Escherichia coli O157:H7 reduction in hamburgers with regards to premature browning of minced beef, colour score and method for determining doneness

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Abstract

This study investigated the effect of premature browning (PMB) on the survival of *E. coli* O157:H7 in beef hamburgers after cooking with respect to interior colour of the hamburger and recommendations to cook hamburgers to a core temperature of 71°C. Assessment of doneness by visual inspection or measurement of internal temperature was compared in terms of survival and the increased relative risk of illness due to PMB was estimated. At the last consume-by-day, hamburgers made from minced meat packaged in 80/20 O_2/CO_2 (MAP hamburger) and from meat minced at retail packaged in atmospheric condition (control hamburger) were inoculated with a *gfp*-tagged strain of *E. coli* O157:H7 (*E. coli* O157:H7*gfp*+). Hamburgers were cooked for different times during assessment of the core temperature every 30 sec and cut in halves after cooking. Doneness was evaluated based on visual judgement of the internal colour using a score chart (C-score) from 'uncooked' (score 1) to 'tan with no evidence of pink' (score 5). An alternative five point score chart (TCC-score) including texture of the meat, clarity of meat juice and internal colour was also developed. Enumeration of viable *E. coli* O157:H7*gfp*+ in cooked hamburgers were based on fluorescent colonies recovered from plates. Results showed that MAP hamburgers developed PMB when compared with controls (P=0.0003) and that the shortest cooking time for the highest C-score was 6 and 11 minutes for MAP and control hamburger, respectively. The mean temperature in the MAP hamburger was then 60.3 ºC. The TCC-score reduced the difference between MAP and control hamburgers. It was also shown that the survival of *E. coli* O157:H7*gfp*+ was highest in MAP hamburgers. The predicted absolute risks for illness were highest for MAP hamburgers for all C-scores and the relative risk associated with PMB increased with doneness. For a C-score of 4 (slightly pink) the predicted relative risks for illness was 300 times higher for MAP hamburger than for controls. A variable pathogen

reduction was observed when cooking hamburgers to temperatures of 70-76ºC (the 5th and 95th percentile range was around 3.3 log CFU). The lower reductions, at the 5th percentile, may, depending on initial contamination levels, not be enough to ensure sufficient and safe inactivation of *E. coli* O157:H7. Efforts to inform consumers about PMB in minced meat packaged in high oxygen packages ($\geq$ 60% O_2) are needed with the aim to make consumers use thermometers correctly or at least not determine doneness based only on meat colour.

Keywords

Bacterial inactivation, Doneness evaluation, Food safety, Modified atmosphere, Relative risk

1. Introduction

Shiga toxigenic producing *Escherichia coli* (STEC) O157:H7 is a foodborne pathogen with severe public health impact caused by haemorrhagic colitis and chronic sequelae, such as haemolytic uremic syndrome (HUS) (Karmali, 2004; Keithlin et al., 2014; Pennington, 2010). Human illness may follow exposure to less than 100 CFU, even after ingestion of one CFU, (Teunis et al., 2004) and disease in humans may thus develop without prior multiplication of the bacterium in food. Minced meat from cattle, or products thereof, are important vehicles for human STEC O157:H7 infections (Pennington, 2010) and are reported as the vehicle in approx. 40 percent of the reported foodborne outbreaks of *E. coli* O157:H7 within the EU and in the US (ECDC and EFSA, 2011; Rangel et al., 2005). Indeed, the first documented outbreak of STEC O157:H7 was linked to hamburgers (Bell et al., 1994). Quantitative risk assessments have shown that cooking preference has an impact on the risk to develop disease, including HUS, and that consumption of raw beef (steak tartar) increases the risk for illness

(Delignette-Muller and Cornu, 2008; Hussein, 2007; Nauta et al., 2001; Signorini and Tarabla, 2009).

Minced meat can be packaged in modified atmosphere (MAP) often consisting of 80/20 or 70/30 O_2/CO_2 mixture to increase shelf life (McMillin, 2008). The consume-by-date is, for example in Sweden, thereby prolonged from one to eight days. During cooking there is, however, a risk of premature browning (PMB) of meat stored in MAP (Hague et al., 1994; Hunt et al., 1999; John et al., 2004; Sorheim and Hoy, 2013). The condition of PMB is influenced by the chemical state of myoglobin in the meat interior during cooking and results in meat developing a well-done appearance earlier than meat not packaged in an 80% oxygen atmosphere (Seyfert et al., 2004). There is thus a risk that the meat develops a well done appearance even if temperatures ensuring inactivation of pathogenic bacteria have not been reached. This implies food safety risks if the consumers base their decision on the meat's doneness exclusively on visual appearance. MAP hamburgers can be perceived as done at temperatures down to as low as 49°C (Hunt et al., 1999; Rossvoll et al., 2014, John et al., 2004). Evaluation of hamburger doneness is most often based on visual judgement (Phang and Bruhn, 2011). Fewer consumers use meat juice clarity and texture of the interior as indicators for doneness whereas only a minor proportion uses a meat thermometer (Mahon et al., 2006; Phang and Bruhn, 2011; Rossvoll et al., 2014). Furthermore, a large proportion of consumers prefer a pink interior of the hamburgers (Altekruse et al., 1999; Phang and Bruhn, 2011; Rossvoll et al., 2014).

The objective of this study was to investigate the effects of PMB on *E. coli* O157:H7 reduction in hamburgers after cooking in relation to interior colour of the hamburger and recommendations on cooking, also taking into account whether judgment of doneness was based on visual inspection of meat colour or measurement of internal temperature. The objective was addressed by: i) comparing reduction of *gfp*-tagged *E. coli* O157:H7 in

hamburgers made of minced meat packaged in modified atmosphere (MAP hamburger) or of meat minced at retail (control hamburger) during cooking, in relation to the interior colour of the hamburger, ii) comparing *E. coli* O157:H7*gfp*+ reduction when visual judgement of doneness was based only on interior colour with reduction when judgment was based on a combination of interior colour, meat texture and clarity of meat juice, iii) developing relationships between *E. coli* O157:H7*gfp*+ log reductions and interior colour and temperature, respectively, for MAP and control hamburgers during cooking, and iv) using these relationships to evaluate the reduction and relative risk of illness for consumers relying on visual inspection of meat colour or measurements of internal temperature depending on recommended final temperatures.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The strain used in this study was a non-pathogenic strain of *E. coli* O157:H7 (verotoxin 1 and 2 negative, *eae*-positive, obtained from the Swedish Institute for Communicable Disease Control, Solna Sweden, registry no. E81186), which was *gfp*-tagged (Alam et al., 2014). This single strain of serotype O157 was selected because the aim of the study was to investigate PMB and not strain variability, and also because it was already available as *gfp*- tagged. More importantly, the serotype O157 accounts for approx. 50% of all human cases of illness caused by STEC (FOHM, 2015). The *gfp*-tagged strain was induced to fluoresce in UV-light when grown on Luria-Bertani (LB, L3022-1kg, Sigma, Stockholm, Sweden) broth or agar supplemented with 100 μg/ml ampicillin and 0.1% L-arabinose. Bacterial cultures were prepared by inoculating a colony into 10 ml of Brain heart infusion broth at 37 ± 1°C for 20 ±

2 h. The final cultures were centrifuged at 3 320 x g for 15 minutes and washed in peptone saline (0.1% peptone in 0.85% NaCl) three times. The pellets were thereafter suspended in peptone saline (0.1% peptone in 0.85% NaCl) to give a target concentration of 10 log CFU per 100 ml of cell suspension. Two 100 ml cell suspensions were made for each trial. The number of bacteria in the cell suspension was confirmed to be 8 log CFU/ml using bacterial enumeration as described below.

