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The development of chlorophyll-based markers in poultry diets to aid detection of fluorescent fecal contamination

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ABSTRACT Incidents of foodborne illness associated with consuming undercooked or raw chicken are often linked to 2 causative pathogens: *Campylobacter* spp. or *Salmonella* spp. Numerous studies have shown that contamination of carcasses results when pathogens are transferred from the intestinal tract or fecal material on feet and feathers to the dressed carcass. Ultraviolet spectral imaging to detect surface fecal and ingesta contamination on poultry carcasses may provide a solution to aid detection. However, poultry diets do not provide sufficiently high levels of natural fluorophores for this system to be reliable. This study investigated the potential of chlorophyll-based feed additives to improve fluorescence of the feces and narrow the excitation and emission wavelengths to aid in the development of a simple visualization system. Twenty-four hens (*Gallus gallus domesticus*) were allocated at random to 1 of 4 treatments: control (C, no marker), Zn chlorophyllin, Mg chlorophyllin, or Fe chlorophyllin. All markers were incorporated into mash before pelleting at a rate

of 1 g/kg of DM. The experiment consisted of two 4 × 4 Latin squares with each period consisting of 2 wk. Feces were collected and extracted in acetone:water (50:50; vol/vol) with fecal fluorescence emission spectra determined using a Jasco FP-6200 Spectrofluorometer with excitation at 382 nm. A main peak evolved at wavelength 670 nm with the total area under the peak used as fluorescence intensity. Following 7 d of marker supplementation, the 3 markers improved the fluorescence intensity by ×14.8, 12.8, and 6.9 for Fe, Mg, and Zn chlorophyllin, respectively, compared with the control. The addition of feces containing Mg chlorophyllin to chicken carcass increased detection of the feces compared with feces with no marker. Also, due to the plain background of chicken skin, a simple image at 675 nm with appropriate thresholds would allow detection of contaminated carcasses at the current slaughter line speed without the need of expensive hyperspectral imaging.

Key words: fecal contamination, fluorescent visualization, chlorophyllin, hazard analysis and critical control points, dietary marker

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INTRODUCTION

The effects of food poisoning costs European countries over €2.4 billion per year, with disease burden of campylobacteriosis estimated to affect 0.35 million disability-adjusted life years (EFSA, 2011). *Campylobacter* alone is responsible for over 80 deaths per year within the United Kingdom. A significant portion (over 65%) is derived from *Campylobacter*-infected chicken. Because the greenhouse gas emissions of poultry are around a quarter of that from beef and lamb, and half the level from pork (Garnett, 2009), the global market for poul-

try meat will continue to grow. Because *Campylobacter* is a global issue and trade in fresh chicken increases, contaminated carcasses will come under greater regulatory pressure and there is a need to reduce pathogens effectively in all agricultural units both at the producer farm level and the slaughterhouse processing level.

Although several factors can influence bacterial contamination of chicken carcasses, one of the leading causes is fecal contamination at the processing plant (Bolder, 2007). Park et al. (2007, 2011) described a hyperspectral approach, whereas Cho et al. (2009) described a laser-induced fluorescence imaging approach to detect fecal contamination on poultry carcasses. Although these technologies have been shown to be effective at detecting feces on carcasses, their industry uptake has been hampered because these systems both produce data of huge size, which is not favored for real-time application, especially when advanced hardware

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and software are not available or cost prohibitive. The nature of the poultry diet, where the level of fluorophores in the feces is low, appears to preclude the use of more real-time UV front-face fluorescence imaging. Front-face fluorescence has been developed for the detection of fecal contamination on carcasses relying on natural dietary fluorophores in ruminants (Ashby et al., 2003). However, even in ruminants, reliance on basal fecal fluorophores of dietary origin resulted in visualization issues. These related to false negative readings (little fluorescent signal with feces present) when the diet had a low fluorophore concentration (Lee et al., 2010, 2013; Burfoot et al., 2011) and false positives (fluorescent signal in the absence of feces) due to natural carcass fluorophores emitting at the excitation wavelength of the detection system (Lee et al., 2010, 2013; Burfoot et al., 2011). Lee et al. (2010) described how the technology could be improved to reduce false negative and positive readings by supplying a dietary marker based on chlorophyll to improve fluorescence of the feces and narrow the excitation and emission wavelengths.

