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Food preservatives influence biofilm formation, gene expression and small RNAs in *Salmonella enterica*

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ABSTRACT

Salmonella enterica is major foodborne pathogens. *Salmonella* persists in the food chain due to its ability to produce biofilms under different conditions. One of the biggest challenges in biofilm research is reproducing real food industry conditions. Also, food microbiologists have the challenge of elucidating the role of small RNAs (sRNAs) in the survival of foodborne pathogens in the food chain. This study evaluated food preservatives (sodium nitrite, sodium sulfite and sodium acetate-citric acid) effect on biofilm formation of ten *S. enterica* strains on two surfaces (polystyrene and stainless steel). The effects of preservatives on transcription of biofilm- and virulence-related genes and sRNAs were evaluated. All *Salmonella* strains produced biofilm in all the conditions evaluated. However, sodium sulfite reduced biofilm formation by *Salmonella* in both surfaces tested. Food preservatives influenced biofilm- and virulence-related genes and sRNAs transcription. This study highlights that *Salmonella* strains can produce biofilms in the presence of food preservatives, representing a public health problem. Fully understanding what metabolic pathways are modified by the presence of preservatives could allow developing new control strategies to prevent foodborne pathogens persistence in the food chain by using effective combinations of preservatives.

1. Introduction

Salmonella spp. are one of the major foodborne pathogens around the world. In 2015, salmonellosis was the second most commonly reported zoonosis in the European Union (EU), with a total of 94,625 confirmed cases and 126 deaths (EFSA, 2016). In the food chain, *Salmonella* have to adapt to a wide range of environmental conditions such as varied temperatures, pH, desiccation, nutrient limitations, and the presence of biocides and food preservatives (Lianou & Koutsoumanis, 2012). The capacity of *Salmonella* to form biofilms helps this foodborne pathogen to persist in those environments. Biofilms are defined as structured monospecies or multispecies communities of microorganisms, enclosed in a polymeric matrix that is adhered to a living or inert surface. Cellulose and curli fimbriae are the two main components of *Salmonella* biofilms, and their production is responsible for the characteristic RDAR (red, dry, and rough) morphotype (Steenackers, Hermans, Vanderleyden, & De Keersmaecker, 2012).

Salmonella biofilm formation relies on a complex genetic network. The biofilm master regulator CsgD is responsible for the production of curli fimbriae and the regulation of cellulose synthesis via AdrA and c-di-GMP. Another key factor that positively contributes to biofilm formation is quorum sensing. This mechanism works through the production of small molecules termed autoinducers, and their receptors, encoded by *luxS* and *sdiA* genes, respectively. Therefore, the transcription of those genes is crucial to produce fully developed biofilms (Steenackers et al., 2012). Another genetic mechanism involved in biofilm formation is small RNAs (sRNAs). These molecules are short transcripts of 50–400 nucleotides characterized by their stability, abundance, rapid synthesis and low energetic cost. sRNAs are defined as post-transcriptional regulators with both activation and repression activity through sRNA–protein or sRNA–mRNA interaction (Mandin & Guillier, 2013). Some studies have observed the potential regulatory activity of determinant sRNAs in biofilm formation through regulation of important components implicated in biofilm formation such as flagella or the biofilm master regulator CsgD (Mandin & Guillier, 2013). Due to their relative novelty, there are very few information on the influence of common food environment conditions and biofilm formation on the transcription of sRNAs.

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Food preservatives are commonly used in the food industry to prevent microbial spoilage and the growth of foodborne pathogens. Nitrites and sulfites are two of the most used food preservatives in meat industry to prevent microbial growth. However, in recent years, nitrites and sulfites have been under the spotlight due to their possible adverse effects on human health, and alternatives such as sodium acetate are considered good options for food preservation (Erickson & Doyle, 2017). Despite the extensive use of these substances, a scarce number of studies have focused on the effect of these food preservatives on the biofilm formation ability and transcription of *Salmonella* strains isolated from the food chain.

Thus, the aim of this study was to evaluate the effect of food preservatives (sodium nitrite, sodium sulfite and sodium acetate–citric acid product) on biofilm formation by *Salmonella* strains on two common food industry surfaces (polystyrene and stainless steel). The effect of those growth media on the transcription of biofilm- and virulence-related genes was also evaluated. Due to the importance of sRNAs as post-transcriptional regulators, this study evaluated the role of sRNAs related to biofilm formation by *Salmonella* strains isolated from the food chain in adaption to food environment conditions.

