total of six samples ($n = 6$) for each treatment across both experiments. Data, including average values and standard deviations, were calculated using Excel software (Microsoft Corp., Redmond, WA, USA). For further statistical examination, SPSS-PC version 15.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) were utilized. The microbiological count data underwent analysis of variance (two-way ANOVA) to determine the significance of differences between treatments over time, followed by Tukey's post hoc test for multiple comparisons. An error probability value (*p-value*) greater than 0.05 was interpreted as statistically significant, indicating a statistically significant difference between the means of the groups being compared.

## 3. Results and discussion

### 3.1. Antimicrobial activity of AOSCs alone and in combination with L-11

Initially, we assessed the antimicrobial activity of selected AOSCs, nisin and the L-11 peptide against five Gram-negative bacterial strains individually. According to our findings presented in Table 1, onion derivatives, PTS and PTSO, exhibited superior antimicrobial activity compared to the garlic-derived compounds allicin, DSA, and DPS, which showed no activity at the highest concentration tested (256 μg/mL). Following this, we investigated the synergistic effects of combining allicin, DSA, DPS, PTS, and PTSO with the L-11 peptide, using nisin as a positive control for synergism (Q. Li et al., 2021).

The most efficacious combinations, as delineated by FICI calculations, are detailed in Table 1. Notably, while synergistic effects between the L-11 peptide and nisin have been documented across a spectrum of Gram-negative pathogens (Li et al., 2021), no synergistic activity was detected in the instances of *Shigella sonnei* CECT 457 and *Yersinia enterocolitica* CECT 754. For *Escherichia coli* and *S. sonnei*, the AOSCs from garlic did not exhibit synergism, in contrast to the observed effect on *Salmonella* strains where the MICs for these compounds were reduced by a factor greater than 16. The synergistic interaction with *Y. enterocolitica* was found to be dependent on the specific garlic-derived AOSC utilized. Further analysis of the onion derivatives, PTS and PTSO, revealed a more pronounced enhancement in antimicrobial activity for PTS, with FICI values ranging from 0.094 to 0.313, compared to 0.177 to 0.5 for PTSO. This discrepancy can be attributed to the inherently superior activity of PTSO when used in isolation. This observation corroborates previous findings indicating that more potent synergistic effects are typically seen when individual agents exhibit lower baseline antimicrobial efficacy (Xia et al., 2021) highlighting the complexity of interactions between peptides like L-11 and AOSCs in combating Gram-negative bacterial pathogens.

According to our results, numerous studies have highlighted the *in vitro* antimicrobial efficacy of these compounds, notably allicin from garlic, which has received extensive coverage (Hu et al., 2023; Z. Li et al., 2022; O'Gara et al., 2000; Zhu et al., 2022). However, compounds from onions, such as PTS and PTSO, have also been recognized for their significant broad-spectrum antibacterial and antifungal activity, with MIC values similar to those obtained in our work (Sorlozano-Puerto et al., 2018, 2020). Indeed, some researchers have documented their *in vitro* antimicrobial activity against various foodborne pathogens and their capability to extend the shelf life of minimally processed vegetables when incorporated into active packaging (Llana-Ruíz-Cabello et al., 2017; Llana-Ruiz-Cabello et al., 2018). Given the outcomes, we proceeded to investigate the efficacy of these combinations for food preservation and safety. Specifically, we assessed the effectiveness of the selected combinations in controlling the tested foodborne pathogens within various contaminated RTE food matrices. The selection of these food matrices was predicated on their optimal organoleptic acceptability to the tested AOSCs. This approach allowed us to determine not only the antimicrobial potential of the combinations but also their

**Table 1**
MIC and synergism test determination. $MIC_a$ represents the MIC for AOSCs and nisin control while $MIC_b$ is for L-11 peptide. $MIC_{ac}$ represent the lower MIC value for AOSCs and controls in conjunction with the lower L-11 concentration ($MIC_{bc}$) that provide synergism. For the FICI calculations twice, the highest concentration tested was used in the cases where the MIC was not reached. Experiments were conducted in triplicate to ensure reliability, and standard deviations were reported as appropriate to quantify variability within the data.

