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IN-PLANT VALIDATION OF TWO ANTIMICROBIAL AGENTS APPLIED DURING PRODUCTION OF FURTHER PROCESSED BEEF PRODUCTS

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Abstract - In 1999, the United States Department of Agriculture – Food Safety and Inspection Service declared *Escherichia coli* O157:H7 as an adulterant in non-intact beef products. As a result, all beef processors are required to address *E. coli* O157:H7 in their hazard analysis, and validate their systems to ensure identified pathogens of concern are adequately controlled. This project was designed to validate in-plant application of two antimicrobial interventions (lactic acid and Beefxide [lactic acid and citric acid mixture]) applied to multiple processing schemes (e.g. single pass or multiple pass tenderization, and marination). Surrogate microorganisms were applied to beef products prior to treatment with an antimicrobial (2.5% Beefxide or 2.9% lactic acid). Following inoculation and antimicrobial spray, products were subjected to a single or multiple pass tenderization and/or marination process. Beefxide and lactic acid treatments resulted in statistically significant ($P < 0.05$) log reductions of surrogate microorganisms on product surfaces for all beef subprimals. Surrogate microorganisms also were recovered from interior samples of all beef product types after mechanical tenderization. These data indicate that (1) Beefxide and lactic acid are similar ($P > 0.05$) in their efficacy as antimicrobial interventions in the production of non-intact beef products, and (2) non-intact processes can transfer microorganisms into the interior of whole-muscle cuts.

I. INTRODUCTION

In 1992-1993, a deadly foodborne outbreak occurred on the nation's west coast involving ground beef contaminated with *Escherichia coli* O157:H7, which caused consumers to question the safety of beef products. In 1994, the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) responded to the outbreak by declaring *E. coli* O157:H7 an adulterant in ground beef under the Federal Meat Inspection Act. USDA also mandated new food safety measures including the development and implementation of a Hazard Analysis and Critical Control Point (HACCP) plan, implementation of sanitation standard operating procedures, and microbiological testing. As part of the HACCP plan, meat processing facilities must identify hazards that are likely to occur and implement critical control points designed to prevent, eliminate, or reduce to acceptable level the pathogen of concern. Various antimicrobial interventions, such as hot water, lactic acid, acetic acid, and other organic acid sprays are applied during harvest to reduce contamination on the carcass. With the declaration of *E. coli* O157:H7 as an adulterant in non-intact beef products, many further processors are also applying antimicrobial interventions. All processors are required to validate their systems to ensure that the pathogens of concern are adequately controlled. This project was designed to validate in-plant application of two different antimicrobial interventions (lactic acid and Beefxide [lactic acid and citric acid mixture]) applied to multiple processing schemes (e.g., single pass or multiple pass tenderization, and marination). These data will help establishments validate in-plant pathogen reduction processes.

II. MATERIALS AND METHODS

Texas A&M University worked with a commercial further-processing establishment in Texas to complete this project. The establishment is federally inspected, so the project was designed to comply with all USDA regulatory requirements related to process validation and use of surrogate microorganisms. According to FSIS [1], "an establishment that chooses to conduct a validation study may use a *surrogate* indicator organism to measure change, but it should do so only after giving careful consideration to specific precautions. These precautions include ensuring that a microbiologist trained in food science and in the design of inoculated-pack studies introduces the non-pathogenic cultures within the establishment. In addition, the establishment should ensure that the introduction of the non-pathogenic cultures does not create an insanitary condition in the facility or cause the food to become adulterated. Finally, establishments should ensure that the non-pathogenic cultures are necessary and proven to be effective for the intended purpose."

A. Product and process parameters

This experiment was conducted in two phases. The first phase utilized the establishment's normal production practices. Three beef products: Boneless Strip Loin (n = 12), Top Sirloin Butt-Cap Off (n = 12), and Bottom Sirloin Flap (n = 12) were selected by the establishment based on availability of product, number of passes through the blade tenderizer, and the marination process. Antimicrobial interventions were applied to each product using a commercial spray cabinet (with conveyor belt) prior to blade tenderization. Bottom sirloin flaps were passed through the tenderizer one (1) time, split, and then marinated (proprietary commercial marinade) using a vacuum-tumbler. The strip loins were passed through the tenderizer two (2) times, and the top sirloin butts were passed through the tenderizer three (3) times. The second experimental phase involved strip loins (n = *6)* that were assigned to one of three non-intact treatments: single tenderization, double tenderization, and triple tenderization. The second phase of this study was designed to help researchers better control the variables that may influence internalization of surrogate microorganisms (i.e. varying subprimal thicknesses and/or non-intact processes).

Phase 1 products were randomly assigned to either Beefxide (lactic acid and citric acid mixture) or lactic acid treatment. Phase 2 product was only exposed to lactic acid spray since the focus of phase two was on microorganism internalization, not antimicrobial effectiveness. The average operating parameters for the antimicrobials were as follows: Beefxide: pH 2.18, temperature 24.4 °C, concentration 2.4%; lactic acid: pH 1.97, temperature 27 °C, concentration 2.9%. To ensure proper coverage with the antimicrobial, all products were placed in a single layer with no overlap. For both study phases, products that were passed through the tenderizer two or three times, did not receive additional antimicrobial treatment, just additional tenderization.

B. Inoculum preparation

Three non-pathogenic *E. coli* Biotype I strains (1427, 1428 and 1430) were obtained from the American Type Culture Collection (ATCC - ww.ATCC.org) for use in this study. These strains then were selected in the Food Microbiology Laboratory at Texas A&M University for their inherent ability to naturally resist rifampicin. Through previous scientific research, these surrogate organisms have demonstrated identical thermal and acid resistance to the human pathogen *E. coli* O157:H7. These marker organisms were designed for use in a "cocktail" to represent possible contamination with enteric pathogens of fecal origin such as *Salmonella* or *E. coli* O157:H7. In previous research [2], these marker organisms demonstrated identical thermal and acid resistance to *E. coli* O157:H7. At 48 h before each collection day, the Rif^R cultures of *E. coli* organisms (1427, 1428, 1430) were propagated by transferring a loop of each stored microorganism from a tryptic soy agar (TSA, Becton, Dickinson and Co., Sparks, MD) slant to a fresh 10 ml tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) tube and incubated aerobically at 37 °C for 18 to 24 h. Each

culture then was transferred individually by pipetting 0.1 ml into Falcon™ (Thermo-Fisher Scientific, Waltham, MA) conical centrifuge tubes containing 10 ml TSB before incubating for 18 h at 37 °C. After incubation at 37°C for 18 h, cells from each culture were harvested by centrifugation at 1,620 × g for 15 min. The supernatant was discarded and the pellet suspended in 10 ml of phosphate buffered saline (PBS). Each cell suspension was centrifuged again (1,620 × g for 15 min) and the procedure was repeated once. The final pellets were suspended in 10 ml of PBS each. Following the final suspension, cell suspensions from each culture were combined to form a cocktail of Rif^R *E. coli* organisms. All beef products were experimentally contaminated with surrogate microorganisms.

C. Product preparation and sampling

Background microbiological samples were taken randomly from six strip loins, top butts, and flaps to show that no naturally occurring rifampicin-resistant microorganisms were present prior to inoculation. All samples for this study were collected by excising two pieces of product (10cm^2 x 2 mm deep) using sterile stainless-steel borer, scalpel and forceps, and compositing them for a total of 20cm^2 sample area. Both sides of each cut were inoculated with: 2 ml (per side) for strip loins and flaps and 1 ml (per side) top butts. Amounts of inoculum used were based on the surface area of the product. Following inoculation of the cuts, 30 minutes was allowed for attachment of microorganisms to the product surfaces. After attachment was achieved, microbiological samples then were collected from both sides of the product, both before and after the intervention/tenderization process. The bottom sirloin flaps also were sampled after 2 additional steps as their production process included splitting and 20-minute marination/vacuum tumbling steps. Each sample was placed in a sterile stomacher bag, inside an insulated container, and transported to the Food Microbiology Laboratory at Texas A&M University. Along with surface samples, the subprimals were transported in insulated containers to the laboratory to accommodate internal microbiological sample collection under aseptic conditions. Once in the laboratory, 99 ml of sterile 0.1% peptone water was added to each sample. The samples then were pummeled for 1 minute at 260 rpm using a Stomacher-400 (Tekmar Company, Cincinnati, OH). For each sample, counts of the surrogate microorganisms were determined by plating the appropriate serial dilutions on pre-poured and dried rifampicin-tryptic soy agar (rif-TSA, Difco, Sparks, MD) plates. The plates were incubated for 24 h at 37 °C. Colonies then were counted, recorded, and reported as log CFU/cm^2.

F. Statistical analysis

Microbiological counts were transformed into logarithms before obtaining means and performing statistical analyses. When counts were found to be below the detection limit for the counting method used, a number between 0 and the lowest detection limit was used to facilitate data analysis. All data were analyzed using JMP Software (JMP Pro, Version 10.0, SAS Institute Inc., Cary, NC). The Fit Model function was used for analysis of variance, determining interactions from the full model, and least squares means comparisons were performed using a Student's t-test.

III. RESULTS AND DISCUSSION

Initial inoculum level was an important consideration for this project, to ensure that the level would be sufficient to measure a reduction; mean initial inoculum level of 7.8 and 8.7 log CFU/cm^2 were used for study phases 1 and 2, respectively. In phase 1, the reduction of surrogate microorganisms from the pre-intervention to post-intervention steps for Beefxide and lactic acid were statistically equal across all subprimal types. Following a 30-minute inoculum attachment period, pre-intervention product surface counts were found to be 5.6 log CFU/cm^2 across subprimals treated with either antimicrobial. Again displaying no difference ($P > 0.05$) in antimicrobial efficacy, post-intervention counts were 4.1 and 4.3 log CFU/cm^2 for Beefxide and lactic acid, respectively. The ability to compare the effectiveness of these antimicrobials was one of the main objectives of this project.

Internalization data from phase 1 of this study are in line with the findings of previous research describing the internalization of *E. coli* O157:H7 in mechanically tenderized meat [3]. While microbiological counts were recovered from the internal surfaces of all subprimals, bottom sirloin flap samples presented significantly higher ($P < 0.05$) counts than the other two subprimal types. It should be noted that the flaps were subjected to marination and vacuum tumbling, whereas the other subprimals were not. This finding as well as findings from other scientific research tell us that microorganisms are pushed further inside the product during further processing steps, such as marination and vacuum-tumbling [4]. To better understand the internalization of the surrogate microorganisms, data from phase 2 of this experiment is presented in Table 1 below.

Table 1. Least squares means for log CFU/cm^2 of Biotype I *Escherichia coli* surrogates for Phase 2 beef strip loins subjected to various blade tenderization applications following lactic acid treatment.

Sampling Interval	Fat surface	Lean surface	Internal
Pre-intervention[1]	6.1 A[2]	5.6 A	NC[3]
Post-intervention			
Single tenderization	5.1 B	5.3 A	2.3 A
Double tenderization	4.8 B	5.1 A	2.2 A
Triple tenderization	4.8 B	4.9 A	1.7 A

[1] Pre-intervention samples were collected following a 30-minute attachment of inoculum.
[2] Means within a column lacking a common letter differ ($P < 0.05$).
[3] NC, not collected.

These data show no differences ($P > 0.05$) in microbiological counts among tenderization treatments (single, double or triple tenderization passes).

IV. CONCLUSIONS

These data indicate that tenderization and marination processes can transfer microorganisms into the interior of whole-muscle cuts, and suggest that Beefxide and lactic acid may be similar in their efficacy as antimicrobial interventions applied during the production of non-intact beef products. Additionally, this project demonstrates the usefulness of surrogate microorganisms in validating an establishment's food safety/HACCP system and provides information that can be used by other further processors to validate their in-plant processes.

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BIOCHIP BASED IMMUNOASSAY AND ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE SCREENING OF MULTIPLE AVERMECTINS IN BEEF MUSCLE AND LIVER

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Abstract – Avermectins are macrocyclic lactone derivatives with potent anthelmintic activity. For consumer protection it is necessary to monitor the levels of these residues in food. The availability of immunoanalytical methods enabling broad recognition of avermectins to facilitate the screening of batches of samples is advantageous. This study reports the analytical performance of a biochip based immunoassay and an enzyme-linked immunosorbent assay (ELISA) for the screening of multiple avermectins in beef muscle or liver. Competitive immunoassays were employed. Biochip array technology is based on the biochip, the solid phase and the vessel where miniaturised chemiluminescent immunoassays take place. The chemiluminescent signals were detected on the Evidence Investigator analyser. For the ELISA the capture antibodies were immobilized and stabilized on the 96-well microtitre plate surface. Absorbances were read at 450nm. On the biochip platform the assay detected emamectin benzoate, eprinomectin, abamectin, ivermectin and doramectin (% cross-reactivity ranging from 75 to 254%); with the ELISA the same compounds were detected (%cross-reactivity ranging from 40 to 174%). The limit of detection in beef muscle was 0.75ppb on the biochip and 5 ppb in beef liver with the ELISA. These immunoassays represent useful analytical tools for the screening of these compounds.

I. INTRODUCTION

The avermectins are a group of chemically related compounds originally isolated from the actinomycete *Streptomyces avermitilis*. They are macrocyclic lactone derivatives with potent anthelmintic activity, but lack antibacterial or antifungal activity (1). The avermectins include the compounds ivermectin, abamectin, doramectin, emamectin, and eprinomectin. The extensive use of anthelmintic drugs in food-producing animals can cause the presence of residues in food. For consumer protection it is necessary to monitor the levels of anthelmintic residues to ensure that they remain within the legally permitted maximum acceptable concentrations. Analytical methods have been developed for the detection of avermectins in different matrices using a range of technologies including high performance liquid chromatography, liquid chromatography-mass spectrometry, liquid chromatography-tandem mass spectrometry, ELISAs (2,3,4,5). Screening methods facilitate the detection of residues in samples at border inspection points, slaughter and import houses as only positive results need to be confirmed with a confirmatory method. The availability of rapid and simple immunoanalytical methods enabling broad recognition of avermectins to facilitate the screening of batches of samples is advantageous and this study reports the analytical performance of a biochip based immunoassay and an ELISA for the screening of multiple avermectins in beef muscle and liver.

II. MATERIALS AND METHODS

Sample preparation

Biochip based immunoassay. Acetonitrile was added to 5 grams of pre-homogenised beef muscle and mixed before addition of sodium chloride and anhydrous magnesium sulphate. The sample was then mixed and centrifuged. After centrifugation, half the volume of the upper solvent layer was transferred to a clean glass test tube and evaporated at +50°C. The sample was then reconstituted with diluted wash buffer and mixed. The reconstituted samples were then diluted to provide an overall sample dilution of 1. The sample volume added to each biochip was 50µl.