Jones et al. (2010) demonstrated the potential of chlorophyll-based markers developed for ruminants when fed mixed into layers mash to significantly increase the fluorescence intensity of poultry feces. The current study examined the potential of these markers supplied in pelleted feed to increase fluorescence of the feces and visualize this contamination on the carcass. Such a system could be used to aid in the detection of fecal contamination and gut spill in the abattoir with simple front-face fluorescence detection cameras. This would provide information on the basal level of contamination entering the plant as a part of hazard analysis and critical control points (HACCP) and also an early warning system of evisceration malfunction resulting in gut spill contamination. An on-line score of carcass cleanliness and an early warning of gut spill will be valuable tools to reduce pathogenic bacteria associated with the poultry carcass.

MATERIALS AND METHODS

Birds, Diets, and Experimental Design

All procedures were carried out under the auspices of the Animal Scientific Procedures Act 1987 of Her Majesty's Britannic Government. Twenty-four chicken (*Gallus gallus domesticus*) layers (Brown hybrids) supplied by Joice and Hill Poultry Ltd. (Eye, Peterborough, UK) with a mean BW of 2.05 ± 0.143 kg were blocked according to live weight into 3 blocks. Layers were chosen as the experimental model to supply clean uncontaminated feces from under their perches and also eggs to determine the extent of marker absorption and deposition, without blood sampling. A bird from each block was then randomly allocated to 1 of 8 treatment groups (3 birds per group). These groups were housed in individual pens, which acted as the ex-

perimental unit and were then allocated at random to 1 of 4 treatments: control (no marker): zinc chlorophyllin (Zn), magnesium chlorophyllin (Mg), or Fe chlorophyllin (Fe). The Zn and Fe chlorophyllins were supplied by Advanced Technology and Industrial Co. (Tai Kok Tsui, Kln., Hong Kong). The Mg chlorophyllin was supplied by British Chlorophyll Company Ltd. (Lincoln, Lincolnshire, UK). All markers were confirmed to be 99.9% pure by International Laboratories (San Francisco, CA). The experiment consisted of two 4×4 Latin squares with each period consisting of 2 wk. The first week had no supplementation of marker and acted as the adaptation and wash-out period with all birds receiving ad libitum pelleted layers mash (Farmgate layer mash, BOCM Pauls Ltd., Wherstead, UK) containing the following: wheat (*Triticum* spp.), wheat-feed, barley (*Hordeum vulgare*), soya (*Glycine max*), calcium carbonate, maize (*Zea mays*), distillers dark grains, sunflower (*Helianthus annuus*) extract, vitamin and minerals (vitamins A, D, E, sodium selenite, and copper sulfate at 7,000, 3,000, 15 IU/kg, and 0.25 and 20 mg/kg, respectively), vegetable oils, dicalcium phosphate, salt, methionine, and sodium bicarbonate to provide oil (3.25%), protein (16.0%), fiber (4.75%), organic matter (87.3%), and methionine (0.30%). At the end of the adaptation period, feces and eggs were collected as the blank samples. In the second week, birds received marker pelleted with the mash at a rate of 1 g/kg of DM. On d 7 of supplementation, feces and eggs were collected as previously stated.

Bird Housing and Measurements

Each group of 3 birds was penned in individual units with free access to a nesting area and perch with dropping board, water, and feed troughs. The pens were floored with sawdust and sand. Fecal collection occurred on 2 occasions within each period at the end of the first adaptation week and at the end of the second supplementation week by removing the feces from the dropping board under the perches and storing at -20°C before analysis. Birds were weighed at the start and end of each experimental period, with mean daily intakes monitored during the feeding of the marker. Samples of the pelleted feed (ca. 100 g) were collected for each period and maintained at -20°C before analysis. Eggs from each pen at the end of each week (adaptation and supplementation) were stored at 4°C before analysis.