2.2. Minced meat used in the trial

Raw minced meat in packages of 1.5-1.7 kg was used. All meat originated from Swedish cattle and was obtained from the same local retail store. Three batches of minced meat packaged in Modified Atmosphere (MA; 80/20 O_2/CO_2) from one supplier were used for the MAP hamburger and three batches of meat minced at the retail store were used for the control hamburger. All minced meat was packaged in plastic trough, covered by plastic foil and kept at 3ºC at retail. The fat content in all batches was around 10%, with a maximum of 12 % according to the manufacturer, and all meat was ground with the same diameter. No additives, such as salt and water, were added. All meat was kept at 5°C until the consume-by-date when inoculations, cooking and analyses were made. The consume-by-date was chosen as consumers may store the minced meat until this date.

2.3. Inoculation, preparation and cooking of hamburgers

Minced beef was weighed aseptically into 13x100 g portions and placed on aseptic plastic plates. Ten ml of cell suspension containing 8 log CFU per ml were added to each 100 g portion resulting in 9 log CFU/hamburger. This is equivalent to 7 log CFU/g which is within the

recommended range of inocula levels in inactivation studies by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 2010). The suspension volume was chosen to facilitate mixing. Each portion was thoroughly mixed by gloved hands for two minutes to ensure homogenous distribution of the organisms and was formed into a hamburger with a diameter of 11 cm and a thickness of 1 cm using a plastic mould custom made at our laboratory. In each trial, 100 g of minced meat inoculated with 10 ml of peptone saline (0.1% peptone in 0.85% NaCl) served as un-inoculated control. The hamburgers were kept at 8°C for a maximum of 3h until cooked in a Teflon-coated skillet with 22 cm diameter at a temperature of 180 ± 5°C on an induction stove (Item No. 9095-1452, Rusta AB). The temperature in the skillet was measured continuously using an infra-red thermometer (No. 405053, accuracy ±1.5°C, Jula AB) and adjusted if needed. The cooking times ranged from 2 to 13 minutes and the hamburger was turned once mid-time. After cooking, the hamburger was removed and placed on a grid for two minutes to simulate the continued cooking that takes place within meat that have been removed from the cooking source (post cooking).

The central core temperature in each hamburger was measured every 30 sec using a digital thermometer (Prima Long, E 905 050-905 052, accuracy <1°C, Amarell Electronic) during cooking and post cooking. Consumers who are using a thermometer when cooking hamburgers presumably measure the central core temperature. The hamburgers were cut in halves after post cooking and two lab-trained investigators (the authors Boqvist and Fernström) evaluated the interior colour using the following five-point Colour score (C-score): 1= uncooked (dark red to purple), 2= bright red, 3= very pink, 4= slightly pink and 5= tan with no evidence of pink (Hunt et al., 1999). An alternative five point score taking Texture and Colour of the meat, and Clarity of the meat juice (TCC-score) into account was also developed.

The TCC-score is a summary of three sub-scores of which the first is the C-score described above. The second describes the texture of the meat, when cutting the hamburger in halves,

using a three-point score: 1= raw (high degree of chewiness and of thread like texture), 2= medium degree of chewiness and of thread like texture, and 3= no evidence of chewiness and of thread like texture. The third score describes the clarity of the meat juice immediately after cooking: 1= bright red, 2= pink, 3= clear with no evidence of pink and 4= no meat juice remaining after cooking. For each hamburger the sum of all sub-scores was calculated, with a minimum of 3 and a maximum of 12, and converted to the TCC-score as shown in Table 1.

The hamburgers were photographed under similar lightning conditions, using a Nikon D50, immediately after the visual assessment, weighed and placed on aluminium foil on ice for rapid cooling. Before the first trial started, a pilot study was conducted to test the experimental set up, and the C- and TCC-scores scores. After each trial the investigators discussed and reviewed the results in the written protocols and compared them with the photographs.

All equipment was thoroughly washed in hot water with detergent followed by disinfection using 70% ethanol between each cooking and each cutting. The risk for cross-contamination was further reduced by starting with the hamburger that was cooked for 13 min and finishing with the hamburger cooked for 2 min. Three separate trials were done using MAP and control hamburgers, respectively. Twelve patties (one for each cooking time) were included in each trial making a total of 36 MAP hamburgers and 36 control hamburgers.

2.4 Bacterial analyses of E. coli O157:H7gfp+ and microbial enumeration of the background flora

Both halves of each MAP and control hamburger, respectively, were mixed with 100 ml (1:1 dilution) of peptone saline (0.1% peptone in 0.85% NaCl) and homogenized for one minute using a Stomacher lab-blender (easyMIX® Lab Blender, AES-Chemunex, Weber Scientific). For hamburgers cooked 2 to 10 minutes and 11 to 13 minutes, serial dilutions 10^{-1}

to 10^{-5} and 10^{-1} to 10^{-3}, respectively, were made. Manual surface plating of each dilution was done on LB agar with ampicillin (100 µg/ml) and arabinose (1 g/ l). All plates were incubated at 37 ± 1°C for 24 ± 2 h before counting fluorescent colonies on the first plate with countable numbers of colonies (that is the lowest dilution) using ultraviolet light (Spectroline, CM-10A, wavelength 365 nm). The six non-inoculated patties (one MAP and one control hamburger from each trial) were subjected to analyses of *Enterobacteriacae* and *E. coli* using NMKL 144.3.2005 and NMKL 125.4.2005, respectively. In all trials, numbers of colonies were transformed into logarithmic numbers (log 10).

2.5 Evaluation of temperature distribution in hamburgers during cooking

A separate trial was made to investigate the temperature distribution in three hamburgers, even if consumers who measure the temperature during cooking of hamburgers likely do this at the central core of the hamburger, as was done in this study. The hamburgers were cooked for 6 min, turned and cooked for additional 6 min at a temperature of 180 ± 5°C. The temperature was measured every 10 sec at three spots in the hamburger (the central core, between the core and the edge, and at the edge) only after turning using a 1 cm long temperature probe (Tinytag Flying Lead Thermistor PB-5009-0M6, Intab) connected to a temperature data logger (Tinytag Plus, IP68, Intab). The data on temperature was analysed using the software programme EasyView Pro5.0 (Intab).

2.6 Analyses of colour scores after cooking

To investigate the potential difference between the colour scores in MAP and control hamburgers the npar1way Wilcoxon rank sum test was used using the software program SAS 9.2. A *P*-value ≤ 0.05 was considered significant.