2. Materials and methods

2.1. S. enterica strains, growth media and colony morphotype

Ten strains isolated from poultry houses and chicken meat were used in this study (Table 1). All strains were isolated from the chicken meat and poultry houses according to ISO 6579-1:2017 (ISO, 2017) and serotyped using the Kauffman–White typing scheme for the detection of somatic (O) and flagellar (H) antigens with standard antisera (Bio-Rad Laboratories, California, USA). Four different growth media were used in this study. Tryptic soy broth (TSB; Oxoid Ltd, Hampshire, United Kingdom) at a concentration of 1/20 (w/v), was used as a reference medium. Sodium nitrite (SN; Sigma-Aldrich, Steinheim, Germany), sodium sulfite (SS; Sigma-Aldrich, Steinheim, Germany) and a product based on the combination of sodium acetate and citric acid (SA; Técnicas Químicas Industriales, Pontevedra, Spain) were also used. Thus, three different formulations were elaborated, by supplement 1/20 TSB with 175 mg/L of SN, 450 mg/L of SS and 10 g/L of SA. The concentrations used of sodium sulfite and sodium nitrite were based on those allowed by the European Union in the Commission Regulation (EU) No 1129/2011 (European Commission, 2011) in meat products. In the case of sodium acetate and citric acid, their use in the products where are allowed is *quantum satis* (European Commission, 2011). Therefore, the concentration of sodium acetate-citric acid used was based in previous studies of inhibition carried out in our laboratory (Lamas, Miranda, Vázquez, Cepeda, & Franco, 2016). *Salmonella* morphotypes were determined according Römling et al. (2003). Briefly, *Salmonella* strain cultures were spread-plated onto Luria–Bertani (LB) plates without salt and supplemented with 40 mg/L of Congo red (Sigma Aldrich, Steinheim, Germany) and 20 mg/L of Coomassie Brilliant Blue (Sigma Aldrich, Steinheim, Germany). Plates were incubated for 96 h, and the morphotypes were determined for each strain at 22 °C. The morphotype RDAR is due to the production of curli fimbriae and cellulose that bind congo red and comassie brilliant blue respectively causing the red, dry, and rough colonies characteristic of this morphotype. The non-production of cellulose and curli fimbriae results in the expression of SAW (smooth and white) morphotype.

Table 1
Salmonella strains used in this study, their source and the morphotype produced at 22 °C.

Strain	Source	Morphotype
S. Bardo LHICA B2	Poultry house	RDAR
S. Enteritidis LHICA ET1	Chicken	RDAR
S. Infantis LHICA I4	Poultry house	RDAR
S. Infantis LHICA I5	Poultry house	RDAR
S. Newport LHICA N5	Poultry house	RDAR
S. Typhimurium LHICA T1	Chicken	RDAR
S. Typhimurium LHICA T4	Poultry house	RDAR
S. Typhimurium LHICA T5	Poultry house	RDAR
S. Typhimurium LHICA T6	Poultry house	RDAR
S. enterica subsp. *salamae* LHICA SA3	Poultry house	RDAR

2.2. Growth of planktonic cells

Growth of the ten *Salmonella* strains used in this study was determined in the four growth media used in this study. Briefly, *Salmonella* strains were grown in nutrient agar (NA; VWR, Barcelona, Spain) overnight. *Salmonella* colonies were spread in 0.85% NaCl solution and adjusted to an optical density (OD) of 0.210, referring to 10^8 CFU/mL. Cell suspensions were diluted four times to obtain a final concentration of 10^4 CFU/mL. One hundred microliters of this dilution was mixed with 10 mL of each of the growth media tested (1/20 TSB, 1/20 TSB with SN, 1/20 TSB with SS and 1/20 TSB with SA) and incubated at 22 °C. Samples were collected after 24 and 48 h and cell counts were enumerated on NA after appropriate dilution.

2.3. Biofilm formation on polystyrene

Biofilm formation on polystyrene by *Salmonella* strains with the four growth media was determined as previously described (Stepanovic, Cirkovic, Ranin, & Švabic-Vlahovic, 2004). Briefly, each well of a 96-well polystyrene microplate (Deltalab, Barcelona, Spain) was filled with 230 µL of growth medium and 20 µL of *Salmonella* 24 h TSB culture containing 10^8 CFU/mL. The microplates were incubated for 48 h at 22 °C. After that time, the microplate liquid was poured off, and the wells were washed three times with 300 µL of distilled water. *Salmonella* cells attached to the microplate walls were fixed by adding 250 µL of absolute methanol (Sigma-Aldrich, Steinheim, Germany) for 15 min, and the plates were emptied and air-dried. After that, the microplate wells were stained with 250 µL of 0.1% crystal violet solution (Panreac AppliChem, Barcelona, Spain) for 5 min. Crystal violet was rinsed off by placing the microplate under running water. Microplates were air-dried, and Crystal violet was resolubilized in 250 µL of 33% glacial acetic acid (Sigma-Aldrich, Steinheim, Germany) per well. The OD was measured at 630 nm with a plate reader (DAS, Roma, Italy). All assays were performed eight times in three independent experiments.

2.4. Biofilm formation on stainless steel

Biofilm formation on stainless steel was determined as described before (Lamas et al., 2018). Briefly, stainless steel coupons (3.5 × 3.5 cm) were placed in the bottom of 125 mL bottles (Deltalab, Barcelona, Spain) with 10 mL of the growth medium and 100 µL of *Salmonella* culture containing 10^8 CFU/mL and incubated for 48 h at 22 °C. After that, stainless steel coupons were washed with distilled water, and *Salmonella* cells attached to the stainless steel were fixed by immersing the coupons in methanol (Sigma-Aldrich, Steinheim, Germany) for 15 min. Then, coupons were air-dried and immersed in a 0.1% crystal violet solution (Panreac AppliChem, Barcelona, Spain) for 5 min. Excess of crystal violet was rinsed off by placing the stainless steel coupons under running water. Coupons were placed in Petri dishes with 10 mL of 33% acetic acid solution (Sigma-Aldrich, Steinheim, Germany) to resolubilize the crystal violet. Finally, 200 µL of these solutions was poured into a 96-well microplate, and the OD was measured at 630 nm with a plate reader (DAS, Roma, Italy). All assays were performed three times in three independent experiments.

2.5. RNA extraction and quantitative RT-PCR

The expression of three biofilm-related genes and two invasion-