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# Scanning Electron Microscopic Investigations into Attachment of Bacteria to Teats of Cows

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**ABSTRACT**

Scanning electron microscopy was used to examine microorganisms on the skin of cows' teats which were artificially contaminated and subsequently stored for different times. From the results it seems that bacteria were not spread uniformly on the surface of the teats. No extra-cellular polymers were observed before storage of the teats, but during storage polymers, in the form of thin fibers, were produced. These fibers became thicker and finally resulted in slime. Multiplication of bacteria during storage involved formation of microcolonies of bacteria in which bacteria were not attached directly to the skin but to other bacteria. This explains the decrease in the differences between bacterial counts obtained by the blending and rinsing methods after long periods of storage.

Bacteria attach readily to the surface of teats of cows as demonstrated by Notermans et al. (*8*). The attachment rate depends, among other factors, on bacterial strain, composition of the medium in which attachment occurs (*7*) and on the attachment surface (*3,10*).

Fletcher and Floodgate (*2*) and Marshall et al. (*6*) stated that the mechanism of attachment involves two consecutive steps, namely a primary attachment followed by a time-dependent secondary attachment. The primary attachment can be caused by different physical forces like London-van der Waals attraction, electrostatic attraction of the two surfaces or by a gain in entropy. Besides these attractive forces, bacteria which attach to the skin of teats may become locked in small holes or in the skin tissue. During secondary attachment the strength of attachment increased, as shown by Notermans et al. (*8*) and by Marshall et al. (*6*). This increment is presumably caused by metabolic activity of the bacteria once attached. Fletcher and Floodgate (*2*) demonstrated that extracellular substances, often composed of secondary acidic polyaccharides are produced by primary attached bacteria. By this process bacteria encapsulated themselves on the skin surface. It may also be possible that bacteria become located or embedded inside the skin tissue.

On the basis of the results of Notermans et al. (*8*) it becomes clear that the S-value (measured as the difference between bacterial counts obtained by the blending and rinsing methods) which increased during the secondary attachment has an optimum. This optimum was reached after 2 - 3 h. After longer storage times colonies of bacteria may be formed which cause a decrease in the S-value.

To learn more about the mechanism of attachment of different bacterial strains to the surface of meat, scanning electron microscopic (SEM) investigations were carried out using teats of cows as surface.

## MATERIALS AND METHODS

*Bacterial strains and their counting media*

In these experiments the following bacterial strains were used: *Pseudomonas* EBT/2/143, *Staphylococcus aureus*, *Klebsiella* sp. and *Salmonella typhimurium*. These bacteria, their growth media and counting media, were the same as described in the previous study of Notermans et al. (*8*) regarding attachment of bacteria to teats of cows.

*Teats*

Teats of cows were obtained from a local slaughter-house. They were cleaned and deep-frozen until experimentation. The teats were thawed by holding them at 4 C for 15 h before experimentation.

*Contamination of the teats*

Teats were dipped in a bath containing 500 ml of attachment suspension. This suspension contained 0.87% NaCl and 0.01 M phosphate buffer with a pH of 7.2. To this suspension a bacterial culture was added so that about $5 \times 10^8$ bacteria were present per ml. The suspension was mixed by forced aeration and maintained at 20 C. After a residence time of 20 min the teats were removed from the bath and washed by dipping and gently moving them in sterile physiological saline solution. This procedure was repeated three times using new sterile solution. After contamination the teats were stored in closed sterile glass jars at 20 C.

*Scanning Electron Microscopy (SEM)*

For fixation of the samples the procedure as described by Karnovsky (*4*) was used. This procedure includes fixation in 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.5 for 24 h. Specimens were rinsed in 0.1 M phosphate buffer (4 C) for 12 h.

Samples were dehydrated through graded ethanol solutions followed by dehydration in graded amyl acetate in ethanol solutions. After dehydration, specimens were dried to critical point with $CO_2$ in a BALZERS critical point drying apparatus. Samples were attached to specimen studs with silver paint and subsequently coated with gold in a BALZERS sputter apparatus. The specimens were viewed with a JEOL-JSM-$U_3$ scanning electron microscope operating at 10-15 kV. One sample was viewed also with a Cambridge 150-S SEM operating under the same conditions.

