

## Abundance and distribution of microbial populations in sheep fleece

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**Abstract** Bacteria were isolated and enumerated from fresh wool samples from sheep flocks throughout New Zealand. Bacterial populations averaged  $10^8$ – $10^9$  colony forming units (cfu)  $g^{-1}$  wool and varied little between flocks. Highest numbers of bacteria were recovered from the back, neck, and rump of the sheep rather than the underside of the animal. Within the fleece, most bacteria (>95% of total) were isolated from the outer portion of the staple. The fleece bacteria could be divided into three broad classes, Gram-positive cocciform bacteria, Gram-positive rods, and Gram-negative rods. Most bacteria from the fleece formed pigmented colonies with colours ranging from yellow to bright red. Gram-positive cocciforms were the most abundant group of bacteria with the most common species corresponding to *Kocuria rosea*, *Micrococcus luteus*, and *M. lylae*. These species form a dominant complex of salt tolerant chromogenic bacteria which are characteristic of the healthy fleece.

**Keywords** sheep fleece; microflora; cocciform bacteria; *Micrococcus* spp.; *Kocuria rosea*

### INTRODUCTION

The skin and surface covering of animals are usually host to a wide ranging microbial flora, and sheep are no exception. The sheep fleece is a complex, dynamic environment providing the microbial flora with nutrients from the skin and secretory glands of the sheep (suint) as well as collecting exogenous nutrients from the environment. Mulcock (1961) recorded a total bacterial count of  $10^6$ – $10^9$  bacteria  $g^{-1}$  of wool but the species were not identified. Lyness et al. (1994), in a study of Australian sheep, reported lower numbers, around  $10^6$  bacteria  $g^{-1}$ , belonging to a range of genera including *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Cryptococcus*, and *Bacillus*. Some of these genera are specifically associated with mammals while others originate from the environment (soil, pasture, and dung) and are capable of surviving and multiplying in the fleece. Other microbes may occur as contaminants without significant multiplication within the fleece.

Microbes can cause fleece and skin problems in sheep. Proliferation of *Pseudomonas aeruginosa* can lead to fleece rot and has also been implicated in the initiation of fly strike (Chin & Watts 1992). Dermatophilosis is caused by multiplication of the bacterium *Dermatophilus congolensis* at the skin surface (Merritt & Watts 1978) and interactions within the microflora of the sheep fleece have also been suggested as a possible cause of fleece discoloration (Mulcock 1965). More recently, Winder et al. (1998) showed that naturally occurring fleece bacteria were involved in the yellow discoloration of wool in the presence of high humidity.

Most sheep, however, harbour large numbers of bacteria in the fleece without detrimental effects on the wool, and fleece and skin problems are unusual. Their sporadic occurrence suggests an imbalance in the microbial population within the fleece. There has been little progress in improving understanding of the microbial ecology of the sheep fleece in New Zealand since studies prior to 1970 (Mulcock & Fraser 1958; Mulcock 1965), yet this

is the arena within which detrimental interactions are taking place. Furthermore, to understand the microbial ecology of the fleece it is important to examine fresh fleece samples rather than stored samples that have been used in most fleece studies. In this paper we report on studies to define the location and structure of the microbial community within the sheep fleece using only freshly removed samples of fleece from apparently normal animals showing no signs of fleece abnormalities.

## MATERIALS AND METHODS

### Sampling of fleece and skin

Wool samples were taken from different body positions from sheep in a number of flocks throughout New Zealand. The fleece was gently parted and a staple cut with sharp scissors, as close to the skin as possible, from an area of about 1 cm<sup>2</sup> and placed in a clean plastic bag. Care was taken in handling and sampling to avoid contamination of the samples and the scissors were disinfected by alcohol dip between each sheep. Samples were held in a cooler and returned to the laboratory for bacterial extraction as soon as possible. If necessary, the sample bags were held under refrigeration at 5°C until extraction, which was no later than 48 hours after sampling.