Fecal and Feed Fluorescence—Spectrofluorometer

The fecal and feed samples were freeze-dried and ground with a Cyclotec 1093 sample mill (Foss, Hillerød, Denmark) to produce a fine powder, of which 0.5 g of DM was then vortexed in a solution of acetone:water (50:50; vol/vol) for 30 s to extract the chlorophyll-based compounds. The samples were centrifuged at $1,200 \times g$

for 10 min (Beckman model J-6B centrifuge, Beckman Coulter Inc., Brea, CA) to obtain a clear supernatant. Following the first extraction, a second extraction was repeated with the same samples and the supernatants were pooled. The supernatants were filtered through a glass microfiber filter paper (GF/A) of 70 mm diameter (Whatman, Buckinghamshire, UK) to remove any insoluble particles. All samples were placed in the dark in an ice box throughout the period of analysis to minimize degradation of the chlorophyll-based compounds before spectrofluorometric analysis. The supernatant obtained from each sample was emptied into a 4.5-mL polystyrene cuvette, 10 mm path length (Fisher Scientific, Kartell, Italy), and fluorescence measurement was carried out using a spectrofluorometer (FP-6200, Jasco Ltd., Essex, UK) with excitation wavelength at 382 nm. The measurement mode was emission, with excitation and emission bandwidth of 5 nm. The response parameter was fast, with data pitch of 1 nm, scanning speed 125 nm/min, and sensitivity option medium. Total area under the 670-nm peak was taken as the measure of fluorescent intensity.

Carcass Visualization— Hyperspectral Camera

The hyperspectral camera was based on a spectral imaging system (V10 E QE, Specim, Teknologiantie 18 A, Oulu, Finland). In our version, the target was replaced by a motorized scanning mirror, which was controlled by a computer (scanning mirror, system integration, and controlling software provided by Gilden Photonics, Clydebank, Glasgow, UK). This allowed the spectrum of each point in an area of interest to be determined by movement of the scanning mirror between acquisitions of successive image lines. The spectral range covered was 377 to 1,000 nm with a resolution of 2.8 nm, 4×4 spectral, and spatial binning was used to reduce image noise and the data volume. This resulted in a 256 waveband record at an average center to center distance of 2.44 nm being recorded for each pixel, with 336 pixels in each line of the image. To ensure a correct aspect ratio in relation to shutter speed, scans were taken of a checkerboard pattern made of 4×4 cm squares in black and white. To remove the effect of accumulation of random electronic noise, a dark offset of the system was recorded in a blacked-out room with the mirror covered and reversed at each exposure time and the average value at each wavelength (because of the variability of sensitivity with wavelength) was subtracted from readings before any other calculations, for both images of areas of interest and spectral calibration data. The response of the hyperspectral imager varies with wavelength because of the response characteristics of the sensor and optical system and the variation in the amounts of energy in photons of different wavelengths. Thus, the relative wavelength sensitivity was

calibrated by collecting an image of a 45×45 cm tile of uniform reflectance of 20% over the wavelengths used (Spectralon SRT-20-180, Labsphere, North Sutton, NH) illuminated by a tungsten lamp of known filament temperature (PF810, 3400K, Philips, Amsterdam, the Netherlands). The relative output of this lamp with wavelength was calculated using Planck's law (Monteith and Unsworth, 2008). A dark zero correction based on the exposure time as described above was applied before sensitivity calculation.

Hyperspectral images were collected of a whole chicken carcass obtained from a local source. It was lit using 2 LEDJ Stage Wash U.V. 36 units (LEDJ106, Pro-light, Darwen, Lancashire, UK) that contained 36×1 W light-emitting diode (LED) bulbs emitting UV radiation at a center wavelength of 395 nm. The LED light source was placed 75 cm from the sample, and the camera was located 80 cm away. The 256 band images collected were 336×566 to 595 pixels. The carcass was then contaminated with 4 spots of feces (feces were combined samples of d 7 feces from the control and Mg groups) and the chicken reimaged. As a control, a microscope slide with bulked control and Mg feces was also imaged alongside the carcass.

Egg Analysis

Egg yolk color was investigated using a Minolta CR-200 Chroma meter (Konica-Minolta, Warrington, UK), calibrated to a standard white plate (Calibration Plate CR A43) to gain precise chromaticity. An 8-mm diameter measuring area, diffuse illumination, from a xenon (pulsed xenon arc lamp, and 0° viewing angle gave precise measurements across the whole sample. The tip of the chroma meter measuring head was placed flat against the surface of a Petri dish, and yolk reflective color was determined from the average of 3 consecutive pulses from the optical chamber of the Chroma meter. Absoluteness of color was measured using $L^*a^*b^*$ coordinates (Dvořák et al., 2009).