| | Antimicrobial | Concentrations (μg/mL) | | | | |
|---|---|---|---|---|---|---|
| | | $MIC_a$ | $MIC_b$ | $MIC_{ac}$ | $MIC_{bc}$ | FICI |
| ***E. coli* CECT 516** | Nisin | 128 | 24 | 8 | 6 | 0.313 |
| | PTS | 64 | | 4 | | 0.313 |
| | PTSO | 26.67 ± 9.23 | | 6.67 ± 2.31 | | 0.500 |
| | Allicin | >256 | | >256 | | – |
| | DAS | >256 | | >256 | | – |
| | DPS | >256 | | >256 | | – |
| ***S. enterica* CECT 7159** | Nisin | 128 | 192 | 16 | 12 | 0.188 |
| | PTS | 64 | | 5.3 ± 2.3 | | 0.145 |
| | PTSO | 64 | | 8 | | 0.188 |
| | Allicin | >256 | | 16 | | 0.094 |
| | DAS | >256 | | 16 | | 0.094 |
| | DPS | >256 | | 16 | | 0.094 |
| ***S. enterica* CECT 7160** | Nisin | 128 | 192 | 32 | 12 | 0.313 |
| | PTS | 64 | | 8 | | 0.188 |
| | PTSO | 64 | | 8 | | 0.188 |
| | Allicin | >256 | | 16 | | 0.094 |
| | DAS | >256 | | 16 | | 0.094 |
| | DPS | >256 | | 16 | | 0.094 |
| ***S. sonnei* CECT 457** | Nisin | 128 | 48 | 128 | 6 | 1.125 |
| | PTS | 42.67 ± 18.47 | | 8 | | 0.312 |
| | PTSO | 32 | | 8 | | 0.375 |
| | Allicin | >256 | | >256 | | – |
| | DAS | >256 | | >256 | | – |
| | DPS | >256 | | >256 | | – |
| ***Y. enterocolitica* CECT 754** | Nisin | 128 | 192 | 128 | 12 | 1.063 |
| | PTS | 32 | | 1 | | 0.094 |
| | PTSO | 16 | | 4 | | 0.313 |
| | Allicin | >256 | | 32 | | 0.125 |
| | DAS | >256 | | 64 | | 0.188 |
| | DPS | >256 | | 128 | | 0.313 |

practical applicability in enhancing the safety of RTE foods without compromising their sensory qualities.

### 3.2. Sensory evaluation of the products

While these compounds have traditionally served as flavor enhancers in a variety of foods, their broad-spectrum antimicrobial properties position them as promising natural food preservative additives. This aligns with contemporary trends towards clean labeling (Choi et al., 2024; Inguglia et al., 2023), highlighting their potential beyond mere taste enhancement. However, their strong sensory profile necessitates their inclusion only in foods that incorporate high doses of garlic or onion in their recipes.

To test if the observed MIC reduction for the AOSCs in the presence of L-11 is enough to reduce their sensorial disadvantages which can render in a broad application of the synergistic combinations in food safety, we performed a sensorial evaluation of the AOSCs using concentrations between 32 and 16 μg/g. Following the completion of the manufacturing process, sensory evaluations were carried out on the RTE foods. Untrained panelists assessed ten attributes of the sauces using a scale ranging from 0 to 10. The outcomes, depicted in Figs. 1 and 2, illustrate the results across various categories. Notably, no significant differences were detected among the samples with respect to appearance characteristics, such as color and homogenization.

The RTE food batches incorporating the L-11 peptide received ratings from panelists that were very similar to those of the control, particularly for attributes related to smell and taste, indicating the minimal sensory impact of this compound on such food matrices. However, for attributes associated with smell, like “garlic and onion smell," and taste factors including “flavor intensity," “persistence," and “garlic and onion taste," significant differences were observed for the AOSCs compared to the control at the highest evaluated doses (32 μg/g). For the AOSCs sourced from garlic (Fig. 1A), even at lower doses, panelists perceived an increased intensity in garlic smell and taste, contrasting with the onion derivatives (Fig. 1B), which at the lower dose garnered assessments similar to both the control and the L-11 peptide groups. Considering the potent flavoring properties of these compounds and the inclusion of garlic and onion in many of the tested food formulations, it was resolved for subsequent food efficacy trials to opt for the lower dosage levels.

### 3.3. Combined effect of Allium organosulfur derivatives and L-11 in different food matrices

The discovery that combinations of AOSCs and L-11 deliver synergistic antimicrobial effects against formidable pathogens such as *E. coli, Salmonella, Yersinia*, and *Shigella* as well as the absence of sensorial changes for some AOSCs at concentrations higher that the observed in the synergism, particularly pronounced with PTS and PTSO, underscores the capacity of natural compounds to bolster food safety keeping sensorial quality. These insights suggest a promising strategy for the food industry and consumers alike: employing these compounds in conjunction with biopeptide L-11 could offer enhanced protection against microbes, even at minimal dosages. To validate this approach, we investigated the synergistic effects in four different food matrices, each contaminated with a distinct pathogen, demonstrating the broad applicability and effectiveness of this natural antimicrobial strategy.

*Combined effect of AOSCs and L-11 in E. coli CECT 516 contaminated salmorejo*. *Salmorejo,* a traditional RTE food from Spain, is crafted from tomato, virgin olive oil, garlic, and bread, occasionally with other vegetables. This non-heat treated food requires cold storage and rapid consumption due to the microbial load from its ingredients, including potential pathogens (Toledo Del Árbol et al., 2015). In the larger food industry, *salmorejo* preservation involves pasteurization, high hydrostatic pressure (HHP) processing, or a combination thereof to prolong shelf life. While heat treatment effectively reduces microbial load, it can also detrimentally affect the nutritional, bioactive, and organoleptic properties of the food (Vioque et al., 2021). Conversely, HHP is preferred for its ability to maintain better food quality compared to thermal methods (Knorr et al., 2011) though its efficacy in microbial inactivation varies with the microorganism type, food composition, pH, and water activity (Alpa et al., 2000). For *salmorejo*, HHP has shown effectiveness against pathogens like *Salmonella* and *Listeria* at lower pressures, but higher pressures are required to eradicate *E. coli* (Toledo Del Árbol et al., 2015).