2.7 Relationships between log reduction and Colour score, and log reduction and temperature

Inactivation of *E. coli* O157:H7*gfp*+ after cooking for a given C-score or internal central temperature was variable. To model log reduction as a function of C-score or temperature, distributions were developed to describe the observed variation. Log reduction of *E. coli* O157:H *gfp*+ after cooking was calculated as $-\log_{10}$ of the relative number of surviving *E. coli* O157:H7*gfp*+ (CFU), i.e. $-\log_{10}(N_t/N_0)$, for each MAP and control hamburger. Log reduction was grouped per C-score for each group of hamburger and described using a triangular distribution including the minimum, median and maximum log reduction (Vose, 2008).

To describe the variable log reduction as a function of core temperature within the hamburgers a linear regression was done on data of log reduction between temperatures of 54 and 76 °C using the R statistical and modelling software (R Development Core Team, 2013). *E. coli* O157:H7*gfp*+ levels below the detection limit (<2 log CFU/g) were assumed to be one log CFU. The 95% prediction interval for the fitted line was estimated and these linear equations were used to define a triangular distribution for the log reduction as a function of the measured internal temperature. The linear equations describing the upper and lower limit of the prediction interval was used, as the minimum and maximum log reduction, respectively, and the fitted line as the most likely log reduction.

247 *2.8 Risk of illness using visual inspection or temperature measurement to decide doneness of*
248 *MAP and control hamburgers*
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250 Risk of illness associated with different C-scores (used as a proxy for consumer
251 preferences) were evaluated for MAP and control hamburgers based on log reductions of *E.*
252 *coli* O157:H7*gfp*+ for different C- scores. The hypothesis is that the risk is greater for MAP
253 hamburgers than for controls as the former appear to be done sooner.
254 To investigate this hypothesis and to evaluate the relative impact of MAP and colour
255 assessment, a reference scenario was simulated in which an initial contamination level of 5
256 log CFU *E .coli* O157:H7*gfp*+/hamburger (equivalent to 3 log CFU/g) was assumed. At lower
257 levels of contamination, the relative impact of MAP and meat colour cannot be properly
258 evaluated since heat inactivation may be sufficient and associated risk would be negligible.
259 The relationships between the C-scores and the distribution of log reductions developed in
260 this study (see previous section) were used to estimate the number of surviving *E. coli*
261 O157:H7*gfp*+ for different C-scores. Since inactivation (log reduction) is variable, the
262 distributions for log reduction were used in a stochastic approach to evaluate the distribution
263 of the relative risk in MAP hamburger compared to controls. The dose, *i.e.* surviving *E. coli*
264 O157:H7*gfp*+ per hamburger, was used as input to an exponential single-hit dose-response
265 model: $p_{illness}$=1-(1-*r*)^dose, where *r* is the probability for illness from a single bacterium
266 (Delignette-Muller and Cornu, 2008). The probability for illness in an adult is modelled and
267 an *r*-value of 0.00113 was used (Strachan et al., 2005). This was done for MAP and control
268 hamburgers, respectively and the relative risk is presented as the $R_{MAP}/R_{control}$ to indicate the
269 increased risk per C-score associated with MAP hamburger. The scenarios were simulated
270 using the Monte Carlo simulation software @Risk (Palisade Corporation, USA) and Latin
271 Hypercube sampling. Each simulation was run using 10.000 iterations.

To illustrate the impact of variable log reduction of *E. coli* O157:H7*gfp*+ at different final internal hamburger temperatures log reductions at temperatures between 70 and 76 °C was simulated using the relationship developed based on our experimental setup (Equation 1).

3. Results

3.1. Experimental conditions

Analyses at the consume-by-date showed that levels of *Enterobaceriacae* ranged from 3.9 to 5.6 log CFU in MAP hamburger and from 2.8 to 4.5 log CFU in control hamburger. The higher levels of *Enterobaceriacae* in MAP hamburgers reflect that the meat had been stored for eight days before analyses at consume-by-date, whereas control hamburgers were minced at retail and analysed on the same day. Levels of *E. coli* were below detection (<2 log CFU/g) in all hamburgers.

Initial measurements of temperature at different locations within the hamburgers showed that the mean temperature difference within a hamburger for all three trials was 5.0°C (SD 1.5 C°), with a minimum temperature difference of 1.8 C° and a maximum of 11.2°C. To mimic consumer behaviour it was decided to monitor temperature only in the central core during the experiments and evaluate the effect of variable temperatures on log reductions within hamburgers by simulation.

For 50% of all MAP and control hamburgers the highest temperature was reached during post-cooking. Only hamburgers cooked ≤ 5 min reached the highest temperature during cooking.

3.2. Colour scores in MAP hamburger and control hamburger

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298 The C-scores for all cooking times ≥ 3 min was higher (*P*=0.0003) for MAP hamburgers
299 compared with control hamburgers, which showed that PMB occurs in the former (Table 2).
300 In MAP hamburgers a maximum mean C-score of 5 was reached after 8 min, whereas the
301 highest mean C-score registered for a control hamburger was 4.3 after 13 min cooking. When
302 using the TCC-score a maximum mean score of 5.0 and 4.7 was reached after 11 and 13 min
303 cooking for MAP and control hamburger, respectively. There was no significant difference
304 (*P*=0.11) in TCC-score between MAP and control hamburgers. The effect of PMB is thus
305 reduced if texture and clarity of meat juice is included in the doneness evaluation of
306 hamburgers.

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308 *3.3. Relationship between E. coli* O157:H7*gfp+ reduction, Colour score and temperature*

309

310 In total, 4 and 22 MAP hamburgers reached a C-score of 4 and 5, respectively (Fig. 1). For
311 control hamburgers 13 and 3 hamburgers reached the corresponding scores. At the C-score 5
312 in MAP hamburgers levels of *E. coli* O157:H7*gfp*+ varied between 0 and 5.9 log CFU, and
313 the maximum core temperature between 60.3°C and 82.6°C (Fig. 1, Fig. 2). The temperature
314 of 60.3°C was reached after 6 min cooking (Table 2). In control hamburgers, the levels of *E.*
315 *coli* O157:H7*gfp*+ for the C-score 5 were between 3.1 and 3.8 log CFU and the temperature
316 was between 80.5°C and 82.9°C.

317 Mean levels of *E. coli* O157:H7*gfp*+ per cooking time were for 9 of 12 cooking times
318 higher in MAP hamburgers whereas internal temperatures were higher in all but one control
319 (Fig. 3 A and B).

Relationships between log reduction at each C-score for MAP and control hamburgers were developed assuming a triangular distribution (Table 3). For all C-scores log reduction was lower for MAP hamburgers compared with control hamburgers.

3.4. Relative risk of illness of MAP hamburgers using Colour score to decide doneness

When evaluating the potential impact of PMB on risk, it was shown that the risk of illness was higher for consumption of contaminated MAP hamburgers for all C-scores compared with the controls (Table 4). As expected, for both MAP and control hamburgers the absolute risk decreased with an increase in C-score preference. However, since absolute risk estimations are associated with great uncertainties the impact was evaluated as relative risk. The predicted relative risk for MAP hamburgers for C-scores of 1-3 was less than four. However, for C-score 4 the relative risk for MAP hamburgers was 297 times greater than for controls. The relative risk could not be estimated for the highest C-score since there was no risk associated with control hamburgers at this score. Thus, the relative impact of PMB increased with increasing C-score.

