

1986

Cryo-Scanning Electron Microscopy of Microorganisms in a Liquid Film on Spoiled Chicken Skin

T. A. McMeekin

D. McCall

C. J. Thomas

Follow this and additional works at: <https://digitalcommons.usu.edu/foodmicrostructure>



Part of the [Food Science Commons](#)

Recommended Citation

McMeekin, T. A.; McCall, D.; and Thomas, C. J. (1986) "Cryo-Scanning Electron Microscopy of Microorganisms in a Liquid Film on Spoiled Chicken Skin," *Food Structure*: Vol. 5 : No. 1 , Article 10. Available at: <https://digitalcommons.usu.edu/foodmicrostructure/vol5/iss1/10>

This Article is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Food Structure by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.



CRYO-SCANNING ELECTRON MICROSCOPY OF MICROORGANISMS IN A LIQUID FILM ON SPOILED CHICKEN SKIN

T.A. McMEEKIN¹*, D. McCALL², and C.J. THOMAS³

¹Department of Agricultural Science, University of Tasmania, Hobart, Tasmania, Australia
²Electron Microscopy Unit, Plant Pathology Research Division, Department of Agriculture for Northern Ireland
³Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand

Abstract

Cryo-scanning electron microscopy was used to examine bacteria which had grown in a liquid film on the skin surface of poultry carcasses. Pits or void zones surrounding bacteria on ethanol-dehydrated, critical point dried (CPD) samples were not found on washed or untreated specimens frozen prior to examination by scanning electron microscopy (SEM). However, pits equivalent to those produced during chemical fixation and dehydration were formed when skin tissue was treated with ethanol prior to freezing. We conclude that the pits are dehydration artifacts caused by the chemical preparative methods employed rather than the result of degradation of film proteins by extracellular enzymes of microbial origin.

Introduction

As a consequence of poultry processing procedures involving water immersion, the skin surface of carcasses becomes covered by a liquid film. It consists of a number of serum proteins, amino acids and other suspended or soluble material and may be 50-60 μm thick. The contents of the film originate either from the processing water or by diffusion from damaged skin tissue. Microscopic details of the liquid film were presented by Thomas and McMeekin (1980).

During storage, the amount and content of the film has been shown to increase and it is in this medium that the spoilage flora develops. Spoilage occurred when numbers of bacteria reached 10^8 cells/cm², while at 10^5 cells/cm² bacterial 'slime' had formed within the liquid film. Microbial penetration and disruption of skin tissue at refrigeration temperatures has not been observed, but bacteria located at the surface of the film on specimens prepared for SEM were surrounded by pits or cleared zones. In attempts to clarify the cause of these pits, microscopic examination of artificially contaminated specimens showed the test bacteria to be located only on the film surface. Consequently, it was not possible to determine whether the pits resulted from enzymatic degradation of film contents by bacteria, or were an artifact of specimen preparation.

Micrographs illustrating details of the liquid film during refrigerated storage were presented by Thomas and McMeekin (1981).

In this paper we report the use of cryo-SEM to examine microorganisms grown in the liquid film on chicken skin in order to determine the origin of the pits and cleared zones observed on ethanol-dehydrated, critical point dried (CPD) specimens. Freezing tissue in liquid nitrogen retains the liquid film *in situ* as does the OsO₄ vapour fixation technique developed by McMeekin et al. (1979) and Thomas and McMeekin (1980, 1981). In addition, liquid nitrogen freezing has the advantage of minimizing shrinkage artifacts caused by chemical dehydration or critical point drying techniques (Boyde, 1972).

Initial paper received November 30 1985
Manuscript received April 24 1986
Direct inquiries to T.A. McMeekin
Telephone number: 61 2 202637

KEY WORDS: Cryo-SEM; chicken skin; pits; shrinkage artifact.

Materials and Methods

Samples

Commercially processed chicken breast pieces (20 x 15 cm²) were stored in polyethylene bags for up to 10 days at 4^o-5^oC at which time spoilage odours were evident. Some specimens were also incubated overnight at 25^oC to encourage bacterial 'slime' formation on the skin surface.

Production of samples for cryo-SEM

Prior to preparation for SEM, samples of skin (4 x 4 cm) were excised from the breast pieces, pinned to dental wax and were either untreated, washed in water, or treated with ethanol. Tissue was washed by agitation in 1 litre of tap water for 30 seconds followed by rinsing in running tap water for a further 30 seconds, or by successive immersion in 3 x 1 litre of tap water followed by rinsing in running tap water for 5 minutes. Other samples were immersed in 70% or 100% ethanol for up to 5 days or simply treated by stepwise addition of ethanol to the skin surface. Untreated control pieces of skin were included also.