Statistical and Image Analysis

For feed and fecal fluorescence area under the main 670 nm peak was taken as the fluorescent intensity and along with egg color scores, bird weight, and DM intake (DMI), statistical analysis was performed using Genstat Release 11.1 (PC/Windows, VSN International, Hemel Hempstead, UK) with treatments as the fixed effect and blocking according to pen + period. For hyperspectral image analysis the ENVI format group of files for each image were processed using Matlab (MathWorks, Cambridge, UK) and the image processing toolbox. The specific programs developed included facilities to apply dark zero and wavelength sensitivity corrections, to plot images at specific wavelengths, and to provide spectra of regions of interest.

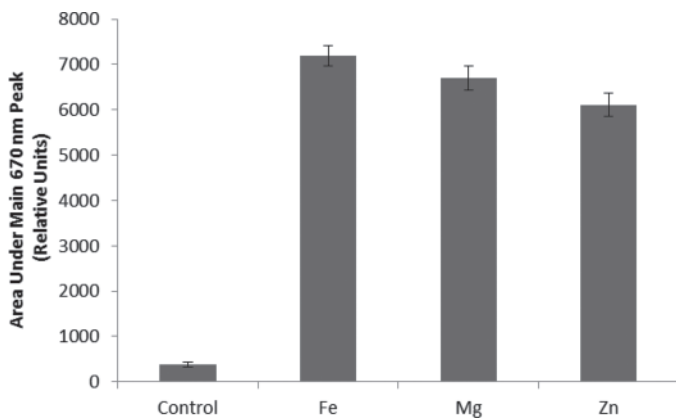


Figure 1. Fluorescent intensity of the pelleted feed.

RESULTS

Bird Measurements and Feed Fluorescence

There were no adverse effects of including the marker in terms of bird health or reduced intake. In fact, there was a significant increase ($P = 0.013$) in intake when birds were offered the Mg diet compared with the other treatment diets: 130.7, 123.2, 121.5, and 125.4 g of DM/d for Mg, control, Fe, and Zn, respectively. Despite the higher intake, there was no significant difference in mean daily live weight change over the course of the experiment with a mean daily increase of 1.20 ± 1.214 g/d. Fluorescence of the feed is shown in Figure 1, with all chlorophyllin treatments significantly higher ($P < 0.001$) than the control and Mg and Fe higher than Zn at 670 nm.

Fecal Fluorescence

Figures 2 and 3 show the fecal fluorescence at d 0 and 7 of receiving the experimental diet. All chlorophyllin treatments resulted in a significant ($P < 0.001$) elevation in fluorescence between d 0 and 7, whereas no difference was seen for the control during the same period. The Fe and Mg had similar mean maximum intensities at d 7, which were significantly higher than Zn. The factorial increase in fluorescent intensities for the 3 chlorophyllin treatments between d 0 and 7 were $\times 14.8$, 12.8 , and 6.9 for Fe, Mg, and Zn chlorophyllin, respectively.

Carcass Visualization

Initial scans of the chicken carcass showed relatively low levels of contamination (Figure 4), but subsequent addition of feces with and without marker showed that feces containing marker could easily and simply be detected because of the uniform background presented by the skin. However, the control feces could not be detected. The carcass was subsequently washed and the fecal material containing feces marker could still easily be detected. The relatively simple background

(white chicken skin) and the application of principal component analysis allowed identification of the carcass in the image and the positions of fecal contamination (Figure 5). Confirmation that the fluorescence detected was due to the marker containing feces was confirmed by visualizing a microscope slide containing feces with and without the marker. Also, a simple image at 675 nm with an appropriate threshold would allow detection of contaminated carcasses as shown in Figure 5c.

Egg Color Analysis

The Mg had no effect on egg yolk color compared with the control, whereas Fe significantly decreased ($P < 0.001$) redness of the yolk and Zn significantly decreased ($P = 0.002$) the lightness of the yolk (Table 1).

