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Inhibition of *Listeria monocytogenes* on bologna sausages by an antimicrobial film containing mustard extract or sinigrin

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ABSTRACT

The ability of *Listeria* (*L.*) *monocytogenes* to convert glucosinolates into antimicrobial isothiocyanates was investigated. Mustard glucosinolates in pure (sinigrin) or extract forms (sinigrin, oriental; sinalbin, yellow mustard) were used in broth media and in a polyvinyl polyethylene glycol graft copolymer (PPG) packaging film with bologna to examine their value as antimicrobial precursors for the control of *L. monocytogenes* viability and extension of bologna shelf-life at 4 °C. During broth tests with deodorized (myrosinase-inactivated) mustard extracts (10 d at 20 °C) or with purified sinigrin (21 d at 20 °C) *L. monocytogenes* was only inhibited when exogenous myrosinase was added. None the less, the organism was able to hydrolyze almost half the pure sinigrin by 21 d in tests without added enzyme. Reductions in sinigrin levels were measured by reversed-phase liquid chromatography, and in the absence of *L. monocytogenes* or added myrosinase the glucosinolate was stable. When pure sinigrin, oriental or yellow mustard extracts were incorporated in PPG films containing 3, 5 and 6% (w/w) of the corresponding glucosinolate and used to package bologna inoculated with 4 log CFU/g *L. monocytogenes*, the pathogen became undetectable in bologna packed with the oriental mustard extract at 52 d storage and remained undetectable at 70 d. The yellow mustard extract was less inhibitory and the pure sinigrin was not antimicrobial. *L. monocytogenes* numbers reached >7 log CFU/g in the film and untreated controls at 17 d storage. At 35 d storage, samples packed with control film contained sufficient numbers of lactic acid bacteria (LAB) (>7 log CFU/g) to be considered spoiled, whereas treatments containing mustard or sinigrin remained <7 log CFU/g LAB for ≤70 d. *L. monocytogenes* played a key role in exerting control over its own viability in bologna by hydrolysis of the glucosinolate in the oriental mustard film, but other antimicrobials in treatments may have contributed.

1. Introduction

Listeria (*L.*) *monocytogenes* is a pathogen that occurs widely in both agricultural and food processing environments (Ryser, 1999). Food poisoning caused by *L. monocytogenes* is usually attributable to contaminated dairy, meat, and poultry products or fruits and vegetables, although ready-to-eat (RTE) meat products are of particular concern due to potential contamination after thermal processing since they are commonly consumed without further cooking (Barmpalia et al., 2005). During the last few decades, the overall incidence of listeriosis has increased in both Europe and North America, and *L. monocytogenes* was identified as the causal agent in at least 13 illness outbreaks associated with RTE meat products from 1987 to 2008 (Couture, 2009). Among RTE foods, deli meats such as bologna sausages were believed to represent the greatest risk of illness and death from this pathogen (Gallagher et al., 2003).

Unlike most bacteria, *Listeria* can survive and sometimes grow on foods stored at refrigeration temperatures. In addition, *L. monocytogenes* is able to grow either aerobically or anaerobically (Nørrung et al., 2009) over a pH range from 4.6 to 9.4 (ICMSF, 1996). Consequently, many refrigerated vacuum-packed meat products (pH 6.3) with long shelf-life are able to support substantial growth of *L. monocytogenes* if contaminated during slicing and packaging. In response to this risk, the United States Department of Agriculture (USDA) adopted a "zero-tolerance" policy for this organism in these products (FDA, 2003), although the European Commission (EC, 2005) and Canada (HC, 2011) established a limit of 2 log CFU/g of *L. monocytogenes* in types of these products that do not support *L. monocytogenes* growth. Thus, techniques to inhibit or prevent the growth of this pathogen are of interest and active packaging systems are an alternative to antimicrobial ingredients addition to food.

