PE6.06 Degradation of Glucosinolates by Bacteria Used in the Production of Dry-Fermented Sausages 207.00

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Abstract—Outbreaks involving Escherichia coli O157:H7 and dry-cured sausages consumption have been reported. Food regulatory agencies mandate a 5-log reduction of E. coli O157:H7 during the manufacturing of these products. Intriguingly, addition of 6% yellow mustard flour lacking the enzyme myrosinase, and therefore unable to produce isothiocyanates, in dry-cured sausage was able to cause very significant reduction of the pathogen in only 6 days. From these results three hypotheses have arisen: 1) intrinsic meat enzymes were able to degrade the glucosinolate and form the isothiocyanate; 2) the mustard present in the spice used (at 10%) in the sausage recipe was causing this effect; or 3) the starter cultures had a myrosinase-like activity and formed the isothiocyanate. Sinigrin and sinalbin were tested to answer these questions and their levels were followed for 6 days using HPLC. Our results showed that neither the meat enzymes nor the spice mix were able to decompose the glucosinolate sinigrin. However, both bacteria used as starter cultures were capable to degrade sinigrin and sinalbin, and perhaps to form isothiocyanates. Similar results were found when a yellow mustard flour broth was used. Staphylococcus carnosus showed higher rates of glucosinolate hydrolysis than Pediococcus pentosaceus. These results suggest that the starter cultures may convert glucosinolates into isothiocyanates and kill E. coli O157:H7 in dry-cured sausages.

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Index Terms — dry-cured sausage, E. coli O157:H7 control, glucosinolate hydrolysis, starter cultures.

I. INTRODUCTION

Escherichia coli O157:H7 has been involved in various food outbreaks in over 30 countries, which were first reported in the early 1980's. Most of these outbreaks have been associated with the consumption of undercooked meats [1]. Dry fermented sausages are traditional products, usually consumed raw and manufactured without thermal processing. Preservation of these products is a result of various characteristics: pH drop produced by the fermenting bacteria commonly known as lactic acid bacteria (LAB), reduction of the water activity (aw) during the drying process, and addition of antimicrobial compounds such as salt, nitrite and spices [2]. Although dry-cured sausages offer diverse hurdles against the growth of microbial pathogens, some of these organisms can overcome these barriers. E. coli O157:H7 is well known to be pH and salt tolerant [3]. In addition, it has been reported to have very low infection doses and high mortality rates, mainly in infants, elderly and immunocompromised people.

In 1994, Escherichia coli O157:H7 sickened 17 individuals in an outbreak related to the consumption of pre-sliced dry salami [4]. Other E. coli O157:H7 outbreaks involving 39 and 143 people were related to the consumption of Genoa salami and Hungarian plus Cervelat salami, respectively [5, 6]. Beef and to a less extent pork meat were the potential sources of E. coli O157:H7 during these outbreaks, and they are major ingredients in dry-cured sausages recipes. The occurrence of these outbreaks led food regulatory agencies in both Canada [7] and the US [8] to adopt very strict rules for the manufacture of fermented meat products, where the process should result in at least a 5-log reduction of the E. coli O157:H7 population. Glucosinolates are substances found within cell compartments of plants belonging to the family Cruciferaceae (e.g. broccoli, mustard, horseradish, wasabi) [7].

When these intracellular compartments are disrupted after damage to some extent (e.g. grinding, milling), the membrane-bound enzyme myrosinase is released and hydrolyzes glucosinolates, resulting in the formation of three main groups of substances: nitriles, thiocyanates and isothiocyanates [8]. The last group contains

diverse compounds with well established antimicrobial activity, such as benzyl isothiocyanate, phenethyl isothiocyanate, allyl isothiocyanate (AIT) and ñ-hydroxy benzyl isothiocyanate (ñ-HBIT) [9, 10, 11]. The last two compounds are the most important for the present study. AIT is formed after hydrolysis of sinigrin by myrosinase and sinalbin generates ñ-HBIT in this same reaction. Sinigrin is the major glucosinolate found in brown and oriental mustard (up to 0.8%), whereas sinalbin is practically the sole glucosinolate found in yellow mustard flour (~2.5%) [12]. Graumann and Holley [13] evaluated the potential antimicrobial effect of 2, 4, and 6% (wt/wt) nondeheated (active myrosinase) yellow mustard powder (Sinapis alba) or 6% (wt/wt) deheated (inactive myrosinase) yellow mustard powder following their addition to dry sausage batter inoculated with E. coli O157:H7 at about 7 log CFU/g. Mustard flour is commonly used as a spice (containing active myrosinase) and/or binder (inactive myrosinase) in dry-cured sausages. Diverse parameters were monitored throughout the study (aw, pH, bacterial population) and it was found that all levels of nondeheated mustard powder tested resulted in significant reductions of E. coli O157:H7 during 30 days of drying.