After each treatment, specimens (~1 cm²) were frozen by plunging into nitrogen/argon slush in the specimen freezing chamber of an EM scope sputter coater (EM Scope Instruments, Kent, England) and transferred under argon to the coating chamber which was then evacuated to 0.05-0.03 torr. Specimens were coated with 20 nm of gold and transferred under vacuum to the cryo stage (pre-cooled to -165^oC) of a Cambridge Stereoscan 600 SEM (Cambridge Instruments, Cambridge, England). The accelerating voltage used was 15 kV and micrographs were recorded on Kodak Technical Pan 2415 film.

Preparation of samples for conventional SEM

Specimens of skin (~1 cm²) were also prepared by solvent dehydration techniques. Samples were fixed in OsO₄ vapour (or by immersion in 2% v/v glutaraldehyde), dehydrated in ethanol and critical point-dried (McMeekin et al., 1979). Specimens were coated with ca. 20 nm of gold and examined in a Cambridge Stereoscan 600 SEM.

Changes in components of the liquid film during storage

Numbers of bacteria, glucose concentration and protein were monitored during refrigerated storage of carcasses at 2^oC for up to 16 days.

Bacteria were enumerated by plating 0.1 ml amounts of suitable dilutions of skin homogenate on the surface of nutrient agar. Skin homogenates were prepared by treating 16 cm² of skin in 100 ml of saline (0.8% w/v NaCl) with a model 400 Colworth Stomacher (A.J. Seward and Co. Ltd., London). Inoculated plates of nutrient agar were incubated at 22^oC for 3 days and colony counts related to numbers of bacteria contained in the liquid film on 16 cm² of skin surface.

Samples of liquid film were obtained by swabbing 16 cm² or 32 cm² areas of skin with a cotton gauze swab. Swabs were then rinsed in 5

ml water and this solution was used to determine glucose and protein levels. Samples to be examined for glucose were first clarified by centrifugation, then freed of protein by treatment with zinc sulphate and sodium hydroxide, followed by further centrifugation to remove zinc hydroxide and precipitated protein. Glucose was then determined by the glucose oxidase-peroxidase method using blood sugar reagents obtained from Sigma Chemicals Co., Ltd. Protein (as albumin) was estimated by a dye binding method (Toro and Ackerman, 1975). Samples (0.2 ml), or standard bovine serum albumin (0.2 ml of 5 g/100 ml solution) were added to 2 ml of bromocresol green reagent (0.01% w/v in 0.1M succinate buffer, pH 4.0, plus 0.3 ml H₂O), mixed and allowed to stand at room temperature. The absorbance of the resultant solution was read at 630 nm.

Results and Discussion

All specimens used in this study were incubated under conditions which allowed spoilage to occur prior to SEM preparation. The bacterial load of samples at this stage was typically 10⁷-10⁸ cells/cm².

Figures 1, 2 and 3 illustrate the typical appearance of pits associated with bacteria in the liquid film on samples of spoiled chicken skin prepared for SEM by conventional procedures. The specimen illustrated in Figure 1 was fixed in OsO₄ vapour, while those shown in Figures 2 and 3 were fixed by immersion in glutaraldehyde solution. This immersion fixation procedure removed much of the film shown in Figure 1, but some portions did remain intact such as that illustrated in Figures 2 and 3. In all specimens where the film remained intact, pits were found associated with bacteria at the film/air interface, but were not evident with those cells just above or below the surface (Figure 3).

Photomicrographs of frozen specimens are shown in Figures 4 and 5. The image shown is the surface of the liquid film, not the skin surface itself. Neither micrograph shows pits associated with the film surface, nor were they discerned in areas of specimen where bacterial colonies were sited just below the film surface (Figure 5). The undulating surfaces evident in Figure 5 represent the outline of bacterial cells growing just underneath the surface. The cause of the larger holes shown is not clear, but they may represent areas of skin devoid of underlying bacteria formed as a result of the coalescence of adjacent colonies. These holes should not be confused with pits.

Washing had little or no effect on the overall appearance of the skin surface of frozen specimens and did not result in production of pits surrounding the cells (Figure 6). The possibility that ice formation obscured the pits seems unlikely, since in several specimens, where this artifact was seen, ice crystals were observed over the entire specimen surface (Figure 7). Similarly, treatment with 70% ethanol for up to 5 days had little effect on pit formation (Figure 8).