*E. coli*, commonly found in the guts of humans and animals, includes strains that are mostly harmless; however, certain pathogenic variants can cause severe foodborne illnesses (Havelaar et al., 2015). In this study, we investigated the impact of combining L-11 peptide and AOSCs on *E. coli* CECT 754-contaminated *salmorejo* stored at 4 °C over 15 days. In the absence of treatment (negative control), *E. coli* populations grew from an initial inoculation of $2.73 \pm 0.01$ to $4.2 \pm 0.35$ $Log_{10}$ CFU/mL after 14 days (Fig. 2). Although no antimicrobial activity was detected *in vitro* at the tested concentration (12 μg/g) of L-11, a significant reduction of 1 $Log_{10}$ in *E. coli* CFU/mL was observed after 14 days of treatment

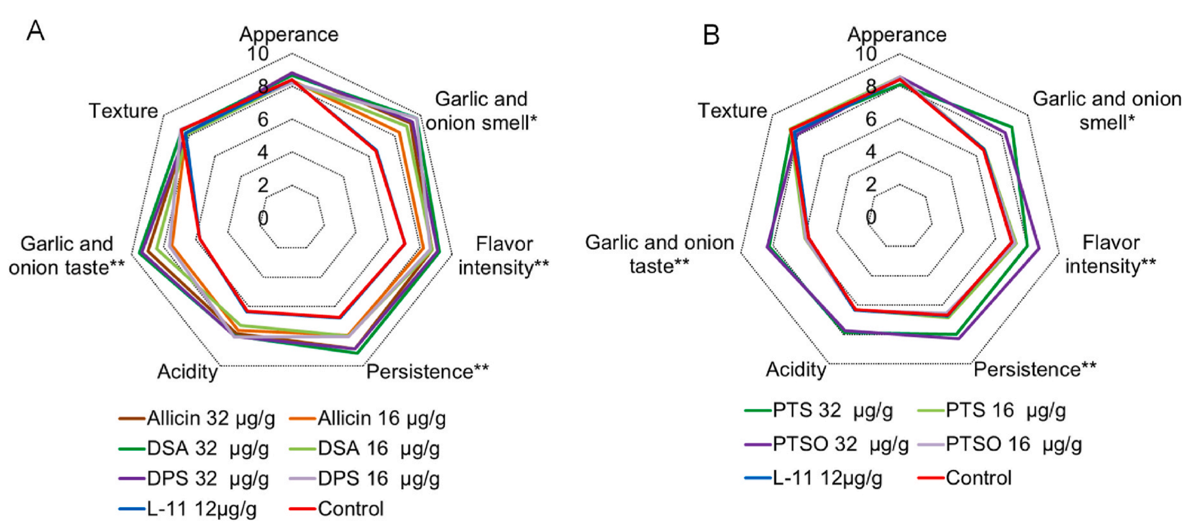


**Fig. 1.** Spider plot of descriptive analysis attributes for sauce samples with AOSCs compounds derived from garlic. A) For garlic-related AOSCs. B) For onion related AOSCs. The data characterised by significant difference are marked with * ($p < 0.05$) or ** ($p < 0.01$).

