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Research Article

The Role of Catalase and Pyruvate on the Recovery of Cold - Shocked Bacteria

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Abstract

The study principally aimed at evaluating different additives to the culture medium for the recovery of cold-shocked bacteria isolated from chilled carcasses. Sixty randomly selected beef carcasses were assigned for investigation from which 630 swabs were collected for assessment of microbiological contamination of carcasses during the processes after skinning and evisceration, along the washing to the chilling stage. Obtained results were analysed by statistical package (SAS 2000, version 9.0) and revealed bacterial counts decreased significantly ($p \leq 0.05$) from skinning to chilling (3.67±0.0 to 1.23±0.10, $p \leq 0.01$). The predominant bacteria isolated from the carcasses were *Staphylococcus* spp. followed by *Enterobacteriaceae* and *Pseudomonas* spp. To test for recovery of cold-shocked bacteria, the bacterial load as CFU was evaluated after treatments with catalase, pyruvate and their combination. The CFU of samples from chilled carcasses increased after treatment with catalase and pyruvate (1.23±0.52 vs. 2.54±0.12 and 2.42±0.33, respectively; $p \leq 0.05$). The results showed that catalase treatment has induced higher bacterial recovery compared to pyruvate alone or the combination of pyruvate and catalase, but the bacteria died when the concentration of catalase and pyruvate was increased. Addition of catalase or pyruvate to plate count agar medium gives true estimates of the bacterial load of chilled carcasses and other cold foods.

***Keywords:** Aerobic plate count, chilled beef carcasses, recovery of cold-shocked bacteria*

Introduction

Chilling can be defined as the fundamental operation in applying cold to meat in order to quickly reduce its temperature (FAO, 1991). The aims of carcasses chilling are: to inhibit autolysis changes, prevent spoilage due to microorganisms and retain the freshly slaughtered state (Doyle, 2002). Storage of chilled meat at – 1.5 ± 0.5 °C could attain the maximum storage life without any surface freezing.

Meat requires adequate storage, processing, packaging and distribution to prevent spoilage and avoid any hazards due to microbial growth (Doulgeraki *et al*., 2012; Húngaro *et al*., 2016). Meat is considered an important source of proteins, essential amino acids, B complex vitamins and minerals. Due to this rich composition, it offers a highly favourable environment for the growth of pathogenic bacteria (Barros *et al.,* 2007; Ncoko *et al*., 2020). In the slaughterhouse, temperatures of 0 to 4°C are applied as a rapid chilling process using advanced refrigeration technology. At the end of the processing chain, chilling is a crucial step with the purpose to avoid pathogen growth within and on the carcasses in order to maintain meat safety (Smith *et al*., 2013).

The effect of cold diluents on viability was first early reported by Sherman and Albus (1923) who indicated that about 95% of the cells were killed within 1 h when the temperature of 4 h culture of *E. coli* was reduced suddenly from 45°C to 10°C. Colee *et al*. (1961) termed this phenomenon as "phoenix" phenomenon due to disappearance of colonies at the recovery media and attributed this to the temperature of the inoculum; a finding which was disqualified by El Sanousi (1975) who explained the phenomenon to be a mere cold shock. This is due to cells being shocked when transferred from high to low temperatures (El Sanousi, 1975).

In a previous investigation, Vorobeva (2004) showed that bacterial growth rate or survival was reduced under low temperature stress (cooling, freezing or deep freezing). Cold shock as defined by El Sanousi (1975) comprises the injury, death or both, which are caused by sudden chilling of the microorganisms. The cold shock phenomenon occurs when growing bacteria are exposed to a sudden temperature drop of at least 10°C, leading to cold shock in susceptible microorganisms (Jones *et al*., 1996). The effect of cold shock is perceived at multiple levels such as; (i) decrease in the membrane fluidity affecting the membrane associated functions such as active transport and protein secretion, (ii) stabilization of the secondary structures of RNA and DNA, leading to reduced efficiency of mRNA translation and transcription, (iii) slow or inefficient folding of some proteins, and (iv) ribosome need to be cold-adapted to function properly at low temperature (Phadtare, 2004). Bacterial injury may simply be defined as the effect of one or more sub-lethal treatment on a microorganism (Hurst, 1984).