DISCUSSION

Marker Delivery and Fecal Output

Chlorophyllins are semi-synthetic derivatives of the natural green pigment chlorophyll. Their use as feed additives and subsequently dietary markers is preferred due to technological advantages over natural chlorophyll, such as greater hydrophilicity, tinctorial power, and stability toward heat, acid, and light. Copper chlorophyllin is most commonly used as a food colorant and has been linked with bioactive properties summarized recently in a review by Tumolo and Lunfer-Marquez (2012). Its use as a fluorescent dietary marker, however, is limited due to its lack of fluorescence in the visible and UV spectrum unlike Mg, Fe, and Zn chlorophyllins, which have been used as dietary markers in ruminants (Lee et al., 2010). All markers, as previously reported (Sagataram et al., 2012b), survived the pelleting process and produced highly fluorescent feed. Palatability of feed additives is essential if their inclusion is not to affect DMI and subsequently animal performance. When offering sheep ad libitum access to a concentrate feed, Lee et al. (2010) recorded no adverse effects on feed intake when chlorophyllin markers were added to the diet. In the present study, no adverse effects were observed in DMI of poultry offered feed containing the chlorophyllin markers. Conversely, birds offered the Mg chlorophyllin treatment showed significantly higher DMI over the control. Sagataram et al. (2012a) reported a similar increase in water intake when Mg chlorophyllin was added to the drinking water of sheep; likewise, Lee et al. (2013) reported a higher intake of concentrate in cattle when a chlorophyll extract was included in the ration.

Lee et al. (2011) reported no effect on egg yolk color when supplementing the diet of laying hens with either Fe or Zn chlorophyllin and a small effect with the addition of Mg chlorophyllin compared with a large effect with a chlorophyll-based extract. The present study showed no effect of Mg chlorophyllin and a small effect of both Fe and Zn. This would appear to agree with the

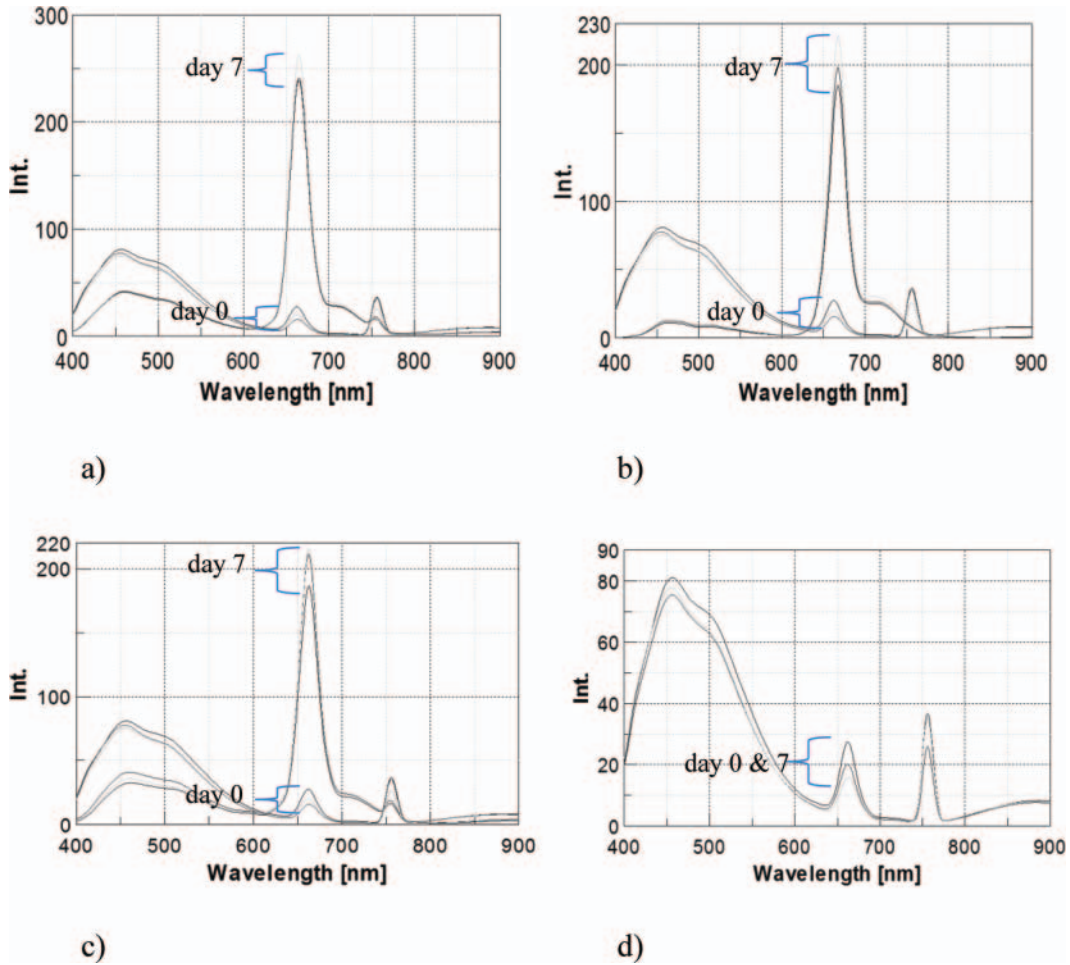


Figure 2. Fluorescent emission spectra (excitation wavelength 382 nm) of the fecal samples from each pen at d 0 and 7 across the experiment: a) Fe, b) Mg, c) Zn, and d) control (y-axis, fluorescence as relative units). Results from individual pens are shown to indicate variance between groups. Int. = intensity. Color version available in the online PDF.