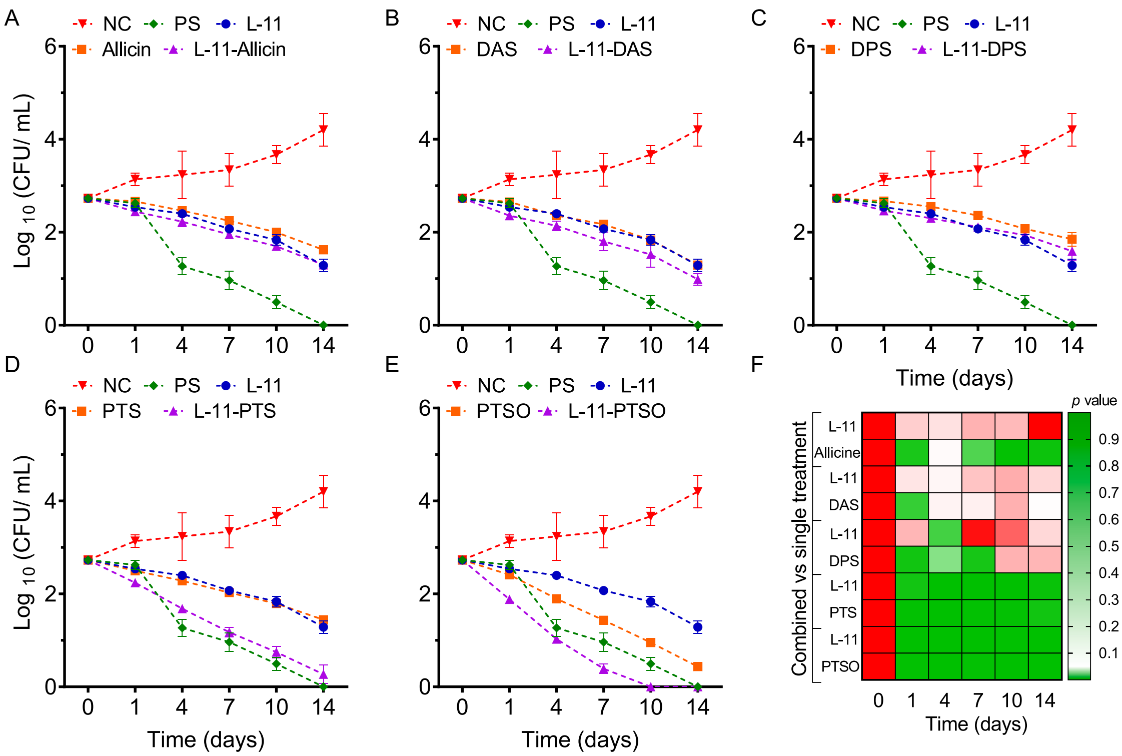


**Fig. 2.** Combined effect of L-11 (μg/g) with AOSCs in *E. coli* CECT 516 contaminated salmorejo. A) L-11 combined with allicin (16 μg/g), B) L-11 with DSA (16 μg/g), C) L-11 with DPS (16 μg/g), D) L-11 with PTS (8 μg/g), and E) L-11 with PTSO (8 μg/g). F) Statistical analysis (ANOVA) comparing the efficacy of combined treatments versus individual treatments at each evaluated time point. NC, negative control. PS, potassium sorbate (1000 μg/g) used as a positive control.

(Fig. 2, L-11 control). However, this did not achieve the complete eradication of the contamination, unlike the positive control, potassium sorbate (PS, 1000 μg/gr), which completely eliminated the *E. coli* contaminant after two weeks of incubation (Fig. 2, PS control).

In evaluating the antimicrobial efficacy of AOSCs against *E. coli* contamination in *salmorejo*, significant differences were noted based on the compounds' source. Garlic-derived AOSCs exhibited less activity compared to those derived from onions. For allicin (Fig. 2A), a cumulative effect led to a reduction in bacterial load by about 1 $Log_{10}$ unit. Similar reductions were also observed for the single treatments of DAS (Fig. 2B) and to a lesser extent, DPS (Fig. 2C). Interestingly, combined treatments of L-11 with garlic AOSCs did not significantly reduce the bacterial load compared to single treatments, with the exception of allicin (Fig. 2E). Conversely, onion-derived AOSCs, PTS and PTSO, demonstrated significant *E. coli* charge reduction (Fig. 2D and E), particularly when combined with L-11. Specifically, PTS and L-11 treatment resulted in a bacterial load reduction comparable to the positive control, potassium sorbate though complete bacterial eradication was not achieved (Fig. 2D). The PTSO and L-11 combination showed an even more pronounced synergistic effect, achieving complete bacterial eradication by the 10th day, thereby surpassing the positive control's effectiveness (Fig. 2E). A significant reduction in bacterial contamination was observed from the first day of treatment for the combined treatments compared to single treatments (Fig. 2F), highlighting the potent antimicrobial potential of combining L-11 with onion-derived AOSCs, and presenting a promising alternative to traditional preservatives like potassium sorbate for enhancing the safety of this RTE product.

*Combined effect of AOSCs and L-11 in S. enterica CECT 7160 contaminated "aioli".* Aioli, a popular Spanish mayonnaise-type emulsion, is distinguished by its pronounced garlic and olive oil flavors. Its microbiological stability is attributed to its high-fat content, the presence of organic acids, and a low pH (<4.5), which inhibit the growth of foodborne Gram-negative pathogens. However, this environment can still support the growth of acid-resistant microorganisms, such as lactic acid bacteria or fungi, that may be introduced during preparation from ingredients, equipment, or air, potentially leading to spoilage (Fialová et al., 2008; Teneva et al., 2021). Additionally, the antimicrobial properties of the sauce are often enhanced by the use of chemical preservatives like potassium sorbate and pasteurized eggs to mitigate health risks from pathogenic bacteria, thereby increasing biosecurity and extending shelf life (Smittle, 2000). Raw eggs, in particular, are frequently implicated in food poisoning outbreaks, mainly due to *Salmonella*, in homemade mayonnaise and aioli preparations (Gavriil et al., 2021; McWhorter et al., 2020, 2021). Salmonellosis ranks as the second most commonly reported gastrointestinal infection zoonosis in humans, accounting for 29 % of cases (in 2022) after campylobacteriosis, and is the leading cause of hospitalization due to foodborne pathogens (Authority (EFSA) & European Centre for Disease Prevention and Control (ECDC), 2023; Scallan et al., 2011).