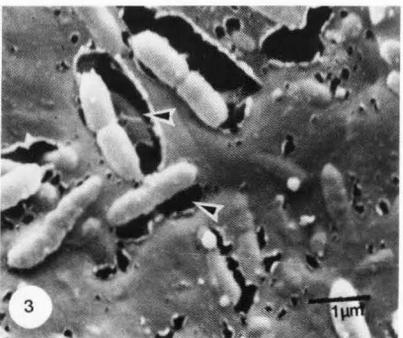
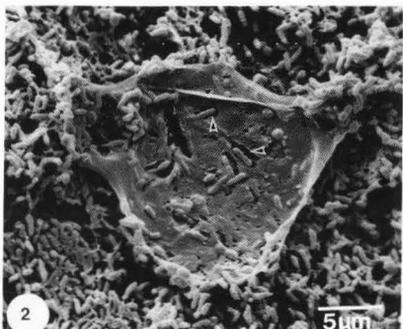
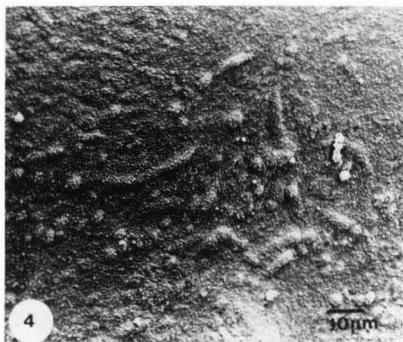
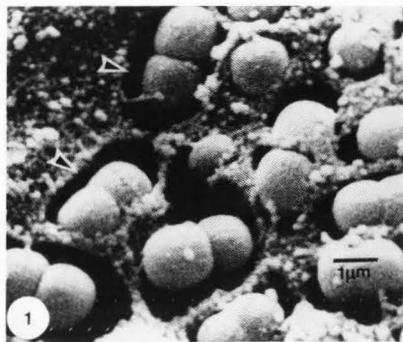


Figure 1 Pits (arrowed) surrounding bacterial cells in liquid film on chicken skin. Specimen was osmium vapour fixed, ethanol dehydrated and critical point dried.

Figure 2 Remains of liquid film on glutaraldehyde immersion fixed specimen. Pits (arrowed) can be seen surrounding cells at film/air interface.

Figure 3 Higher magnification of liquid film area in Figure 2. Pits (arrowed) occur around cells at film/air interface but not on those just underneath the surface.

Figure 4 General appearance of the surface of the liquid film on a frozen specimen. No bacteria or pits are evident.

Figure 5 Liquid film overlying a heavily colonized area on a frozen specimen. The undulations shown represent the outline of bacterial cells growing in a colony beneath the surface of the liquid film. Note the larger "holes" (arrowed) which should not be confused with "pits".

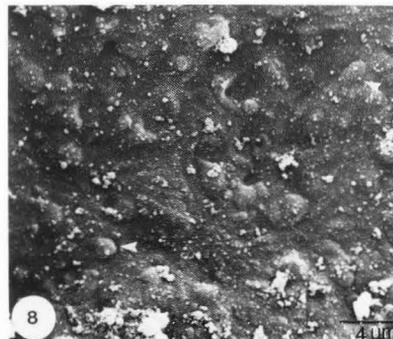
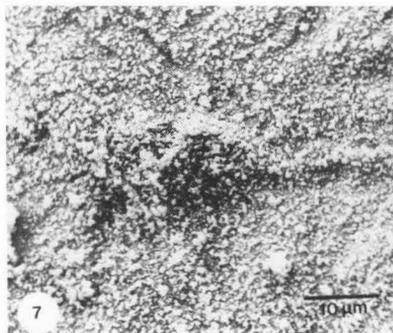
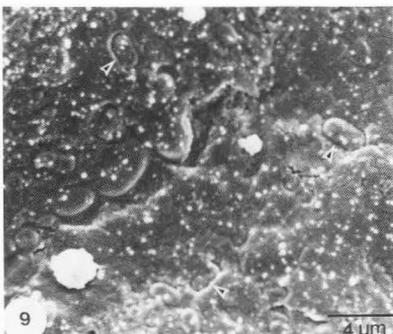


Figure 6 Surface of the liquid film in a specimen washed prior to freezing. No pits are evident but outlines of bacterial cells (arrowed) can be seen under the film surface.

Figure 7 Ice crystal formation on the surface of a frozen specimen. The artifact is obvious.

Figure 8 Specimen treated in 70% ethanol prior to freezing. No pits are evident, but bacterial outlines (arrowed) can be seen under the surface of the liquid film.

Figure 9 Specimen treated by dropwise addition of 100% ethanol prior to freezing. Note development of pits (arrowed) surrounding the cells in the liquid film.