Skin washings were carried out using methods described previously (Chin & Watts 1992). A 25 mm diameter cylinder with a silicone greased end was placed directly and firmly onto the skin, after trimming off a section of the fleece, and a 10 ml sterile solution of 0.01% Tween 20 in 0.05 M NaCl was syringed onto the skin surface. The liquid was used to wash any bacteria from the skin and was retrieved into a sterile universal.

### Extraction and identification of bacteria

Subsamples of approximately 0.1 g of fleece were teased from the staples and accurately weighed before being added to a 250 ml flask containing 100 ml sterile 0.05% Tween 20 in water or dilute saline solution. For some experiments the fleece was divided into outer, mid, and inner zones (Fig. 1). The outer zone was visibly discoloured and often matted, the mid zone was relatively clean unmatted wool, and the inner zone occupied 20–30% of the staple closest to the skin. The samples were agitated for 1 min and placed in an ultrasonic bath for 3 min (Julabo, Allentown, PA, USA; Model USR3; Frequency 35 kHz). Serial dilutions of the

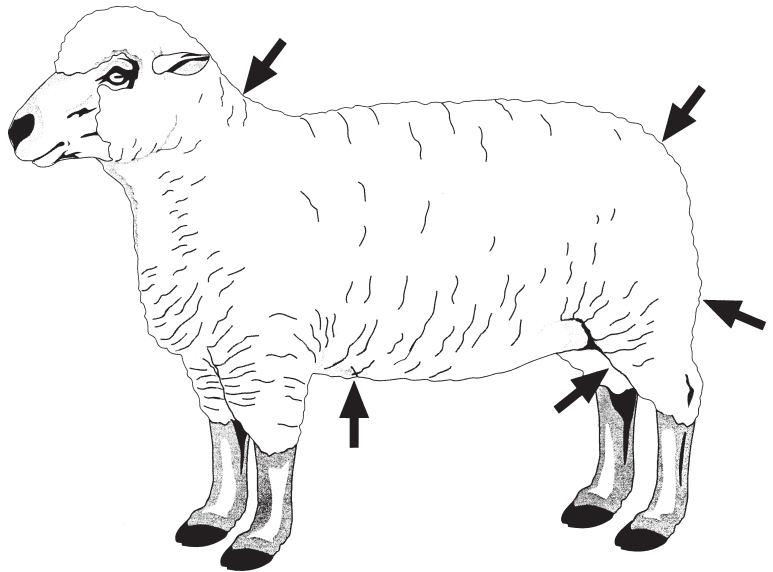


Fig. 1 Wool staple showing three zones sampled.

staple and skin samples were plated onto Nutrient Agar (Merck) (NA) plates. The plates were incubated at 30°C for 3–7 days and resulting colonies counted in order to estimate the bacterial population which was expressed as colony forming units (cfu) g<sup>-1</sup> wool.

To determine the composition of the bacterial population, colonies were numerically assessed on the basis of growth form and colour followed by removal and characterisation of the predominant strains. Alternatively, 25 randomly selected colonies per sample were transferred to fresh NA plates and cultured overnight before various biochemical tests were performed to identify the bacteria to genus level. Tests included: Gram staining and microscopic determination of morphology; catalase and oxidase tests; motility; Ziehl-Neelsen's method for

**Fig. 2** Diagram of a sheep showing locations from which fleece samples were collected.



acid fast stain; Hugh and Leifson's method for oxidation or fermentation of glucose under aerobic and anaerobic conditions; and spore stain (modified version of Moeller's spore stain). Tests were conducted as described in Barrow & Feltham (1993). Bacterial isolates were also pasteurised (85°C for 10 min) and plated on NA for isolation of spore-forming bacteria. For identification of *Micrococcus* species, single colonies were purified on nutrient agar and profiled using ID 32 STAPH identification system (bioMerieux sa, Lyon, France).

#### **Enumeration of bacterial populations on different areas of body**

Wool staple samples were collected from five body positions (neck, rump, belly, inside leg, and crutch) (Fig. 2) on each of three Coopworth lambs randomly selected from a flock of 3-month-old lambs at Templeton, Canterbury, New Zealand. Samples were processed as described above.