observations of Smart et al. (1954) that chlorophyllin absorption and hence deposition is limited. However, more recent investigations have shown that the deesterification of phytol from chlorophyll resulting in the compounds such as chlorophyllin significantly increased their transfer to intestinal epithelial cells during digestion (Gandul-Rojas et al., 2009). Gomes et al. (2009)

reported a high deposition in the serum, liver, and kidneys of Cu chlorophyllin in rats (*Rattus norvegicus*) fed supplemented diets. Work at Aberystwyth University has recently shown a high digestibility of Mg chlorophyllin across the small intestine of cattle and whole digestive tract of sheep (S. Sagataram, PhD thesis in preparation, Aberystwyth University, Aberystwyth, UK). Such deposition of the marker into muscle tissue would result in background fluorescence and limit the usefulness of a marker to detect feces on the carcass. However, the lack of muscle fluorescence in cattle fed chlorophyll-rich diets (Lee et al., 2013) and the small effect on egg yolk color in the present study would suggest a high level of catabolism of the marker in the liver and little deposition into egg and tissue. Future work using animal carcasses from birds offered the markers would be needed to confirm the lack of deposition of the marker into the poultry carcass. Although total recovery of the marker across the gut was not measured in the present study, the addition of the markers for 7 d significantly enhanced the fluorescence of feces, showing an effective dose was 1 g/kg of DM. The period of 7 d was chosen to simulate the typical coccidiostat withdrawal feed offered to broilers before slaughter

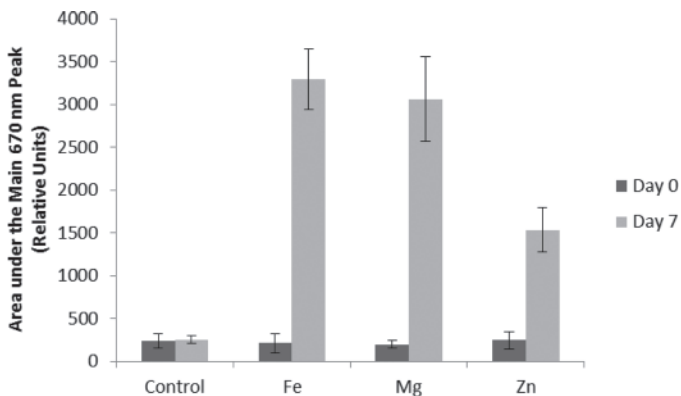


Figure 3. Fecal fluorescence at d 0 and 7 of offering the different pelleted diets.