Active packaging materials are designed by deliberately incorporating components that are released into food (EC, 2009) to extend shelf-life or to maintain or improve packed food quality by being antimicrobial or by enhancing chemical stability (Quintavalla and Vicini, 2002). Among substances permitted for use in packaging, food

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additives such as organic acids and their salts, bacteriocins and nitrites are unpopular with consumers (Han, 2000). For this reason, naturally derived antimicrobials (essential oils and spices) seem of greater importance as they represent lower perceived risk to the consumer (Appendini and Hotchkiss, 2002; Nicholson, 1998).

Although they have no direct antimicrobial effects, glucosinolates are considered natural antimicrobials since they are known precursors of isothiocyanates (ITCs) which are potently antimicrobial (Luciano and Holley, 2011; Palop et al., 1995; Verkerk, 2002). Glucosinolates are compartmentalized within cells of plants belonging to the *Brassicaceae* family (i.e. broccoli, mustard, horseradish and wasabi; Nielsen and Rios, 2000). The enzyme β-thioglucosidase (EC 3.2.1.147), or myrosinase, which catalyzes the hydrolysis of glucosinolates, is physically separated from its substrate in intact plant cells. When physical injury occurs, the enzyme comes in contact with the glucosinolates and breakdown products such as isothiocyanates, nitriles, oxazolidinethiones, thiocyanates and epithionitriles are formed (Verkerk, 2002). Among these degradation products, ITCs are known to be the most biologically active, being reported to possess broad spectrum antimicrobial activity against bacterial and fungal pathogens, nematodes, insects and weeds (Brown and Morra, 1997; Lin et al., 2000; Manici et al., 2000; Rosa and Rodriges, 1999).

Oriental mustard (*Brassica juncea*) and yellow mustard (*Sinapis alba*) seeds and powder contain high level of glucosinolates sinigrin and sinalbin, respectively, forming the ITCs, allyl isothiocyanate (AIT) and p-hydroxybenzyl isothiocyanate (p-HBIT), in the presence of moisture and myrosinase, respectively (Delaquis and Mazza, 1995). Thermal treatment of ground mustard (in the absence of moisture) to inactivate plant myrosinase leaves the glucosinolates unaffected, but the resulting deodorized powder can be used as a flavorless, odorless, and high protein food ingredient (Luciano et al., 2011).

Interestingly, some bacteria have been found to possess myrosinase-like activity and are able to convert glucosinolates into ITCs in absence of the plant enzyme. Examples include *Lactobacillus agilis* R16 (Palop et al., 1995), unidentified strains of *Bacillus* and *Staphylococcus* (Brabban and Edwards, 1994), *Bacteroides thetaiotaomicron* (Elfoul et al., 2001), and more recently *Pediococcus pentosaceus* UM 116P, *Staphylococcus carnosus* UM 109M, *Escherichia coli* O157:H7 (Graumann and Holley, 2008), *Lactobacillus curvatus* and *Lactobacillus plantarum* (Luciano et al., 2011), *Salmonella* Typhimurium (02:8421), *Enterococcus faecalis* ATCC 7080 and *L. monocytogenes* GLM-4 (Herzallah et al., 2011).

Since some microorganisms can degrade glucosinolates and convert them into ITCs in the absence of plant myrosinase, the present study evaluated the ability of *L. monocytogenes* to convert glucosinolates present in deodorized oriental and yellow mustard, as well as pure sinigrin, into their respective ITCs, during in vitro tests and on sliced bologna vacuum-packed with films containing glucosinolates. Since *Lactobacillus sakei* and *L. curvatus* along with *Leuconostoc* spp. are often the main causes of offensive and commercially important spoilage of cooked processed meats (Davies et al., 1999; Korkeala et al., 1988), it was also of interest to follow changes in numbers of lactic acid bacteria (LAB).