#### **Bacterial populations on flocks from different geographical regions**

Staple samples were taken from the midside of 1-year-old Romney hoggets from four flocks in three locations, Ruakura (Waikato) (two flocks), Lincoln (Canterbury), and Gore (Southland), during early summer. Between 17 and 24 sheep were sampled from each flock. Bacteria were isolated and

enumerated as described above. Bacteria isolated from the Gore flock were characterised for predominant colony types and the most common species identified.

#### **Position of bacteria on the staple**

Wool staples were taken from the sides of 10 Coopworth lambs in a Lincoln flock and divided into outer, mid, and inner zones. Bacteria on the skin surface were enumerated as described above.

Five fleece staples were randomly sampled from the midside of each of eight randomly selected ewes at Winchmore, Canterbury. The sheep were 2- to 5-year-old Coopworth ewes, that had never been drenched or dipped, with 9 months wool growth. Samples were cut into outer, mid, and inner sections and the zone samples pooled for each sheep. Analysis of microbial population and composition was carried out as a randomised block design with eight sheep  $\times$  three wool zones.

## **RESULTS**

#### **Bacterial populations on different parts of the sheep**

Total bacterial numbers on the fleece samples ranged from  $10^8$  to  $10^9$  bacteria  $g^{-1}$ . Highest numbers occurred on the neck and rump of the

animals, while numbers on the lower portions of the fleece were generally fewer (Table 1). Cocci-form bacteria predominated, with *Acinetobacter* sp., *Bacillus* spp., and yeasts also present.

### Bacterial populations on flocks from different regions

Bacterial numbers on sheep from the Gore flock were significantly higher than from flocks in the other regions ( $P < 0.01$ ) (Table 2). The predominant bacteria from 17 of the 20 sheep sampled from the Gore flock, comprising an average of 39% of the total bacterial flora, formed regular, circular, orange colonies on NA plates and were identified as Gram-positive cocciform bacteria belonging to the family Micrococcaceae. Predominant species from the remaining sheep were Gram-negative and formed orange or yellow colonies.

### Position of bacteria on the staple

In the Lincoln flock, the total number of bacteria/staple was  $2.8 \times 10^7 \text{ g}^{-1}$  (weighted average from the three zones). Most bacteria (>95%) were found in the outer zone of the fleece (Table 3) which was darkened and stained. Relatively few bacteria were isolated from the inner zone or associated with the skin. Bacterial density at the skin surface was similar to that in the inner zone of the fleece.

A similar pattern was found in fleece from the 2-year-old flock at Winchmore. The total microbial population on the Winchmore fleeces was  $1.5 \times 10^8 \text{ g}^{-1}$  (a weighted average from the three zones). The microbial population showed a similar pattern in relation to fleece structure to the previous sampling, with 97% of bacteria found in the outer zone of the fleece (Table 4). As shown in earlier sampling, most bacteria were pigmented with colours

**Table 1** Bacterial colony forming units (cfu)  $\text{g}^{-1}$  according to fleece location (mean of three sheep).

Location	Mean $\times 10^8 \text{ cfu g}^{-1}$	
	Back transformed	Square root
Neck	34.57	5.88
Rump	9.54	3.09
Belly	6.60	2.57
Leg	1.32	1.15
Crutch	1.04	1.02
LSD (5%)	2.6	

**Table 2** Average bacterial number ( $\pm$  SEM)  $\text{g}^{-1}$  fleece of sheep from four flocks from different regions of New Zealand. cfu, colony forming units.

Flock	No. sheep sampled	cfu $\text{g}^{-1}$ ( $\pm$ SEM) fleece
Gore	20	$24.00 (\pm 1.83) \times 10^8$
Lincoln	24	$5.06 (\pm 0.64) \times 10^8$
Ruakura 1	17	$5.11 (\pm 0.87) \times 10^8$
Ruakura 2	17	$3.41 (\pm 0.52) \times 10^8$

**Table 3** Numbers of bacteria ( $\pm$  SEM)  $\text{g}^{-1}$  in outer, mid, and inner zones of the fleece and on the skin surface of Coopworth lambs. cfu, colony forming units.