In response to consumer demand for mayonnaise-based sauces that are lower in fat, free from chemical preservatives, and retain traditional flavors, the food industry faces significant challenges in maintaining food safety and quality. To address these consumer preferences, there is an increasing interest in utilizing naturally occurring preservatives. In this context, AOSCs present a promising solution, offering dual benefits of microbiological preservation and enhancement of aioli's sensory characteristics. Consequently, this study investigates the synergistic effects of combining L-11 peptide and AOSCs in aioli contaminated with *Salmonella* enterica, aiming to explore innovative approaches to preserving the traditional qualities of aioli while ensuring food safety.

In this investigation, aioli was intentionally contaminated with approximately 3 $Log_{10}$ CFU/g of *Salmonella enterica* CECT 7160, and the progression of contamination was monitored over 30 days of storage at 4 °C, with potassium sorbate serving as the preservative-positive control. According to the data presented in Fig. 3, the bacterial load

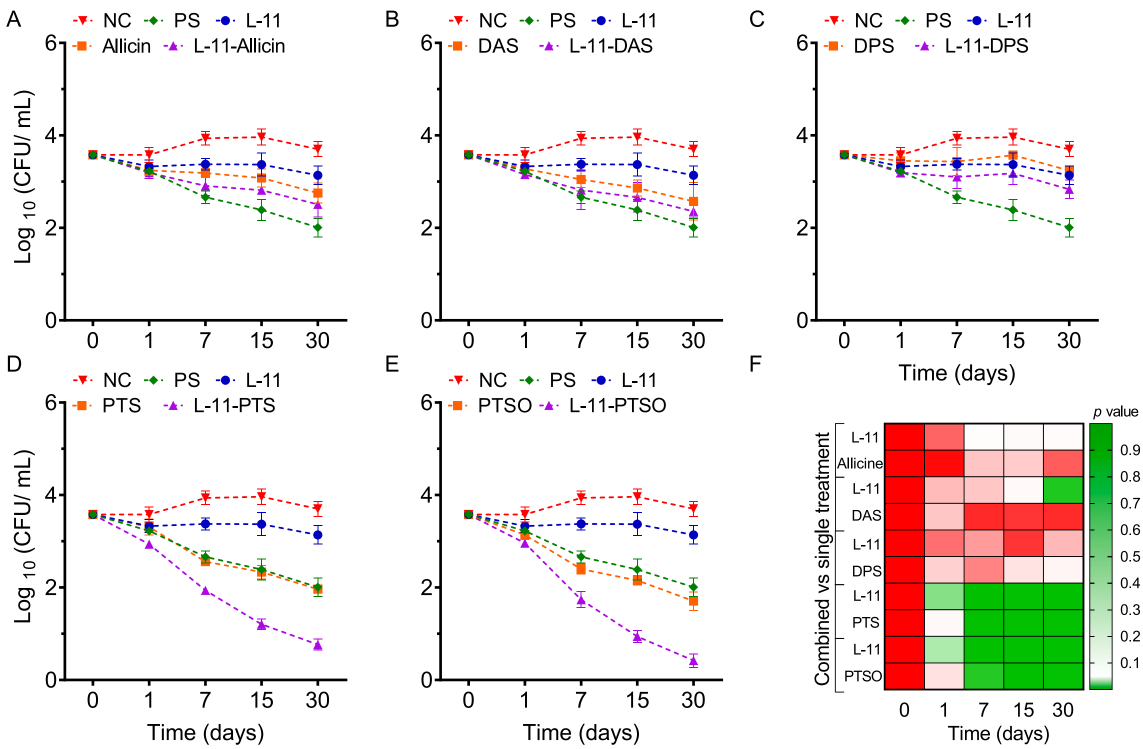


**Fig. 3.** Combined effect of L-11 (12 μg/gr) with AOSCs in *S. enterica* CECT 7160 contaminated aioli. A) L-11 combined with allicin (16 μg/gr), (B) with DSA (16 μg/gr), (C) with DPS (16 μg/gr), (D) with PTS (16 μg/gr) and (E) with PTSO (16 μg/gr). F) Statistical analysis comparing the combined treatment concerning the individual treatments at each time point. NC, negative control. PS, potassium sorbate (1000 μr/gr) used as a positive control.