Injured cells are cells which can form colonies on enriched medium, but not on stressing media (Clark and Ordal, 1969). The presence of injured microorganisms in food has recently gained an increasing significant public health concerns. As reported by Alissa *et al*. (2009) injured cells may initially go undetected during routine quality control checks and at critical control points during manufacturing.

Improved recovery of injured *Staphylococcus aureus, Pseudomonas fluorescens, Salmonella typhimurium* and *E. coli* has early been reported when media selective for these microorganisms were supplemented with either catalase or sodium pyruvate (Martin *et al*., 1976). Pyruvate is known to be one factor that promotes resuscitation of viable but nonculturable, VBNC cells (Vilhena *et al*., 2019). It has been early reported that addition of pyruvate and TDPA (3, 3'-thiodipropionic acid), improved the recovery of injured cells in both selective and non-selective media (McDonald *et al*., 1983).

Sodium pyruvate gives energy to the cell while additions of compounds such as catalase or pyruvate to the media increases the chance of recovering stressed bacteria (McDonald *et al.,* 1983). Former reports indicated that exogenous addition of various supplements, most often catalase or sodium pyruvate can improve the detection of microbes under stress conditions (Chang *et al*., 1993). Calabrese and Bissonnette (1990) demonstrated that additions of sodium pyruvate, as well as combinations of sodium pyruvate and catalase were effective in recovering sub lethally-injured cells and increasing the detection of total heterotrophic bacteria from acid mine water.

A study by Ridina (2013) proved that addition of pyruvate to the media was 30.7% more effective compared to catalase enzyme. The medium which was supplemented with both catalase enzyme and pyruvate has performed better in recovering cold-shocked bacteria than catalase and pyruvate added separately (Wigdan, 2013). Gamer (2017) succeed in recovering the cold-shocked bacteria by applying trace elements as well as pyruvate and catalase.

Jones *et al*. (2008) claimed that the most important pathogenic psychrotrophs for the meat industry are *Listeria monocytogenes* and *Yersinia enterocolitica,* both can grow at temperatures as low as 1.1°C.

Several studies indicated increments in levels of bacterial loads on refrigerated carcasses. Bolton *et al*. (2002) reported an increase in total viable bacterial counts from 3.8 to 4.5 $\log_{10}$cfu/cm^2 on carcasses at the refrigeration stage. In fact, as the temperature decreases, the bacterial lag phase extends whereas the growth rate decreases and the ultimate cell numbers may decrease (Beales, 2004).

The objective of the presented study was to evaluate the true number of bacteria in the chilled beef carcasses.

Materials and Methods

This work has been performed in one government's slaughterhouses which is utilized for export of beef and mutton.

Samples collection

In this study a total of 630 swabs were collected from the carcasses surfaces, sixty fresh beef carcasses were randomly selected from an export slaughterhouse. The slaughterhouse was visited 12 times to obtain data regarding the microbiological contamination of carcasses during their processing from evisceration, along the dressing line to chillers. Post-chiller swabbing was performed after 24 h chilling. During each sampling visit to the slaughterhouse, ten beef carcasses were randomly selected. Sampling of beef carcasses were performed in cattle prepared for exporting.

Sampling procedure

At each carcass, a moistened swab (NaCl solution) was wiped in both horizontal and vertical directions across the sampling site (100 cm^2). The total area swabbed on each carcass was 400 cm^2. Sampling was carried out at three different operational points of slaughtering in this continuum: before washing, after washing, and after chilling on the same carcasses, then held in a plastic whirl bag. Samples were placed in closed cooled containers to prevent microbial growth and labelled according to the corresponding carcass number and stage of collection then transported on the same day to the laboratory for further microbiological analysis.

