ANTIBIOTIC AND ALLYL ISOTHIOCYANATE RESISTANCE AMOUNG ENTEROCOCCI ISOLATED FROM FERMENTED DRY SAUSAGES

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Abstract – The study investigated susceptibilities of enterococci from dry sausages to 12 antibiotics and allyl isothiocyanate (AIT), a natural antimicrobial isolated from oriental or brown mustard seeds. Susceptibility tests and the minimum inhibitory concentration (MIC) of the antibiotics were performed using sterile 96-well microtiter plates. The MIC of AIT was determined using broth macro-dilution using screw-capped tubes. A total of 26 enterococci were isolated from 50 commercially fermented dry sausages. The highest incidence of resistance was to clindamycin (92%), followed by tetracycline hydrochloride (65%), tylosin (58%), erythromycin (42%), streptomycin and neomycin (19%), chloramphenicol (15%), penicillin and ciprofloxacin (12%) and gentamicin (4%). None of the isolates was found resistant to vancomycin or ampicillin. Twenty four of the 26 strains were resistant to more than one drug tested. The highest MIC of AIT was 2.5mM. It is possible that fermented dry sausage may act as a reservoir for multi-drug resistant enterococci, but frequencies of their resistance to the clinically important drugs (ampicillin and vancomycin) were low. Resistance of the enterococci to AIT was similar to that of other Gram positive bacteria.

Key Words – food safety, MIC, multi-drug resistant bacteria, enterococci.

I.   INTRODUCTION

Enterococci are Gram-positive, facultatively aerobic bacteria ubiquitous in the gastrointestinal tract of humans and animals. They are not generally considered pathogenic but for immunocompromised individuals they may cause severe disease including endocarditis, wound and urinary tract infections [10, 11]. Enterococcal resistance to antimicrobials can be intrinsic or acquired. They are resistant to a wide variety of antibiotics commonly administered in human medicine and as well as those that are used for animal growth promotion or for treatment and control of animal diseases. Enterococci are also known for their capacity to exchange genetic information by conjugation [3]. They may spread antibiotic resistance among other non-pathogenic enterococci or enhance the virulence of other pathogens [5, 7]. Thus there is concern about their presence in fermented meats that are not heat-treated before consumption because they may be a vehicle for transferring antimicrobial resistant bacteria from the indigenous animal microflora to the human gastrointestinal tract [13]. There is a growing interest in using plant-derived antibacterial compounds such as extracts of spices and herbs for food preservation [16]. Allyl isothiocyanate (AIT) a component in the essential oil of mustard contributes to its hot spiciness. In both its vapor and liquid forms it has high bactericidal activity, and thus has been tested for its ability to eliminate pathogenic bacteria from meat and fermented meat products [2, 9].

In the present work, broth micro-dilution of antibiotics and broth macro-dilution of AIT were used to assess the level of resistance of 26 enterococci strains isolated from commercial fermented dry sausages. The objectives of this study were to generate, by means of phenotypic susceptibility tests, a representation of antibiotic and AIT resistance patterns of enterococci present in commercially prepared dry fermented sausages.

II.   MATERIALS AND METHODS

Bacterial strains and culture conditions.
Enterococci in fermented dry sausage samples purchased at retail (Winnipeg, MB, Canada) were detected by PCR using genus specific primers derived from 16S rDNA sequences and then isolated by culture-based methods. The strains were confirmed to be enterococci at the species level by API 20 Strep strips (BioMérieux, Marcy l'Etoile, France) and sequencing using universal primers. The isolates were identified as *Enterococcus faecalis* (n=14), *Enterococcus faecium* (n=11) and *Enterococcus gallinarum*

(n=1). From KF- Streptococcus agar plates (Difco, Fisher Scientific, Edmonton, AB, Canada), isolated colonies were inoculated into Mueller-Hinton broth (MH, Fisher Scientific) and incubated overnight at 35ºC. The bacterial density was adjusted using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England) at 600 nm to achieve a concentration near 7.4 log CFU ml^{-1}. The cultures were further diluted in sterile normal saline to obtain a final concentration of approximately 5 X10^{-5} CFU ml^{-1} [4].

Antimicrobial drugs and AIT.
Ten antibiotics currently registered in Canada for use in food animals plus vancomycin and ciprofloxacin were used in this study. Antibiotic powders of known potencies were obtained from Sigma-Aldrich, Canada Ltd. (Oakville, ON). The ranges of antibiotic concentration used were 0.125 to 64 μg ml^{-1} for erythromycin, clindamycin and tylosin; 0.250 to 128 μg ml^{-1} for ampicillin, penicillin G, chloramphenicol, tetracycline and vancomycin; 16 to 8192 μg ml^{-1} for gentamicin; 32 to 16384 μg ml^{-1} for streptomycin and neomycin; and 0.0625 to 32 μg ml^{-1} for ciprofloxacin. Antimicrobials were dissolved in distilled water and filter sterilized through 0.20 μm pore-sized syringe filter units (Fisher Scientific), except for tetracycline which was dissolved in ethanol (25%, v/v) as a solubility mediator.
Concentrations of AIT (Aldrich Chemical Co., Milwaukee, WI, USA) used were from 0.5 mM to 5mM.