remained relatively unchanged over the incubation period for both the samples treated solely with L-11 and the untreated controls. Conversely, the positive control, which included potassium sorbate, exhibited a reduction in bacterial load by approximately 1.5 $Log_{10}$. The application of single treatments with allicin or DAS resulted in a 1 $Log_{10}$ CFU/g reduction in bacterial contamination, while DPS had no observable impact (Fig. 3A, B, and 3C). Despite the *in vitro* evidence of synergism against *Salmonella* strains (Table 1), the combined treatments with L-11 and these AOSCs displayed only modest improvements compared to single-agent treatments. Notably, none of these treatments outperformed the positive control, and no significant differences were noted relative to samples treated with AOSCs alone (Fig. 3A, B, 3C, and 3F). In line with observations made in *salmorejo* studies, PTS and PTSO when used alone in aioli, exhibited a significant protective effect, which was notably enhanced by combining with L-11. This combination reduced the *S. enterica* load to near-complete eradication levels (Fig. 3D, E, and 3F). The effectiveness of the PTS/PTSO and L-11 combinations surpassed that of the potassium sorbate positive control, indicating that such combinations could not only inhibit *Salmonella* growth in aioli but potentially also lead to its complete removal.

*Combined effect of AOSCs and L-11 in Shigella sonnei CECT 457 contaminated pumpkin cream. Shigella,* a Gram-negative bacterium that is almost genetically identical to *E. coli* and closely related to *Salmonella*, has been increasingly implicated in foodborne outbreaks. This bacterium is particularly associated with foods that undergo manual processing or preparation, receive minimal heat treatment, or are served or delivered raw to consumers (Warren et al., 2006). Distinct from other foodborne Gram-negative pathogens, *Shigella* exhibits resistance to acidic conditions, tolerance to salt, resilience against low concentrations of organic acids, and can become pathogenic at relatively low infective doses (Warren et al., 2006). This combination of traits makes *Shigella* a formidable pathogen in the context of food safety, necessitating vigilant control measures, especially in foods prone to contamination that are consumed without further cooking or processing.

As depicted in Fig. 4, *Shigella sonnei* demonstrated growth under the tested conditions, with the L-11 peptide alone showing no antimicrobial effect. Consistent with the *in vitro* results (Table 1) and similar observations with *E. coli* (Fig. 2), no antimicrobial activity was detected for allicin, DAS, and DPS either as standalone treatments or in combination with L-11 (Fig. 4A, B, 4C, and 4F). However, the combinations of PTS and particularly PTSO with L-11 significantly reduced the contamination by *S. sonnei* (Fig. 4D, E, and 4F), with PTSO in combination with L-11 nearly eradicating the bacteria from the food matrix within just one day (Fig. 4E). Additionally, PTSO demonstrated effective antimicrobial activity when used alone at the tested concentration, approaching total bacterial eradication after 20 days of incubation (Fig. 4E). This indicates the potent antimicrobial potential of PTSO, both as a standalone agent and in synergy with L-11, against *S. sonnei* contamination in food products.

*Combined effect of AOSCs and L-11 in Yersinia enterocolitica CECT 754 contaminated spinach cream.* Yersiniosis ranked as the third most reported zoonosis in European countries, following infections caused by Shiga toxin-producing *E. coli* (STEC) and L. monocytogenes. Primarily attributed to *Yersinia enterocolitica*, yersiniosis manifests as fever, abdominal pain, and often bloody diarrhoea, particularly in young children (Authority (EFSA) & European Centre for Disease Prevention and Control (ECDC), 2023; Gupta et al., 2015). Transmission of *Y. enterocolitica* typically occurs via the fecal-oral route through the consumption of contaminated foods, predominantly of animal origin (Gupta et al., 2015). A notable cross-border outbreak involving *Yersinia*-contaminated spinach in Denmark and Sweden underscored the importance of recognizing various vehicles for *Y. enterocolitica* outbreaks beyond animal-derived foods (Espenhain et al., 2019). In light of this, the efficacy of proposed combinations for controlling this bacterium in a spinach-based cream was examined.