ELISA. Acetonitrile was added to 5 grams of pre-homogenised beef liver and mixed before addition of sodium chloride and anhydrous

magnesium sulphate. Following shaking, hexane was added. The sample was then mixed and centrifuged. After centrifugation, 2ml of the lower acetonitrile layer was transferred to a clean glass test tube, dimethyl sulfoxide (DMSO) was added then dried down at +50^{0}C. The sample was then reconstituted with sample buffer and mixed. The reconstituted samples were then diluted 5-fold. The sample volume added to each well was 25µl.

Biochip based immunoassay

The biochip based immunoassay for the determination of avermectins is competitive. The avermectins present in the standard and/or sample compete with horseradishperoxidase labelled conjugate for specific sites in the antibodies on the biochip surface. The biochips (9mm x 9mm) were supplied in carriers (3x3 biochips per carrier); a carrier handling tray is provided with the system and allows the simultaneous handling of 6 carriers (54 biochips). 150µl of assay diluent was applied to the biochips, followed by 50µl of calibrator or sample. After incubation for 30 minutes at +25°C and 370 rpm, 100µl of conjugate was added into each biochip. After incubation for 60 minutes at +25°C and 370rpm, the biochips were washed. Signal reagent was then added and the chemiluminescent signal output generated on the biochips was then captured using digital imaging technology on the Evidence investigator analyser. The system incorporates dedicated software, which automatically processes, reports and archives data generated.

ELISA

The avermectins ELISA presented the 96-well microtitre plate precoated with the capture antibody. 25 µl assay buffer/sample buffer was applied to each well, followed by 25 µl of calibrator/sample and 75 µl of conjugate. The duration of the assay was 1 hour and 30 minutes at a temperature of +15-+25^{0}C.

The assay is a direct competitive ELISA where the avermectins present in the standard and/or sample compete with horseradishperoxidase labelled conjugate for specific sites in the antibodies coated to the plate. The colorimetric visualization of the reaction is carried out by addition of tetramethylbenzidine (TMB) as substrate reagent and the absorbance measured at 450 nm is inversely proportional to the concentration of the analyte.

Performance evaluation parameters

Specificity / Cross-reactivity. Specificity, expressed as %cross-reactivity(%CR) was calculated as follows:

% CR = [(IC50 (analyte) / IC50 (cross-reactant)] x 100. The half maximal inhibitory concentration (IC50) for each analyte and cross-reactant tested was calculated by taking 50% of the signal of the zero calibrator and reading this value from the x-axis (ppb) off the corresponding calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Limit of Detection (LOD). LOD was calculated as the mean + 3 SD from the data for a minimum of 20 negative beef muscle or liver samples. This represents the lowest concentration of each analyte that can be distinguished due to matrix effects.

Precision. For the biochip intra-assay precision was determined for each analyte by fortifying controls at 3 levels spanning the calibration range and then assaying 20 replicates of the three different levels over three runs. For the ELISA the intra-assay precision was determined at 6 levels and assaying 12 replicates. Results were expressed as %C.V.

III. RESULTS AND DISCUSSION

Performance evaluation of the biochip based immunoassay and ELISA for avermectins.

Both immunoassays were standardised to ivermectin and presented broad cross-reactivity profile (Table 1). The avermectins emamectin benzoate, eprinomectin, abamectin, ivermectin and doramectin were detected with % cross-reactivity ranging from 75 to 254% for the biochip based immunoassay and from 40% to 174% for the ELISA. This increases the screening capacity of the tests for these compounds.

Table 1. Specificity data of the biochip based immunoassay and ELISA for avermectins

Compound	Biochip % Cross-reactivity	ELISA % Cross-reactivity
Ivermectin	100	100
Emamectin benzoate	254	174
Eprinomectin	191	137
Abamectin	178	148
Doramectin	75	40

The LOD in beef muscle was 0.75ppb (calibration range 0-96 ppb) for the biochip based immunoassay. The LOD in beef liver was 5 ppb for the ELISA (calibration range: 0-10 ppb). These values are below Maximum Residue Limits (MRLs) available for different countries, as for instance 10ppb-40ppb in muscle and 70-100ppb in liver (6).

The intra-assay precision data, expressed as %C.V., showed values typically ≤12% for different concentration levels with the biochip immunoassay and <10% with the ELISA (Tables 2 and 3).

Table 2. Intra-assay precision of the biochip based immunoassay for avermectins

Compound	Coefficient of variation (%)
Level 1	9
Level 2	12
Level 3	11

Table 2. Intra-assay precision of the ELISA for avermectins

Level	Coefficient of variation (%)
Level 1	3.7
Level 2	2.6
Level 3	3.1
Level 4	3.7
Level 5	8.1
Level 6	3.1

The biochip based immunoassay was applied to the Evidence Investigator analyser and with this system fifty four biochips' including nine calibrators and up to forty five samples can be handled at a time. The time to result for forty five samples is 4 hours including sample preparation. With the ELISA forty samples can be analysed at a time and the time to result for the forty samples is 4 hours including sample preparation.

IV. CONCLUSION

In this study two immunoassays for the screening of avermectins on different platforms were evaluated with regard to their applicability to the broad recognition of these compounds in beef matrices. Both immunoassays detected multiple avermectins: abamectin, doramectin, emamectin, ivermectin and eprinomectin. The data indicates optimal analytical performance. These immunoassays represent useful analytical tools for the screening of avermectins in batches of beef muscle or liver samples and reduce the quantity of samples to be assessed by confirmatory analysis.

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CROSS-CONTAMINATION OF *AEROMONAS* SPP. FROM CHILLED PORK TO *BRASSICA CHINENSIS* UNDER DIFFERENT FOOD-HANDLING SCENARIOS

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Abstract - The purpose of this paper was to quantify the cross-contamination of *Aeromonas* spp. from chilled pork to Chinese cabbage（*Brassica chinensis* ）through cutting boards, knives and hands in the kitchen. Transferring experiments were performed to mimic the food preparation process of consumer under laboratory conditions. The pork sample was inoculated with *Aeromonas* spp. before splitting, to determine the transfer rate of *Aeromonas* spp. from chilled pork to cutting boards, knives and hands, respectively. Meanwhile, transfer rates from cutting boards, knives and hands to *B. chinensis* under various food-handling scenarios were also determined. Scenario 1(after cutting pork, cutting boards, knives and hands were also used for cutting *B. chinensis* without any cleaning) was taken to simulate cross-contamination. The results showed that each set of transfer rates varied over experiments significantly ($p<0.05$) and the analogue simulation showed that cross-contamination of foodborne pathogens from raw meats to ready-to-eat foods might bring some potential risks for consumers. Combined with cooking stage assessment and dose-response relationship, these results could provide theoretical references for complete establishment of *Aeromonas* spp. risk assessment.

I. INTRODUCTION

Food-contact surfaces are highly contaminated with foodborne pathogens. According to World Health Organization[1], 32% of foodborne outbreaks are closely associated with cross-contamination events involving deficient hygiene practices, contaminated equipment, or inadequate storage. *Aeromonas* spp. is one of the dominant spoilage bacteria in chilled pork [2], also proved to be a kind of bacteria that can cause gastroenteritis and septicemia [3]. Yet, studies on *Aeromonas* spp. is relative lack, especially in the field of cross-contamination.

The objective of our study was to assess the cross-contamination and transfer rates of *Aeromonas* spp. from contaminated chilled pork to Chinese cabbage *Brassica chinensis* through cutting boards, knives and hands to *B. chinensis* under various food-handling scenarios and take scenario 1(after cutting pork, cutting boards, knives and hands were also used for cutting *B. chinensis* without any cleaning) for example to simulate cross-contamination. We expected that our study could provide a theoretical reference for building a complete risk assessment system of *Aeromonas* spp.

II. MATERIALS AND METHODS

The test organism used in this study was

Aeromonas spp. (CICC 23564) and the food products were chilled pork and *B. chinensis*, which were purchased from local supermarket in Shanghai. P.R. China. The food contact surfaces included cutting boards and stainless steel knives and hands.

We evaluated the cross-contamination and transfer rates of *Aeromonas* spp. from contaminated chilled pork to *B. chinensis* through cutting boards, knives and hands under various food-handling scenarios. Firstly, approximately 25 g chilled pork were surface inoculated with *Aeromonas* spp. and the inoculum was spread by turning over the chilled pork pieces for several times, then the inoculated chilled pork pieces were allowed to place under a bio-hood for 10 min to let the bacteria attach to the surface. Secondly, inoculated chilled pork pieces were placed on a sterilized cutting board (5×5cm^2) and cut 10 times using a sterile knife. During this process, *Aeromonas* spp. on chilled pork was transferred to cutting board, knives and hands, respectively. Before cutting *B. chinensis*, six food-handling scenarios were considered as follows: Scenario 1, cutting board、knife and hand used to cut chilled pork were also used for cutting *B. chinensis* without any washing; Scenario 2, cutting board、knife and hand were washed with 500mL sterile water separately after cutting chilled pork; Scenario 3, after cutting chilled pork, contaminated cutting board was taken away and a new sterile one was used to cut *B. chinensis*; Scenario 4, contaminated knife was changed for a new one as scenario 3; Scenario 5, contaminated hand was washed thoroughly before cutting *B. chinensis*; and scenario 6, cutting board、knife and hand used to cut chilled pork were washed thoroughly using washing-up liquid before cutting *B. chinensis*.

The initial contamination level of chilled pork was quantified by sampling inoculated chilled pork pieces immediately after the attachment of *Aeromonas* spp. [4]. Recovering method used for quantifying bacterial transfer between surfaces is the most popular non-destructive method, namely swabbing method [5].The contamination level of *B. chinensis* was also determined by Chinese national standard [4].

The transfer rates were calculated as follows:

$$T\% = \frac{Nr}{No} * 100 \qquad (1)$$

where *T*: transfer rates; *No*: CFU on source (CFU/g or CFU/cm^2); *Nr*: CFU on destination(CFU/g or CFU/cm^2).

III. RESULTS AND DISCUSSION

Table 1 shows the mean, standard deviation and variation range of % transfer rates that occurred from chilled pork to cutting boards, knives and hands. Through the test of significance, we found that T_{MB} and T_{MH} showed no significant difference (P > 0.05), but T_{MH} and T_{MB} were significantly different to T_{MK} (P < 0.05), which meant the transfer rate from chilled pork to cutting boards and hands were much higher than to knives.

Table1 Transfer rates of *Aeromonas* spp. from chilled pork to cutting boards, knives and hands

T type	T (%)	range
T_{MB}	16.35±12.33a	1.74-36.57
T_{MK}	1.16±0.62 b	0.62-2.85
T_{MH}	12.37±9.69 a	4.01-45.70

Notes: T_x: Transfer rate The subscript x: M: Chilled pork B: Board H: Hand For example: T_{MB} stands for the transfer rate from chilled pork to board

Values in the same column that are followed by the same uppercase letter are not statistically significantly different (p>0.05).

The % transfer rates of *Aeromonas* spp. from cutting boards, knives and hands to *B. chinensis* under six different food-handling scenarios were shown in Table 2. These results showed that washing these contaminated medium in water could remove a large population of *Aeromonas* spp. and as a result lower numbers of organisms (compared to scenario 1) were transferred to the *B. chinensis*. Besides , when boards, knives and hands were washed thoroughly with vigorous mechanical scrubbing using washing-up liquid and brush, none *Aeromonas* spp. were detected on *B. chinensis* (scenario 6). Changing the new boards, knives or washing our hands thoroughly after spliting chilled pork were not ideal methods for reducing cross-contamination for the average % transfer rates to *B. chinensis* could as high as 16.50%, 25.72% , 27.07%, respectively.

Table 2 Transfer rates of *Aeromonas* spp. from cutting boards, knives and hands to *B. chinensis* under different scenarios

Different scenarios	T type	T(%)	range
1	T_{BL1}	18.59±8.80 abc	6.41-34.84
	T_{KL1}	5.30±4.62 d	1.12-13.10
	T_{HL1}	8.86±4.93 cd	3.16-16.89
2	T_{BL2}	0.38±0.21 d	0.10-0.81
	T_{KL2}	0.09±0.07 d	0.01-0.24
	T_{HL2}	0.43±0.52 d	0.12-1.79
3	T_{KHL}	16.50±10.07 bc	5.85-38.49
4	T_{BHL}	25.72±12.18 ab	11.19-46.05
5	T_{BKL}	27.07±18.61 a	11.85-69.42
6	--ND	--ND	--ND

Note：--ND : Not Detected

L: *B. chinensis* For example: T_{BL1} stands for the transfer rate from board to *B. chinensis* in scenario 1; T_{KHL} stands for the transfer rate from knife and hand to *B. chinensis*.

The initial contamination level of *Aeromonas* spp. in chilled pork was from -0.8(lg(CFU/g)) (5% confidence interval) to 7.7(lg(CFU/g)) (95% confidence interval) [6] and it was shown in fig.1.

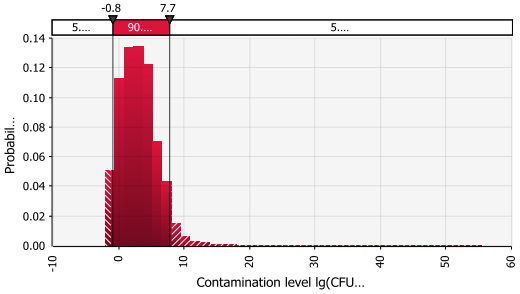

Fig.1 Initial contamination level of *Aeromonas* spp. in chilled pork

Optimal distribution of each transfer rates for scenario 1 were fitted using @Risk 5.5 (Palisade, USA). Then we used Monte Carlo sampling method to simulate the input parameters, and the final contamination level of *Aeromonas* spp. on *B. chinensis* was shown in Fig.2.

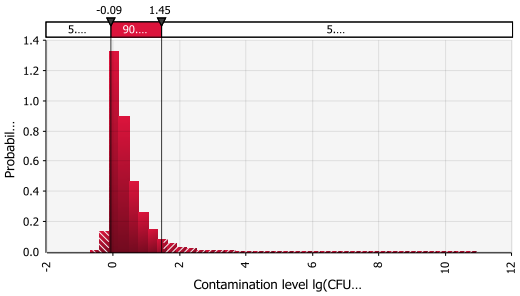

Fig.2 Contamination level of *Aeromonas* spp. on *B. chinensis*

As we can see from Fig.2, the contamination level of *Aeromonas* spp. on *B. chinensis* was from -0.08(lg(CFU/g)) (5% confidence interval) to 1.45(lg(CFU/g)) (95% confidence interval), which indicated that the *Aeromonas* spp. could be partly transformed from chilled pork to *B. chinensis* through cutting boards, knives and hands. Meanwhile, the cross-contamination between food-contact faces might resulted a high

contamination level of *Aeromonas* spp. on *B. chinensis* occasionally.