Antimicrobial susceptibility testing and MIC determination.
Fifty μL of double-strength sterile MH broth were placed into each well of 96-well microtiter plates (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, NJ, USA). To the first wells 50 μl of antibiotic solutions were added and serial two-fold dilutions were made to the desired concentrations. Wells were then inoculated with 50 μl of bacterial suspension, giving a total volume of 100 μl. Plates were covered and incubated overnight at 35ºC. The trials were conducted in triplicate. The MICs for enterococci were determined according the Clinical and Laboratory Standards Institute (CLSI) [4], the European Food Safety Authority (EFSA) [6] and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) [1].
For AIT, 0.1 ml cultures were added to screw-capped tubes containing 9.9 ml of MH broth. Then AIT was added to each tube to give the concentrations noted above. The tubes were incubated at 35 °C with a shaker speed of 200 rpm (Junior Orbit Shaker; Lab-Line Instruments Inc., Melrose Park, IL, USA) for 24 h. Absence of growth (no increase in measured OD) was considered to be the MIC.

III. RESULTS AND DISCUSSION

It was found that 24 of the 26 enterococci isolates exhibited resistance to at least two antibiotics, but none was resistant to ampicillin or vancomycin (Table1). Only some strains of *E. faecium* were resistant to ciprofloxacin (27%) or penicillin (27%). Nine of 11 *E. faecium* strains were resistant to clindamycin (82%), followed by erythromycin (72%), tylosin (63%), tetracycline (45%), streptomycin (27%), and chloramphenicol (18%). In contrast, all 14 *E. faecalis* strains were resistant to clindamycin (100%) followed by tetracycline (71%), tylosin (64%), neomycin (21%), streptomycin (14%), chloramphenicol (14%) and gentamicin (7%).
Results here showed that only *E. faecium* strains were resistant to penicillin, which is consistant with the historical observation that *E faecium* strains were more frequently resistant to penicillin than those of *E. faecalis* [14]. More frequent resistance toward erythromycin among *E. faecium* than *E. faecalis* strains might be explained by the presence of an erythromycin resistant plasmid or transposons in these strains [14]. Occurrence of chloramphenicol resistance among these sausage enterococci is consistent with an earlier observation that food enterococci were resistant to chloramphenicol with varied frequency [8, 17]. It is of interest that three strains of each of *E. faecalis* and *E. faecium* were resistant to more than 4 antibiotics. Although the enterococci in the present study showed resistance to a number of antibiotics, they did not show resistance to the clinically relevant antibiotics ampicillin or vancomycin, and had a low frequency of resistance towards ciprofloxacin and gentamicin.

These food enterococcal strains were largely still susceptible to the clinically relevant antibiotics.

Table 1 Antibiotic resistance of *E. faecalis*, *E. faecium* and *E. gallinarum* strains isolated from commercial dry fermented sausages

Antibiotics	Resistant E. faecalis strains		*Resistant E. faecium strains*		Resistant E. gallinarum strains	
	Strains (n=14)	%	Strains (n=11)	%	Strains (n=1)	%
PG		0	S9, S15, S31	27		0
Te	[a]S5, S13, S14, S18, S25, S36, S39, S40, S41, S48	71	S15, S27, S30, S34, S50	45	S19	100
Cm	S13, S40	14	S15, S30	18		0
Em	S13, S36, S41	21	S9, S15, S22, S27, S28, S29, S31, S50	72		0
Sm	S13, S36	14	S9, S15, S27	27		0
Gm	S41	7		0		0
Ci		0	S6, S15, S27	27		0
Ne	S13, S36 S41	21	S15, S27	18		0
Ty	S10, S11, S13, S18, S25, S36, S38, S41, S48	64	S6, S22, S28, S29, S30, S31, S50	63		0
Cl	S3, S5 S10, S11, S13, S14, S18, S25, S36, S38, S39, S40, S41, S48	100	S6, S9, S15, S22, S27, S29, S30, S34, S50	82	S19	100

[a](S) Enterococci isolated from fermented dry sausage; ciprofloxacin (Ci), Chloramphenicol (Cm) clindamycin (Cl), erythromycin (Em), gentamycin (Gm), neomycin (Ne), penicillin G (PG), streptomycin (Sm), tetracycline (Te) and tylosin (Ty).