3.5. Evaluation of log reduction at different recommended internal temperatures

To describe the variable log reduction as a function of the measured central core temperature the linear equations illustrated in Fig. 4 were developed. The lines in the figure represent the best fit of a linear regression and the upper and lower limits of the log reduction 95 % prediction interval. For comparison a relationship previously reported (Cassin et al., 1998), and based on data in (Juneja et al., 1997), is also shown in Fig. 4.

To evaluate the predicted log reduction at different recommended core temperatures, the linear relationships in Fig. 4 were used to simulate minimum, most likely, and maximum log reduction of *E. coli* O157:H7*gfp*+ at different temperatures using the following Triangular(min; most likely; max) distribution:

RiskTriang(-12.094 + 0.209*T;-9.425 + 0.205*T;-6.756 + 0.201*T) Eq (1)

In Table 5, the mean, 5^{th} and 95^{th} percentiles of the simulated log reduction at different temperatures are shown. Estimated log reductions ranged between 4.9 and 6.2 for temperatures between 70 and 76°C and there was a variation in log reduction of approx. 3.3 between the 5th and 95th percentile for all temperatures.

4. Discussion

Results from this study support other findings showing that MAP hamburgers develop PMB (Hague et al., 1994; John et al., 2004; Seyfert et al., 2004; Sorheim and Hoy, 2013). In the present study the MAP hamburgers had a well done appearance at a core temperature of 60.3°C, which is similar to results reported in other studies (Hunt et al., 1999; Rossvoll et al., 2014). The effect of PMB combined with results from studies showing that between 20 and 43% of consumers prefer undercooked hamburger (Altekruse et al., 1999; Lyon et al., 2000; Phang and Bruhn, 2011; Rossvoll et al., 2014) emphasise the health risks MAP hamburgers might constitute if doneness is based only on visual judgement. In these cases the core temperatures may be too low to inactivate pathogenic bacteria.

To ensure the safety of hamburgers and avoid foodborne illness a core temperature of 71.1°C in hamburgers is recommended (FDA, 2011). However, most consumers (27-83%) determine hamburger doneness based on colour of the meat, fewer by colour of the meat juice (11-38%) and texture of the meat (16%) whereas only a few percentages (0.2-6%) use a

thermometer (Mahon et al., 2006; Phang and Bruhn, 2011; Rossvoll et al., 2014). Rossvoll et al. also reported that 83% of participants in a food safety survey did not know what the core temperature in a hamburger should be. This together with the poor compliance of the recommendation to use a thermometer makes the advice to use a thermometer less effective. The prescribed cooking time of hamburgers in recipes available on the internet varies from, for example, two to six minutes on both sides depending on if the hamburgers should be medium rare or well done. Some recipes mention that the hamburger should be cooked well-done, but surprisingly many do not. Based on results from this study, these recommendations are not safe, particularly if minced meat packaged in modified atmosphere that develops PMB is used.

In the present study, we used a previously developed colour score (in this study called the C-score) based on the interior colour of the hamburger to judge doneness (Hunt et al., 1999). In addition to this, we developed an alternative score (the TCC-score) based on visual inspection of the interior colour of the hamburger, texture of the meat when cutting the hamburger in halves and clarity of the meat juice, to investigate if this score would reduce the effect of PMB. For example, a thread like chewy texture and red meat juice was evaluated as the hamburger being not well done even if the colour had a well done appearance.

This study showed that the use of the developed TCC-score may reduce the effect of PMB, which would have positive effects on food safety. However, the TCC score presented in this study was developed and tested within the present study yielding promising results, but will need more evaluation and verification in future studies.

Extensive temperature variation within hamburgers observed in this study has also been reported by Rhee et al. (2003). They suggest that internal temperature differences may explain prolonged survival of *E. coli* O157:H7 as parts of the hamburger may not reach temperatures high enough to inactivate bacteria despite a high central core temperature. This suggestion is

supported by the variable reduction of *E. coli* O157:H7*gfp*+ observed in the present study and described in Equation 1. In the study of Rhee et al. (2003), it is shown that the temperature variations could be due to cooking practices, such as cooking on one or two sides and number of turnings of the patties (the patties were turned at 30 s intervals). It is possible that internal temperature variations can explain why portions of some control hamburgers in this study remained slightly pink at central core temperatures exceeding 71C°. This also agrees with findings showing that the internal colour of a hamburger remained somewhat red even at a cooking temperature of 79°C (John et al., 2004).

To compare bacterial reduction between MAP and control hamburgers a high inoculation level (9 log CFU/hamburger, equivalent to 7 log CFU/g) was used, which is according to recommendations given for inactivation studies (NACMCF, 2010). Our results showed that the mean bacterial reduction of *E. coli* O157:H7*gfp*+ in MAP hamburger after cooking to 71°C was 5.1 log, which corresponds with other reported results (Juneja et al., 1997; Rhee et al., 2003; Rossvoll et al., 2014). It is interesting to note that although the experimental set-up was different from that reported by Cassin et al. (1998), the relationship between temperature and log reduction was similar. Taking the observed variation in log reductions and temperature into consideration it is clear that reduction even at a central core temperature of 71 °C (recommended temperature) may not, depending on initial contamination levels, be enough to ensure safety for hamburger consumers.

The log reduction of *E. coli* O157:H7*gfp*+ was lower in MAP hamburgers compared with control hamburgers for all C-scores, which most likely can be explained by PMB resulting in shorter cooking times for MAP hamburgers. It was also observed that the same cooking times resulted in slightly higher core temperatures in the control hamburgers, which may be difficult to explain. All meat included was minced with the same diameter and had, according to the manufacturer, the same fat content. However, during the preparation of the hamburgers there

appeared to be certain differences in texture during manual handling, which may be the reason for the variation in heat penetration in MAP hamburgers. However, as we used the C-score as an evaluation of doneness instead of cooking time, this observation is not believed to invalidate the conclusion on the effect of lower log reduction in MAP hamburgers displaying PMB. Further the results indicate that advice in terms of cooking times that will achieve similar log reductions in MAP as in control hamburgers, would under the present conditions be more than 8 minutes.

The inactivation of *E. coli* O157:H7*gfp*+ in hamburgers observed in the present study was lower compared to what is indicated by predictions of the inactivation model in ComBase predictive models (ComBase, 2013). For instance, the time for a one-log reduction (D-value) at 64.5°C, the maximum temperature of the ComBase model, is 0.26 min. This translates to 3.8 log reductions per minute which is more than observed in our study. The model in ComBase is based on inactivation studies carried out in liquid broth and it can be suggested that temperature is more homogenously distributed in broth than within hamburgers. Another reason could be that *E. coli* O157:H7 is more heat-resistant in ground beef with a high fat content (Ahmed et al., 1995; Smith et al., 2001) and a third reason is a combination of both. van Asselt and Zwietering (2006) estimated D-values and z-values for several bacteria based on a systematic approach using published thermal inactivation data in different matrixes. Based on the variable inactivation they reported D-values for *E. coli* (mean value and upper 95% prediction interval, respectively) which would correspond to 0.7 and 10 minutes at 64.5°C and 0.2 and 3 minutes at 70°C which are more in line with observations in the present study.