IV. CONCLUSION

The results showed that each set of transfer rates varied over experiments significantly ($p<0.05$) and the analogue simulation of cross-contamination showed that cross-contamination of foodborne pathogens from raw meats to ready-to-eat foods suggesting some potential risks for consumers. Our study also proved that proper washing of contaminated kitchen implements could remove *Aeromonas* spp. effectively.

ACKNOWLEDGEMENTS

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GROWTH OF *PSEUDOMONAS AERUGINOSA* SINGLE CELLS AND CELL POPULATIONS

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Abstract: In this article, a single cell growth image system was used to study the single cell growth of *Pseudomonas Aeruginosa*. A stochastic modelling was applied as a simulation process to connect the growth from *P. Aeruginosa* single cells to cell populations. Bacteria growth counts using different inoculum sizes were generated experimentally to validate the simulation process. Results demonstrated that the agreement between simulations and bacteria growth counts were good at both 25℃ and 35℃. This method made it possible to predict bacterial population growth with considering microbial single cells which useful for food safety control.

Ⅰ. INTRODUCTION

Traditional predictive microbiology uses deterministic models to describe the growth of large microbial populations without considering the microbial single cells. Since food contaminations caused by harmful microbes usually occur due to small amounts which consequently aggravate the effect of uncertainty and variability on the population growth of microorganisms.

Methods used to measure the growth of microbial individual cells could be traced back to the early 1930s when Kelly et al.[1] put a slice of inoculated solid agar under a microscope to study the growth rate of individual cells. For the last several decades, facilities have emerged in endlessly. Wakamoto et al.[2] suggested a method that could transfer a single bacterium from one well to another using microfluidic devices. Fritzsch et al.[3] invented an integrated microfluidic lab-on-a-chip system which allowed for isolation, contactless cultivation, and time-resolved analysis of single cells in a micro-flow. Elfwing et al.[4] designed a flow chamber to monitor the consecutive divisions of a single cell in which the cells attached to a solid surface and grew in a continuous flow environment.

Monitoring the growth of bacterial single cells plays an increasingly important role in predicting the growth of microbial populations, especially when the inoculum was small. How to establish the connection from microbial single cells to cell populations was also a fundamental issue. Inspired by the method suggested by Elfwing et al.[4], in this article, a flow chamber was improved as a whole system with time-lapse microscopic photography to study the single cell growth of *P. Aeruginosa*. Then a stochastic modeling process was applied as a simulation process to connect the growth from *P. Aeruginosa* single cells to cell populations.

Ⅱ. MATERIALS AND METHODS

One colony of *P. Aeruginosa* strain was picked from an agar plate and inoculated into a flask containing 100 mL sterile phosphate-buffer saline (PBS) (8g/L NaCl, 0.2g/L KCl, 0.24g/L KH_2PO_4, 1.44g/L Na_2HPO_4; pH 7.5). Another colony of *P. Aeruginosa* strain was picked and inoculated into a flask containing 300 mL of sterile Nutrient Broth (NB) (10g/L,

5g/L veal infusion, 10g/L NaCl; pH 7.2). This flask was shaken for 18h at 37°C until the culture reached its stationary phase.

The tubing system was first washed with 75% ethyl alcohol for at least 15 minutes and then washed by sterile PBS for another 15 minutes. 150μL of *P. Aeruginosa*-PBS suspension prepared before was inoculated onto a sterile slide surface and placed horizontally in the laminar flow cabinet for at least 30 minutes. Main parts of flow chamber were assembled orderly from "a" to "c" and tightly combined with pipe duct tape. Details were shown in Fig. 1.

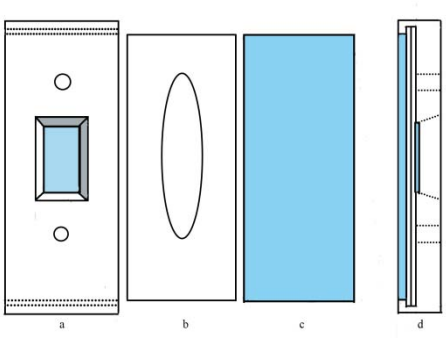


Fig.1 Main parts of flow chamber (a. Top block of flow chamber with cover slide in the middle and inlet and outlet pipes; b. polymer spacer made by qualitative filter paper; c. microscope slide; d. combination of flow chamber)

A single cell growth image system was established as showed in Fig. 2. Since *P. Aeruginosa* single cells were restrained in PBS, growth initiated when the valve changed from PBS to nutrient broth. The flow medium gave an average linear velocity of approximately 1.1cm/s. The driver (NIS-Elements 3.2) software installed in computer was used for taking pictures for every 3~5 minute intervals. Growth data of *P. Aeruginosa* single cells was expressed using pixels which acquired through image processing (Image-Pro Plus 6.0).

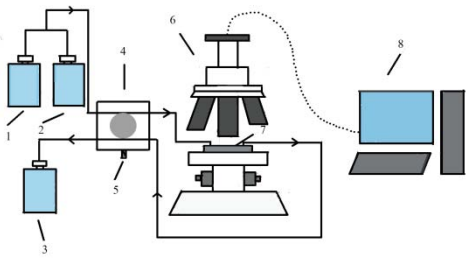


Fig.2 Single cell growth image system

(1. Nutrient broth; 2. phosphate buffer; 3. waste liquid; 4. peristaltic pump; 5. switch button; 6. microscope; 7. flow chamber; 8. computer)

The first *k*th generation times of 30 *P. Aeruginosa* single cells were recorded at both 25℃ and 35℃. Population growth simulation was carried out on the basis of binary fission multiplication and with an initial inoculum size of N_0 cells. The time for each generation was assigned accordingly to the distribution time acquired from single cell growth image system. After the *k*th division, the time for which beyond the *k*th generation was assigned by the *k*th generation time (The value of *k* was usually around 3 or 4)[5]. The iteration continued until the population reached a given number of cells (N_t), and then we could obtain a series of relevant time changing along with the multiplication of *P. Aeruginosa.* The simulation process was carried out by a created Java program and the result was dealt with by fitting the model of Gompertz as follows[6] :

$$\log N = N_0 + C * \exp\{-\exp[-B*(t-M)]\}$$

where log *N* was the decimal logarithm of

microbial counts [lg(CFU/mL)] at time t, N_0 was the asymptotic log count as time decreases indefinitely [lg(CFU/mL)], C was the log count increment as time increases indefinitely [lg(CFU/mL)], B was the relative maximum growth rate at time M (min^{-1}), M was the time required to reach the maximum growth rate (min).

Bacteria growth counts using different initial inoculum sizes [0 lg(CFU/mL), 1 lg(CFU/mL), 2 lg(CFU/mL)] were generated experimentally to validate the simulation process (Matlab®R2009b, Mathworks Company, USA).

Ⅲ. RESULTS AND DISCUSSION

Daughter cells with weaker adhesive abilities were flowed away by the shear force of nutrient broth. Pixels of a *P. Aaeruginosa* single cell were extracted from pictures obtained through the single cell growth image system. Fluctuated tendency of pixels as a function of time for a *P. Aaeruginosa* single cell were showed in Fig. 3. Each sudden drop represented an end of the cell division. Moreover, the time for each *P. Aaeruginosa* single cell began to divide was different. It suggested a stochastic growth property of *P. Aaeruginosa* single cells.

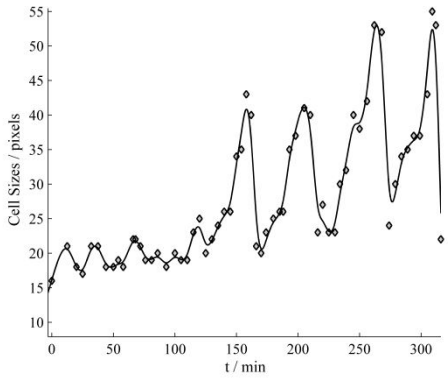
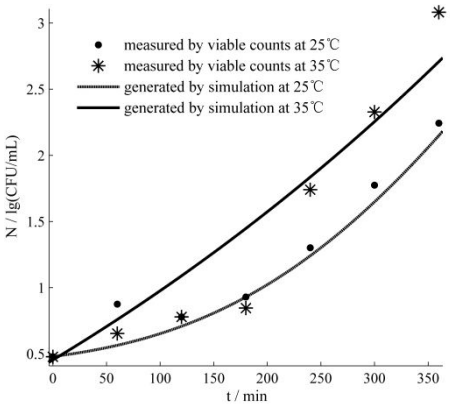
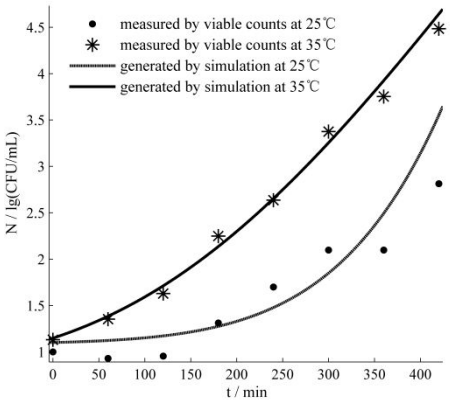


Fig.3 Changes of a *P. Aaeruginosa* single cell size

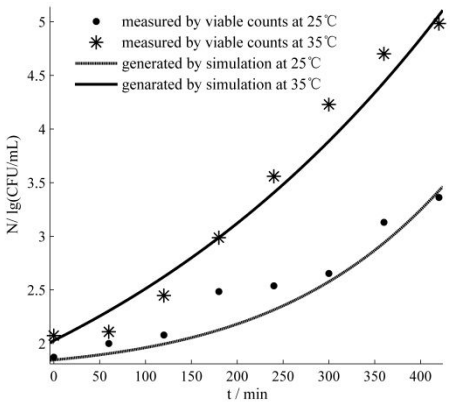
Growth of *P. Aaeruginosa* single cells in the flow chamber was obviously different from that under the optimal conditions. Because the flow liquid taking away some signaling molecules along with the daughter cells, it might weaken the effect of quorum sensing[7]. However, the results got from our experiment (Fig. 4) demonstrated an agreement result between bacterial growth counts and simulation curves at both 25℃ and 35℃. Results indicated that when the inoculum size was relatively small, the influence caused by quorum sensing might be ignored. In fact, it was more important to control food which contaminated with small harmful microorganisms instead of those already spoiled.



（A）



(B)



(C)

Fig.4 Simulation and validation of *P. Aaeruginosa* growth with different inoculum sizes (The initial inoculum size is about 0 lg(CFU/mL) (A), 1 lg(CFU/mL) (B), 2 lg(CFU/mL) (C))

Ⅳ. CONCLUSION

A stochastic growth property of *P. Aeruginosa* single cells was verified by using the single cell growth image system. The simulation process developed the relationship of the growth from *P. Aeruginosa* single cells to cell populations, and this method might provide a more accurate way to predict bacterial population growth.

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DEVELOPMENT OF A RAPID PCR METHOD TO QUANTIFY *E. COLI* (O157 AND O26) IN BOVINE FAECES

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Abstract – Bovine animals are reservoirs for a range of VTEC, including VTEC belonging to serogroups O157 and O26. Whilst shedding dynamics of the pathogens is highly variable, it is recognised that 'super-shedders' may excrete high numbers (>10,000 colony-forming units (CFU)/g) in faeces subsequently increasing the risk of VTEC contamination at farm and slaughter level. The frequency and underlying causes of super-shedding are poorly understood due to the lack of robust and sensitive enumeration methods in screening studies. This method consists of a short pre-enrichment in modified TSB followed by a spin column DNA extraction and finally detection and quantification of O157 and O26 serogroups by real-time PCR in bovine faeces as a tool for investigating shedding dynamics. Independent assays for *E. coli* O157 and O26 were developed in parallel, and resulted in the generation of calibration curves by plotting PCR Ct values against the starting concentration in the swab sample for each serogroup. A calibration curve for *E. coli* O157 with a R^2 value of 0.85 was achieved whilst for *E. coli* O26 the R^2 value was 0.87 demonstrating the developed method is effective for collection of quantitative data on *E. coli* O157 or O26 serogroups found in bovine faecal samples.

I. INTRODUCTION

Verocytotoxigenic *E. coli* (VTEC) is a zoonotic pathogen that has caused major outbreaks worldwide. Cattle are recognised as a principal reservoir for VTEC [1]. These pathogens are generally transient members of the intestinal micro-flora and only rarely do they cause disease in young, weakened calves. While cattle have been shown to harbour this pathogen on occasion in their rumen, it is found more frequently in the distal portion of the bovine gastro intestinal tract, with the rectal anal junction identified as the predominant colonisation site for O157: H7 [2, 3]. Shedding of VTEC by cattle is generally intermittent with herd members remaining negative for months with only a proportion sporadically becoming positive for a few weeks at a time [4, 5]. It has also been hypothesised that high level carriage of these microorganisms is a consequence of intestinal colonisation while low levels within individual animals may be a result of environmental exposure with no significant colonisation [6, 7]. The typical pattern of shedding in a herd is sporadic with intense periods of shedding interspersed with periods of non-shedding [8]. Ogden *et al* (2004) have also reported that concentrations of *E. coli* O157 being shed in the faeces of positive cattle were highest during summer months [9]. The number of VTEC (CFU g^{-1}) being shed in the faeces of individual animals is considered important in the context of hide, environmental and subsequent carcass contamination. The phenomenon of 'super shedding' animals (those shedding $>10^4$ CFU/g faeces) is thought to be a significant contributor in the dissemination of O157 VTEC within and between herds and within abattoirs [10-12]. However, quantitative data and the frequency of super-shedders are few relative to prevalence data, as the routine detection methods generally employed in large surveys, are designed to yield data only on presence of the pathogen and not on the numbers present. Generation of quantitative data has been hampered by a lack of enumeration methods which are robust and sensitive enough to be applied in large scale animal screening studies.

In our study we set out to develop a strategy to detect and quantify *E. coli* O157 and O26 in bovine faeces through the combination of a short enrichment period and a quantitative real time PCR, with a developed calibration curve to correlate starting concentration (CFU) in the sample with the PCR Ct value to screen and identify super-shedders of these serogroups.