MIC results from tests of AIT against the 26 enterococcal strains are presented in Table 2. It was found that 3 *E. faecium* strains were only sensitive to 2.5mM AIT, which was high compared to *E. faecalis* isolates. Eleven of 14 *E. faecalis* strains had an MIC of 2.0mM/L. The lowest MIC of 1.0 mM was found with one *E. faecium* strain. In earlier studies Gram positive *Listeria monocytogenes* at 1.42 mM AIT showed greater resistance than *Escherichia coli* O157:H7 and *Salmonella* Montevideo. It was concluded that Gram positive bacteria tend to have more resistance towards AIT than Gram negatives [12]. In another study [15]., MIC values for the Gram positives *Streptococcus pyogenes* (0.63mM) and *Staphylococcus aureus* (0.15) were much lower than the lowest MIC observed in the present study for the enterococci.

Table 2 MIC values of AIT for enterococcal strains isolated from commercial dry fermented sausages

AIT (mM)	*E. faecalis* strains	*E. faecium strains*	*E. gallinarum* strains
	Strains (n=14)	Strains (n=11)	Strains (n=1)
1.0		S50	
1.5	[a]S3, S5, S13	S6, S9, S31, S34	
2.0	S10, S11, S14, S18, S25, S36, S38, S39. S40, S41, S48	S15, S22, S30	S19
2.5		S27, S28, S29	

[a](S) Enterococci isolated from fermented dry sausage

IV.   CONCLUSION

The frequent occurrence of antibiotic resistant enterococci in commercially produced fermented dry sausage may be of concern because these products may be food vehicles for dissemination of bacteria with multiple, transferable antibiotic resistance. It is unlikely that the natural antimicrobial AIT would be useful to control enterococci in fermented sausage because of their resistance to this agent.

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REFERENCES

1. Bager, F. (1998). Consumption of antimicrobial agents and occurrence of antimicrobials in bacteria from food animals, food and humans in Denmark. Danish Veterinary Laboratory, Copenhagen. Available at: http://www.danmap.org/pdfFiles/Danmap_1998.pdf.

2. Chacon, P. A., Muthukumarasamy, P. & Holley, R. A. (2006). Elimination of *Escherichia coli* O157:H7 from fermented dry sausages at an organoleptically acceptable level of microencapsulated allyl isothiocyanate. Applied and Environmental Microbiology 72:3096–3102.

3. Clewell, D. B., & Dunny, G. M. (2002). The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, D.C.

4. Clinical and Laboratory Standards Institute. (2002). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard M31-A2:1-80. Clinical and Laboratory Standards Institute, Wayne, PA.

5. Cocconcelli, P.S., Cattivelli, D. & Gazzola, S. (2003). Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. International Journal of Food Microbiology 88: 315–323.

6. European Food Safety Authority. (2008). Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. Question no. EFSA-Q-2008-004 adopted on 18 June 2008. EFSA J. 732:1–15.

7. Fisher, K & Phillips, C. (2009). The ecology, epidemiology and virulence of *Enterococcus*. Microbiology 155:1749-1757.

8. Franz, C. M. A. P., Muscholl-Silberhorn, A. B., Yousif, N. M., Vancanneyt, M., Swings, J. & Holzapfel, W. H. (2001). Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. Applied and Environmental Microbiology 67: 4385–4389.

9. Graumann, G. H. & Holley R. A. (2008). Inhibition of *Escherichia coli* O157:H7 in ripening dry fermented sausage by ground yellow mustard. Journal of Food Protection 71: 486–493.

10. Hummel, A., Holzapfel, W. H., & Franz, C. M. A. P. (2007). Characterization and transfer of antibiotic resistance genes from enterococci isolated from food. Systemic and Applied Microbiology 30: 1-7.

11. Lanthier, M., Scott, A., Zhang, Y., Cloutier, M., Durie, D., Henderson, V. C., Wilkes, G., Lapen, D. R. & Topp, E. (2011). Distribution of selected virulence genes and antibiotic resistance in *Enterococcus* species isolated from the South Nation River drainage basin, Ontario, Canada. Journal of Applied Microbiology 110: 407-421.

12. Lin, C.-M., Preston, J. F. & Wei, C.-I. (2000). Antibacterial mechanism of allyl isothiocyanate. Journal of Food Protection 63: 727–734.

13. Mathur, S. & Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria—a review. International Journal of Food Microbiology 105: 281– 295.

14. Murray, B. E. (1990). The life and times of the *Enterococcus*. Clinical Microbiology Reviews 3:46–65.

15. Palaniappan, K. & Holley, R. A. (2010). Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. International Journal of Food Microbiology 140: 164–168.

16. Shan, B., Cai, Yi-Z., Brooks, J.D. & Corke, H. (2007). The *in vitro* antibacterial activity of dietary spice and medicinal herb extracts. International Journal of Food Microbiology 117:112–119.

17. Teuber, M., Meile, L. & Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. Antonie van Leeuwenhoek 76:115–137.