*Exposures taken by SEM*

In the first experiments exposures were made of the surface of the

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teat without artificial contamination. Next, exposures were made of the surface after artificial contamination. For these experiments teats were contaminated as described above with, respectively, *Ps.EBT*/2/143, *S. aureus*, *Klebsiella* sp. and *S. typhimurium*. Using *Ps. EBT*/2/143 and *S. aureus*, exposures of the samples were made immediately after attachment, after a storage time of 3 h and after a storage time of 12 h. Using *Klebsiella* and *S. typhimurium*, exposures were only made after a storage time of 3 h.

## RESULTS

In Fig. 1, an exposure of the surface of the teat is shown with the natural flora generally present. The natural flora of the teat was spread very non-uniformly over the skin. Some of the bacteria were present in small niches of the skin. On the basis of the other exposures it was clear that only a very small part of the surface was covered with bacteria and that the exposure in Fig. 1 shows an extremely contaminated area.

To study the mechanism of attachment, artificially contaminated teats were used. Different exposures were made of every sample. The most representative are presented in this paper. In Fig. 2 (a,b,c,d), surfaces with *Ps. EBT*/2/143, after different storage times, are shown.

Figure 2a shows the bacteria on the teat surface immediately after contamination. The bacteria were non-uniformly spread over the teat. After storage of 3 h at 20 C (Fig. 2b) production of extracellular polymers in the form of thin fibers could be observed. The amount of those fibers and their thickness increased during storage. Slime was also noted (Fig. 2c). After 12 h of storage microcolonies were observed (Fig. 2d). In the microcolonies it could be seen that more and more bacteria were attached to each other and not to the skin of the teat. Some of the colonies were covered with slime while others contained only a very small amount of slime.

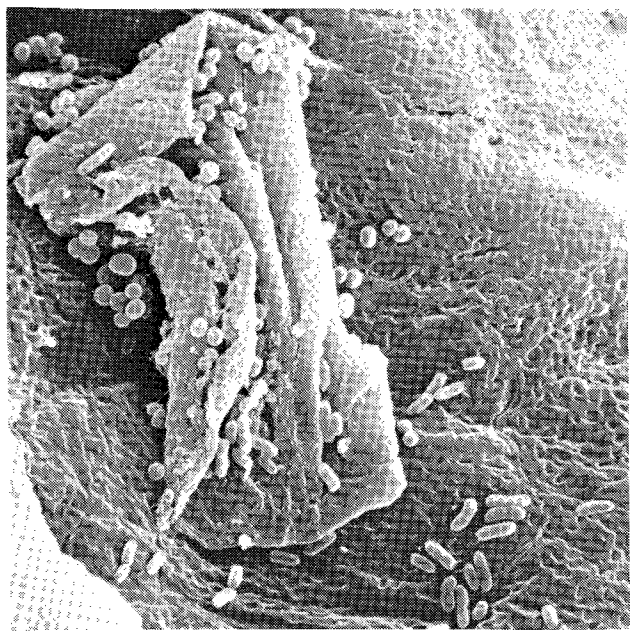

Figure 1. *The natural flora generally present on the skin of teats (× 3000).*

Figure 3 (a, b, c, d) shows teats artificially contaminated with *S. aureus*. The behavior of *S. aureus* was similar to that observed for *Ps.EBT*/2/143, i.e. immediately after inoculation the bacteria were spread non-uniformly and no extracellular material could be observed. During storage extracellular materials produced by *S. aureus* looked different from those produced by *Ps.EBT*/2/143 (less fibrous).

Inoculation of teats with *Klebsiella* sp. for 3 h brought similar results, i.e. production of extracellular fibers followed by a small amount of slime.

A picture of *S. typhimurium* attached to teat skin after 3 h of storage is presented in Fig. 4 (a, b). This bacterium also produced extracellular fibers (4b), which are absent on teat skin without bacteria. The cells of this bacterium were much longer than those of other bacteria examined and they tended to grow in short chains of 2-3 cells. This seemed to interfere with attachment of this bacterium to the skin (Fig. 4a).

## DISCUSSION

Teats of cows were found to be extremely suitable for examining the attachment of microorganisms with a SEM. The surface of the cleaned teat seen in the electron microscope was smooth and did not contain fibers. SEM experiments made with chicken skin (5) showed that those surfaces were covered with fibers. This made it difficult to distinguish between those fibers and the fibers produced by the bacteria during storage.