Zone	Bacterial no.	% of total bacteria isolated (cfu)	Length of zone (mm)	Estimated bacterial density ( $\text{mm}^3$ )
Outer	$2.1 (\pm 0.49) \times 10^7 \text{ g}^{-1}$	$95.07 \pm 2.11$	$40.0 \pm 2.06$	2234
Mid	$8.6 (\pm 3.04) \times 10^5 \text{ g}^{-1}$	$3.84 \pm 2.04$	$35.5 \pm 1.77$	92
Inner	$4.5 (\pm 3.79) \times 10^4 \text{ g}^{-1}$	$1.09 \pm 1.00$	$18.5 \pm 0.93$	4.8
Skin	$0.37 \pm 0.11 \text{ cm}^{-2}$	–	–	3.7

**Table 4** Distribution of bacterial population through the fleece of eight Coopworth ewes.

Zone	Length of zone mm ( $\pm$ SEM)	No bacteria $\text{g}^{-1}$ ( $\pm$ SEM)	% Gram +ve cocci	% Gram	% Gram	% Gram
				–ve rods	–ve rods	+ve rods
Outer	$51.5 (\pm 3.3)$	$5.52 (\pm 1.69) \times 10^8$	100	41	27	32
Mid	$54.4 (\pm 3.1)$	$1.03 (\pm 7.68) \times 10^7$	96	39	28	29
Inner	$31.9 (\pm 1.6)$	$6.71 (\pm 3.53) \times 10^5$	100	35	29	36
LSD	–	–	8.1	10.7	3.5	12.3

ranging from yellow through orange to bright red. Gram staining and microscopic examination indicated that the bacteria growing on NA could be divided into three main groupings: Gram-positive cocci, Gram-negative pseudomonad-like rods, and Gram-positive rods. Similar proportions of each grouping were found within each of the zones (Table 4).

### Identification of bacteria

In all samples cocciform bacteria predominated, with most bacteria forming distinctive pigmented colonies on NA. More than 30% of the total bacteria from the Winchmore flock were pigmented (orange-red), Gram-positive cocci with biochemical characteristics corresponding to *Kocuria rosea* and *Micrococcus luteus* (Table 5). Similar proportions of *Kocuria rosea*, *Micrococcus luteus*, or *M. lylae* were isolated from the Gore flock, and bacteria of the same colour and characteristics were found on all fleeces sampled.

Gram-negative rods, where identified, were *Pseudomonas* spp. or *Flavimonas oryzihabitans*. Several isolates morphologically similar to *Pseudomonas* could not be identified to species level. The most commonly isolated Gram-negative rod, present in virtually every sample, formed orange-pigmented, glossy colonies and was identified as *Pseudomonas vesicularis*.

Colonies of Gram-positive rods were present in a range of colony forms and colours. They exhibited characteristics of diptheroid bacteria: non-sporeforming, catalase positive, oxidase negative.

Biochemical tests and commercial kits did not identify isolates from these colonies to a satisfactory level, although they shared similarities with *Corynebacterium* spp. Endospore-forming *Bacillus* spp. were uncommon and could only be isolated after pasteurisation of the samples.

### DISCUSSION

Enumeration of bacteria from different areas of the fleece showed that bacteria were distributed throughout the sheep fleece, but appeared to be most abundant on the upper part of the body. Numbers averaged  $10^8$ – $10^9$  bacterial cfu g<sup>-1</sup> of fleece, and numbers in this range were consistently recovered from different flocks and at different times of the year during this study. Similar numbers were recorded by Mulcock (1961) but, in Australia, Lyness et al. (1994) estimated that total bacterial populations were lower by approximately two logs, averaging  $10^6$  cfu g<sup>-1</sup>. In another study, Merritt & Watts (1978) estimated approximately  $10^7$  bacteria g<sup>-1</sup> from Australian fleece. While the methods used were different, these results suggest that differences in environmental conditions between the two countries may influence the total bacterial numbers in the fleece.