However, only concentrations of 6% were able to achieve the 5-log reduction required by food regulatory agencies. Longer fermentation periods were necessary for a 5-log E. coli O157:H7 reduction by 2 and 4% yellow mustard powder. On the other hand, populations of the starter cultures used were not affected by the mustard powder. Interestingly, the 6% deheated mustard powder treatment resulted in the most rapid reductions of E. coli O157:H7 (significant drop after 6 days).

This result was not expected since the “cold” or tasteless flour lacks the enzyme myrosinase and, therefore, is not able to produce isothiocyanates intrinsically. The aim of the present paper is to understand, at least partially, the mechanism by which the “cold” flour is able to kill E. coli O157:H7.

II. MATERIALS AND METHODS

Bacterial Strains. Experiments used a five strain mixture of E. coli O157:H7. The strain LCDC 7283 (pathogenic, hamburger isolate) was provided by Dr. R. Khakria, Laboratory Centre for Disease Control, Ottawa, Canada. Strains 02-0628, 02-0627, 00-0351 and 02-0304 (non-pathogenic, human isolates) were supplied by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health, Winnipeg, MB, Canada. The Staphylococcus carnosus (UM 109M) and Pediococcus pentosaceus (UM 116P) were isolated from commercial lyophilized dry-sausage starter culture preparation (Trumark LTII and Trumark LTIIM, respectively; Rector Foods Ltd., Mississauga, Ontario, Canada). Chemicals.

Sinigrin and myrosinase were purchased from Sigma Chemical Co (St. Louis, MO, USA); sinalbin from Glucosinolates (Copenhagen, DK); and the deheated yellow mustard flour (inactivation of the enzyme myrosinase was done using high temperatures) from Newly Weds Foods (Chicago, IL, USA) Microbial Growth Conditions. All bacteria were incubated in broth for 16h at 25oC prior to the experiments. E. coli O157:H7 (5-strain cocktail) and Staphylococcus carnosus grew in tryptic soy broth; Pediococcus pentosaceus grew in deMan, Rogosa and Sharpe (MRS) broth. After the incubation period, 0.1 ml of each bacterial culture was used as the inoculum for subsequent experiments. The final volume of 10 ml in screw-capped tubes was used during all studies. Sinigrin, Sinalbin and Yellow Mustard Flour Broth. Doses of 0.1% sinigrin were used in preliminary studies. This selection was made because sinigrin is commercially available in higher amounts than sinalbin in its purified form and results for both glucosinolates were found to be very similar. Levels of sinigrin were followed for 6 days (samples collected on days 1, 2, 3 and 6) in different sets of experiments: 1) Bacterial growth in minimal salts (M9) [14] containing 0.1% sinigrin and 0.1% glucose was examined. 2) Then, the effect of fresh and cooked meat exudates was evaluated in the presence of 0.1% sinigrin dissolved in M9 media (1:10). 3) The spice mix used by Graumann and Holley [13] was found to contain up to 10% of mustard powder and, therefore, could contain the enzyme myrosinase, causing the conversion of glucosinolates to isothiocyanates. A level of 0.44% of the spice mix, which is used in dry-cured sausage recipes, was added to M9 media + 0.1% sinigrin. 4) Degradation of sinigrin (0.1%) by bacteria growing in Muller-Hinton broth (MHB) was also analyzed. Experiment 4 was repeated replacing sinigrin by 0.1% sinalbin. A broth was prepared containing 10g of deheated yellow mustard flour (YMF) in 1 L of

water. The mixture containing natural sinalbin was boiled for 10 min to extract sinalbin and filter-sterilized. Bacteria were grown in either 100% YMF broth or 50% YMF broth + 50% MHB. Levels of sinalbin were followed for 6 days by HPLC. HPLC Analysis.