*Y. enterocolitica* demonstrated the capability to replicate at 4 °C, with

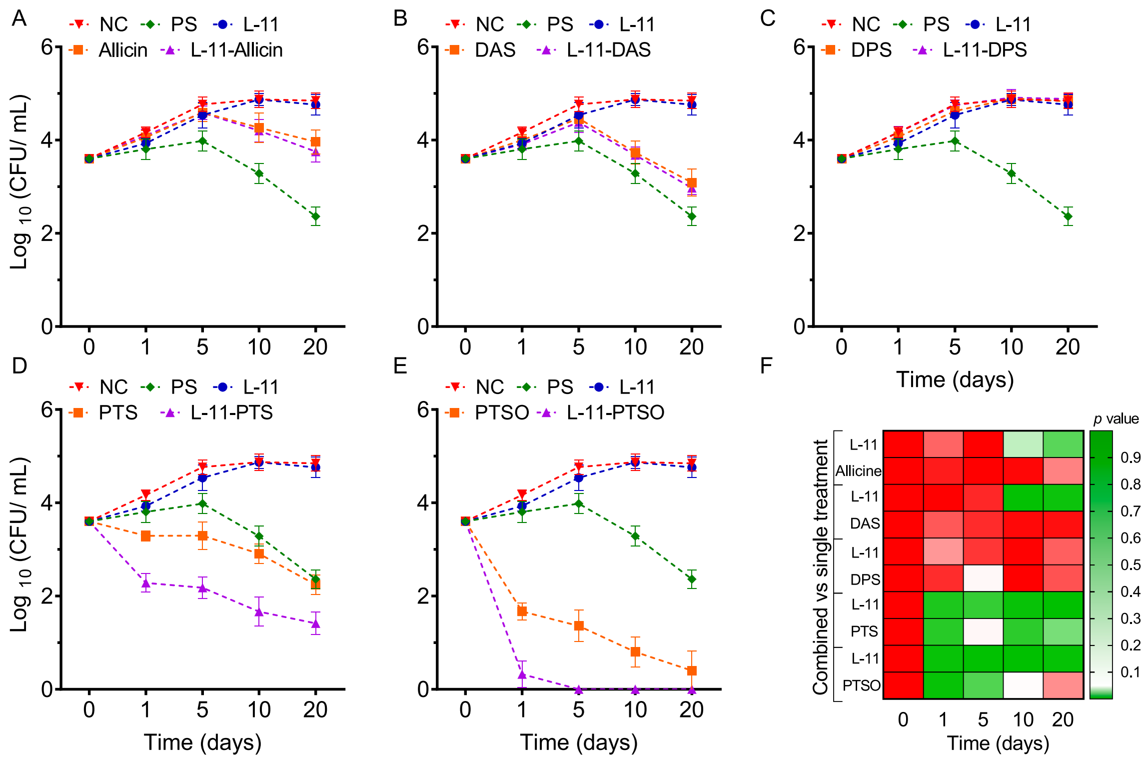


**Fig. 4.** Combined effect of L-11 (12 μg/gr) with AOSCs in *S. sonnei* CECT 457 contaminated pumpkin cream. A) Combined effect of L-11 and allicin (16 μg/gr), (B) DSA (16 μg/gr), (C) DPS (16 μg/gr), (D) PTS (16 μg/gr), and (E) PTSO (16 μg/gr). F) Statistical analysis comparing the combined treatment concerning the individual treatments at each time point. NC, negative control. PS, potassium sorbate (1000 μr/gr) used as a positive control.

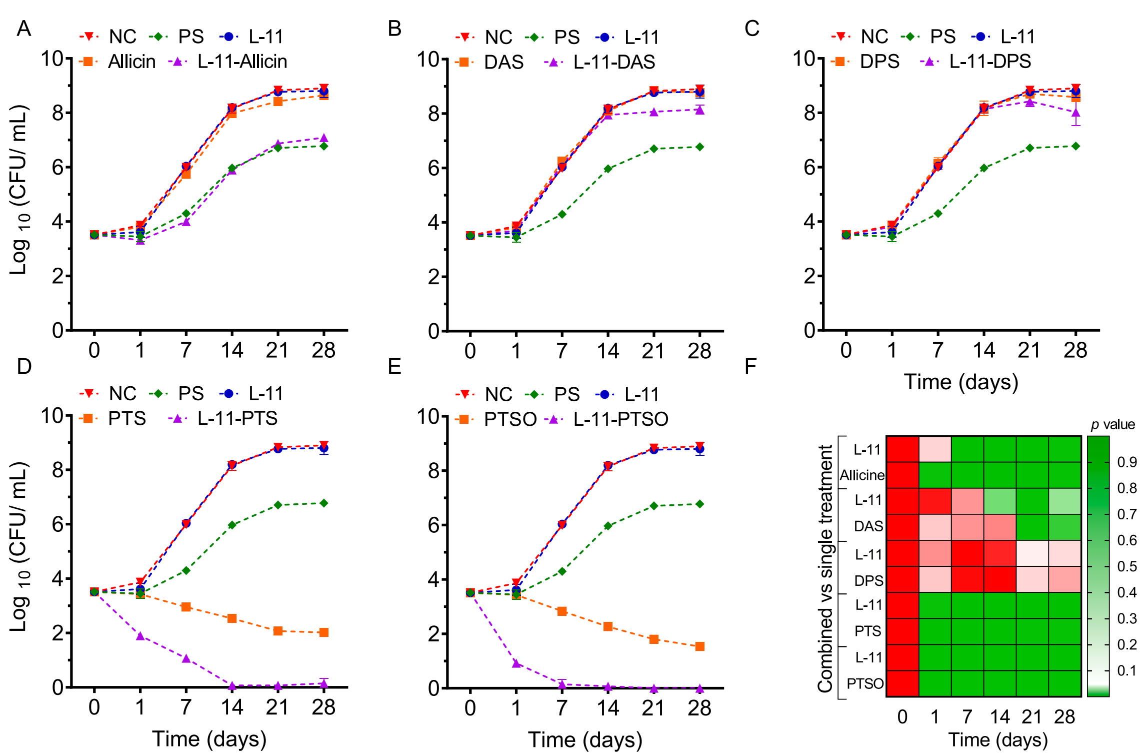


**Fig. 5.** Combined effect of L-11 (12 μg/gr) with AOSCs in *Y. enterocolitica* CECT 754 contaminated pumpkin cream. A) Combined effect of L-11 and allicin (16 μg/gr), (B) DSA (16 μg/gr), (C) DPS (16 μg/gr), (D) PTS (16 μg/gr) and (E) PTSO (16 μg/gr). F) Statistical analysis comparing the combined treatment concerning the individual treatments at each time point. NC, negative control. PS, potassium sorbate (1000 μr/gr) used as a positive control.

