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Thiol-reactive natural antimicrobials and high pressure treatment synergistically enhance bacterial inactivation

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ABSTRACT

Bacterial inactivation by high pressure (HP) treatment can be strongly enhanced in the presence of antimicrobial compounds, but the mechanism of this synergy is poorly understood. In the present study, screening of thirteen natural antimicrobial compounds (NACs) led to the identification of six NACs that exhibited synergy with HP treatment against eight Gram-negative and two Gram-positive bacteria. The strongest synergy was found with α,β-unsaturated aldehydes (t-cinnamaldehyde, t-2-hexenal, dimethylfumarate), isothiocyanates (allyl isothiocyanate, sulforaphane) and other electrophilic compounds (reuterin). The antibacterial activity of these compounds and their synergistic interaction with HP was linked to thiol reactivity based on (1) their reaction with cysteine in vitro, (2) their reduced minimal inhibitory concentration in an *Escherichia coli gshA* mutant which is defective in glutathione synthesis, and (3) the loss of synergy with HP in the presence of excess cysteine. These novel insights in the mechanisms leading to synergy will support the development of more effective hurdle technology applications of HP treatment and natural antimicrobials.

Industrial relevance: HP treatment is an emerging mild processing technology that offers a better balance between microbiological safety and stability and quality retention compared to traditional heat-based pasteurization methods. The efficacy of microbial inactivation by HP treatment is improved in the presence of natural antimicrobials, but the basis of this synergistic interaction is poorly understood. In the present work, a specific antibacterial mode of action is shown to result in synergy, and this insight will facilitate the development of more effective combined treatments.

1. Introduction

High pressure (HP) treatment is a relatively young technology that has been developed as a non-thermal alternative for thermal food pasteurization and that has been increasingly adopted in the food industry since the late 1990's. Generally, HP treatment allows achieving a considerable shelf-life extension with a minimal loss of nutritional and sensorial properties and natural freshness (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007; Rendueles et al., 2011). Examples of successful applications include cooked and sliced meat products, fruit and vegetable pieces, juices, and purees, and ready-to-eat meals or meal components. However, despite these commercial applications, there is still a need for more data and a better understanding of HP microbial inactivation and the factors that influence it, in order to develop a reliable quantitative framework that can be used by food producers to design and optimize HP processes. An aspect of particular importance that is hindering further implementation of HP technology in a wide range of food products is bacterial HP resistance.

Two different types of resistance have been described. The first is intrinsic resistance, this is the property of certain strains to be more resistant than others. Natural strain variations in HP sensitivity, and the occurrence of natural highly HP resistant isolates have been documented in several food bacteria including *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Campylobacter jejuni* and *Salmonella enterica* (Alpas et al., 1999; Benito, Ventoura, Casadei, Robinson, & Mackey, 1999; Chen, Neetoo, Ye, & Joerger, 2009; Liu, Betti, & Gaenzle, 2012; Maitland, Boyer, Eifert, & Williams, 2011; Robey et al., 2001; Simpson & Gilmour, 1997; Tay, Shellhammer, Yousef, & Chism, 2003; Whitney, Williams, Eifert, & Marcy, 2007). A phenomenon that is closely linked to this strain variation is the ability of some bacteria to become extremely HP resistant –even into the gigapascal range – by a mechanism of mutation and selection (Hauben et al., 1997; Vanlint, Rutten, Michiels, & Aertsen, 2012; Vanlint et al., 2011).

The second type of resistance depends on the properties of the matrix in which the bacteria reside during HP treatment and is therefore referred to as extrinsic resistance. Matrix properties that enhance bacterial HP resistance include reduced water activity due to solutes like salt and sugar (Goh, Hocking, Stewart, Buckle, & Fleet, 2007; Molina-Hoppner, Doster, Vogel, & Ganzle, 2004; Scheyhing, Hormann,