The process of attachment of bacteria to the teats may also be divided into two sequential processes, as stated by Fletcher and Floodgate (2) and Marshall et al. (6) who used other surfaces. After primary attachment a time-dependent secondary attachment appeared during storage of the samples. No production of extracellular materials could be seen after the primary attachment. However, the non-uniform spread of the natural flora, as well as of all other bacteria with which the teats were contaminated, could indicate an interaction between bacteria and certain microecosystems on the surface. The shape of bacteria could also play a role in the primary attachment (Fig. 4a). The mechanism of primary attachment could not be explained by these experiments.

During secondary attachment, production of extracellular polymers was observed for all bacteria examined. Similar results, i.e. production of extracellular polymers by attached bacteria, was also found by Marshall et al. (6) and by Fletcher and Floodgate (?), using marine bacteria. At the beginning of secondary attachment, production of thin fibers was observed. These fibers became thicker during storage. In this stage the bacteria tended to produce slime. The fibers, as well as the slime produced by different bacteria, looked different and may have been composed of different polymers. The shape of bacteria and their fit to the surface seems also to increase the strength of attachment.

Some of the microcolonies formed were covered with slime (Fig. 2c and 3c), while others contained only small

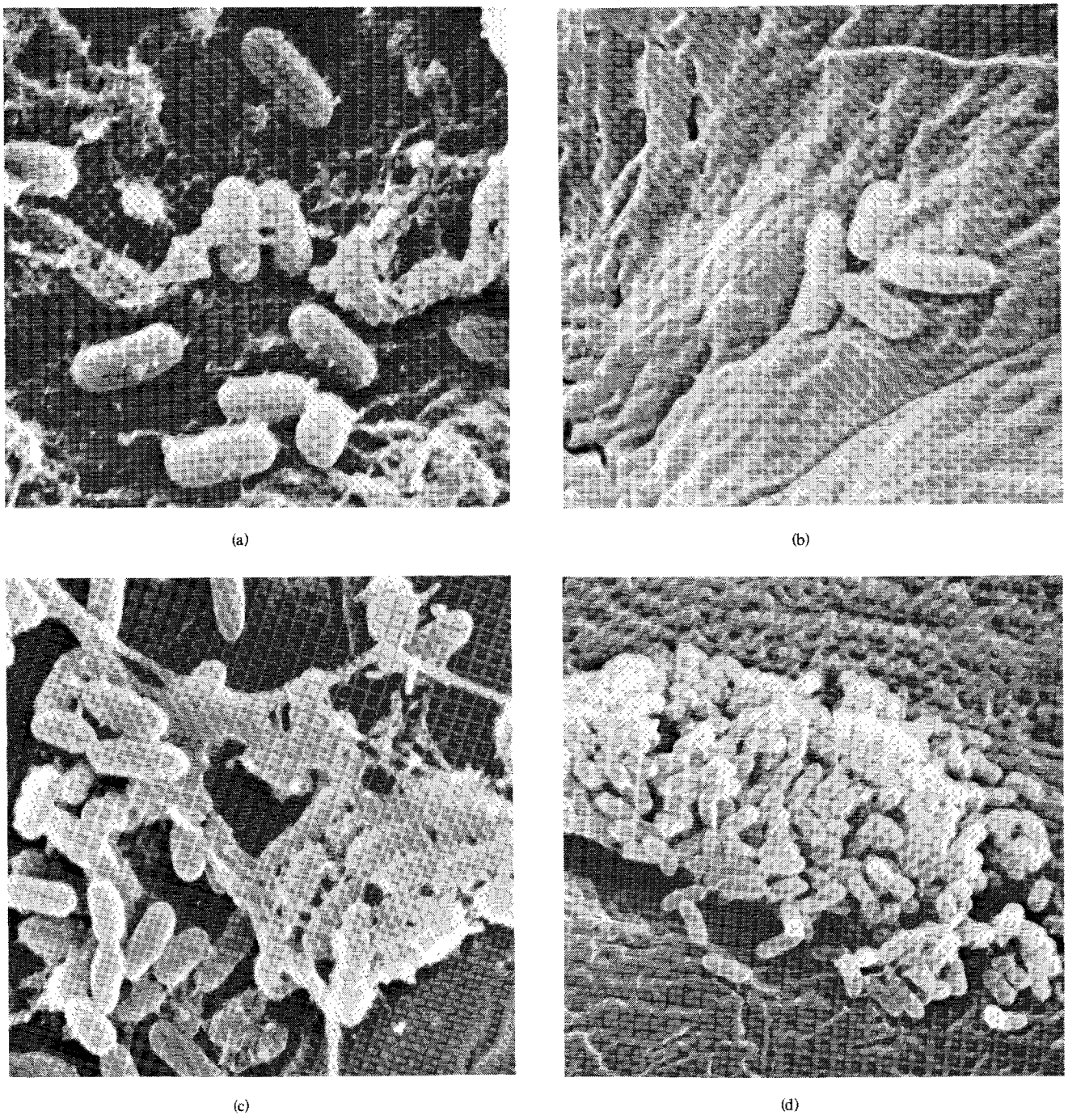

Figure 2. *Teats artificially contaminated with* Ps.EBT*/2/143:* (a) *immediately after contamination* (× *10,000*), (b) *after 3 h of storage* (× *10,000*), (c) *slime production after 12 h of storage* (× *10,000*), *and* (d) *micro-colonies formed after 12 h of storage* (× *6,000*).

amounts of slime (Fig. 2d and 3d), probably due to differences in available nutrients. Zobell (*9*) stated that slime production by attached bacteria appeared to be influenced mainly by the micro-ecosystem. He found that more slime was produced in ecosystems in which it was difficult for bacteria to survive. Costerton et al. (*1*) stated that the polymers (glicocalyx) produced by bacteria may not only position the bacteria but also conserve and concentrate the digestive enzymes and serve as a food reservoir for the bacteria.

Results obtained in the work with the SEM support the experimental data obtained previously (*8*). The increase in S-value during the first 2-3 h of storage could be explained by the formation of the extracellular fibers. The decrease in strength of attachment found after longer storage was probably due to formation of microcolonies in which more of the bacteria were attached to each other and not directly to the skin, and so could be removed easily.