II. MATERIALS AND METHODS

Independent real time assays for *E. coli* O157 and O26 were developed in parallel in pure culture. The PCR primers used for the amplification of O antigen specific genes for *E. coli* O157 and O26 were *rfbE* and *wzx* respectively as described in ISO13136:2012. In order to determine the optimal enrichment time samples spiked with different initial concentrations of *E. coli* O26 or O157 (Log_{10} 1 CFU to Log_{10} 6 CFU) were enriched in modified tryptone soya broth (mTSB) for various periods of time to assess when a differential Ct could be obtained, the enrichment times trialled were 4, 5, 6 and 8h at 41.5°C and the DNA was extracted with Qiagen Blood and Tissue Kit. The Ct value being the value that measures the linear relationship between the crossing point of fluorescence produced and the log of the input DNA start molecules. The objective was to ascertain if a correlation existed between starting concentration of the pathogen (CFU) and the PCR Ct value .To validate the assay, swabs were then taken from the bovine recto-anal junction (RAJ) in cattle presented for slaughter. These were inoculated with various levels (< log_{10} 1 CFU/g to > log_{10} 6 CFU/g) of either *E. coli* O157 (n= 81) or *E. coli* O26 (n=77). Three strains of each serogroup were used. The swabs were enriched in mTSB prior to DNA extraction. These DNA samples were used as a template in real time PCR reactions using the serogroup specific primers and probes outlined in ISO13136:2012. A calibration curve was constructed for each serogroup, plotting PCR Ct value against the starting concentration (CFU) in the swab sample.

III. RESULTS AND DISCUSSION

Cattle are recognised as a principal reservoir for VTEC [1] and Rhoades et al (2009) extensively reviewed the prevalence of VTEC in the beef chain and the faecal prevalence of *E. coli* O157 in cattle and showed it varied from 0% to 48.8% [13]. However, quantitative data are few relative to prevalence data as the routine detection methods generally employed in surveys, are designed to yield data only on presence of the pathogen and not on the numbers present. A study by [14] examined faeces and hide for concentrations of six VTEC serogroups and showed that the vast majority of samples had counts below the limit of detection of the count method and samples with detectable counts ranged from 60 to 100 CFU/cm^2 on hide and 100 to 1300 CFU g^{-1} in faeces. A UK abattoir study found that 70% of *E. coli* O157-positive animals shed <100 cfu g^{-1}of faeces but in some individual concentrations could be as high as 10^6 cfu g^{-1} of faeces [15]. These authors also showed that the 9% of the animals shedding *E. coli* O157:H7 at slaughter produced over 96% of the total O157:H7 faecal load for the group. The phenomenon of 'super shedding' animals (those shedding >10^4 CFU/g faeces) is thought to be a significant contributor in the dissemination of O157 VTEC within and between herds and within abattoirs [10-12] but there is limited information on how often this occurs in cattle, with such studies limited by lack of a robust method to rapidly screen, detect and enumerate key VTEC serogroups in bovine faeces.

Therefore, in this study we aimed to develop a method based on quantitative PCR to rapidly detect and enumerate *E. coli* O157 or O26 in bovine faeces. In the first part of the study, it was shown that following five hours enrichment in mTSB there was a direct correlation between the initial number of *E.*

coli O157 or O26 (CFU/ml) in the broth and the PCR Ct value for that sample. This demonstrated that a standard calibration (r^2 value of 0.99) could be set up relating CFU and PCR Ct value and used to calculate the number of VTEC in the tested sample. This approach based on enrichment, real time PCR and the use of a standard calibration curve, is the similar to the one used by Krämer *et al*, (2011) to detect and enumerate *Salmonella* in porcine samples. The approach has the advantage of giving a higher sensitivity than previous quantitative approaches which were based on direct application of the PCR to the sample [16, 17].

The approach was further tested using swabs taken from the bovine rectal junction that were inoculated with O157 and O26. From the results obtained it was found that each assay provided satisfactory correlations between the Ct values obtained in the PCR reactions and the starting concentration of VTEC in the swab samples. For *E. coli* O157 an R^2 value of 0.85 was achieved whilst for *E. coli* O26 the R^2 value was 0.87. These constructed calibration curves allow the estimation of the number (CFU) VTEC O26 or O157 in the sample from the PCR Ct value. This compares to an r^2 value of 0.94 reported for the enumeration of *Salmonella* in porcine samples using a similar approach (Kramer *et al*, 2011). This developed method will support large scale screening studies on the numbers of *E coli* O157 and O26 serogroups shed by bovine animals and will progress investigations on shedding dynamics of VTEC and on the frequency and causes of the super shedding phenomenon. Additionally, as the start of the assay, ie. the enrichment procedure is similar to that used in ISO13136:2012 (presence/ absence test), any samples testing positive by the developed method can then be reverted back into the ISO method for cultural isolation of the strains.

IV. CONCLUSION

The developed method provides a rapid qualitative and quantitative method for *E. coli* O157 and O26 in bovine faecal samples and will support studies on shedding of these serogroups in cattle including super shedding dynamics.

ACKNOWLEDGEMENTS

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MORPHOLOGICAL AND FUNCTIONAL CHARACTERIZATION OF *CARNOBACTERIUM MALTAROMATICUM* ISOLATED FROM VACUUM-PACKED BEEF WITH LONG SHELF LIFE

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Abstract – The aim of this study was to perform a morphological and functional characterization of a *Carnobacterium maltaromaticum* strain with a potential bioprotective effect isolated from vacuum packaged long shelf life beef. The morphological, biochemical and enzymatic profiles, the influence of different temperatures and atmospheres, and the microbial stability of fresh beef inoculated with the *C. maltaromaticum* strain were evaluated. The isolated *C. maltaromaticum* strain presented similar morphological, biochemical and enzymatic profiles as those of two reference strains (LMG 11393 and LMG 22902). The growth of *C. maltaromaticum* was slower in an atmosphere containing O_2 and CO_2. Vacuum packing is therefore suitable for this bacterium. An antimicrobial effect against *Enterobacteriaceae* was highlighted on inoculated fresh meat stored under N_2. The functional characterization of this isolate will be further pursued by a genotypic characterization to better understand its potential bioprotective effect.

I. INTRODUCTION

In order to limit chemical, enzymatic and microbial mechanisms responsible for the deterioration of meat, the use of cold chain during distribution and storage is mandatory. In practice, lower temperatures are often applied to extend the shelf life. A temperature near the freezing point of meat (~ −2 °C), associated with vacuum packaging, allows the preservation of this product up to several months (1), which makes possible the meat trade across the planet without resorting to freezing. Other the type of packaging and the storage temperature, the shelf-life of meat is directly related to its initial microbiological ecosystem (2) and its evolution.

Carnobacterium maltaromaticum is a lactic acid bacterium, and many lactic acid bacteria associated with meat are known for their bactericidal or bacteriostatic activity against other strains, species or genera of bacteria. Some *C. maltaromaticum* strains have been reported to produce class I and II bacteriocins, in addition to circular bacteriocins (3). Bacteriocin production, however, is not a pre-requisite for the biopreservative efficacy of *Carnobacterium* (4).

In this way, the presence of certain lactic acid bacteria adapted to a low temperature in fresh meat could extend the shelf life and improve the microbial stability and safety of this product. Nevertheless, undesired effects of *Carnobacterium* on food quality have been reported, e.g., the production of a malty/chocolate like aroma due to 3-methylbutanal from the catabolism of leucine (5).

The aim of the present study was to perform a morphological and functional characterization of *C. maltaromaticum* with a potential bioprotective effect isolated from vacuum packaged long shelf life beef.

II. MATERIALS AND METHODS

Sample: One strain of *C. maltaromaticum* (CFAUS2/DLC/4/E1) isolated from a vacuum packaged *longissimus dorsi*, displaying a shelf-life of 140 days, obtained from a food wholesaler located in the Walloon Region of Belgium.

Morphological, biochemical and enzymatic profiles: Macroscopic and microscopic observations, Gram staining, catalase and oxydase tests were performed. The biochemical and enzymatic profiles of the strain was evaluated using API 50CH and API ZYM galleries (bioMérieux®).

Influence of different atmospheres on growth: Minced pork meat sterilized by irradiation, used as model of sterile meat, was inoculated with a 10^5 CFU/mL suspension of *C. maltaromaticum* (1 % v/w). Eighty grams of inoculated meat were repackaged in polypropylene trays sealed with a polypropylene film (52 µm thick, oxygen permeability of 110 $cm^3/m^2 \cdot 24$ h at +23 °C and 0% RH) containing a modified atmosphere – 100 % N_2, 70 % O_2:30 % CO_2 or 30 % O_2:70 % CO_2 –, and stored up to 7 days at +4 °C, +8 °C or +12 °C. Bacterial counting was performed on PCA at +25 °C on days 0, 3 and 7.

Microbiological stability of beef inoculated with C. maltaromaticum: bovine *psoas major* samples were supplied by a food wholesaler located in the Walloon Region of Belgium 16 days after slaughter. In the lab, 3 cm thick steaks were cut and inoculated on surface with a 10^5 CFU/mL suspension of *C. maltaromaticum* (1 % v/w). They were repackaged under vacuum and stored at −1 °C during 7 days (day 7). Then, they were repackaged in polypropylene trays sealed with a polypropylene film (52 µm thick, oxygen permeability of 110 $cm^3/m^2 \cdot 24$ h at +23 °C and 0 % RH) containing a modified atmosphere – 100 % N_2 or 70 % O_2:30 % CO_2 –, and stored up to 7 days at +4 °C (day 14). Total viable count (TVC), lactic acid bacteria (LAB), *Enterobacteriaceae* (EB), *Pseudomonas* spp. (PS) and *Brochothrix thermosphacta* (BT) counts were performed on PCA (+22 °C), MRS (+22 °C), VRBG (+30 °C), CFC (+25 °C) and STAA (+22 °C), respectively.

Statistical analysis: Experimental data for each response variable was analyzed by ANOVA using the GLM procedure. Whenever a *post-hoc* test was suitable, Tukey test was performed.

III. RESULTS AND DISCUSSION

Morphological, biochemical and enzymatic profiles: The colonies of *C. maltaromaticum* presented the following characteristics: circular, convex, entire, ø < 1 mm, smooth, translucent, unpigmented and odorless. Microscopic examination revealed Gram positive bacillus shaped cells arranged in pairs. The strains were catalase and oxydase negative. The API 50 CH system showed that the *C. maltaromaticum* strain could ferment the following carbohydrates and derivates: glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, gentiobiose, D-turanose and potassium gluconate. In addition, the API ZYM test revealed the activity of the following enzymes: esterase (C4), esterase lipase (C8), valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. These profiles were similar to those of the two reference strains of *C. maltaromaticum* (LMG 11393 and LMG 22902).

Influence of different atmospheres on growth: The concentration of *C. maltaromaticum* immediately after inoculation of irradiated minced pork meat was 3.3 $\log_{10}$ CFU/g. At +4 °C a weak growth of *C. maltaromaticum* was observed. At +8 °C, only the atmosphere without oxygen (100 % N_2) allowed *C. maltaromaticum* to reach a high concentration (7.7 $\log_{10}$ CFU/g) in less than one week. At +12 °C, the 70 %-CO_2 atmosphere produced a partial bacteriostatic effect on *C. maltaromaticum*, and the 30 %-CO_2 atmosphere did not inhibit its growth. Altogether, among the studied conditions, a higher temperature (+12 °C) and an atmosphere

poor in oxygen were the optimal conditions for the growth of *C. maltaromaticum* (Figure 1). These conditions are, however, not applicable in practice.

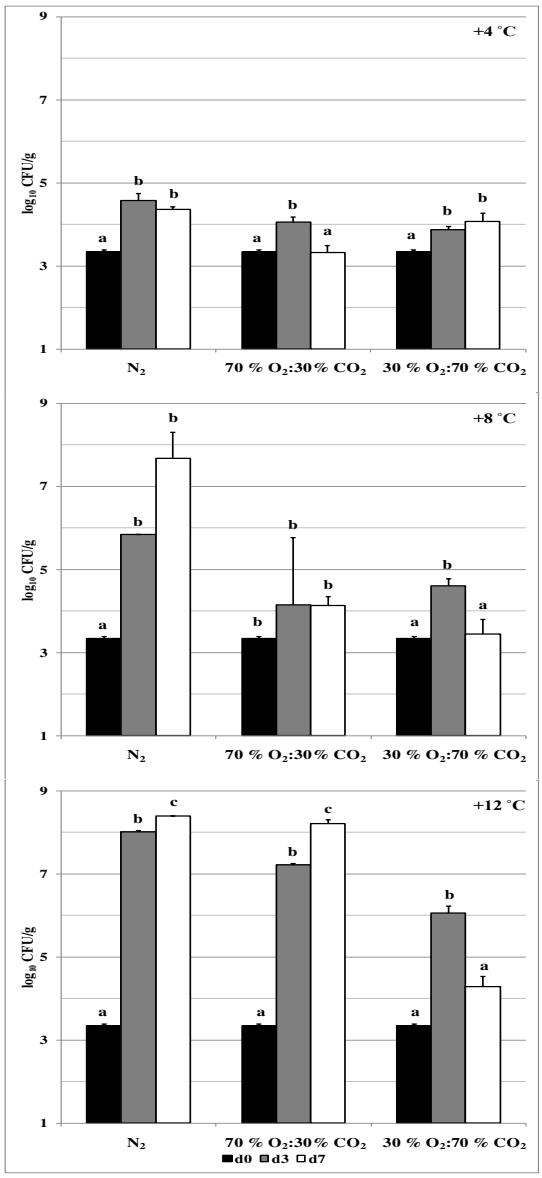

Figure 1 Growth of *Carnobacterium maltaromaticum* in sterilized minced pork meat. Different letters indicate significant differences ($P < 0.05$).

Microbiological stability of beef inoculated with C. maltaromaticum: Two different vacuum-packaged *psoas major* samples were used to evaluate the microbial stability of beef inoculated with *C. maltaromaticum* under two different modified atmospheres. An initial counting before inoculation was performed (Table 1).

Table 1 Initial microbial counts of *psoas major* samples before inoculation with *C. maltaromaticum*. Results are expressed in $\log_{10}$ CFU/cm²

	Sample 1	Sample 2
Atmosphere	100 %N_2	70 % O_2 /30 % CO_2
TVC	5.6 ± 0.0	5.7 ± 0.0
LAB	3.1 ± 0.0	3.5 ± 0.0
EB	2.5 ± 0.1	1.2 ± 0.3
PS	2.5 ± 0.1	1.3 ± 0.4
BT	2.1 ± 0.7	< 1.0

After inoculation and 7 days of storage under vacuum, no effect was observed on the total viable count and on the count of lactic acid bacteria. A reduction of *Pseudomonas* sp. and *B. thermosphacta* was observed during the first week of storage under vacuum conditions (Figures 2 and 3). *Pseudomonas* sp. counts remained lower than the counting threshold after inoculation.