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Ehrmann, & Vogel, 2004; Smiddy et al., 2005; Van Opstal, Vanmuysen, & Michiels, 2003) and some bivalent cations and anions (Black, Huppertz, Fitzgerald, & Kelly, 2007; Hauben, Bernaerts, & Michiels, 1998). Both intrinsic and extrinsic HP resistance can reach levels that potentially compromise the effectiveness of commercial HP treatments. An approach that has been explored to overcome both intrinsic and extrinsic bacterial HP resistance is the use of antimicrobials in combination with HP treatment. Studies on a wide range of vegetative bacteria and both in buffer and foods have found that antimicrobials can considerably enhance bacterial inactivation by HP treatment, even when they are used at sublethal concentrations (de Alba, Bravo, & Medina, 2013; Garcia-Graells, Van Opstal, Vanmuysen, & Michiels, 2003; Hauben, Wuytack, Soontjens, & Michiels, 1996; Ogihara, Yatuzuka, Horie, Furukawa, & Yamasaki, 2009).

In the current study, we focus on antimicrobial compounds from herbs and spices, which have generated a wide interest as potential food biopreservatives (Burt, 2004). The effectiveness of combinations of HP and plant essential oils and antimicrobial compounds thereof has been recently reviewed, and this leads to the conclusion that many combinations are synergistic (Gayan, Torres, & Paredes-Sabja, 2012). However, the environment and the treatment conditions may determine whether or not a synergistic effect is found. Karatzas, Kets, Smid, and Bennik (2001) reported a synergistic effect of high pressure and 3 mM carvacrol as the combined treatment reduced the bacterial count by 3.6 log more than the sum of the inactivation levels by the individual treatments. However, the synergistic effect was strongly reduced (0.9 log) when the treatment was done in milk. More recently, Espina, Garcia-Gonzalo, Laglaoui, Mackey, and Pagan (2013) investigated the inactivation of *L. monocytogenes* EGDe and *E. coli* O157:H7 by HP in combination with nine essential oils and thirteen pure essential oil compounds in buffer at pH 4.0 and 7.0 and concluded that synergy was both strain- and pH-dependent. Of the pure compounds, (+)-limonene and carvacrol had the strongest synergistic effect (up to 4.2 log) on both organisms and in both buffers. (+)-Limonene was also effective in combination with HP treatment to inactivate *E. coli* O157:H7 in apple and orange juices. Other compounds that exhibited a synergistic effect up to 4.2 log were α-terpinyl acetate and linalool. However, camphor, borneol and eucalyptol showed no or only very limited synergy with HP on both bacteria. The finding that some antimicrobials exert a synergistic effect with HP and some don't led us to hypothesize that synergy may require a specific mode of action of the antimicrobial.

To investigate this hypothesis, we screened a set of thirteen natural antimicrobial compounds (NACs) for synergy with HP on the inactivation of a panel of eight Gram-negative and two Gram-positive bacteria. Bacteria were selected to allow analysis of both species and strain variability of synergistic effects. Compounds represented different functional groups such as aldehydes (t-cinnamaldehyde (CIN), reuterin (REU)), an isothiocyanate (allyl isothiocyanate (AIT)) and a variety of monoterpenoids (linalool (LIN), carvacrol (CAR), citral (CIT), eucalyptol (EUC), eugenol (EUG), geraniol (GER), linalool oxide (LOX), (−)-α-pinene (PIN), α-terpineol (TER) and thymol (THY)). This selection includes several compounds for which the combination with HP has not yet been reported (CIN, EUG, GER and LOX). The results allowed us to identify thiol reactivity as a property that results in synergy with HP treatment.