Samples were cultured onto plate count agar (Oxoid Ltd.). Plates count agar were incubated aerobically for 24 h at 37ºC to determine bacterial count, Enterobacteriaceae and Staphylococcus count. Biochemical tests were performed for identification of isolates according to Barrow and Feltham (2003). All counts were converted to log (CFU/cm^2 or CFU/g) prior to statistical analysis to determine microbial load.

The viable count (Colony Forming Unit)

The viable count of the isolated microorganisms was carried out according to the method of Miles and Misra (1938). Ten-fold serial dilution of the samples was prepared, then 20µl was placed onto the plate count agar for bacterial enumeration. Each swab sample was homogenized in 1 ml sterile saline according to (Legg *et al*., 1999). A sample of 1 ml suspension was transferred to 9 ml Ringer's solution, and the mixture was vortexed for 60 second for homogenization (all samples were diluted three times). Samples (20µl) of the appropriate 10-fold serial dilutions were spread on the surface of the growth medium on Petri dishes for enumeration of colony forming unit (CFU) on plate count agar and was incubated at 37ºC for 24 h.

Recovery of cold-shocked bacteria

Stressed bacteria do not grow on media when exposed to cold (low temperature). To recover these organisms' pyruvate and catalase were added to the growth medium. In the first batches 1% pyruvate was added to plate count agar medium and compared with non-addition (the control). In the second batches 1% catalase was added to plate count agar medium. In the third batches 1% catalase plus 1% pyruvate were added. Samples after chilling were cultured on plate count agar, plate count agar plus 1% pyruvate, plate count agar plus 1% catalase, plate count agar plus 1% catalase and 1% pyruvate.

Statistical analysis

All bacterial counts were transformed to colony forming units per square centimeter of surface sampled ($\log_{10}$cfu/cm^{-2}), and log $\log_{10}$cfu/ml of water sampled for analysis and means were calculated. The data were analysed using the statistical programme SAS software (SAS 2000, version 9.0). Data presented are means and standard deviations. Statistically significant differences among means are indicated by different letters at a probability level of ($P \leq 0.05$).

Results

The bacterial isolates obtained in this investigation were classified on the basis of their cultural characteristics, cell morphology, Gram-stain reactions and their biochemical properties as described by Barrow and Feltham (2003). The isolates identified in the current study consisted of Gram-positive and Gram-negative bacteria. They were Acinetobacter, Aeromonas, Alcaligenes, Bacillus, Brochothrix, Enterococcus, *Escherichia coli*, Klebsiella, Lactobacillus, Listeria, Micrococcus, Proteus, Pseudomonas, Staphylococcus, and Streptococcus.

The effect of chilling temperature on bacterial count of beef carcasses

Our results indicated a decrease in CFU of bacteria after chilling (Fig. 1)

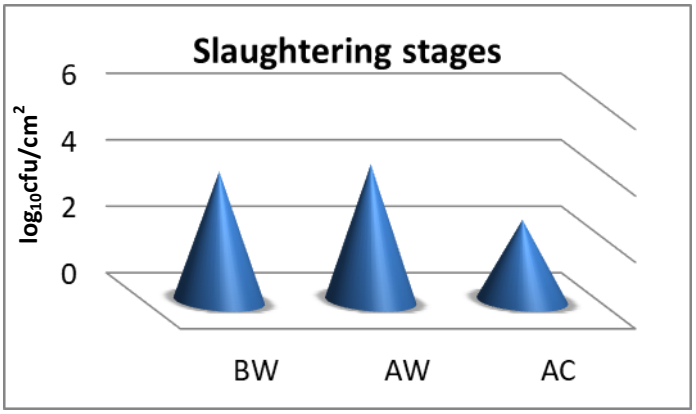

Fig. 1. *Comparison of bacterial count ($\log_{10}$cfu/cm^2) of beef carcasses among the three different slaughtering stages (BW: before washing, AW: after washing and AC: after chilling).*

The recovery of cold-shocked bacteria in chilled beef carcasses during sampling days using catalase and pyruvate separately or combined.

