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# Control microbial growth on fresh chicken meat using pinosylvin inclusion complexes based packaging absorbent pads

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ABSTRACT

Fresh chicken is a potential source of human infection due to its high microbial load, making the issues regarding its microbiological safety, especially the control of *Campylobacter* spp., of paramount relevance. Pinosylvin has recently proved effective against this major food pathogen. In this work, we evaluated the efficacy of absorbent pads containing pinosylvin (PS) inclusion complexes (ICs) for the control of fresh chicken meat microbial contamination, focusing on its anti-*Campylobacter* activity. Coated pads were evaluated *in vitro* against *C. jejuni*, followed by *in vivo* testing in chicken exudates and meat, assessing their anti-*Campylobacter* activity as well as their efficacy in inhibiting poultry microbiota. Pads exhibited a bactericidal activity in liquid medium at a concentration of 0.08 mg/cm2. At 4 °C, pads with 0.4 mg PS/cm2 exhibited anti-*Campylobacter* activity in chicken fillets and exudates. Regarding spoilage bacteria, active coated pads were not able to reduce pseudomonads but caused reductions in LAB, psychrotrophs and total viable counts. The present work demonstrates that absorbent pads coated with pinosylvin inclusion complexes possess an efficient antimicrobial activity not only against *C. jejuni* but also against chicken spoilage bacteria, enabling the use of these active pads to control microbial growth in packaged chicken meat.

## 1. Introduction

According to the data from the US National Chicken Council, the consumption of chicken meat continues on the rise, being almost equivalent to the consumption of red meats (http://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/). Therefore, poultry industry is facing a new challenge regarding the processing and distribution of fresher, higher quality and safer meat which led to the development of strategies able to increase poultry meat shelf-life either by decreasing product oxidation or its microbial loads (Silva, Domingues, & Nerin, 2016). Reducing poultry microbial load has gained interest in recent years due to the fact that chicken is the leading cause of foodborne outbreak-associated illness in the US (CDC, 2015), as these animals are natural reservoirs of pathogens such as *Salmonella* or *Campylobacter* (Marcus et al., 2007). According to the latest EFSA report, the overall incidence of *Campylobacter* contamination in broiler meat in EU countries is 35.5%, although this prevalence value differs widely from country to country, with several countries reporting very high (> 50%) or extremely high proportions (> 70%) of positive samples (EFSA & ECDC, 2016). Furthermore, 444 foodborne *Campylobacter* outbreaks were reported, representing an 8.5% of the total reported foodborne outbreaks in the EU (EFSA & ECDC, 2016). In fact, the emergence of *Campylobacter* as a zoonotic agent has led EFSA Biohazard panel to suggest that a maximum level of *Campylobacter* contamination by gram of meat should be established (Hazards, 2011).

Besides these pathogens, other aerobic spoilage bacteria such as *Pseudomonas*, Enterobacteriaceae or psychrotrophic bacteria such as lactic acid bacteria will also grow in packed poultry meat making it a significantly perishable food (Silva et al., 2016).

In recent years, the meat packing industry has evolved towards the development of active packages, with antioxidant and/or antimicrobial activities that could effectively increase fresh meat shelf-life. In the development of all these active packaging strategies, the incorporation of natural compounds instead of synthetic food preservatives should be the preferred alternative, as the use of synthetic compounds is currently restricted (Nguefack et al., 2009).

As natural compounds, stilbenes can be potentially used in the development of antimicrobial food packaging systems (Plumed-Ferrer et al., 2013; Valimaa et al., 2007) due to their inherent antimicrobial activity, from which pinosylvin stands out as the most active stilbene

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against *Campylobacter* spp. (Plumed-Ferrer et al., 2013; Silva, Nerin, & Domingues, 2015). However, to be able to use stilbenes in food applications, one must overcome their low stability and water solubility. For this, stilbenes have been included in encapsulating agents such as liposomes, metal nanoparticles and cyclodextrins, among others (Augustin, Sanguansri, & Lockett, 2013; Santos, Veiga, & Ribeiro, 2011). Regarding the use of cyclodextrins as encapsulating agents, hydroxypropyl-derived cyclodextrins are currently preferred over natural ones, as they are less toxic than native ones and exhibit higher water solubility values (Gould & Scott, 2005; Veiga & Ahsan, 1999). These hydroxypropylated derivatives are formed through the substitution of the hydroxyl groups on the native CD structure by 2- hydroxypropyl groups and have been shown to improve stilbene photostability (Silva, Figueiras, Gallardo, Nerin, & Domingues, 2014).

Although chicken packages contain a wrapping film, they also contain absorbent pads, mainly the ones intended for chicken breast packages due to the fact that chicken breast meat has a low water retention capacity (Bowker & Zhuang, 2015), resulting in the production of exudate fluids (Bowker & Zhuang, 2013). Regarding antimicrobial absorbent materials for pads, research is still scarce, with only a few studies describing the use of coatings such as essential oils or silver (Fernandez et al., 2009; Oral et al., 2009) while most of the studies have instead focused on the development of new composite absorbent materials by combining silver or copper and cellulose (Fernandez, Picouet, & Lloret, 2010; Llorens, Lloret, Picouet, & Fernandez, 2012) yielding pads with intrinsic antimicrobial properties. However, none of these pads was evaluated regarding their antimicrobial activity against *Campylobacter* spp.