Eating uncooked/very pink hamburgers, either made from meat minced at retail or from minced meat packaged in modified atmosphere, may constitute safety risks. However, when comparing MAP and control hamburgers the effect of PMB would be small for uncooked to

very pink hamburgers (C-scores 1-3) since the additional increase in risk for illness due to PMB was small (<4) compared with the substantial risk associated with control hamburgers with this heating regime. However, for hamburgers that were slightly pink (C-score 4) the relative risk for illness due to PMB was almost 300 times higher for MAP hamburgers than the control. It should be pointed out that although there was a huge increase in the relative risk of illness between the C-scores 3 and 4 the absolute risk is greater at C-score of 3 and that the potential health impact will depend on the initial levels of *E. coli* O157:H7 contamination. No risk could be calculated for the well done hamburgers due to complete inactivation in the control hamburgers.

The magnitude and range, 0.03 to 0.5, of estimated risks for gastrointestinal illness for MAP hamburgers estimated in the present study was based on a high initial contamination. These risks are, however, similar to the estimated risk for HUS among children under the age of 5 assuming contamination levels from a French outbreak Delignette-Muller and Cornu (2008). Interestingly, the relative increase in HUS risk going from a preference from rare, medium to well-done (19 times, 0.113/ 0.006) is similar to the estimated gastro-intestinal illness risk going from a preference from C-score 1 to 5 (17 times, 0.5/0.03).

Stressed induced treatments, such as storage of meat until consume-by date, may have an effect on subsequent survival of bacteria during cooking (Shen et al., 2014), but was not evaluated in the present study since bacteria were inoculated into hamburgers just before cooking. The stability of the GFP plasmid is a crucial factor in this study as the result on bacterial survival is based on enumeration of gfp-marked *E. coli* O157:H7*gfp*+ bacteria. The strain included has been used regularly at the Swedish University of Agricultural Sciences since 2009 and there have been no reports of bacteria losing the plasmid (personal communication, B. Alsanius, SLU, Sweden). In a previous study it has been found that the GFP plasmid was stable in *E. coli* O157:H7 and that the plasmid had insignificant effect on

growth of the bacteria (Ma et al., 2011). It has also been shown that *gfp*-tagged cells remain fluorescent following stress, such as starvation, and that they are detectable in all growth phases (Tombolini et al., 1977; Lowder et al., 2000).

The advantage with minced meat packaged in high oxygen packages is that the colour of the meat remains red, which is appealing for the consumer, and that it prolongs shelf life. However, consumers need to be informed about PMB to deal with the increased risk for exposure of pathogenic bacteria, such as of *E. coli* O157:H7, due to risks of insufficient core temperatures to ensure bacterial inactivation when evaluating doneness of MAP hamburgers.

5. Conclusion

The present results support previous findings that MAP hamburgers appear to be cooked at a lower temperature compared with fresh minced meat and that this may be associated with increased risk of illness. Under the present conditions up to a three-hundredfold increased relative risk was estimated. From a food safety perspective optimal behavior from the consumers would be a preference for well-done hamburgers and to use thermometers to control that recommended internal temperatures are reached. However, acknowledging that most consumers do not use a thermometer, our results indicate that basing decisions on doneness not only on meat color but also on meat texture and the clarity of meat juices may improve safety. Further, observed reduction of *E. coli* O157:H7*gfp*+ was variable between hamburgers and results indicate that food safety concerns may remain even when consumers use a thermometer and cook hamburgers to recommended central core temperatures. Thus, information directed at consumers of risks associated with cooking of MAP hamburgers is needed and the present results may help to inform such efforts.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Ahmed, N.M., Conner, D.E., Huffman, D.L., 1995. Heat-resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. Journal of Food Science, 60, 606-610.

Alam, M., Ahlström, C., Burleigh, S., Olsson, C., Ahrné, S., El-Mogy, M.M., Molin, G., Jensén, P., Hultberg, M., Alsanius, B.W., 2014. Prevalence of *Escherichia coli* O157:H7 on spinach and rocket as affected by inoculum and time to harvest. Scientia Horticulturae, 165, 235-241.

Altekruse, S.F., Yang, S., Timbo, B.B., Angulo, F.J., 1999. A multi-state survey of consumer food-handling and food-consumption practices. American Journal of Preventive Medicine, 16, 216-221.

Bell, B.P., Goldoft, M., Griffin, P.M., Davis, M.A., Gordon, D.C., Tarr, P.I., Bartleson, C.A., Lewis, J.H., Barrett, T.J., Wells, J.G., Baron, R., Kobayashi, J., 1994. A Multistate Outbreak of *Escherichia coli* O157:H7 associated bloody diarrhea and hemolytic-uremic-syndrome from hamburgers - the Washington experience. Journal of the American Medical Association, 272, 1349-1353.

Shen, C., Geornaras, I., Belk, K.E., Smith, G.C., Sofos, J.N., 2011. Thermal inactivation of acid, cold, heat, starvation, and desiccation stress-adapted *Escherichia coli* O157:H7 in moisture-enhanced nonintact beef. Journal of Food Protection, 74, 531-538.

Cassin, M.H., Lammerding, A.M., Todd, E.C., Ross, W., McColl, R.S., 1998. Quantitative risk assessment for *Escherichia coli* O157:H7 in ground beef hamburgers. International Journal of Food Microbiology, 41, 21-44.

ComBase, 2013. (http://www.combase.cc/index.php/en/). Accessed January 28, 2015.