To test for the recovery of cold-shocked bacteria, CFU were evaluated after treatments with catalase, pyruvate or a combination of both. Based on the data highlighted in Table (1), it is quite obvious that treatment with 1% catalase caused an increase in CFU to 2.36±2.08 (4.07×10^4), 3.82±0.89 (2.72×10^4), 2.38±0.38 (3.67×10^2), 2.12± 1.55 (9.76×10^3), 2.62±0.25 (2.90×10^4) and 2.20±1.21 (3.86×10^3) $\log_{10}$cfu/cm^2. Such effect was noted across all sampling days.

Similar results were obtained upon treatment with 1% pyruvate where the mean logs were 2.04±1.92 (2.52×10^4), 3.64±1.00 (1.54×10^4), 2.16±0.52 (2.15×10^2), 2.16±1.59 (8.89×10^3), 2.63±0.24 (5.04×10^2) and 2.14±1.39 (3.18×10^3) $\log_{10}$cfu/cm^2, respectively. For the treatment of combination of pyruvate and catalase the mean log was 1.21±1.90 (1.67×10^4), 3.88±0.78 (2.63×10^4), 1.71±0.84 (1.30×10^2) , 2.08±1.61 (9.51×10^3), 2.55±0.26 (4.31×10^2) and 1.81±1.56 (4.38×10^3) $\log_{10}$cfu/cm^2.

Table 1. Recovery of cold-shocked bacteria in beef carcasses using catalase and pyruvate, either single or together.

Days	Treatment	Mean ±SD
Day 1	AC	0.72 ± 1.38^{g}
	ACC	2.36 ± 2.08^{bcd}
	ACPY	2.04 ± 1.92^{bcd}
	ACCPY	1.21 ± 1.90^{efg}
Day 2	AC	2.68 ± 0.90^{bc}
	ACC	3.82 ± 0.89^{a}
	ACPY	3.64 ± 1.00^{a}
	ACCPY	3.88 ± 0.78^{a}
Day 3	AC	0.78 ± 1.00^{g}
	ACC	2.38 ± 0.38^{bcd}
	ACPY	2.16 ± 0.52^{bcd}
	ACCPY	1.71 ± 0.84^{def}
Day 4	AC	1.08 ± 1.62^{fg}
	ACC	2.12 ± 1.55^{abcd}
	ACPY	2.16 ± 1.59^{bcd}
	ACCPY	2.08 ± 1.61^{bcd}
Day 5	AC	1.19 ± 1.07^{efg}
	ACC	2.62 ± 0.25^{b}
	ACPY	2.63 ± 0.24^{b}
	ACCPY	2.55 ± 0.26^{b}
Day6	AC	1.17 ± 1.18^{efg}
	ACC	2.20 ± 1.21^{bcd}
	ACPY	2.14 ± 1.39^{bcd}
	ACCPY	1.81 ± 1.56^{cde}

AC= after chilling, **ACC**= after chilling + catalase enzyme, **ACPY**= after chilling + sodium pyruvate, **ACCPY**= after chilling + catalase enzyme + sodium pyruvate

Catalase treatment has induced higher bacterial recovery as indicated by the number of bacteria being higher compared to those under the other two treatments (pyruvate alone or pyruvate and catalase combined). (Fig. 2)

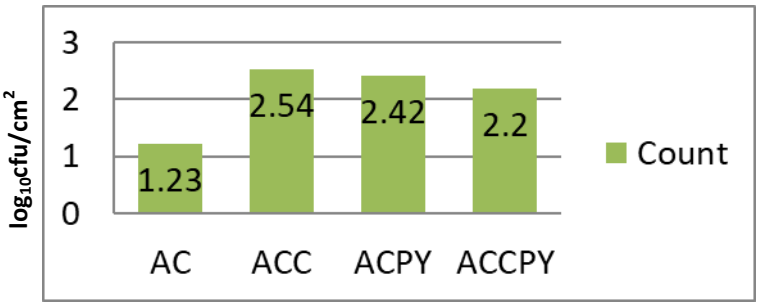

Fig. 2. *Comparison of bacterial enumeration ($log_{10}cfu/cm^2$) of cold-shocked bacteria after recovery with catalase (ACC), pyruvate (ACPY) and catalase and pyruvate combined (ACCPY).*

Recovery of cold-shocked bacteria in chilled beef carcasses after chilling stage using different concentrations of catalase and sodium pyruvate separately or together.