2. Materials and methods

2.1. Chemicals

Hot or spicy (with active myrosinase) oriental/brown mustard was obtained from Sakai spice (Calgary, AB, Canada) and cold or deodorized (myrosinase-inactivated) yellow mustard (#615) was obtained from G.S. Dunn Limited (Hamilton, ON, Canada). Sinigrin, myrosinase (thioglucosidase from *S. alba*, white/yellow mustard seed), DL-lactic acid and 2-phenylethanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sinalbin was obtained from Cerilliant Analytical Reference Standards (Round Rock, TX, USA). Allyl isothiocyanate (94%) was obtained from Acros Organics (Morris Plains, NJ, USA). Tetrabutylammonium hydrogen sulfate (98%) was purchased from J. T. Baker (Phillipsburg, NJ, USA) and acetonitrile HPLC grade and ethanol (95%) synthesis grade were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). The polymeric solution for film making (polyvinyl polyethylene glycol graft copolymer, PPG) was obtained from BASF (Ludwigshafen, Germany).

2.2. Bacterial strains and inoculum preparation

L. monocytogenes GLM-4 was obtained from the culture collection of the Food Science Department, University of Manitoba (Winnipeg, MB, Canada). The strain was kept on Brain Heart Infusion (BHI, Oxoid Ltd., Basingstoke, England) agar at 4 °C and streaked on agar plates on a weekly basis in fresh media for further experiments. The stock culture was prepared by transferring a single colony of *L. monocytogenes* from BHI agar to BHI broth (Oxoid Ltd.) and incubated overnight at 36 °C (first generation). Then 0.1% (v/v) of overnight culture was transferred to fresh BHI broth and incubated as described previously, reaching 9 log CFU/ml prior to the experiments (second generation).

2.3. Mustard extract preparation and HPLC characterization

A two cm layer of hot oriental mustard in an aluminum foil tray (20×20 cm) was treated for 15 min at 115 °C to inactivate the enzyme myrosinase. Then 300 g of autoclaved oriental or commercial deodorized yellow mustard was added to 3 l distilled water at room temperature, stirred for 1 h at 350 rpm, centrifuged for 20 min at 4200×*g* at 4 °C, and then filtered through a Whatman no. 4 filter. The filtrate was boiled for 30 min at 100 °C (±1 °C) to precipitate mucilage. The mixture was centrifuged and filtered again as described before. The filtrate was frozen overnight at −20 °C, then freeze-dried for 48 h and kept at 4 °C in a tightly closed container.

Analysis of the glucosinolates sinigrin and sinalbin from oriental and yellow mustard extracts, respectively, was based on the method used by Luciano and Holley (2010, 2011). Samples were dissolved in distilled water and filtered (0.22 μm, Millipore, Cork, Ireland) before HPLC analysis. The stability of sinigrin and sinalbin after extraction confirmed the lack of myrosinase activity. Separation and quantification of the glucosinolates were performed using reversed-phase liquid chromatography (RP-HPLC, Waters 2695, Waters Corporation, Milford, MA, USA) equipped with a C18 column (Gemini-NX, 150×4.60 mm, 5 μ; Phenomenex, Torrance, CA, USA) and a security guard cartridge (Gemini C18, 4×3 mm; Phenomenex). Elution was carried out isocratically at room temperature for 20 min at a flow rate of 1 ml/min, using a solvent system containing 20% acetonitrile and 80% tetrabutylammonium hydrogen sulfate buffer (0.02 M, pH 5.5). (pH 5.5). The injection volume was 10 μl. A UV detector (Waters, model 486) was used to measure the absorbance at 227 nm.

2.4. In vitro antimicrobial activity and glucosinolate degradation

A modified BHI broth was prepared containing the ingredients present in bologna sausages (1.9% (w/v) NaCl (HyGrade, Sifto Canada Corp., Mississauga, ON, Canada), 0.30% (w/v) proprietary pickle cure concentrate as a source of nitrite (6.25% $NaNO_2$, 1% sodium bicarbonate, with added NaCl and glycerol; Canada Compound Corp., Winnipeg, MB, Canada), 0.20% (w/v) polyphosphates, and 0.05% (w/v) sodium erythorbate (Canada Compound Corp.) (pH = 6.2). After autoclaving, a filter (0.22 μm) sterilized solution of D-glucose (Sigma Chemical Co.) was added to achieve a final concentration of 0.1% (w/v), since it was found that heated or autoclaved glucose yielded compounds that had growth-inhibitory effects on bacteria (Suortti and Malkki, 1984; unpublished, this lab).