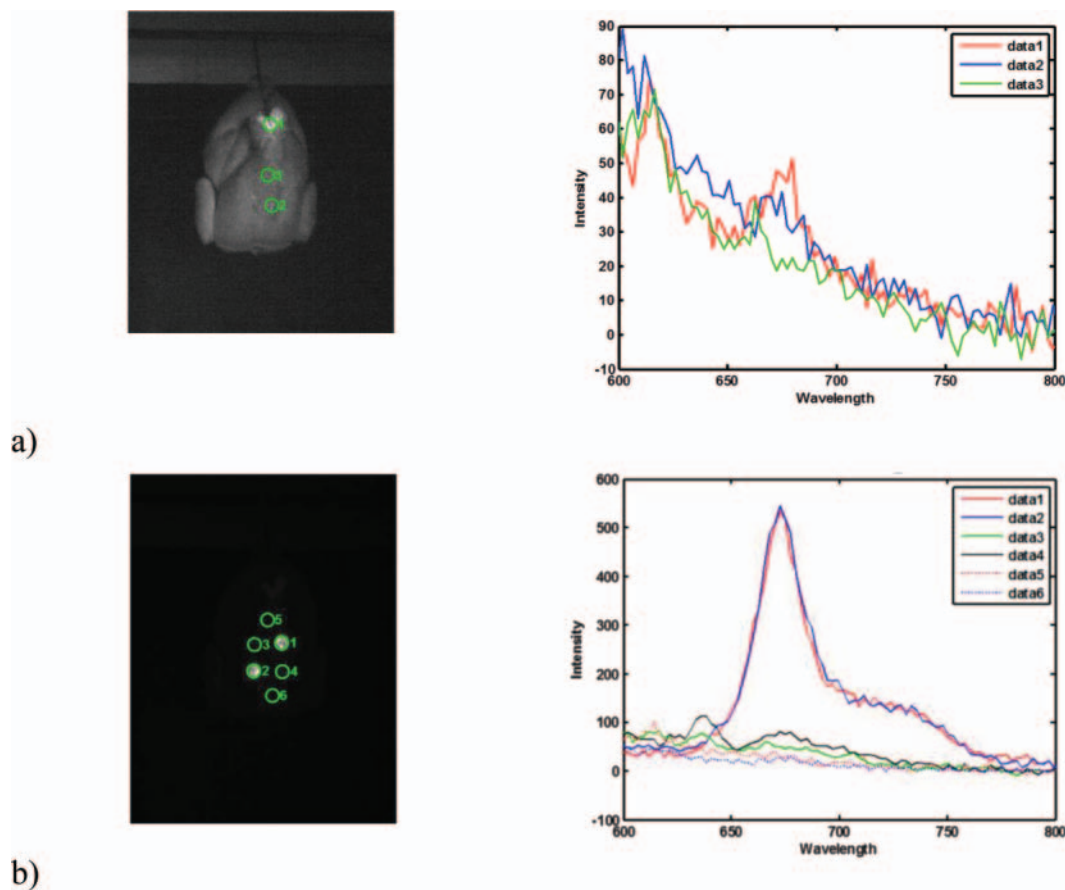


Figure 4. Measurements on a chicken carcass, left column: images at 675 nm, right column: spectra taken from areas marked on images. a) Images of chicken as received, showing low level of contamination; b) fecal material containing marker (Mg) applied to points 1 and 2, fecal material without marker (control) applied to points 3 and 4, and control areas with no feces points 5 and 6. Color version available in the online PDF.

(Damron and Christmas, 1997). Although in the present study, feed withdrawal was not simulated before removing the d 7 feces to test marker clearance through the birds, marker has been seen to remain in feces for a period of up to 3 to 4 d postfeeding and hence the

use of a 7-d clear-out in the current study between diet changeover. Future work is required to determine the exact gut clearance rate of the marker, but considering that birds are slaughtered typically within 8 to 12 h after feed withdrawal (Joe Lawson, Moy Park, Dungan-

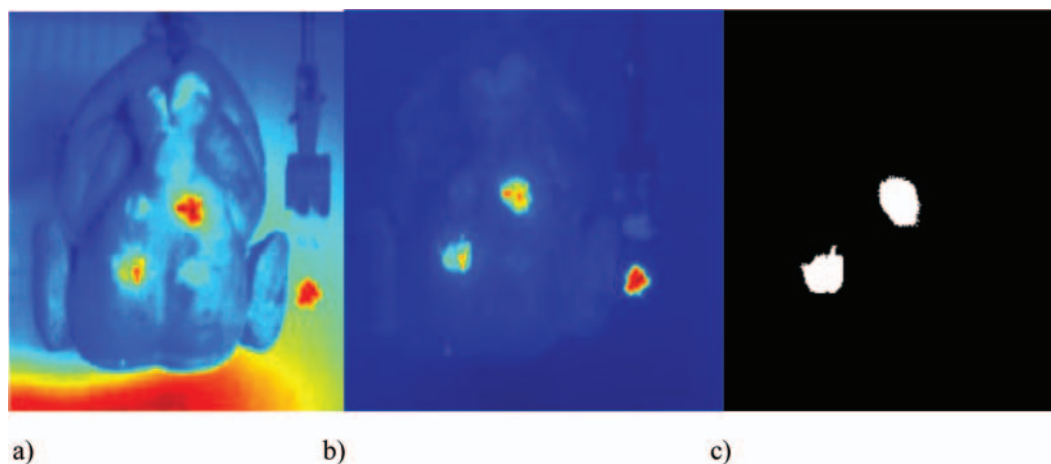


Figure 5. Principal component analysis of chicken images with fecal contamination, layout as in Figure 4. A microscope slide with a sample of fluorescing fecal material has been added on the right of the carcass for a and b. a) Principal component 1, showing outline of chicken and some areas, including contaminated areas (black spot with white outlining) and part of the background; b) principal component 2 identifying regions of the image contaminated by feces containing marker; c) threshold image at 675 nm identifying positions of contamination. Color version available in the online PDF.