522 Delignette-Muller, M.L., Cornu, M., 2008. Quantitative risk assessment for *Escherichia coli* O157:H7 in frozen
523 ground beef patties consumed by young children in French households. International Journal of Food
524 Microbiology, 128, 158-164.
525 ECDC, EFSA, 2011. Joint Technical Report: Shiga toxin/verotoxin-producing *Escherichia coli* in humans, food
526 and animals in the EU/EEA, with special reference to the German outbreak strain STEC O104 . Stockholm,
527 Sweden: ECDC.
528 FDA, 2011. Food facts: Safe Food Handling.
529 (http://www.fda.gov/downloads/food/foodborneillnesscontaminants/ucm257049.pdf). U.S. Food and Drug
530 Administration. Accessed January 28, 2015.
531 FOHM, 2015. (http://www.folkhalsomyndigheten.se). Accessed June 15, 2015.
532 Hague, M.A., Warren, K.E., Hunt, M.C., Kropf, D.H., Kastner, C.L., Stroda, S.L., Johnson, D.E., 1994.
533 Endpoint temperature, internal cooked color, and expressible juice color relationships in ground beef patties.
534 Journal of Food Science, 59, 465-470.
535 Hunt, M.C., Sorheim, O., Slinde, E., 1999. Color and heat denaturation of myoglobin forms in ground beef.
536 Journal of Food Science, 64, 847-851.
537 Hussein, H.S., 2007. Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and
538 their products. Journal of Animal Science, 85, 63-72.
539 John, L., Cornforth, D., Carpenter, C.E., Sorheim, O., Pettee, B.C., Whittier, D.R., 2004. Comparison of color
540 and thiobarbituric acid values of cooked hamburger patties after storage of fresh beef chubs in modified
541 atmospheres. Journal of Food Science, 69, 608-614.
542 Juneja, V.K., Snyder, O.P., Williams, A.C., Marmer, B.S., 1997. Thermal destruction of *Escherichia coli*
543 O157:H7 in hamburger. Journal of Food Protection, 60, 1163-1166.
544 Karmali, M.A., 2004. Infection by Shiga toxin-producing *Escherichia coli*: an overview. Molecular
545 Biotechnology**,** 26, 117-122.
546 Keithlin, J., Sargeant, J., Thomas, M.K., Fazil, A., 2014. Chronic sequelae of *E. coli* O157: systematic review
547 and meta-analysis of the proportion of *E. coli* O157 cases that develop chronic sequelae. Foodborne
548 Pathogens and Disease, 11, 79-95.
549 Lowder, M., Unge, A., Maraha, N., Jansson, J.K., Swiggett, J., Oliver J.D., 2000. Effect of Starvation and the
550 Viable-but-Nonculturable State on Green Fluorescent Protein (GFP) Fluorescence in GFP-Tagged
551 *Pseudomonas fluorescens* A506. Applied and Environmental Microbiology, 66, 160-3165.

552 Lyon, B.G., Berry, B.W., Soderberg, D., Clinch, N., 2000. Visual color and doneness indicators and the
553 incidence of premature brown color in beef patties cooked to four end point temperatures. Journal of Food
554 Protection, 63, 1389-1398.
555 Ma, L., Zhang, G.D., Doyle, M.P., 2011. Green fluorescent protein labeling of *Listeria*, *Salmonella*, and
556 *Escherichia coli* O157:H7 for safety-related studies. PloS One, 6:e18083.
557 Mahon, D., Cowan, C., Henchion, M., Fanning, M., 2006. Food-handling practices of Irish beef consumers.
558 Journal of Food Safety, 26, 72-81.
559 McMillin, K.W., 2008. Where is MAP going? A review and future potential of modified atmosphere packaging
560 for meat. Meat Science, 80, 43-65.
561 NACMCF, 2010. Parameters for determining inoculated pack/challenge study protocols. National Advisory
562 Committee on Microbiological Criteria for Foods. Journal of Food Protection, 73, 140-202.
563 Nauta, M.J., Evers, E.G., Takumi, K., Havelaar, A.H., 2001. Risk assessment of Shiga-toxin producing
564 *Escerichia coli* O157 in steak tartar in the Netherlands. Report 2578510036/2001. Rijksinstituut voor
565 Volksgezondheid en Milieu, Biltoven, the Netherlands.
566 Pennington, H., 2010. *Escherichia coli* O157. Lancet. 376, 1428-1435.
567 Phang, H.S., Bruhn, C.M., 2011. Burger preparation: What consumers say and do in the home. Journal of Food
568 Protection, 74, 1590-1781.
569 Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M., Swerdlow, D.L., 2005. Epidemiology of *Escherichia coli*
570 O157:H7 outbreaks, United States, 1982-2002. Emerging Infectious Diseases, 11, 603-609.
571 Rhee, M.S., Lee, S.Y., Hillers, V.N., McCurdy, S.M., Kang, D.H., 2003. Evaluation of consumer-style cooking
572 methods for reduction of *Escherichia coli* O157 : H7 in ground beef. Journal of Food Protection, 66, 1030-
573 1034.
574 Rossvoll, E., Sorheim, O., Heir, E., Moretro, T., Olsen, N.V., Langsrud, S., 2014. Consumer preferences,
575 internal color and reduction of shigatoxigenic *Escherichia coli* in cooked hamburgers. Meat Science, 96, 695-
576 703.
577 Seyfert, H.M., Mancini, R.A., Hunt, M.C., 2004. Internal premature browning of cooked ground beef patties
578 from high-oxygen modified-atmosphere packaging. Journal of Food Science, 69, 721-725.
579 Signorini, M., Tarabla, H., 2009. Quantitative risk assessment for verocytotoxigenic *Escherichia coli* in ground
580 beef hamburgers in Argentina. International Journal of Food Microbiology, 132, 153-161.

581 Smith, S.E., Maurer, J.L., Orta-Ramirez, A., Ryser, E.T., Smith, D.M., 2001. Thermal inactivation of *Salmonella*
582 spp., *Salmonella typhimurium* DT104, and *Escherichia coli* O157 : H7 in ground beef. Journal of Food
583 Science, 66, 1164-1168.
584 Sorheim, O., Hoy, M., 2013. Effects of food ingredients and oxygen exposure on premature browning in cooked
585 beef. Meat Science, 93, 105-110.
586 Strachan, N.J., Doyle, M.P., Kasuga, F., Rotariu, O., Ogden, I.D., 2005. Dose response modelling of Escherichia
587 coli O157 incorporating data from foodborne and environmental outbreaks. International Journal of Food
588 Microbiology, 103, 35-47.
589 R Development Core Team, 2013, R: A language and environment for statistical computing. R Foundation for
590 Statistical Computing, Vienna, Austria.
591 Teunis, P., Takumi, K., Shinagawa, K., 2004. Dose response for infection by *Escherichia coli* O157:H7 from
592 outbreak data. Risk Analysis, 24, 401-407.
593 Tombolini, R., Unge, A., Davey, M.E., de Bruijn, F. J., Jansson, J.K., 1997. Flow cytometric and microscopic
594 analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiology Ecology, 22, 17-28.
595 van Asselt, E.D., Zwietering, M.H., 2006. A systematic approach to determine global thermal inactivation
596 parameters for various food pathogens. International Journal of Food Microbiology, 107, 73-82.
597 Vose, D., 2008.Risk Analysis - A quantitaitve guide, third ed. John Wiley & Sons, Ltd, Chichester, England.

598 **Captions:**

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Table 1

Description of the TCC-score based on meat Texture and Colour as well as meat juice Clarity after cooking hamburgers. The score is based on the sum of the three sub scores.

Sub scores included in the TCC score				
Interior colour of the hamburger [1]	Texture of the hamburger	Clarity of the meat juice	Sum of the sub-scores[1]	TCC score
1= uncooked (dark red to purple)	1= raw (high degree c chewiness and of thread like texture)	1= bright red	3	1= uncooked
2=bright red	2= medium degree of chewiness and of thread like texture	2= pink	4-5	2= rare
3= very pink	3= no evidence of chewiness and of thread like texture.	3= clear with no evidence of pink	6-8	3= medium rare
4= slightly pink	-	4= no meat juice remaining after cooking	9-10	4= medium
5= tan with no evidence of pink	-	-	11-12	5= well done

[1]Hunt, M.C. et al. 1999. Journal of Food Science, 64, 847-851

Table 2

Observed C-scores (based on internal colour of the hamburger) and TCC scores (based on texture of the meat, internal colour of the hamburger and the meat and clarity of the meat juice scores) after cooking hamburgers made of minced meat packaged in modified atmosphere (MAP hamburger) and hamburgers made of meat minced at retail (control hamburger).