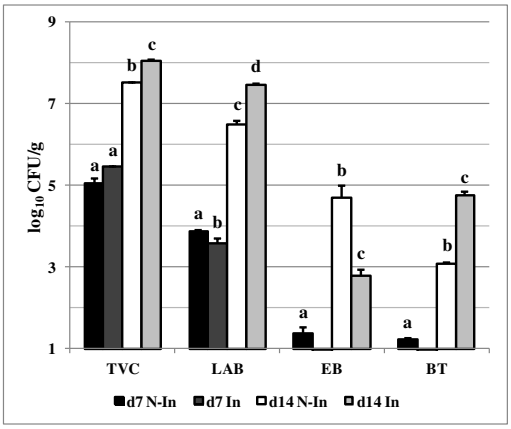

Figure 2 Microbial counts of samples after inoculation with *C. maltaromaticum* and storage under vacuum conditions at −1 °C for 7 days, and then under 100 % N_2 at +4 °C for 7 days. N-in: non inoculated, In: inoculated. Different letters indicate significant differences ($P < 0.05$).

In the samples stored under N_2, the presence of the inoculant favored the growth of *B.*

thermosphacta. On the other hand, an inhibiting effect of the inoculant on the growth of *Enterobacteriaceae* was observed.

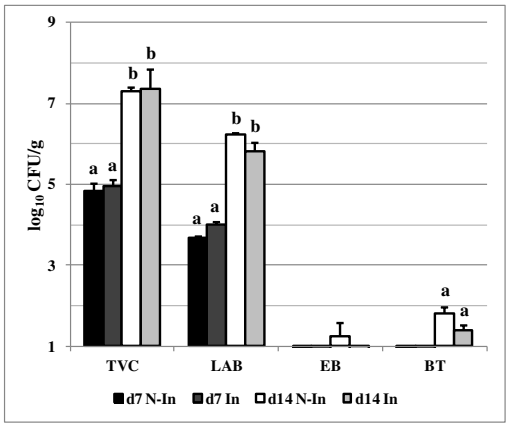

Figure 3 Microbial counting of samples after inoculation with *C. maltaromaticum* and storage under vacuum at −1 °C for 7 days, and then under 70 % O_2 / 30 % CO_2 at +4 °C for 7 days. N-in: non inoculated, In: inoculated. Different letters indicate significant differences ($P < 0.05$).

The growth of *Enterobacteriaceae* and *B. thermosphacta* was limited by the presence of CO_2. No effect of the inoculant was observed when an atmosphere 70 % O_2:30 % CO_2 was applied.

IV. CONCLUSIONS

Morphological, biochemical and enzymatic profiles of the *C. maltaromaticum* strain (CFAUS2/DLC/4/E1) isolated from vacuum packaged beef samples with extremely long shelf life were similar to those of two reference strains. The evaluation of the influence of different atmospheres showed that the growth of *C. maltaromaticum* was slower in an atmosphere containing O_2 and CO_2. Vacuum packaging and low temperatures are therefore more suitable for the growth of this bacterium. An antimicrobial effect against *Enterobacteriaceae* was highlighted on inoculated fresh meat stored under N_2.
The functional characterization of this strain will be further pursued by genotypic characterization and its potential bioprotective effect will also be studied.

ACKNOWLEDGMENTS

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CONTROL OF FOODBORNE PATHOGENS ON FRESH BEEF BY JENSENIIN G, A BACTERIOCIN PRODUCED BY *PROPIONIBACTERIUM THOENII (JENSENII)* P126

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Abstract- The combined effect of jenseniin G, a bacteriocin produced by *Propionibacterium thoenii (jensenii)* P126, with an organic acid mixture or an EDTA treatment against *Listeria monocytogenes* or *Escherichia coli* was examined on fresh beef cuts to increase inhibition spectrum of the bacteriocin. While combination of jenseniin G and the organic acid mixture reduced the number of *L. monocytogenes* attached to lean beef tissues from 4.78 to 3.49 log CFU/g, combination of 20 mM EDTA with jenseniin G did not show a significant reducing effect on the number of *E. coli* over the 3-day period.

I. INTRODUCTION

Meat and meat products are subjected to foodborne illnesses due to possible contamination with pathogenic bacteria [1,2]. Application of bacteriocins as food protection and preservation agents have prompted a promising approach to control growth of pathogenic bacteria associated with meat and meat products. The antibotulinal activity of jenseniin G, a bacteriocin produced by *Propionibacterium thoenii (jensenii)* P126; its heat and pH stability suggest its usefulness as a biological food preservative in thermally processed foods. One limitation for jenseniin G as a food preservative, as observed in other applications, is its narrow spectrum of activity. Jenseniin G is only active against related propionibacteria and some lactic acid bacteria and is sporostatic to botulinal spores [3,4].

Combination of jenseniin G with sublethal treatments that impair the barrier functions of the cell wall of Gram-positive and Gram-negative bacteria resulted in the sensitivity of organisms normally resistant to jenseniin G [5]. Similar results were obtained when sublethally injured Gram-positive and Gram-negative bacteria were treated with pediocin AcH and nisin [6].

The objective of this study was to determine whether combining jenseniin G with an acid or an EDTA treatment would increase its spectrum against two foodborne pathogens, *L. monocytogenes* and *E. coli* on fresh beef samples.

II. MATERIALS AND METHODS

Cultures and growth conditions: The jenseniin G producer *Propionibacterium thoenii* P126 and the jenseniin G-sensitive indicator *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 were grown in sodium lactate broth (NLB) and lactobacillus MRS broth, respectively, as previously described [5]. *Esherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 15313 were propagated in brain heart infusion (BHI) broth (BBL, Microbiology Systems, Cockeysville, MD) at 37˚C for 18-20 h. The identity of the strains was confirmed by Gram staining and biochemical assays. All cultures were stored in the appropriate growth medium containing 20% glycerol at -70^{0}C. Viable counts were performed according to standard methods on BHI, NLA, or MRS agar, as appropriate, and expressed in log10 CFU/ml.

Jenseniin G preparation: Partially purified preparations of jenseniin G were obtained as described by Ekinci and Barefoot [7]. Jenseniin G activity was detected by a spot-on-lawn method and quantitated by a modification of the critical dilution assay as previously described [5,7]. The assay culture was the jenseniin G-sensitive indicator, *L. delbrueckii*

subsp. *lactis* ATCC 4797. Bacteriocin titers were expressed as the reciprocal of the highest dilution exhibiting detectable inhibition and reported in activity units (AU) per milliliter [7].

Sample Preparation: Lean beef muscle tissue was obtained from a local supermarket. Meat was aseptically cut into 2.5 x 2.5 x 1.5 cm pieces, sterilized by U.V. light (2 x 15-watt germicidal bulbs, 35 cm distance from meat pieces, 60 min), stored in sterile whirlpack bags at -20^0C, and thawed at room temperature for 20-30 min before use. *L. monocytogenes* ATCC 15313 and *E. coli* ATCC 25922 were grown in BHI broth for 18 h at 37^0C and diluted to 10^3 to 10^4 CFU/ml with 0.1% peptone. Individual pieces of meat were inoculated by submersion in 10 ml of solution containing approximately 10^3 to 10^4 CFU/ml of each culture for 15 min at 25^0C and mixed manually at 5 min intervals. Each piece of inoculated tissue was submerged into 10 ml of sterile test solution for 30 min at 25^0C, attached to sterile clips, hung in a covered, sterile beaker, and held at 4^0C. Solutions were: a) control (0.1% peptone); b) organic acids (40% lactic acid, 16% propionic acid, and 16% acetic acid, pH 5.5) for *L. monocytogenes* (ACID) and 20 mM EDTA in 0.1% peptone for *E. coli* c) jenseniin G (JG) (10000 AU/ml); and d) Jenseniin G (10000 AU/ml) and mixture of organic acids for *L. monocytogenes* and jenseniin G (10000 AU/ml) and 20 mM of EDTA in 0.1% peptone for *E. coli*. Randomly selected pieces of beef tissue (each weighing approximately 7 g) were blended in a stomacher blender with 63 ml of 0.1% peptone for analysis at days 0, 1, and 3.

III. RESULTS AND DISCUSSION

Activity of jenseniin G in the presence of 20 mM EDTA against *E. coli* inoculated on the surface of beef cuts was tested during 3 days storage at 4°C. Although EDTA+jenseniin G combination reduced *E. coli* viable counts as compared to the other groups and to the initial count on the first day of storage ($p<0.05$), this effect was not apparent on day 3 (Fig. 1). This very limited activity of EDTA+jenseniin G combination in the present study might be due to relatively low concentration of the bacteriocin used for application on the beef surface. The storage period in the current study was also limited given the 3 day period. Further studies are needed to determine the effect of the higher concentrations of the bacteriocin jenseniin G on meat surfaces.

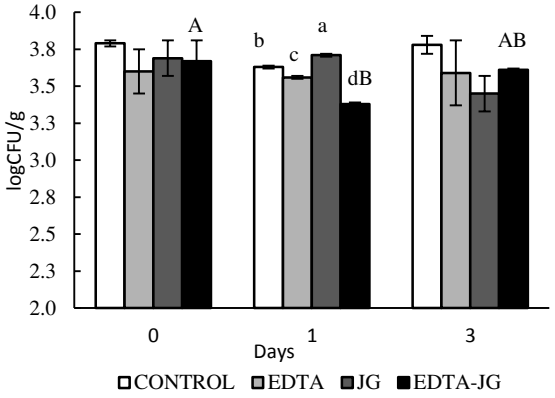

Fig. 1. *E. coli* counts (log CFU/g) of beef cuts during 3 days storage at 4°C. Bars represent the mean value ± standard error. (a-c): Within a day between sample groups, bars having common letters are not statistically different ($p>0.05$). (A, B): Within a sample group between storage days, bars having common letters are not statistically different ($p>0.05$).

Combination of lactic acid and acetic acids were selected for preservation treatments because they are generally recognized as safe (GRAS) food additives [8]. Combinations of lactic and acetic acids can be used for increased microbial inhibition and shelf-life extension [9,10]. Treatments based on organic acids are widely used in decontamination of carcasses [11]. In a previous study by Baker et al. [5], in vitro treatments of *L. monocytogenes* with jenseniin G and organic acid mixture caused 4.5 log reductions in the population of *L. monocytogenes* after seven days storage at 4˚C. In this study the effect of jenseniin G and an organic acid mixture against *L. monocytogenes* ATCC 15313 attached to lean beef tissue was examined at 4°C for up to 3 days (Fig. 2). While jenseniin G and the organic acid mixture alone were not effective on *L. monocytogenes*, the combination of them reduced the number of *L. monocytogenes* over the 3 day period from 4.78 to 3.49 CFU/g.

There could be many reasons for the inability of this treatment to reduce as much populations of *L. monocytogenes* attached to meat compared to in vitro treatments [5].

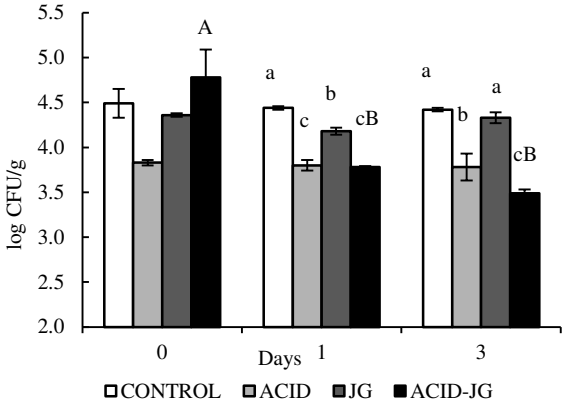

Fig. 2. *L. monocytogenes* counts (log CFU/g) of beef cuts during 3 days storage at 4°C. Bars represent the mean value ± standard error. (a-c): Within a day between sample groups, bars having common letters are not statistically different (p>0.05). (A, B): Within a sample group between storage days, bars having common letters are not statistically different (p>0.05).

Bacteriocins may be degraded by endogenous proteases associated with meat or may bind to adipose [12]. Concentration of bacteriocin on meat surface is also important. However, the amount of jenseniin G on the meat surface was not determined in this study after the treatment or storage for 3 days.

IV. CONCLUSIONS

An acid mixture or EDTA was used to increase antimicrobial activity of jenseniin G against *L. monocytogenes* or *E. coli* on fresh beef cuts, respectively. A slight effect of EDTA + jenseniin G combination against *E. coli* and a comparatively greater, but still not significant, effect of acid mix+jenseniin G against *L. monocytogenes*, was determined. Even though the enhanced antimicrobial activities of jenseniin G *in-vitro* has been reported in previous studies, the same effect, when applied on the fresh beef surface, was not observed in the current study. Meat has a very complex food matrix. In most cases, although activity of an antimicrobial agent was observed during in vitro studies, this activity might be decreased in real food applications, likely due to characteristics of the product and the possible interaction between the active agent and food components. There is a need for further investigation of higher concentrations of jenseniin G against foodborne pathogens in meats over longer storage periods.

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PRESENCE OF *ESCHERICHIA COLI* O157:H7 IN FECES OF GRAZING LAMBS READY FOR SLAUGHTER IN URUGUAY

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Abstract – *Escherichia coli* O157:H7 is an important human foodborne pathogen being cattle the main reservoir. Recent studies have indicated that sheep are also potential carriers. The aim of this study was to detect the presence of *E. coli* O157:H7 and associated virulence factors in feces of grazing lambs intended for slaughter in Uruguay. Individual fecal samples were collected from grazing lambs (n=220) in four research units of the National Institute of Agricultural Research (INIA) and sent refrigerated to the microbiology unit of the Technological Laboratory of Uruguay (LATU). Samples were analyzed for the presence of *E. coli* O157:H7 and genes encoding virulence factors (*eae*, stx_1, stx_2) following standard procedures. Samples positives for *E. coli* O157:H7 were identified in two of the four research units participating in the study. Overall, 9 of 220 (4.1%) fecal samples were *E. coli* O157:H7-positive. All isolates carried genes encoding intimin and Shiga toxins 1 and 2. This study revealed the presence of pathogenic *E. coli* O157:H7 in the feces of grazing lambs ready for slaughter in Uruguay. It suggests that feces can be a source of contamination of lamb carcasses with *E. coli* O157:H7.