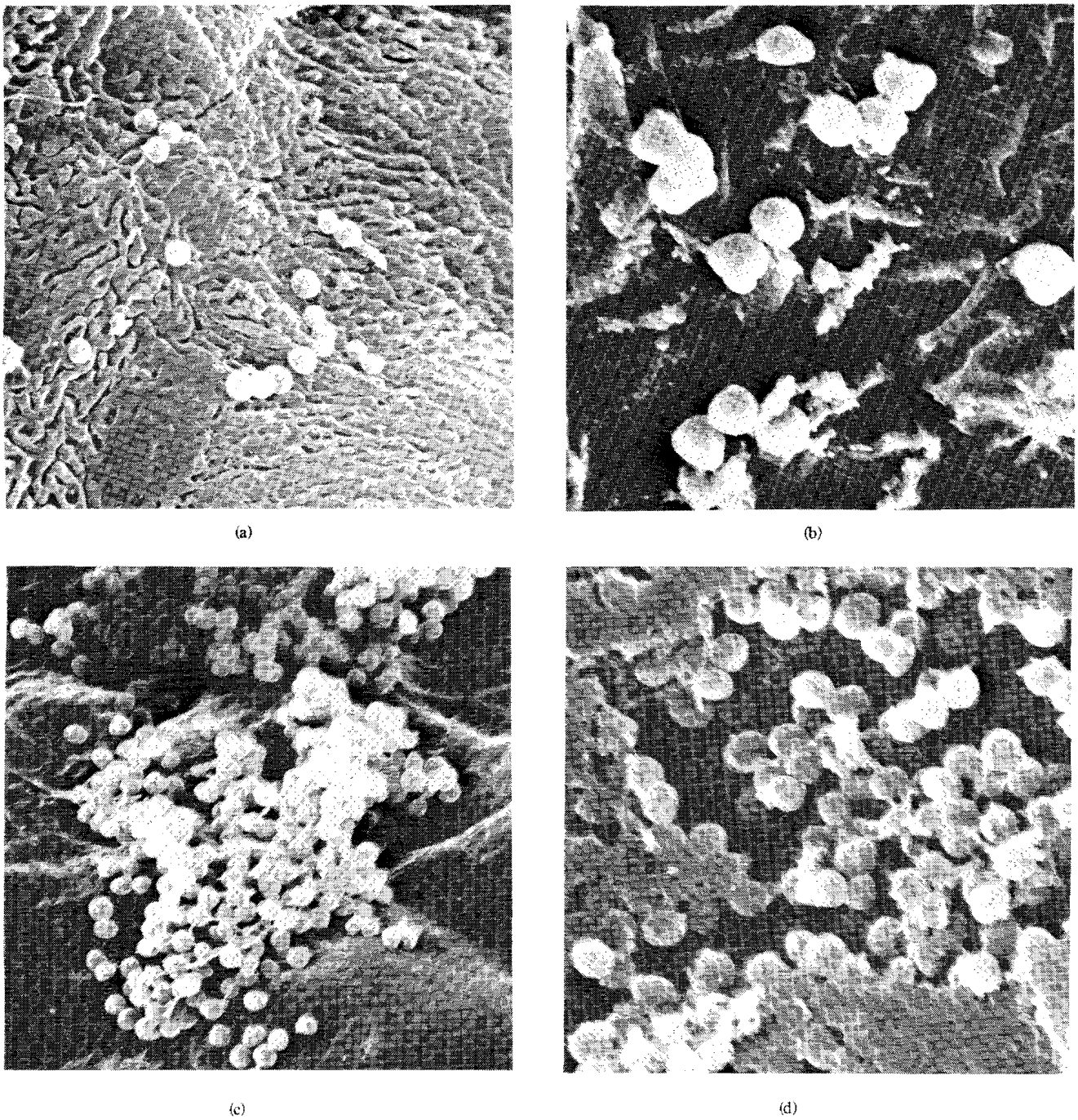

Figure 3. *Teats artificially contaminated with* S. aureus: (a) *immediately after contamination* (× *6,000*), (b) *after 3 h of storage* (× *10,000*), (c) *slime production after 12 h of storage* (× *10,000*), *and* (c) *micro-colonies formed after 12 h of storage* (× *6,000*).