growth increasing by approximately 6 $Log_{10}$ (Yehualaeshet et al., 2013) (Fig. 5). None of the garlic-derived AOSCs effectively controlled bacterial growth alone or in synergy, with the exception of allicin in combination with L-11, which significantly reduced the growth rate by about 3 $Log_{10}$, aligning with the performance of the positive control (Fig. 5A, B, 5C, and 5F). This outcome was anticipated based on the *in vitro* synergism tests, where allicin exhibited the most favorable synergistic interaction (Table 1). Contrary to previous observations, combinations of PTS and PTSO demonstrated the most effective synergistic effect in the conducted tests. PTS and PTSO alone managed to control bacterial growth in the spinach-based cream, reducing CFU/g by 1.5–2 $Log_{10}$, respectively, after 28 days of treatment. Remarkably, combining PTS and PTSO with L-11 achieved complete bacterial eradication after 14 and 7 days, respectively, with a significant reduction in bacterial load observed just one day post-treatment (Fig. 5D and E), highlighting the potent antimicrobial potential of these combinations against *Y. enterocolitica* in food matrices.

The results presented in this study highlight the significant potential of *Allium*-derived compounds in controlling foodborne pathogens. Our investigation specifically focused on the antimicrobial properties of these compounds in combination with L-11, demonstrating notable efficacy against a range of pathogens. Although it is essential to acknowledge that this research focuses solely on the antimicrobial aspect, future studies should include a broader range of wild isolates in pathogen-specific cocktails and evaluate the impact of these compounds on various food technological properties, such as pH levels, overall microbial ecology, and the shelf life of treated foods. Additionally, exploring the synergistic effects of these compounds when combined with other preservation technologies could provide comprehensive insights into their practical applications.

Furthermore, extensive research, both *in vitro* and in vivo, has consistently shown that these *Allium* compounds do not exhibit acute or subchronic toxicity (Cascajosa-Lira et al., 2022; Lira et al., 2020; Mellado-García, Puerto, Pichardo, et al., 2016). The absence of genotoxicity has also been confirmed in rodent studies (Cascajosa-Lira et al., 2021; Mellado-García, Puerto, Prieto, et al., 2016) thereby reinforcing their safety profile. These findings support the potential use of *Allium*-derived compounds as safe and promising alternatives to traditional food preservation methods, paving the way for innovative strategies in food safety.

## 4. Conclusions

In this work we demonstrated of the synergistic antimicrobial effects of AOSCs and the biopeptide L-11 against Gram-negative foodborne pathogens in various RTE food matrices. This study addresses a critical need in the food industry for natural and effective antimicrobial strategies that ensure the safety and quality of RTE foods without compromising their sensory attributes. The research findings highlight that while individual AOSCs and L-11 showed minimal antimicrobial activity against target bacteria such as *Escherichia coli, Salmonella enterica, Yersinia enterocolitica*, and *Shigella sonnei*, their combinations exhibited remarkable synergistic effects. This synergy was particularly notable for combinations including PTS and PTSO, which effectively suppressed bacterial proliferation within RTE food matrices. In these cases, the reduction in the MIC for PTS/PTSO was enough to avoid their sensory impact on food, addressing the potential challenge of these compounds altering the sensory characteristics of food products. This outcome suggests a promising avenue for the development of natural, effective, and consumer-acceptable food preservation strategies, offering a viable alternative to synthetic preservatives. This work represents a significant advancement in the field of food safety and preservation, offering a novel and effective strategy for enhancing the safety of RTE foods. The findings not only address key challenges in food safety and public health but also open new avenues for research and development in natural food preservation methods. This research could contribute to advancing the food industry's approach to food safety and preservation, aligning with consumer preferences for natural, minimally processed food products.

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## CRediT authorship contribution statement

**Juan José Ariza:** Investigation, Formal analysis, Data curation. **J. David García-López:** Validation, Software, Investigation. **María Arántzazu Aguinaga-Casañas:** Software, Methodology, Investigation. **Alberto Baños:** Writing – original draft, Resources, Methodology, Conceptualization. **Federico García:** Writing – review & editing, Visualization, Resources. **Oscar P. Kuipers:** Writing – review & editing, Resources, Funding acquisition. **Rubén Cebrián:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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