These absorbent pads are of highly relevance in chicken packaging, as nowadays, they are the staple package for chicken sold in pieces (Richman et al., 2010). Taking into consideration that the current model package for chicken breast has absorbent pads, we intended to develop coated absorbent pads with antimicrobial activity both in chicken exudates and meat. In this work, we assessed the potential of a new coating for absorbent pads used in chicken packages containing pinosylvin inclusion complexes with hydroxypropyl-β-cyclodextrin and hydroxypropyl-γ-cylodextrin as antimicrobials and tested their efficacy against *Campylobacter jejuni* and common chicken microbiota both *in vitro* and *in vivo* using chicken fillets and exudates.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Two *Campylobacter* reference strains were used in this study: *Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559 as well as one clinical isolate: *Campylobacter jejuni* 930/12. Strain *C. jejuni* 930/12 was isolated from human clinical samples and belongs to the collection of *Campylobacter* strains of the Department of Infectious Diseases from Instituto Nacional de Saúde Dr. Ricardo Jorge in Lisbon, Portugal. All *Campylobacter* strains were stored at −80 °C in the form of a concentrated suspension in Brain Heart Infusion (BHI, Scharlab, Spain) broth with 20% (v/v) glycerol. Prior to experiments, bacterial strains were subcultured firstly on *Campylobacter* blood-free selective medium [modified CCDA – Preston (Oxoid, England)] and, afterwards, on blood agar plates [Blood Agar Base 2 (Oxoid, England) supplemented with 5% (v/v) defibrinated horse blood (Scharlab, Spain)] at 37 °C in gas-tight containers under microaerobic conditions using microaerophilic generating pouches [Campygen Compact (Oxoid, England)]. *Campylobacter* growth in liquid medium was performed at 37 °C under microaerobic conditions, as described above, using Mueller Hinton (MH) broth (Oxoid, England).

### 2.2. Preparation of the coating containing pinosylvin-cyclodextrin complexes

Pinosylvin (PS; Sequoia Research Products Limited, U.K.) inclusion complexes (ICs) with hydroxypropyl-β-cyclodextrin (HP-β-CD; KLEPTOSE® HP, Roquette Freres S.A., Lestrem, France) and hydroxypropyl-γ-cyclodextrin (HP-γ-CD, Sigma-Aldrich, St. Louis, MO) were prepared and quantified by HPLC-DAD as described previously (Silva et al., 2014). The coating was prepared by dissolving 20% (v/v) of these concentrated ICs solutions in hydroxyethylcellulose (Tylose® H4000 NG4, SE Tylose GmbH & Co. KG, Germany) in order to reach a more homogeneous spread of the coating on the surface of the pads as it yielded a more viscous and extendible solution. The final PS concentration attained in the 1% (w/v) Tylose® solution was 8 mg/mL.

### 2.3. Active absorbent pads

Perforated cellulose/polypropylene absorbent pads (Linpac Packaging, Spain) were cut in 1 × 1 cm pieces and sterilized under UV light for 30 min on each side before being coated. Afterwards, the pads were coated with different volumes (10, 20, 50, 70 and 100 μL) of 1% Tylose solutions containing hydroxypropyl-beta-cyclodextrin and hydroxypropyl-gamma-cyclodextrin inclusion complexes with pinosylvin at a concentration of 8 mg/mL and allowed to dry at room temperature for 24 h. When dried, the pads were used for the corresponding antimicrobial assays.

### 2.4. In vitro antimicrobial activity of active absorbent pads

A preliminary evaluation of the antimicrobial efficacy of active pads was performed by disk diffusion using *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559. The agar diffusion method was used to determine the bacterial sensitivity of *Campylobacter* species to the active absorbent pads. One hundred microliters of a bacterial suspension of each microorganism containing 4–5x$10^8$ CFU/mL were spread on the surface of blood agar plates. Afterwards, 1 × 1 cm pieces of the active coated pads with 0.08–0.8 mg/$cm^2$ of pinosylvin were placed on top of the inoculated agar plates and incubated at 37 °C in microaerophilic conditions for 48 h. After incubation, the inhibition halos were measured with a digital ruler. Disk diffusion assays were performed, at least, in triplicate.

The antimicrobial testing of the active absorbent pads containing different concentrations of ICs and its active compound, pinosylvin, in liquid medium was performed as described by Fernandez and collaborators (Fernandez et al., 2009) with some alterations. Absorbent pads were tested against *C. jejuni,* the most predominant *Campylobacter* species in poultry meat (Luber & Bartelt, 2007). Two hundred microlitres of a bacterial suspension containing approximately 1 × $10^9$ CFU/mL were added to the active pads and incubated at 37 °C in microaerophilic conditions for 48 h in tubes with 2 mL of MH broth. After incubation, 3 mL of buffered peptone water (Scharlab, Spain) were added to the samples that were gently vortexed for 1 min. Serial dilutions were made in peptone water and the microorganism suspensions were drop-plated on Blood Agar plates (Chen, Nace, & Irwin, 2003). Colony counts were performed after incubation at 37 °C for 48 h in microaerophilic conditions. According to the plated volume (20 μL), the detection limit for this plating technique was 50 CFU/mL.

The results were expressed as CFU/mL. A further control experiment was carried out with absorbent pads coated with 1% Tylose® without inclusion complexes.

### 2.5. Antimicrobial activity of active absorbent pads in chicken exudates

Chicken exudates were kindly provided by NUTRECO España. Chicken exudates were filtered (0.2 μm syringe filters) on a safety cabinet to ensure sterility. The assays with chicken exudates were