Table 1. Egg yolk color from chickens offered pelleted mash with or without 3 chlorophyll-based markers¹

Item	Control	Fe	Mg	Zn	SED	P-value
Chroma	36.8	35.3	38.7	36.0	1.60	NS
Lightness (L*)	60.7 ^a	61.3 ^a	60.9 ^a	59.9 ^b	0.34	0.002
Redness (a*)	7.44 ^a	5.30 ^b	7.20 ^a	7.26 ^a	0.171	<0.001
Yellowness (b*)	36.1	34.9	38.0	35.2	1.61	NS

^{a,b}Differing superscripts within a row are different at $P < 0.05$.

¹Fe, iron chlorophyllin; Mg, magnesium chlorophyllin; Zn, zinc chlorophyllin.

non, Northern Ireland, personal communication), the loss of the marker from feces and gut content will not be an issue. The low level of fluorescence observed from d-0 fecal samples and from the control diet (Figures 2 and 3) show the typical level of fluorescence associated with poultry feces and the need for alternative detection systems when not using fluorescent markers such as hyperspectral, near-infrared reflectance spectroscopy, and laser-induced fluorescence imaging reported by Park et al. (2007), Chao et al. (2008), and Cho et al. (2009), respectively.

Visualization

Visualization of fecal markers was investigated using a hyperspectral approach, as first suggested by Lawrence et al. (2001) and further developed by Park et al. (2007, 2011), which allowed the carcass to be screened for fluorescent signal with and without feces and in the presence and absence of marker to fully validate the potential of the marker approach. Hyperspectral imaging has been shown to be able to detect fecal and digesta contamination from birds consuming a fluorophore low content diet such as maize and soya. The initial scan of the purchased carcass (Figure 4a) showed a low level of contamination, confirming the ability of the hyperspectral approach to detect contamination of poultry carcasses. However, Windham et al. (2002) noted a disparity in accuracy between different diets. This was evident once feces were added to the carcass with and without Mg chlorophyllin marker. Marker significantly enhanced the intensity signal compared with the feces from the control diet, which were hardly discernible over the background skin intensity (Figure 4b). Park et al. (2011) reported the ability to identify fecal spots as little as 10 mg in size on carcasses at a high-speed processing line (140 birds per minute) using a real-time detection, hyperspectral imaging system. However, with current line speeds approaching 250 birds per minute and the requirement to detect minute levels of contamination (in the ppm), a system designed to improve fluorescence of the feces such as the use of dietary markers may also be needed for hyperspectral imaging approaches (Figure 5a and 5b).

The aim of the current study was not only to determine the potential of a dietary marker to increase fluorescence of subsequent feces to aid detection, but to determine whether the feces containing marker could

be detected by a simple UV front-face fluorescence system more tuned to on-line detection of a current high line speed (200–250 birds per minute) abattoir. Our results have shown that detection of a chlorophyll-based marker (chlorophyllin) can be achieved at a simple threshold of 675 nm (Figure 5c). This should allow an appropriate detection system to be set up using an UV LED light source emitting at 395 nm to achieve adequate uniformity over the carcass. Subsequent fluorescence from contaminated areas could be detected by a monochrome camera as birds passed through a darkened covering to reduce ambient light, without a near red filter, but supplied with a filter transmitting between 660 and 690 nm and exposure time in the milliseconds. The simplicity of this process makes it attractive to fit modern high-speed digital imaging and computing systems to achieve the required throughput. This approach could inform the abattoir of basal levels of contamination arriving in the plant as part of quality assurance monitoring but also as an early detection system for evisceration malfunction and gut spill contamination as part of HACCP.

The current project has indicated the potential use of chlorophyll markers to aid detection of feces using simple front-face UV fluorescent approaches. To determine the full potential of this approach, the next step would involve testing the system on carcasses of broilers offered the marker and processed through an abattoir.

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