Bacteria were most abundant on the outer zone of the fleece, which accounted for >95% of the total population. Lower numbers were present in the mid zone, with very low densities in the inner zone and on the skin of these healthy sheep. Similarly low densities (< $10^2$  cfu cm<sup>-2</sup>) of Gram-positive cocci

**Table 5** Physical and biochemical characteristics of the two predominant species isolated from fleece. +, present; –, not present; D, depends on strain.

Characteristic	<i>Kocuria rosea</i>	<i>Micrococcus luteus</i>
Colony form	circular, smooth	circular, smooth
Colour	orange-red	yellow
Cell size	1–1.5 µm	1–1.5 µm
Cell configuration	pairs to clusters	pairs to clusters
Endospore	–	–
Gram test	+ve	+ve
Oxidase test	–	+
Aerobic acid from glucose	+	–
Nitrate to nitrite	+	–
Growth at 37°C	+	+
Growth on nutrient agar with 7.5% NaCl	+	+
Urease	–	D
β-Galactosidase	+	+
Arginine arylamidase	+	+
Pyrrolidonyl	–	+

were recovered from merino skin (Merritt & Watts 1978), but bacilliform bacteria were more abundant on the skin of the Australian sheep. Bacilliform bacteria were only a minor proportion of the total bacteria found in this study.

The sheep fleece is highly absorbent and will readily accumulate soil and faecal matter from the environment. This led to the suggestion that the bacteria associated with the sheep fleece are exogenous contaminants arising from the environment (Michalska 1957). High numbers of bacteria on the outer portion of the fleece lend weight to this suggestion. The composition of the bacterial population throughout the different fleece zones, however, is remarkably consistent and suggests the alternative hypothesis; that fleece bacteria are specifically associated with sheep. It is interesting that >80% of bacteria cultured from the fleece formed pigmented colonies on NA with colours ranging from deep red to yellow. Only a small proportion of bacteria recovered were cream, white, or colourless, which are much more common colours for soil and faecal bacteria. Lower numbers of bacteria on the legs and belly of the sheep also suggest that exogenous contamination is not the primary determinant of bacterial numbers.

The predominant bacteria isolated from the fleece were characterised as Gram-positive cocciforms of the Micrococcaceae. Most species in this family produce carotenoid pigments (Heinz Schleifer 1986) which are responsible for their colour range. Pigmentation has been shown to provide resistance to ultra-violet radiation in environmental bacteria (Cerdeña-Olmedo et al. 1996; Sundin & Jacobs 1999), which is a necessary attribute for bacteria colonising the fleece. The predominant bacterium isolated from many of the samples was an orange-red cocciform, identified as *Kocuria rosea* (formerly *Micrococcus roseus*) (Stackebrandt et al. 1995). While the primary habitat of the Micrococcaceae is mammalian skin (Heinz Schleifer 1986), *Kocuria rosea* is described as being isolated from soil and water (Heinz Schleifer 1986; Stackebrandt et al. 1995). The presence of this species in such high proportions on the sheep fleece suggests that sheep, and perhaps other mammals, are in fact the primary habitat for this bacterium with its presence in soil and water being coincidental. Growth at 37°C, a high level of salt tolerance, and carotenoid pigmentation are all factors that would suggest a mammalian surface

environment as the natural habitat for this bacterium.

These studies of the microbial population of the sheep fleece suggest that the fleece contains a specialised microflora with a carrying capacity in the healthy fleece of  $10^8$ – $10^9$  bacteria  $g^{-1}$ . Mulcock (1965) suggested that the fleece is a niche for microorganisms that can be detrimental to wool. Our studies suggest that the fleece contains a specialised microflora which poses no threat to wool quality but that disturbance of the microflora may allow proliferation of detrimental bacteria leading to fleece problems. The apparent differences in susceptibility of sheep from a single blood line to fleece rot and fly strike (Chin & Watts 1992) may owe more to variation in the microbial composition of the healthy fleece than any inherent differences in the sheep. The role of the endogenous microflora in suppressing fleece disorders such as fleece rot and fly strike should be investigated. If significant, a probiotic approach involving inoculation of sheep with beneficial microbes could be used.

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