I. INTRODUCTION

Escherichia coli O157:H7 is an important human foodborne pathogen that can cause syndromes such as bloody diarrhea, vomiting, hemolytic uremic syndrome, and in some cases, death [1]. *E. coli* O157:H7 strains commonly carry virulence factors such as Shiga toxins (encoded by stx_1 and stx_2 genes) and factors for attachment to the host mucosa, including intimin (encoded by the *eae* gene) [2]. Early studies have indicated that cattle represent the main reservoir of *E. coli* O157:H7 [3, 4]; however, recently sheep have been proposed as carriers and sources for human infection on a number of occasions [5, 6].

Due to the lack of competitiveness of extensive sheep production systems, traditionally oriented to wool production, high quality meat derived from lambs has gained in importance among sheep producers in Uruguay. This product is characterized by a young animal at slaughter (milk teeth female or male lamb), with a minimum of 34 kg of live weight and 3.5 value in the corporal condition scale from 1 to 5 [7]. In the last agricultural year (July 2012-June 2013) Uruguay exported 25.000 tons of mutton and lamb meat, which ranks it only behind Australia and New Zealand globally [8]. The aim of this study was to detect the presence of *E. coli* O157:H7 and associated virulence factors in feces of grazing lambs intended for slaughter in Uruguay.

II. MATERIALS AND METHODS

Four Research Units (RU) of the National Institute of Agricultural Research (INIA) finishing lambs on pastures were visited once between July 2012 and June 2013. In three RU sixty lambs (30-40 kg live weight) were chosen at random for sampling while in the remaining RU forty lambs were sampled (n=220). Fecal samples were collected rectally from each animal, placed in sterile bags, and transported refrigerated to the microbiology unit of the Technological Laboratory of Uruguay (LATU).

Fecal samples pools (25 g) were created by combining 5 g of individual samples from 5 lambs within each RU (n=44). Pooled samples were enriched, incubated and processed through the polymerase chain reaction (PCR) BAX System assay (Q7 Qualicon Dupont). Pools with positive results were open and individual samples integrating the pool were subjected to immunomagnetic separation (Dynal) and plated onto CHROMagar 157. Plates were incubated overnight (37ºC) and typical mauve colored *E. coli* O157 colonies were picked up and tested for agglutination with *E.coli* O157:H7 latex agglutination test reagents (Oxoid) followed by biochemical confirmatory tests. Finally, confirmed *E. coli*

O157:H7 isolates were tested by PCR using specific primers for the presence of genes encoding toxins (stx1, stx2) and adherence factors (intimin).

III. RESULTS AND DISCUSSION

Lamb fecal samples from a total of four research units of INIA located in the East (n=2), North and South-West of Uruguay were analyzed for the presence of *E. coli* O157:H7. Samples positives for *E. coli* O157:H7 were identified in two of the four research units participating in the study. Overall, 9 of 220 (4.1%) fecal samples were *E. coli* O157:H7-positive. Previous studies in different countries have reported prevalence estimates in sheep between 0.2% and 8.7% [9, 10]. The sample size in the present study was inadequate to definitively estimate the prevalence of the pathogen at the national level in the lamb population. While cattle are generally regarded as the main reservoir of *E. coli* O157:H7 infection, the results of this study indicate that lambs may also be a significant contributing source. Most farmers that keep lambs also have cattle which may contribute to the survival and recycling of *E. coli* O157:H7 within grazing herds [5].

Different virulence genes, such as stx_1 and stx_2 and their variants which encode Shiga toxins, and *eae* which encodes the bacterial outer-membrane protein intimin, have been targeted to assess the presence of pathogenic *E. coli* O157:H7. In the present study, all confirmed isolates (n=9) carried genes encoding intimin and both Shiga toxins. Particularly important was the presence of stx_2 which appears to be more cytopathic than stx_1 in animal and in vitro models and is more frequently associated with severe forms of human morbidity [11].

IV. CONCLUSION

This study revealed the presence of pathogenic *E. coli* O157:H7 in the feces of grazing lambs ready for slaughter in Uruguay. It suggests that feces can be a source of contamination of lamb carcasses with *E. coli* O157:H7. By improving the understanding of the on-farm epidemiology of *E. coli* O157:H7, methods of prevention and/or control at the pre-slaughter/harvest level can be identified in order to increase the safety and competitiveness of the Uruguayan red meat chain.

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THE USE OF LACTIC ACID AND OZONE FOR THE REDUCTION OF THE BACTERIAL COUNT IN OVINE CARCASS SURFACES

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Abstract – The effect of ozone in low concentrations (2.4 mg × m^{-3} × h^{-1}) and/or a warm lactic acid solutions (50mL/carcass, 3% m/m, 45 °C) over the Total Plate Count (TPC) and Enterobacteria count (EB) in 60 ovine carcass surfaces after 24 hours of post sacrifice maturation was studied. The TPC was not affected by any treatment. On the other hand, only the carcasses treated with ozone showed a reduction in the EB count (-1.08 log ufc / cm^2; p≤0.05).

I. INTRODUCTION

A wide variety of antimicrobial treatments have been tested in order to reduce the microbial count in bovine and ovine carcass surfaces (1). Internationally, organic acids, such as lactic acid, and hot water or steam are the most common. In Uruguay, the use of lactic acid has been recently introduced due to EU regulation N° 101/2013 (2). At the end of the slaughter line the carcass is washed with tap water, and even though this is not meant to improve the microbial count, it contributes to wash out the bacteria and to prevent meat superficial color deterioration and dry out (1,3). Another USDA authorized compound is ozone, which is an effective pathogen reducer either in aqueous solution or as a gas (4). It is widely used in the food industry because it does not leave any toxic residues, making it versatile and compatible with HACCP programs. As a result, it has been used in slaughter plants as disinfectant for facilities and equipment and in other industries, such as vegetable and fish, for microbial reduction during processing as well (5). Two of these studies were carried out locally by our group (6, 7). No references were found regarding the use of lactic acid in the slaughter line of bovines or ovines. The purpose of this paper was to evaluate the effect of the use of lactic acid in the slaughter line as well as ozone exposure during post sacrifice maturation.

II. MATERIALS AND METHODS

The study was carried on in a local slaughterhouse (FRICASA). The sources of variation in the initial microbial count in carcass surfaces are several, from operational practices (of the slaughter line) and personal hygiene habits, to original microbial charge of the fleece (depending on the establishment of origin and the transport), being all of them of great incidence. For this paper it was chosen to cancel as much of these variation factors as possible, because any of them have influence in the effectiveness of the treatments, only in the initial microbial counts. In order to this, sixty ovine carcasses from a lot (single producer, single day) were used (Corriedale ewes, 17.26 ± 0.51 kg). Carcasses and treatments: 1-15: AL 3%; 16-30: ozone; 31-45: AL3%+ozone; 46-60: blank. In order to emphasize the effect of the treatments used, no routine washing of the carcasses was performed at the end of the slaughter line. This was a conservative approach, because if the treatments turned out to be effective, they would be much more in normal operational conditions. And besides, it was conducted this way for two purposes: to avoid the possibility of getting final (post-treatment) counts of zero ufc/cm^2 (that cannot be included in statistical analysis), and to protect the confidentiality of the microbiological baseline of the slaughter process of the exporter establishment. For the ozone exposure studies, the ozone-production equipment was installed inside one of the chillers (LER S.A., 2.4 mg × m^{-3} × h^{-1}). For the blank and the carcass treated only with lactic acid a different chiller was used. But for what it counts for this study, the parameters of maturation of both chillers were the same (dimensions, chilling equipment performance, and number of carcasses inside). For lactic acid applications, a backpack fumigation unit with a solution of food grade lactic acid in

warm water was employed (50mL/carcass, 3% m/m, 45 °C). The 60 carcasses were subjected to microbiological analysis before the treatments and after chilling for 24 hours. For each assay, four delimited zones (80 cm^2 total) were sampled in each case, two by side, forequarters and hindquarters. The sample collection was made using a sponge (non-destructive). The cell recovery was in peptone water and the growth in 3M ® petrifilms for TPC (ISO 4833) and for EB (ISO 2158-2) during 24 hours at 37 °C. Two sets of data were obtained for TPC and EB. Each set made of the counts before the treatments and after chilling for 24 hours. For the statistical analysis, viable cell count reduction (R) was selected as a variable, expressed in $\log_{10}$ base (being ufc colony forming units):

$$R = \log_{10}[\text{initial}(\text{ufc} / \text{cm}^2)] - \log_{10}[\text{final}(\text{ufc} / \text{cm}^2)],$$

A variance analysis was performed for the reduction in each case, and the variance homogeneity was tested.

III. RESULTS AND DISCUSSION

The TPC was not affected by any treatment ($p > 0.10$). The figure 1 shows the initial and final counts for the lactic acid treatment. On the opposite of what it was expected, there was an increment in the microbial counts for most cases. Considering that the concentration and temperature used are within the limits of the cited reference (2), this could be due to the application method of the acid, more volume of solution per carcass or an arch with pressurized nozzles should be tested in order to improve the treatment.

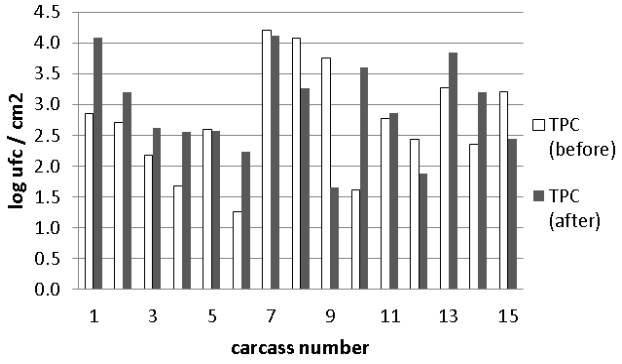

Fig. 1. Variation in the Total Plate Count for the 3% lactic acid treatment, before and after the 24 hour maturation.

The table 1 shows the effect of both treatment, and their interaction, in the TPC. As it can be seen the R turned out to be negative (the counts increased after the treatments and maturation), although the R values of the ozone treatments were less negative than the lactic acid ones. A Tukey (5%) comparison was made, and no significant difference was detected. These results could be due to variability [$\mu = 2.8 \pm 0.74$ $\log(\text{ufc/cm}^2)$], and high values [up to 4.2 $\log(\text{ufc/cm}^2)$] of the initial counts of the TPC, which is expectable for a microbiological indicator and for a superficial sampling, in which different (adjacent) places are sponged every time, and also could be due to the shield effect of such high concentration of microorganisms per superficial unit, which interferes with the reaching of the gas and with the contact of the acid solution. Both of these last effects could be eliminated by reducing the initial count by means of the routine washing of the carcasses on the line, as discussed in the methodology.

Table 1. Effect of both treatments in the TPC reduction.

Lactic	Ozone	Reduction log(ufc/cm2)	(± St.dev)	Tukey 5%
0%	No	-1.35	0.49	a
0%	Yes	-0.79	0.49	a
3%	No	-0.21	0.49	a
3%	Yes	-1.17	0.49	a

Different letters indicate significant difference, with $p \leq 0.05$.

The EB was affected only by the ozone treatment. The carcasses treated with ozone showed a reduction of 1.08 units ($p \leq 0.05$). Table 2 presents the reduction comparison for the ozone treated group and the blank.

Table 2. Effect of the ozone treatment in the EB reduction.

Treatment	Reduction ± st.dev. log(ufc/cm2)
Blank	0.20 (± 0.18) a
Ozone	1.08 (± 0.18) b

a,b: $p \leq 0.05$.

The results of Table 2 show that the use of ozone during the post sacrifice chilling of the carcasses reduces the microbial count. This is in agreement with international (8, 9) and national literature (6, 7).

The variations in the EB count for both treatments (ozone and blank) are shown in Figures 2 and 3. Note that the initial counts are variable, but not as variable nor as high as the TPC discussed before. This is expected because EB count is not an indicator, and is normal to find it in lower counts. In this study, this was an advantage.

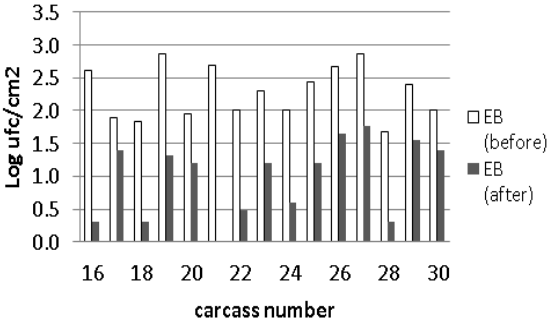

Fig. 2. Variation in the enterobacteria count for the ozone treatment, before and after the 24 hour exposure.

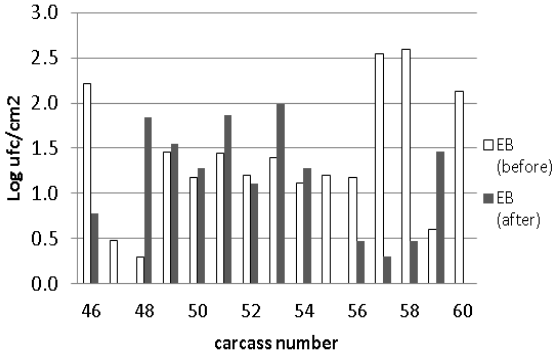

Fig. 3. Variation in the enterobacteria count for the blank, before and after the 24 hour maturation.

Nevertheless, considering that the variable selected for the analysis is a subtraction, the effect of the variation in the initial count is cancelled, and the result of the treatment is evidenced. After the 24 hour exposure period, the reductions reached levels as high as 2.5 log (ufc/cm^2). This is a very promising result, and demands further investigation to improve the ozone treatment parameters inside the chiller, either during the maturation process or afterwards.

On the other hand, more studies should be done regarding the use of lactic acid. Different application methodologies should be tested. And real process conditions, such as washing routines of the carcasses, should be accounted in the experimental designs for both, ozone and lactic acid, treatments.

IV. CONCLUSION

The anti-microbial effect of the ozone treatment is promising. However, the effect on the quality of the meat products has not been studied locally yet as it has been internationally (5). Clearly, it is necessary to explore the use of different alternatives besides ozone, such as ozonized water (10), and to compare with other sanitization products, such as hot water and lactic acid in different temperatures and concentration combinations. The possible effects on different quality parameters and consumer preferences should also be investigated.