Separation and quantification of the glucosinolates was performed using an HPLC equipped with a C18 column (Waters Co., 4.6 x 250 mm i.d. 5 ìm). Elution was carried out isocratically for 18 min at a flow rate of 1 mL/min, using a solvent system containing 20% (v/v) acetonitrile and 80% water + 0.02M tetrabutylammonium. The injection volume used was 10 ìl. A detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of the glucosinolates.

III. RESULTS AND DISCUSSION

Neither the meat exudates nor the spice mix were able to reduce the levels of sinigrin in the media. These results suggest that the enzymes present in meat are not able to hydrolyze sinigrin to form allyl isothiocyanate. In addition, the mustard present in the spice mix had very little or no myrosinase activity. When the bacteria were put in the presence of 0.1% sinigrin, they had 0.1% glucose as the only source of energy. This media (M9) was selected to try and force the bacteria to use sinigrin as an energy supply. Very little consumption of the glucosinolate was found after 6 days. However, when the same experiment was done using MHB to improve bacterial growth (Fig. 1), S. carnosus produced a 17.01% (or 425 ìM) reduction of the sinigrin levels and P. pentosaceus dropped the levels by 11.89% (or 297 ìM). The E. coli 5-strain cocktail did not cause a decline of the glucosinolate concentration. When S. carnosus was grown in MHB with 0.1% sinigrin and re-inoculated into fresh MHB with the same amount of sinigrin, it was able to generate faster hydrolysis of the glucosinolate (29.16% or 729 ìM in 6 days).

Previous studies have shown that the minimum inhibitory concentration of allyl isothiocyanate against E. coli O157:H7 at pH levels found in dry-cured sausages (pH 4.9 to 5.5) was ~ 250 ìM [15]. Formation of isothiocyanates by the starter cultures is hard to follow due to the high instability of this class of compounds in aqueous systems. Conversion of glucosinolates to isothiocyanates by myrosinase was shown to have a yield of up to 90% [16]. If the decomposition of sinigrin generated by the starter cultures follows the same pattern, this reaction will indeed exert an extra hurdle against E. coli O157:H7 growth in the dry-sausage environment, and could possibly cause the death of the pathogen. Similar results were found when sinigrin was replaced by sinalbin (Fig. 2). Staphylococcus carnosus and Pediococcus pentosaceus caused 13.82% (326 ìM) and 13.25% (313 ìM) reduction of sinalbin, respectively. No effect on sinalbin concentration was found when the bacteria grew in YMFB. However, S. carnosus caused a 19.75% (233 ìM) reduction when the YMF broth/MHB mixture was used. Myrosinase-like activity in bacteria has been described before [14, 17, 18, 19]. The starter cultures, more importantly S. carnosus, also showed the capacity to degrade glucosinolates. Yellow mustard flour has 2.5% of sinalbin on average, and this results in 3.53 mM of sinalbin in a dry sausage containing 6% of YMF. This concentration should be sufficient for the formation of ñ-hydroxy benzyl isothiocyanate by the starter cultures in order to kill E. coli O157:H7.

IV. CONCLUSION

The results presented in this paper suggest that the starter cultures P. pentosaceus UM116P and, more importantly, Staphylococcus carnosus UM109 may convert significant amounts of glucosinolates to isothiocyanates. This conversion may help to understand why deheated yellow mustard flour is able to kill E. coli O157:H7 in dry-cured sausages [13]. S. carnosus adapted to the presence of sinigrin showed higher yields of glucosinolate hydrolysis, and perhaps could have better efficiency in killing E. coli O157:H7 when applied to dry-cured sausages formulated with “cold” yellow mustard flour. Understanding the mechanisms of glucosinolate-to-isothiocyanate formation and selection of bacteria with higher capacity to conduct this conversion could be the answer to achieve the 5-log reduction of E. coli O157:H7 required by food regulatory agencies.

ACKNOWLEDGEMENT

This project was funded by the Natural Science and Engineering Research Council of Canada (NSERC). We also would like to thank Alison Ser for her technical assistance with the HPLC apparatus. F.B. Luciano also expresses his gratitude to the University of Manitoba and the Government of Manitoba (Canada) for financial support through

the University of Manitoba Graduate Fellowship and the Manitoba Graduate Scholarship, respectively.

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