In this study, the results showed that the viable bacterial count decreased when added high concentrations (2, 3%) of catalase and pyruvate (either single or together) to resuscitate the cold-shocked bacteria. (Table 2)

Table 2. Recovery of cold-shocked bacteria in chilled beef carcasses after chilling stage using different concentrations of catalase and sodium pyruvate separately or together.

Treatments	Mean ±SE
AC	1.17 ± 0.18^{bc}
ACC1%	2.20 ± 0.19^{a}
ACC2%	1.70 ± 0.25^{ab}
ACC3%	1.70 ± 0.26^{ab}
ACPY1%	2.14±0.22a
ACPY2%	1.12 ± 0.24^{bc}
ACPY3%	0.53 ± 0.19^{d}
ACCPY1%	1.81 ± 0.24^{a}
ACCPY2%	1.21 ± 0.25^{bc}
ACCPY3%	0.73 ± 0.19^{cd}
P value	**0.000**

AC= after chilling, **ACC**= after chilling + catalase enzyme, **ACPY**= after chilling + sodium pyruvate, **ACCPY**= after chilling + catalase enzyme + sodium pyruvate.

Discussion

The present study was conducted in an export abattoir. In this study and for determination of bacterial viable counts (CFU) a swabbing method was employed since these carcasses were assigned for exportation, and according to restricted regulations of the abattoir we were not allowed to perform the excision procedures. Therefore, we used the swab sampling method as an adequate method as reliably used by others (Lindblad, 2007) who stated that sampling by swabbing was found to be beneficent than the excision method. Sampling by swabbing was found to be more beneficent than the excision method as stated by Lindblad (2007).

The present study also investigated the effect of chilling treatment on the bacterial load. Our findings revealed that chilling resulted in a further significant reduction. These findings were similar to Smith *et al*. (2013) who demonstrated that chilling temperature is used to delay meat alteration through the development of spoilage bacteria.

Our results showed that the mean number of bacteria was reduced and tends to be zero, indicating that it was exposed to cold shock stress, as described by El sanousi (1975) who explained the phenomenon to be a mere cold shock which comprised the injury, death or both, which are caused by sudden chilling of the micro-organisms. Viable but nonculturable (VBNC) state, which is a survival strategy adopted by cells exposed to adverse environmental conditions (Oliver, 2000; Vilhena *et al*., 2019). The VBNC bacteria do not form colonies in standard medium, but otherwise retain their metabolic activity and can express toxic proteins.

Early reports indicated that exogenous addition of various supplements, most often catalase or sodium pyruvate can improve the detection of microbes in stress (Chang *et al*., 1993).

It is worth-noting that catalase treatment has induced higher bacterial recovery as indicated by the number of bacteria being higher compared to those under the other two treatments (pyruvate alone or pyruvate and catalase combined). This finding is in contradiction to the finding of Ridina (2013) and Wigdan (2013) who found that pyruvate was more induced the recovery of cold-shocked bacteria than catalase.

In the current investigation we managed to improve the recovery of injured bacteria by incorporation of catalase and pyruvate or separately, a result similar to that early reported by Martin *et al*., (1976) and Chang *et al*., (1993) who noted improved recovery of injured *S. aureus, Pseudomonas fluorescens, Salmonella typhimurium* and *E. coli* when media selective for these microorganisms were supplemented with either catalase or sodium pyruvate.

Conclusion

The present findings further showed that the combination of catalase and pyruvate were less effective in the recovery of cold-shock bacteria compared the conditions when catalase and pyruvate were added separately. In contrast, Wigdan (2013) and Gamer (2017) found that media supplemented with both catalase enzyme and pyruvate together improved the recovery of cold-shocked cells better than the addition of each one separately.

Conflict of Interest

The authors do not have any conflict of interest.

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