This paper shows that ozone could be an option as a disinfectant when it comes to slaughter lines and carcass maturation processes, and it could be a mayor improvement in the meat export industry. The reduction of the superficial microbial count at a previous step to the deboning and packing process, or even before the introduction of the fresh meat to the local market, could mean an extension in the shelf life of meat products.

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EFFECT 0F CHITOSAN, FRESH GARLIC AND PEDIOCIN PA-1 PRODUCER (*Pediococcus pentosaceus* TISTR 536) ON *Staphylococcus aureus* IN NHAM (THAI FERMENTED MEAT) MODEL BROTH

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Abstract – The study informs the beneficial effect of 5% of fresh garlic on the recovered of pediocin PA-1 producer (*Pediococcus pentosaceus* TISTR 536) as starter in the high concentration of Chitosan (CS, 5000 ppm) during Nham model broth (NMB) fermentation. Besides, the effect of CS (5000 ppm) combined with 5 % fresh garlic and *P. pentosaceus* TISTR 536 on *S. aureus* were also determined in NMB, it was found that these 3 ingredients in NMB exhibited an interactive effect on *S. aureus* and could diminish this pathogenic microorganism in NMB within 42 h. The results implied that CS and pediocin PA-1 producer can be used in Nham production in order to produce a safety of traditional Thai fermented meat product.

I. INTRODUCTION

Chitosan (CS) has been reported to be possessed as various functional properties such as intestinal lipid binding and serum cholesterol lowering effects [1, 2], water binding [3], antioxidative and preservative effects in muscle foods [4] and emulsifying capacity [5]. Chitosan has also been informed the inhibitory effect on various gram negative and gram positive bacteria including Salmonellae and *Staphylococcus aureus* [7, 8]. The main objective of our earlier study was to investigate whether CS (concentrations of 100, 500 and 1000 ppm) has antimicrobial effect on some pathogens (*Salmonella* Anatum, *Salm.* Derby and *Staphylococcus aureus*) which are associated with Nham (a popular fermented sausage in Thailand, mainly composed of lean pork, sliced cooked pork rind, cooked rice, garlic and salts) during fermentation. The results from our earlier study informed that higher concentration of chitosan (500 and 1000 ppm) in Nham model broth (NMB) exhibited higher inhibitory effect on all pathogens than NMB with 100 ppm of chitosan [8]. The study for effect of CS on some lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, which mostly associated in various traditional Thai fermented meat products [9], and *Pediococcus pentosaceus* TISTR 536, which is pediocin PA-1 producing strain isolated from Nham [10] by using spot-on-lawn method and Nham Model Broth (NMB) has revealed that, between two studied LAB strains, *P. pentosaceus* TISTR 536 exhibited a higher resistance to Chitosan than *L. plantarum* ATCC 14917 [11]. Hence, this study is to further report the interactive effect among chitosan (5,000 ppm), fresh garlic (5%) and pediocin PA-1 starter cultures (10^6 cfu/ml) on *S. aureus* in Nham model broth (NMB).

II. MATERIALS AND METHODS

Microorganisms : *Pediococcus pentosaceus* TISTR 536, which is pediocin PA-1 producing strain isolated from Nham [10], was used as starter culture for Nham model broth (NMB). *Staphylococcus aureus* ATCC 12600, a food pathogenic bacterial strain, was used for this study.

Medium :
Nham model broth (NMB) : NMB without nitrite, which simulated the conditions of Nham production (a_w. 0.970, pH 6.3, micro-aerophilic condition with paraffin oil) [12], was used as a model instead of Nham product. *P. pentosaceus* TISTR 536 starter culture at a level of 10^6 cfu/ml and 5 % fresh sterilized garlic, was used for the study of their inhibitory effect on *S. aureus* ATCC 12600 at the level of 10^6 cfu/ml in NMB. The samples of each studied condition in NMB were left to ferment at 30°C for 2 days after *P. pentosaceus* TISTR 536 and *S. aureus* ATCC 12600 inoculation. The pH and percentage of lactic acid were investigated in NMB every 6 h, the growth of *P. pentosaceus* TISTR 536

and *S. aureus* ATCC 12600 in NMB were determined every 12 h [12].

MRS broth : medium modified was used as cultivation medium for *Pediococcus pentosaceus* TISTR 536. Pure cultures of *P. pentosaceus* TISTR 536 was transferred to MRS broth and incubated at 30 ^{o}C for 24 h.

Trypticase soy broth (TSB) : the medium was used as a cultivation medium for *Staphylococcus aureus* ATCC 12600. Pure cultures from trypticase soy agar (TSA) slant was transferred to TSB and incubated at 37 ^{o}C for 24 hours.

Preparation of fresh sterile garlic for NMB : Fresh sterile garlic was prepared by the method recommended by Swetwiwathana et al. [12] and contained in NMB for 5 % (w/v).

III. RESULTS AND DISCUSSION

The study of the interactive effect of CS salts (5,000 ppm sodium), 5 % fresh garlic and LAB starter cultures (*P. pentosaceus* TISTR 536 at 10^6 cfu/ml) on 10^6 cfu/ml of *S. aureus* ATCC 12600 was performed. The results (Fig. 1) showed that *S. aureus* could grow in NMB and gradually grow from 10^6 cfu/ml up to 10^8 cfu/ml after 48 h of incubation. Five percent fresh garlic could retard the growth of *S. aureus* at the early stage of NMB fermentation, while *P. pentosaceus* TISTR 536 could also retard the growth of this pathogen and reduce the cell number of *S. aureus* after 24 h of NMB fermentation due to the higher amount of lactic acid produced by *P. pentosaceus* TISTR 536 which led the pH of NMB decreased from 6.15 to 4.60 after 24 h of fermentation (Table 1). Among the 3 studied factors, NMB with 5,000 ppm CS showed the best inhibitory effect on *S. aureus.* Chitosan could gradually diminish all cells of *S. aureus* in NMB after 48 h of NMB fermentation. Additionally, CS with *P. pentosaceus* TISTR 536 as starter in NMB and CS, fresh garlic and *P. pentosaceus* TISTR 536 as starter showed the best results in diminishment of *S. aureus* cells from NMB after 42 h of NMB fermentation.

This can be explained because CS alone itself can exhibit an inhibitory effect on *S. aureus* as reported by many researchers [7, 8]. The use of CS together with *P. pentosaceus* TISTR 536 as starter culture and fresh garlic, *P. pentosaceus* TISTR 536 can grow and produce higher lactic acid during fermentation (data not shown) which led to the reduction of pH in NMB (Table 1). With the reduction of pH in NMB, CS exhibited a higher inhibitory effect on *S. aureus,* even more than the higher pH [13]. Besides, by the report of *P. pentosaceus* TISTR 536 which is known as pediocin PA-1 producing strain [10] and fresh garlic which contained allicin and exhibited an effect on *S. aureus* [14], the best inhibitory effect on *S. aureus* had been exhibited when these 3 studied factors were combined in NMB when compared to NMB with CS alone.

IV. CONCLUSION

The results from this study revealed the inhibitory effect of Chitosan (CS), fresh garlic and pediocin PA-1 producer (*P. pentosaceus* TISTR 536) as starter culture on *S. aureus* in Nham model broth (NMB) fermentation. Thus CS and pediocin PA-1 producer can be used in Nham production in order to produce a safety of traditional Thai fermented meat product.

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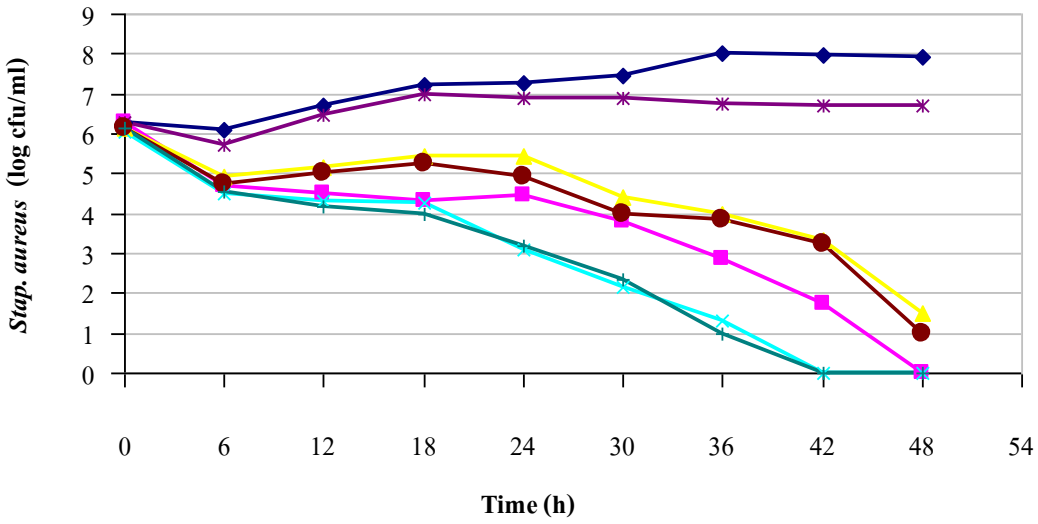

Figure 1 : Effect of chitosan (CS) 5000 ppm , *P. pentosaceus* TISTR 536 (10^6 cfu/ml) and 5% fresh garlic (w/v) on *S. aureus* (10^6 cfu/ml) in NMB : *S. aureus* ( ), *S. aureus*+ CS ( ), *S. aureus* + *P. pentosaceus* ( ), *S. aureus* + *P. pentosaceus* + CS ( ), *S. aureus*+ Garlic ( ), *S. aureus* + *P. pentosaceus* + Garlic ( ) and *S. aureus* + *P. pentosaceus* + Garlic+ CS ( )

Table 1. Effect of chitosan (CS) 5000 ppm , 5 % fresh garlic, *P. pentosaceus* TISTR 536 (10^6 cfu/ml) and *S. aureus* (10^6 cfu/ml) on the change of pH and percentage of lactic acid during 48 h of NMB fermentation.at room temperature (30 ^{o}C)

sample	pH					Lactic acid (%)				
	Incubation period (h)					Incubation period (h)				
	0	12	24	36	48	0	12	24	36	48
Pp	6.15	5.29	4.49	4.02	3.91	0.23	0.39	0.50	0.60	0.64
PpCS	6.10	6.07	5.17	4.85	4.51	0.26	0.29	0.45	0.54	0.58
PpSt	6.15	4.84	4.60	4.38	4.00	0.23	0.42	0.51	0.52	0.60
PpStCS	6.14	6.00	5.53	5.50	4.88	0.26	0.30	0.32	0.32	0.50
PpStG	6.10	4.80	4.50	4.32	3.98	0.23	0.44	0.56	0.61	0.65
PpStGCS	6.15	5.98	5.01	4.81	4.45	0.23	0.32	0.35	0.42	0.53

Pp - NMB with *P. pentosaceus* TISTR 536
PpCS - NMB with *P. pentosaceus* TISTR 536 and CS 5000 ppm
PpSt - NMB with *P. pentosaceus* TISTR 536 and *S. aureus*
PpStCS - NMB with *P. pentosaceus* TISTR 536, *S. aureus* and CS 5000 ppm
PpStG - NMB with *P. pentosaceus* TISTR 536 , *S. aureus* and 5 % fresh garlic
PpStGCS - NMB with *P. pentosaceus* TISTR 536 , *S. aureus*, 5 % fresh garlic and CS 5000 ppm

DECONTAMINATION INTERVENTIONS OF EDIBLE BYPRODUCTS FROM SHEEP

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Abstract - Norwegian abattoirs have introduced different decontamination interventions for edible byproducts from sheep used for dry fermented sausages. The aim of the study was to investigate the effect of heat treatments of both steam and hot water pasteurisation, and also the effect of cold water bath after the heat treatment. Included in the study were 126 hearts and 60 diaphragms from sheep. Both steam and hot water pasteurisation lasted for 40 s ± 3 s. The products were sampled at three sites in two commercial abattoirs; directly from the slaughter line before heat treatment, after heat treatment, and subsequently from cold water bath. The products were analyzed as pooled samples. The results showed that there was a small reduction in *Escherichia coli, Enterobacteriaceae* and aerobic plate count (APC) after decontamination treatment with steam on hearts and hot water pasteurisation on diaphragms, but the differences were not significant. The number of pooled samples was small and a new study with a larger number of samples is planned.

I. INTRODUCTION

In Norway, edible byproducts from sheep such as hearts and diaphragms have been widely used in dry fermented sausages. In 2006, an E. coli O103:H25 outbreak occurred in Norway. Dry fermented mutton sausages were found to be the vehicle of infection. One child died and 17 children became ill, most of them with symptoms of haemolytic-uraemic syndrome (HUS). After this outbreak, the meat industry has been cautious with using sheep byproducts in dry fermented sausages. Dry fermented products are normally not heat-treated before consumption, and thus might involve a larger risk for food poisoning. During the last few years some abattoirs have introduced decontamination interventions of the sheep byproducts after they are separated from the carcasses on the slaughter line. Both steam and hot water are used for decontamination before freezing of the products. The effect of the decontamination interventions has not been investigated and published in Norway and we have not been able to find international studies on edible byproducts and offal from sheep. The aim of this study was to investigate the effect of steam and hot water pasteurisation of hearts and diaphragms from sheep and subsequent cold water bath.

II. MATERIALS AND METHODS

The study was performed in two commercial abattoirs in October 2013. The abattoirs used different slaughter techniques. In abattoir A, the carcasses were suspended by the forelegs during evisceration, and rodding of oesophagus was not performed, as the oesophagus was cut just above the diaphragm. In abattoir B, rodding of oesophagus by a clip and bagging of rectum in a plastic bag were performed while the carcasses were hanging by the hind legs.

The hearts and diaphragms included in the study were sampled from the slaughter lines as the products were removed from the plucks. The slaughter line speed in both abattoirs was approximately 250 sheep per hour.

In abattoir A, steam was used for decontamination of hearts for 40 s ± 3 s. The hearts were put on a metal net band that transported them into a cabinet with steam nozzles. On the other side of the cabinet, the hearts dropped into a cold water bath. In abattoir B, both hearts and diaphragms were decontaminated for 40 s ± 3 s. Abattoir B used a cabinet with hot water deluging the byproducts hanging on hooks on a conveyor. In the end of the cabinet cold water was sprayed on the by products. The product then fell into a cold water bath. In both abattoirs the byproducts were taken from the cold water baths and frozen into 20 kg

blocks and sold to dry fermenting sausage manufacturers.