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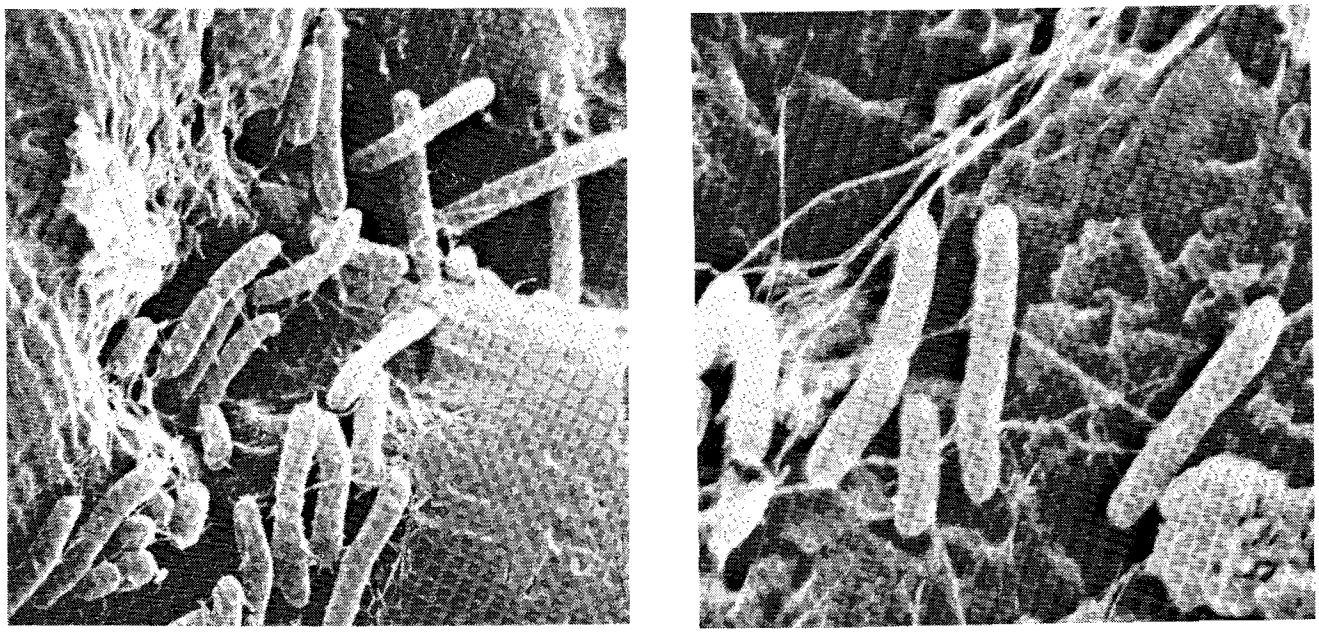

(a) (b)

Figure 4. *Teat artificially contaminated with* S. typhimurium *after 3 h of strage:* (a) *arrangement of* S. typhimurium *on the skin* (× *4,000*), *and* (b) *fibers produced by* S. typhimurium (× *10,000*).

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