2. Materials and methods

2.1. Antimicrobials

AIT (93%), CIT (≥96%), EUC (≥99%), EUG (≥98%), GER (≥97%), t-2-hexenal (HEX) (98%), LIN (≥97%), LOX (≥95%), PIN (≥97%), sulforaphane (SUL) (≥90%), TER (≥96%) and THY (≥99%) were purchased from Sigma-Aldrich (Diegem, Belgium). CAR (≥98%), CIN (99%) and dimethyl fumarate (DMF) (>98%) were purchased from SAFC (St. Louis, MO, USA), Acros Organics (Fairlawn, NJ, USA) and TCI (Tokyo, Japan), respectively. Stock solutions of all compounds at 1.0 M were prepared in 99.9% ethanol, and diluted down to working concentration in potassium phosphate buffer (10 mM, pH 7.0) just before use. REU (3-hydroxy propionaldehyde) is relatively unstable and not commercially available, and was therefore produced by *Lactobacillus reuteri* ATCC 55730 essentially as described by Talarico, Casas, Chung, and Dobrogosz (1988). Cells from a stationary phase culture (22 h, 30 °C) of this strain in De Man, Rogosa, Sharp broth (MRS, Oxoid, Hampshire, U.K.) were collected by centrifugation (4000×*g*), washed with water and resuspended in one fourth volume of 250 mM glycerol. The suspension was incubated at 30 °C for 4 h, and the supernatant obtained after centrifugation (30 min, 8000×*g*) was stored at 4 °C. The concentration of REU in this solution was determined indirectly by its antimicrobial activity against *E. coli* MG1655 and expressed in activity units per ml (AU/ml) (Schaefer et al., 2010). One unit is defined as the amount of REU needed for total growth inhibition, measured by optical density (600 nm, Multiscan RC, Thermo Scientific, Franklin, MA, USA) (OD < 0.1). HPLC analysis with an Aminex HPX 87H column (Bio-Rad Laboratories, Richmond, CA, USA) using a 60:40 mixture of water and acetonitrile revealed that a major part of the available glycerol had been converted to REU, and that only minor quantities of other compounds were present (Fig. S1).

2.2. Preparation of bacterial suspensions for NAC–HP treatment

The following bacteria were used for assessment of NAC–HP synergy in this work: *Pseudomonas aeruginosa* PAO1, *Escherichia fergusonii* ATCC 35469, *Salmonella* Typhimurium LT2, *Salmonella* Enteritidis ATCC 13076, *E. coli* BL21 DE3, *E. coli* MG1655, *Listeria innocua* CIP 8012, *E. coli* O157:H7 ATCC 43888, *S. aureus* LMG 8064 and *E. coli* LMM1010. For HP and antimicrobial treatments, stationary phase cultures were grown for 22 h with shaking (200 rpm) in lysogeny broth (LB) composed of tryptone (10 g/l; LabM, Lancashire, U.K.), yeast extract (5 g/l; Oxoid) and sodium chloride (5 g/l) at 37 °C, except for *L. innocua* which was grown in brain heart infusion broth (BHI; Oxoid). Cells were harvested by centrifugation at 4000×*g* for 5 min, washed and resuspended to a concentration of approx. 9.5 log cfu/ml in sterile 10 mM potassium phosphate buffer (pH 7.0) with or without added antimicrobial compound. Potassium phosphate buffer was chosen since it is non-toxic and because the pH change caused by a pressure of ≤300 MPa remains limited (0.4 per 100 MPa) (Gayan, Condon, Alvarez, Nabakabaya, & Mackey, 2013).

2.3. Treatment with HP

HP treatment (15 min, 20 °C, 150–300 MPa) was carried out in an eight-vessel (8 ml volume each) HP equipment (HPIU-10,000, 95/1994, Resato, Roden, The Netherlands). The temperature of the vessels was maintained by a water circuit connected to a cryostat. A mixture of glycols (TR15, Van Meeuwen, Weesp, The Netherlands) was used as pressure-transmitting liquid. Samples were treated in 1-ml heat-sealed sterile polyethylene bags. The number of survivors was determined by spotting 5-μl samples of 10-fold dilution series on tryptone soy agar (TSA; Oxoid) and counting colonies after 24–48 h of incubation at 37 °C. The lower detection limit with this method was 2.3 log cfu/ml.

The degree of synergy for each antimicrobial in combination with HP was quantified by subtracting the reduction achieved by the compound (NAC) and by HP separately from the reduction achieved by the combined treatment (HP + NAC), with all reductions expressed in logarithmic units (log cfu/ml):

$$\text{Synergy} = N_0/N_{HP+NAC} - [N_0/N_{HP} + N_0/N_{NAC}.]$$

In this equation, N_0 is the plate count of the untreated sample (always approx. 9.5 log cfu/ml), $N_{HP\ +\ NAC}$is the plate count after the combination treatment, $N_{HP}$is the plate count after HP treatment and $N_{NAC}$is