In both abattoirs 20 hearts were sampled before heat treatment and another 20 hearts after heat treatment. In abattoir B also 20 diaphragms were sampled before and another 20 diaphragms after heat treatment. From the cold water baths 10 hearts untreated and 10 heat-treated were sampled and in abattoir B also 10 diaphragms untreated and heat treated were sampled from cold water baths. The samples were stomached for 30 s with sterile peptone water. Pooled samples of five were analyzed for APC on pour plate agar by NMKL method No. 88, and *Enterobacteriaceae* by NMKL method No. 144 [1]. *E. coli* was analyzed by SimPlate Coliforms & E. coli (BioControl Systems Inc, Bellevue, WA, USA)

Sensors for temperature logging were attached to the products as they were transported through the cabinets. Also temperature reductions in the cold water baths were logged.

Stata IC version 12 for windows (StataCorp, College Station, Texas) was used for statistical analyses. Descriptive statistics were performed and differences between groups were tested by t-tests. The level of significance was set at $P \leq 0.05$.

In abattoir A, a pilot study was performed which included three hearts before heat-treatment and three hearts after treatment. The heat treatment in a steam cabinet was similar to the description of the main study.

III. RESULTS AND DISCUSSION

The results from the study of steam decontamination of sheep hearts in abattoir A, showed a small reduction of APC, *Enterobacteriaceae*, and *E. coli.* However, the differences were not significant. A pilot study with three hearts heat treated and three hearts untreated in abattoir A were included in Figure 1.

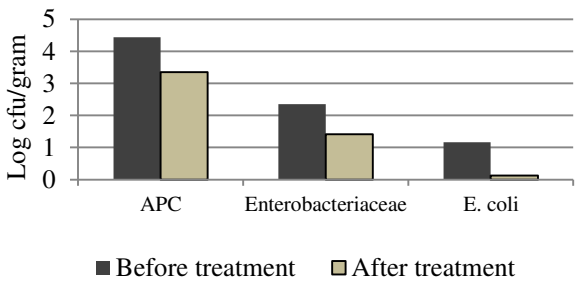

Fig. 1. Results for hearts before and after steam treatment in a cabinet in abattoir A. Results from a pilot study was included (n=3 in each group) so the total number of samples both before and after heat treatment was 23.

In abattoir B there was no difference in the levels of APC, *Enterobacteriaceae, and E. coli* between the hearts with and without hot water pasteurisation.

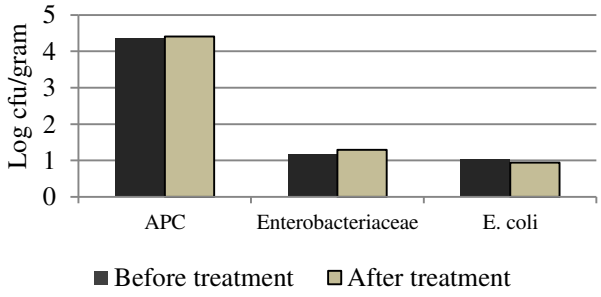

Fig. 2. Results for hearts before and after hot water pasteurisation in a cabinet in abattoir B.

Logging of the air temperature in the cabinets in abattoir A and B showed a temperature of 80-90 °C. When logging the heart temperature, the sensor was attached immediately under the surface, showing a maximum temperature of approximately 61 °C in abattoir A and 55 °C in abattoir B.

The maximum temperature on the surfaces of the hearts was lower in abattoir B than abattoir A (approximately 6 degrees lower). This might be due to the positions of the nozzles spraying hot water only at the lower part of the hearts. The cabinet was made of steel and it was not possible to see the spraying of water.

The diaphragms had a larger difference in microbial contamination between groups treated and not treated with hot water pasteurisation. But the differences were not significant. The diaphragms were hanging on the same hooks on the conveyor in the cabinets, but they had a larger size and thus hang much lower. The hot water spraying out of the nozzles might hit the diaphragms better than the hearts.

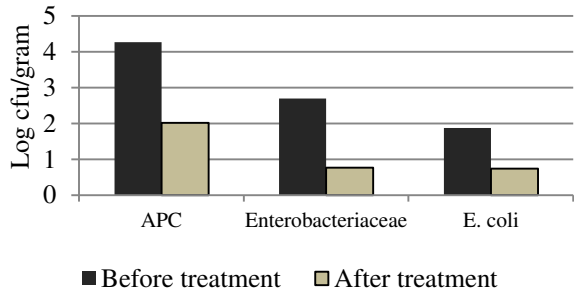

Fig. 3. Results for diaphragms before and after hot water pasteurisation in a cabinet in abattoir B.

The difference in slaughter method between abattoir A and B might affect the contamination level of the hearts. There is assumed that abattoir A has a higher risk of contamination of hearts than in abattoir B, because rodding of oesophagus was not performed. A small pressure on the rumen can push the rumen content out and contaminate the hearts and other offal. The results are therefore expected to vary widely in bacterial levels from sample to sample in abattoir A. However, the results from hearts before heat treatment in abattoir A showed that the initial contamination level was the same as in abattoir B.

IV. CONCLUSION

The results showed a tendency of a reduction of contamination measured by APC, *E. coli,* and *Enterobacteriaceae* on sheep hearts by application of steam pasteurisation and on sheep diaphragms by application of hot water pasteurisation in cabinets. However, the number of pooled samples was low and there were no significant differences between groups of treatment. A new study with a larger number of samples is needed, to get more reliable results.

ACKNOWLEDGEMENTS

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OTM-BEEF RECOGNITION USING CHROMATOGRAPHIC DATA PROCESSED BY METALEARNING BASED CLASSIFIER

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Abstract – In order to control bovine spongiform encephalopathy (BSE), a strategy called OTM was devised (thirty months). It requires the disposal of bovines older than thirty months from the food chain. Although bovine age can be estimated through dentition, this method cannot be applied to processed meat. Because of this, volatile organic compounds (VOCs) have been presently used for OTM (over thirty months) beef sample recognition. VOCs released by meat were sampled through gas chromatography. This was done from a set of more than 500 chromatograms (each one with 17 fully identified VOCs) of vacuum sealed, chilled and fresh meat. A classifier was developed using metalearning optimization methods and neural networks as the principal learner.

The optimized configuration of the neural network allowed it to discriminate between OTM and UTM (under thirty months) meat gathered from cattle, with a precision near 90%. The results were contrasted with traditional statistical methods like the linear discriminant analysis (LDA), soft independent modeling of class analogy (SIMCA), partial least squares discriminant analysis (PLS-DA) and support vector machine (SVM).

In conclusion, volatile organic compounds can be used for the recognition of bovine OTM meat in various presentations (fresh, chilled or vacuum sealed).

I. INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a serious neurodegenerative disease affecting a significant number of the domestic cattle. This disease reached epidemic proportions in several European countries, as well as in Japan and North America [1]. There is also a variant of the Creutzfeldt- Jakob disease (vCJD) that is caused by the oral exposition to the BSE's agent [2]. With the purpose of controlling this disease, a strategy called OTM (over thirty months) was created. This strategy requires the disposal of bovines older than thirty months from the food chain. An OTM based regulation banned the sale of beef older than thirty months.

Processed meat, is difficult to label as OTM or UTM (under thirty months), because the animal's age can be best estimated by dentition (the processed meat does not have teeth). This situation is a common classification problem, where adequate information can be used for the training of mathematical algorithms and generates decision rules. Previous works have informed that the age of mammals could be correlated with their volatile profile [3] and the volatile organic compounds (VOC) can be used to discriminate between OTM and UTM [4].

Artificial neural networks (ANN) have been successfully used as a tool for the modeling and study of complex problems in the area of biology and biochemistry [5, 6, 7, 8].

Previous investigations have used the information generated by gas chromatography (GC/MS) for development of high precision classifiers for the recognition and discrimination of UTM and OTM [4]. In this present work, we have extended this investigation with fresh meat, chilled meat and vacuum sealed meat and a complete characterization of the bovine meat volatile release profile considering 17 VOCs. The information of more than 500 volatile profiles was used for the development of a binary sorter for the discrimination between OTM and UTM using ANN.

II. MATERIALS AND METHODS

1. Samples

The meat samples were obtained from *M. longissimus dorsi* Holstein (*Bos taurus*). In the analyzed samples there are male and female cattle with 5 different dentitions (0, 2, 4, 6 and 8 definitive incisive teeth). More of 500 samples are sorted for 7 types of meat: fresh meat, chilled meat for 3, 5 and 7 days; and vacuum sealed meat for 15, 30 and 60 days. A total of 525 chromatograms (one for each sample) were obtained which were considered as OTM if the sample came from a bovine with 2 or more definitive incisive teeth [9].

2. Volatile organic compounds

We analyzed the release of volatile molecules from beef by means of a GC/MS-SPME (Gas chromatography / mass spectrum - Solid phase micro extraction). We used the method described previously by [4].
Potential emanations were analyzed by using the Finnigan Xcalibur Software (Thermo Electron Corporation) matching mass spectrums with those saved in the NIST MS Spectral Library 2008. Selected chromatographic peaks were checked with their respective chemical standards and retention indexes.

3. Classification

A binary sorting was made (classes labeled as UTM or OTM) through the use of chromatographic profiles by using ANN. This was contrasted with other methods such as the linear discriminant analysis (LDA), soft independent modeling of class analogy (SIMCA), partial least squares discriminant analysis (PLS-DA) and support vector machine (SVM). The SIMCA and PLS-DA calculations were made using the SIMCA-P software (Umetrics) and for SVM the LibSVM software was used with a gaussian kernel [10].
For the entire set of data (525 chromatograms), we applied random sampling with cross validation [11] toward the generation of training sets and tests in a 50/50 proportion. This selection was repeated thirty times using a uniformly distributed random selection procedure. This gives rise to thirty different data sets used for the classification (by each one of the methods previously indicated).

4. *Neural Network*

The neural network used, is a feed forward artificial neural network (FFANN). This network is constituted by three layers including a hidden layer. With a multilayer neural network like this one, it is possible to model highly nonlinear decision surfaces. This is a requirement for complex classification problems [12].
Our FFANN was trained with the backpropagation algorithm [13, 14], due to its general simplicity and good performance in a variety of classification and modeling of difficult problems [15]. The input layer is composed of 17 neurons, one for each volatile organic compound (VOC). In basic neural network configuration, the amount neurons in the hidden layer are adjusted through a manual iterative process. The basic neural network configuration (before optimization) is used as a basic classifier. In the output layer there is only one neuron taking in account that we are interested in only one class (OTM or UTM). In our implementation of a basic FFANN, the learning rate and the impulse were fixed with a floor of 0.01 for each, this was a reasonable empirically obtained value [16]. A maximum of 2.5 million iteration were used for the training algorithm after an ad-hoc experimental process that was performed toward the determination of a reasonable number of iterations in this problem context.

5. Neural network based metalearning

Meta-learning is a machine learning methodology where the learning algorithms are applied to the metadata related with an automatic learning process. The main goal of meta-learning is to improve the performance of existing learning algorithms. In the literature there are different definitions available of what is “meta-learning”. In [17] meta-learning is defined as the capacity of changing a learner with the objective of achieving a better performance. In [18] meta-learning is defined as learning algorithms that improve their dynamic tendency through the experience of the accumulation of meta-knowledge. In our approach, we have optimized the neural network described before, adjusting the number of neurons in the hidden layer, the learning rate and the momentum using genetic algorithms (GA). The fitness function used is the neural network’s classification success rate. The meta-learning process (namely a GA) tries to determine which is the best parameter vector with the goal of finding the best configuration for the neural network.

III. RESULTS AND DISCUSSION

An exploratory analysis of the chromatography data took place by using a simple LDA [19]. The average precision was close to 67 %. This preliminary analysis indicated the presence of a complex data distribution and the impossibility to use a simple classification procedure for the discrimination between UTM and OTM.
The strategy used in [4] allows an excellent discrimination between UTM and OTM meat using a PLS-DA and SIMCA based parallel classifier [20]. This classifier was more precise

than SVM [21]. However, only fresh meat was tested. In this present work, we have extended the investigation with three bovine meat types (fresh meat, chilled meat and vacuum sealed meat). The results obtained with LDA, PLS-DA, SIMCA and SVM are informed in Table 1. The obtained results with the methods mentioned before weren't enough to get a satisfactory discrimination between UTM and OTM meats. This motived our investigation about the use of neural networks (ANN) that were optimized by meta-learning.

Table 1. Accuracy for all classifiers evaluated on the test-set.

	Average	Minimum	Maximum
LDA	67.3 (±1.69)	65.0	70.7
SIMCA	63.5 (±2.77)	56.4	69.2
PLS-DA	66.4 (±1.71)	63.5	69.9
SVM	76.6 (±1.91)	73.4	79.9
FFANN	81.9 (±2.10)	78.4	87.6
OML-NN	84.3(±1.74)	81.1	89.2

LDA: Linear Discriminant Analysis; SIMCA: Soft Independent Modeling of Class Analogy; PLS-DA: Partial Least Squares Discriminant Analysis; SVM: Support Vector Machine; FFANN: Feed Forward Artificial Neural Network; OML-NN: Optimal Meta Learner

The precision of the ANN was significantly higher than SVM (see Table 1). However, with an average precision close to 82%, the FFANN was still low for using as an appropriate classifier.

In our present optimization approach, we used the meta-learning process described before with the purpose of generating thirty optimal configurations for the learner (neural network).

Starting from the obtained 30 optimal configurations 30 neural networks were trained (with each training/test pair) for every Meta-learner configuration. From these meta-learners we can select the most adequate for each data set; these are labeled as Optimal Meta Learner (OML-NN). The best results of the OML configuration in an approximate precision of 90 % (see table 1).

In every used data set the accuracy of OML-NN was better than FFANN, which indicates that in the 100% of the cases the accuracy of the OML-NN was significantly higher than FFANN.

IV. CONCLUSION

In conclusion, the volatile organic compounds can be used for the recognition of bovine OTM meat in any presentation (fresh, chilled or vacuum sealed meat).

The best configuration for OTM meat recognition sorters is OML-NN, with a precision near to 90%. As can be seen in Table 1, it was a significantly better result compared with other sorting methods.

The meta-learner optimized neural network allows to develop a more accurate sorter than others methods, including LDA, SIMCA, PLS-DA, SVM and basic FFANN. These sorting methods are well known and widely used with success in a variety of classifiers but for the problem at hand, they have failed to reach a good precision, even after careful manual tuning process. This speaks of the inherent difficulty present in the classification problem that may be due to the data's multidimensional complexity at hand. To the best of our knowledge, our current approach based in the optimization of chromatographic neural networks is the first to address the important issue of the food safety in relation to meat. This application could be of benefit to industry as well as regulators in order to assist traceability systems currently in place.

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