		Cooking time (min)											
		2	3	4	5	6	7	8	9	10	11	12	13
MAP hamburger													
C-score[1]	Trial 1	1	2	3	3	4	4	5	5	5	5	5	5
	Trial 2	1	1	2	4	5	5	5	5	5	5	5	5
	Trial 3	1	2	3	4	5	5	5	5	5	5	5	5
	Mean	**1.0**	**1.7**	**2.7**	**3.7**	**4.7**	**4.7**	**5.0**	**5.0**	**5.0**	**5.0**	**5.0**	**5.0**
TCC-score[2]	Trial 1	1	2	2	3	3	4	4	4	5	5	5	5
	Trial 2	1	1	2	3	4	4	4	5	5	5	5	5
	Trial 3	1	2	2	3	4	3	4	4	4	5	5	5
	Mean	**1.0**	**1.7**	**2.0**	**3.0**	**3.7**	**3.7**	**4.0**	**4.3**	**4.7**	**5.0**	**5.0**	**5.0**
control hamburger													
C-score	Trial 1	1	2	2	3	3	3	4	4	4	5	5	5
	Trial 2	1	1	2	2	2	3	4	4	4	4	4	4
	Trial 3	1	1	2	2	2	3	3	3	4	4	4	4
	Mean	**1.0**	**1.3**	**2.0**	**2.3**	**2.3**	**3.0**	**3.7**	**3.7**	**4.0**	**4.3**	**4.3**	**4.3**
TCC-score	Trial 1	1	2	2	3	3	3	4	4	4	5	5	5
	Trial 2	1	2	3	3	3	3	4	4	4	4	4	4
	Trial 3	1	1	2	2	2	3	3	3	4	4	4	5
	Mean	**1.0**	**1.7**	**2.3**	**2.7**	**2.7**	**3.0**	**3.7**	**3.7**	**4.0**	**4.3**	**4.3**	**4.7**

[1]C-score 1= uncooked (dark red to purple), 2= bright red, 3= very pink, 4= slightly pink and 5= tan with no evidence of pink.

[2] TCC-score 1= uncooked, 2= rare, 3= medium rare, 4= medium and 5= well done

Table 3

Observed log reduction (-log10(N_t/N_0)) of *E. coli* O157:H7*gfp*+ after cooking per colour score (C-score) for hamburgers made of minced meat packaged in modified atmosphere (MAP hamburger) and from meat minced at retail (control hamburger).

		Log reduction of *E. coli* O157:H7*gfp*+ after cooking (log_{10})		
C- score [1]	hamburger	median	min	max
1	MAP	2.2	2.1	2.3
	control	3.2	2.2	3.3
2	MAP	2.3	2.3	2.3
	control	3.4	2.4	3.7
3	MAP	2.5	2.4	2.9
	control	4.0	2.7	5.4
4	MAP	3.1	2.6	3.4
	control	5.7	4.6	8.0
5	MAP	4.8	3.1	8.0
	control	5.7	5.2	5.9

[1]C-score 1= uncooked (dark red to purple), 2= bright red, 3= very pink, 4= slightly pink and 5= tan with no evidence of pink.

Table 4

Mean risk and mean relative risk of illness per serving of one hamburger following consumption of *E. coli* O157:H7*gfp*+ contaminated hamburgers (5 log CFU/hamburger, equivalent to 3 log CFU/g) made from meat packaged in modified atmosphere (MAP hamburger) and from meat minced at retail (control hamburger) based on interior colour of the hamburger (C-score)

C- score [1]	Mean risk of illness		Mean relative risk of illness
	MAP hamburger	control hamburger	MAP/control hamburger
1	0.5	0.3	2.2
2	0.4	0.2	2.6
3	0.3	0.1	3.5
4	0.1	0.0004	297
5	0.03	0	NA

[1]C- score 1= uncooked (dark red to purple), 2= bright red, 3= very pink, 4= slightly pink and 5= tan with no evidence of pink.

NA= not available

Table 5

Simulated variable *E. coli* O157:H7*gfp*+ log reduction in hamburgers made from contaminated meat packaged in modified atmosphere depending on final hamburger core temperature using equation 1.

Log CFU *E. coli* O157:H7*gfp*+ reduction	Final central internal temperature						
	70°C	71°C	72°C	73°C	74°C	75°C	76°C
Mean	4.9	5.1	5.3	5.5	5.7	6.0	6.2
5%; 95% percentile	3.3; 6.6	3.5; 6.8	3.7; 7.0	3.9; 7.2	4.1; 7.4	4.3; 7.6	4.5; 7.8

Fig 1. Levels of *E. coli* O157:H7*gfp+* (log CFU/hamburger) in relation to the interior colour (C-score) in hamburgers made from minced meat packaged in modified atmosphere (MAP hamburger) and from meat minced at retail (control hamburger). C-score 1: dark red to purple (MAP: N=4, control: N=5), 2: bright red (MAP: N=3, control: N=8), 3: very pink (MAP: N=3, control: N=7), 4: slightly pink (MAP: N=4, control: N=13) and 5: tan with no evidence of pink (MAP: N=22, control: N=3).