Figure 10 Specimen treated by immersion in 100% ethanol prior to freezing. Film shrinkage and pits (arrowed) surrounding individual cells are evident.

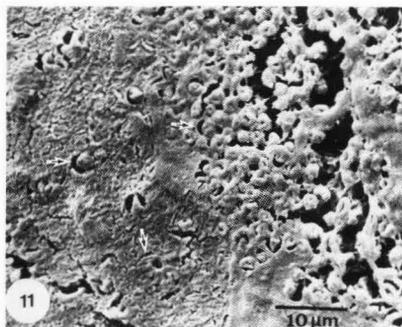


Figure 11 Higher magnification of edge of colony shown in Figure 10.

However, dropwise addition of 100% ethanol prior to freezing caused channel formation around cells (Figure 9) and immersion for 2 hours or more produced pits equivalent to those formed on critical point dried material (Figures 10, 11).

These results demonstrate positively that pits observed around bacteria growing in a proteinaceous film can be induced by treatment of the sample with ethanol. This is consistent with the hypothesis that these features are artifacts of the methods of preparation rather than the result of bacterial activity.

Other evidence is available to support this conclusion. For example, pits associated with microbial cells in the film on skin from carcasses stored 4 days at 2°C have the same dimensions as those on spoiled samples incubated 16 days at 2°C. This observation is not consistent with ongoing proteolytic degradation (Thomas and McMeekin, 1981). Also, protein degradation by bacteria prior to spoilage is not consistent with accepted biochemical events associated with spoilage, in which proteases are repressed in the presence of small molecular weight carbohydrates and amino acids (Dainty et al., 1975; Gill, 1976). We have demonstrated an increase in glucose content of the surface film on skin for up to 8 days at 2°C. This occurs apparently as a result of diffusion of film components from underlying tissues and net utilization of glucose occurred only after this time (Table 1). Protein levels remained constant over a 16 day sampling period at 2°C. Furthermore, the predominant type of bacteria present in the film is actively motile *Pseudomonas* spp. Since the majority of these bacteria are not associated with the skin surface but are located within the liquid film, they would be unlikely to elaborate discrete, individual zones of film degradation (Thomas and McMeekin, 1981).

Specimen shrinkage is known to result both from ethanol dehydration prior to critical point drying (Boyd, 1972, 1978) and the

Table 1. Changes in numbers of bacteria, glucose and protein levels in breast skin washings of chicken carcasses stored at 2°C.

	Storage interval				
	0 days	4 days	8 days	12 days	15 days
Log bacteria per 16 cm ² skin	4.81	4.91	6.00	7.56	9.58
Glucose µg/32 cm ²	370	450	882	475	116
Protein (as albumin) ₂ mg/32 cm ² skin	9.96	10.53	9.10	10.26	12.43

Each value represents the mean of 3 estimates.

critical point drying process itself (Gusnard and Kirschner, 1977; Schneider, 1976). In this study we have not distinguished quantitatively between the effects of solvent dehydration and critical point drying. However, it was evident that pits can be initiated by ethanol treatment and that elimination of solvent dehydration procedures by cryo-techniques prevented pit formation.

Other reports of SEM observations of food associated microorganisms have demonstrated structures similar to the pits/void spaces described in this paper. 'Pockets' were noted by Kalab (1978; 1979) surrounding lactic acid bacteria in SEM preparations of yogurt and cottage cheese. These were considered to be the result of bacterial enzyme action on the casein micelle and Kalab et al. (1983) have presented further results to support the initial findings.

The results presented in this paper demonstrate a situation different to that in milk gels and indicate pits in the liquid film on spoiled chicken skin prepared for SEM are an artifact of specimen preparation rather than the result of bacterial enzymatic activity. In view of these results, we suggest a re-evaluation of the cause of "erosion troughs" associated with *Pseudomonas putrefaciens* observed on Os₄ vapour-fixed samples of pork skin (Butler et al. 1979) may be necessary.

References

- BOYDE A. (1972). Biological specimen preparation for the scanning electron microscope - an overview. *Scanning Electron Microsc.* 1972: 257-264.
- BOYDE A. (1978). Pros and cons of critical point drying and freeze drying for SEM. *Scanning Electron Microsc.* 1978; II: 303-314.
- BUTLER JL, STEWART JC, VANDERZANT ZL, CARPENTER L, SMITH GC. (1979). Attachment of microorganisms to pork skin and surfaces of beef and lamb carcasses. *J. Food Prot.* 42, 401-406.
- DAINTY RH, SHAW BG, de BOER KA, SCHEPS ESJ. (1975). Protein changes caused by bacterial growth on beef. *J. Appl. Bacteriol.* 39, 73-81.

GILL CO. (1976). Substrate limitation of bacterial growth at meat surfaces. *J. Appl. Bacteriol.* **41**, 401-410.

GUSNARD D., KIRSCHNER RH. (1977). Cell and organelle shrinkage during preparation for scanning electron microscopy: effects of fixation, dehydration and critical point drying. *J. Microsc.* **110**, 51-57.

KALAB M. (1978). Milk gel structure. VIII. Effect of drying on the scanning electron microscopy of some dairy products. *Milchwiss.* **33**, 353-358.

KALAB M. (1979). Scanning electron microscopy of dairy products: an overview. *Scanning Electron Microsc.* 1979; III: 261-267.

KALAB M., ALLEN-WOJTAS P., PHIPPS-TODD BE. (1983). Development of microstructure in set-style non-fat yoghurt - a review. *Food Microstruct.* **2**, 51-66.

McMEEKIN TA, THOMAS CJ, McCALL D. (1979). Scanning electron microscopy of microorganisms on chicken skin. *J. Appl. Bacteriol.* **46**, 195-200.

SCHNEIDER GB (1976). The effects of preparation procedures for scanning electron microscopy on the size of isolated lymphocytes. *Amer. J. Anat.* **146**, 93-100.

THOMAS CJ, McMEEKIN TA. (1980). Contamination of broiler carcass skin during commercial processing procedures: an electron microscopic study. *Appl. Environ. Microbiol.* **40**, 133-144.

THOMAS CJ, McMEEKIN TA. (1981). Spoilage of chicken skin at 2°C: electron microscopic study. *Appl. Environ. Microbiol.* **41**, 492-503.

TORO G, ACKERMAN PG. (1975). *Practical Clinical Chemistry*. Little, Brown and Co., Boston, 182-186.

Discussion with Reviewers

S. Humphreys: Are the pieces of skin from the same part of the chicken? Skin from different parts of the body may appear differently.

Authors: The skin pieces were all taken from the breast area of commercially processed carcasses. It is important to realize that the image by SEM is that of the surface of the liquid film on top of the skin, not the skin per se.

S. Humphreys: Tap water is not isosmotic with avian skin - can lysed cellular material fill pits?

Authors: Tap water was used to simulate commercial processing conditions. Again it is important to note that the film surface is being observed and not the skin surface.

S. Humphreys: How dry was the skin surface when it was frozen? Could ice have filled the "pits" and obscured them?

Authors: No particular precautions were taken to dry the skin before freezing. Excess water was not removed as we were concerned to maintain the film intact with minimal disturbance of the film and bacteria. The possibility that ice formation obscured the pits seems unlikely since in several specimens where this artifact was seen ice crystals were observed over the entire specimen surface (see Figure 7).

S. Humphreys: What do these pits look like in sections?

Authors: In sections, pits appear as cleared zones surrounding bacterial cells. See Thomas and McMeekin (1981), *Appl. Environ. Microbiol.*, **41**, 492.

M. Kalab: I was surprised to learn that to study the effect of dehydration, unfixed samples (of chicken skin) were immersed (directly) in 70 or 100% ethanol. I wonder why no attempts had been made to fix parallel samples before subjecting them to dehydration in ethanol?

Authors: In all our previous work with conventional SEM preparations, pits were observed after chemical fixation and dehydration. Therefore it seemed unlikely that prior fixation would prevent pit formation. Our intention was to fix by liquid nitrogen freezing with or without ethanol treatments.

M. Kalab: Is the liquid film already some kind of a degradation product?

Authors: The liquid film is not a degradation product. It results from the processing procedures used and is the substrate for bacterial growth and activity. There is very little bacterial action associated with the actual skin compared to that in the overlying film.

B. Skura: Rather than creating an artifact due to shrinkage, is it not possible that the ethanol washed away components of the liquid film around the bacterial cells while the intact components of the liquid film farther removed from the bacterial cells were not washed away by the ethanol and remained in place?

Authors: This is very unlikely. If the products of protein degradation were washed away by ethanol treatment, they would also be eluted by the severe aqueous washes described.

B. Skura: Are there special precautions that should be taken when freezing chicken skin in liquid nitrogen for cryo-scanning electron microscopy?

Authors: The major concern was to preserve the liquid film in situ on the skin surface. This means that minimal disturbance prior to freezing is important. During freezing, coating and observation, it is important to monitor temperature, vacuum and argon flushing procedures at various stages.