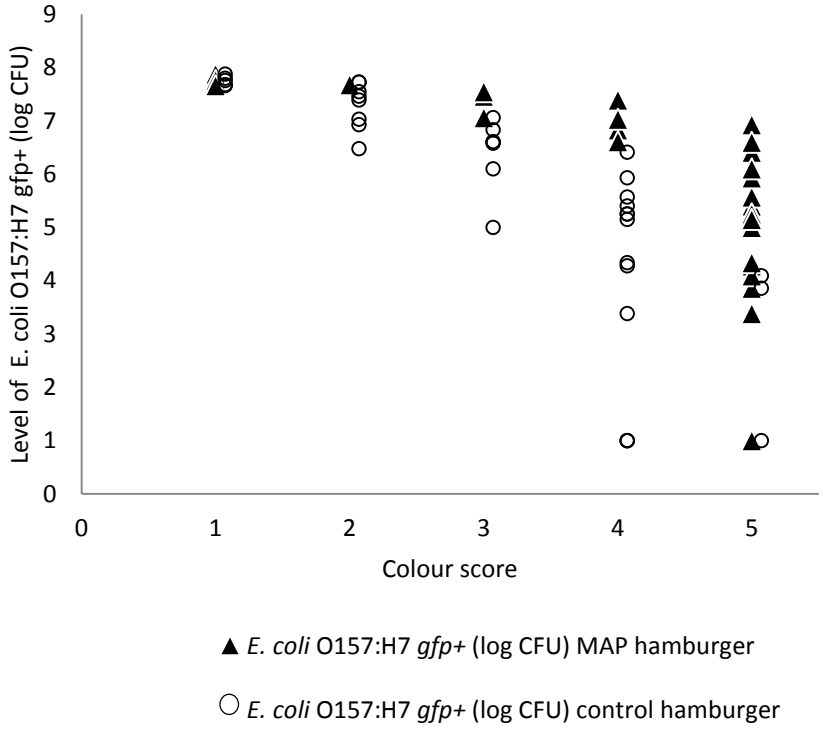
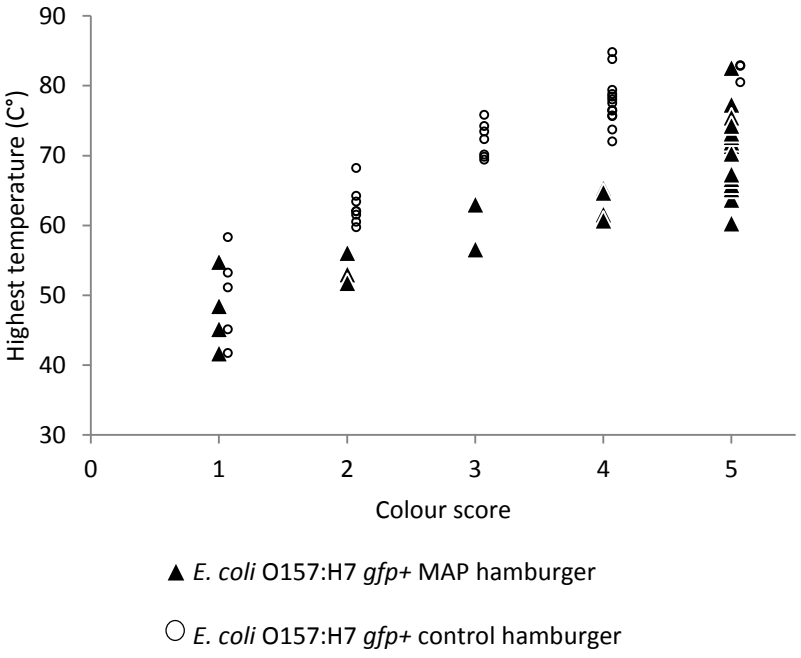


Fig 2. Maximum central core temperature in relation to interior colour (C-score) in a hamburger made from minced meat packaged in modified atmosphere (MAP hamburger) and from meat that were minced at retail (control hamburger). C-score 1: dark red to purple (MAP: N=4, control: N=5), 2: bright red (MAP: N=3, control: N=8), 3: very pink (MAP: N=3, control: N=7), 4: slightly pink (MAP: N=4, control: N=13) and 5: tan with no evidence of pink (MAP: N=22, control: N=3).



688 **Fig 3.** A) Mean levels of *E. coli* O157:H7*gfp*+ (log CFU) and, B) mean of the highest core temperatures (mean
689 of three trials) in relation to cooking time in hamburgers made from minced meat packaged in modified
690 atmosphere (MAP hamburger) and from meat minced at retail (control hamburger). Error bars represent standard
691 errors.
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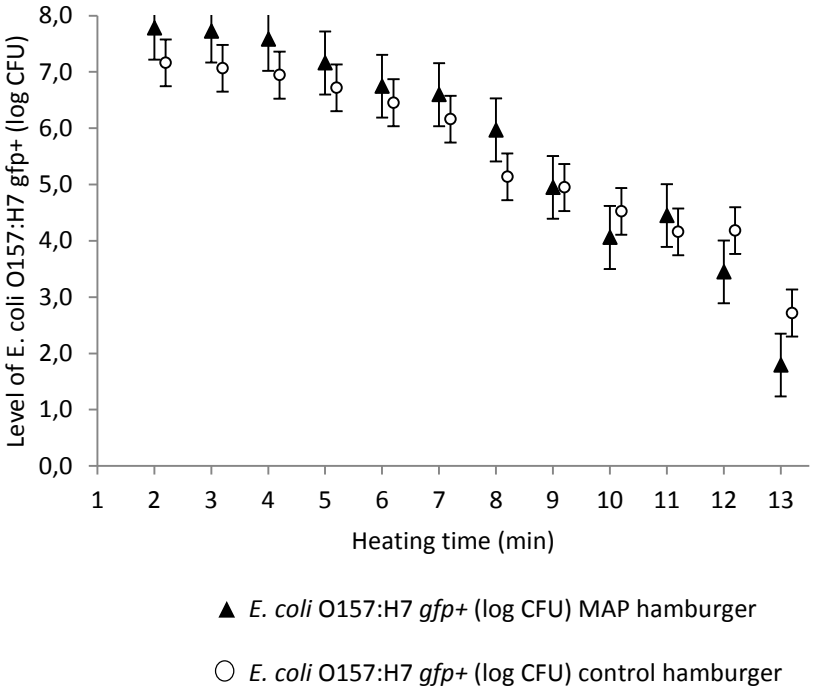
693 A



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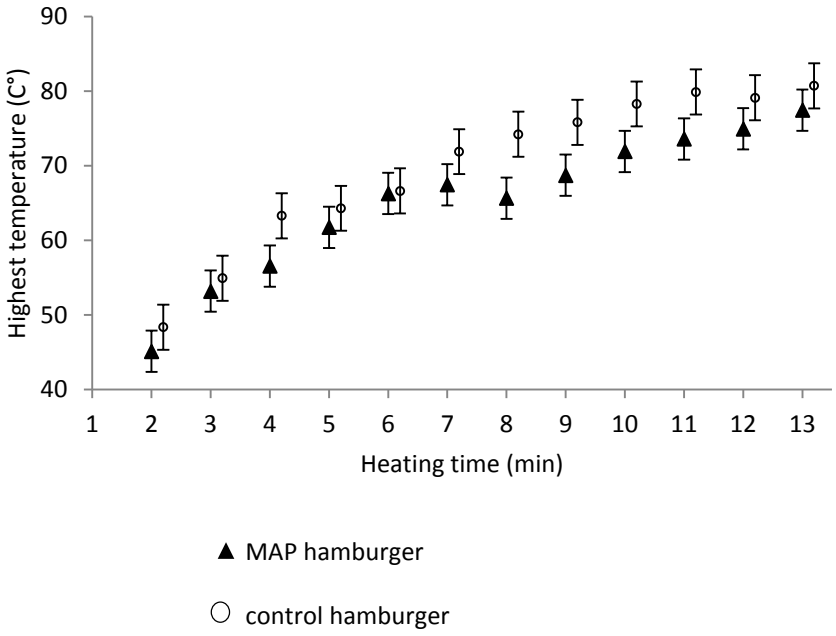

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Fig 4. Relationship between observed central core temperature of MAP hamburgers and log reduction of *E. coli* O157:H7*gfp*+. The solid line is the best fit of a linear regression to the data (black circles); log Reduction = 0.205 * T – 9.425, R^2=0.59. The dotted lines represent the upper and lower prediction intervals, and the striped line is a relationship reported in Cassin *et al*., (1998) for comparison.