New Method To Study Bacterial Adhesion to Meat

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A new method was developed for the study of bacterial adhesion to meat surfaces. Thin slices of meat (40 μm thick) were inserted into a specially designed observation chamber. The meat slices were then exposed to a bacterial suspension (ca. 10^6 CFU · ml^{-1}) to initiate adhesion (20 min of contact time) and subsequently rinsed to eliminate nonadherent bacteria. Because of the special chamber design, the disruptive force exerted on the bacteria during rinsing (shear stress) was uniform over the whole surface of the meat slices, was constant, and could be varied from 0 to 0.08 N · m^{-2}. After being rinsed, the meat slices were stained with basic fuschin and observed under light microscopy to determine the number and distribution of adherent bacteria. This new method was used to study the adhesion of *Acinetobacter* strain LD2, a *Lactobacillus* sp., and *Pseudomonas fluorescens* to slices of beef fat and tendon. At 25°C, most (≥99.9%) of the cells of the *Lactobacillus* sp. deposited on the meat were washed off the surface during rinsing (0.05 N · m^{-2}), whereas a large number (ca. 10^5 CFU · cm^{-2}) of *Acinetobacter* strain LD2 and *P. fluorescens* cells remained adherent. The extent of adhesion was similar on fat and tendon, and adherent bacteria were distributed evenly over the whole surface of the slices. This preliminary study indicates that the combined use of thin slices of meat and of the observation chamber provides us with the means to more accurately study bacterial adhesion to meat surfaces.

The surface of meat carcasses is always contaminated with microorganisms during slaughter and subsequent handling. Some of the contaminating organisms will grow at refrigeration temperatures and cause spoilage. A better understanding of the association of bacteria with meat surfaces could suggest ways to limit the initial contamination and to decontaminate meat surfaces, resulting in a delay of spoilage.

The association of bacteria with meat surfaces is best documented on chicken. Most of the organisms growing on chicken are dispersed in the water film remaining on the meat after spray cleaning and immersion chilling (22). These bacteria are easily washed off with water. In contrast, a fraction of the organisms associated with the chicken surface are not displaced by rinsing. Some of these bacteria are thought to be entrapped in channels and crevices formed during immersion chilling of the chicken carcass (13, 19, 20, 22, 23), while others are believed to be adherent to the meat surface (1, 2, 5, 6, 11, 12, 17–19, 21), although the precise mechanisms of adhesion have not been elucidated.

In the absence of a simple method to enumerate entrapped and adherent bacteria separately, little is known about their respective numbers on the meat surface. Consequently, it is still debated whether bacteria actually adhere to meat in large numbers (1, 6, 11, 12, 17) or not (22, 24). To clarify the situation, we developed a new microscopic method to evaluate the number and distribution of bacteria adhering to meat. The method is based on the fact that thin slices of meat are transparent to light. An observation chamber into which meat slices are inserted, exposed to bacterial suspensions, and rinsed under controlled conditions before the adherent bacteria are counted by light microscopy was specially designed. We report on the design and characteristics of the observation chamber and illustrate its use in a study of the adhesion of three meat spoilage organisms on slices of beef tendon and beef fat.

MATERIALS AND METHODS

Design and operation of the observation chamber. An expanded view of the observation chamber is presented in Fig. 1. The main body of the chamber is composed of two polycarbonate plates (C and D) screwed tightly to an aluminum base plate (E). A shape is cut out from the central plate (D) to create a channel through which a fluid can be circulated. To prevent leakage, gaskets (not shown in Fig. 1) are inserted between the central plate and the adjacent plates. The gaskets are cut from Parafilm sealing material (American Can Co., Greenwich, Conn.), with the central plate as a template. The chamber is completed with the insertion of windows in the external plates. Each window consists of a glass cover slip (24 by 60 by 0.15 mm) (B) glued to a polycarbonate frame (A) with silicone sealant. One window is fixed to the chamber with silicone sealant and remains in place permanently. Samples of tendon or fat are inserted on the cover slip of the other window. This window is sealed with electrician's tape to permit easy removal and replacement. Once both windows are in place, the chamber is leakproof. The meat sample can therefore be observed by light microscopy when the chamber is filled with a bacterial suspension (Fig. 2).

Liquids are introduced into the observation chamber by gravity through a distribution line composed of Tygon tubing (internal diameter, 1.6 mm) and polycarbonate valves. The distribution line connects the chamber to one of three reservoirs. The first two reservoirs (10 ml each) are used to deliver bacterial suspensions and colorant, respectively, to the chamber. The third reservoir (500 ml) is a Mariotte flask which contains the rinsing solution. The fluid is released at a constant pressure, and the flow rate in the chamber is adjusted by raising or lowering the Mariotte flask.

Flow characteristics in the observation chamber. Since the velocity profile across a closed channel is symmetrical (14), the fluid velocities along the surfaces of the top or bottom cover slips (v_s) are identical. To measure v_s, we placed the observation chamber under a microscope and focused on the
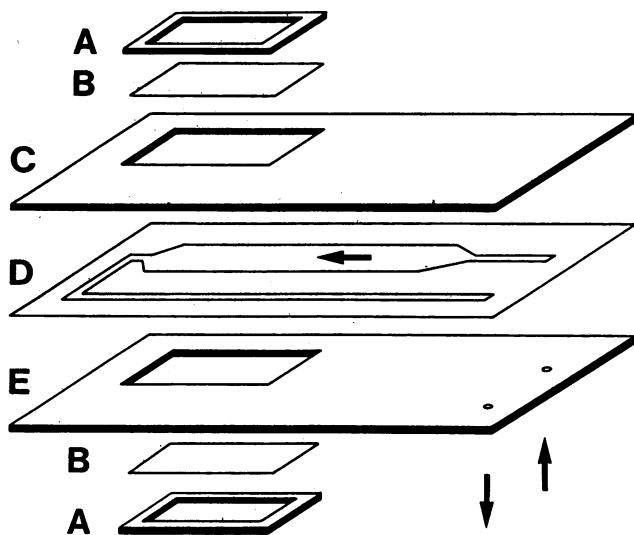
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FIG. 1. Expanded view of the observation chamber. Windows: A, window frame; B, glass cover slip. Main body: C, external polycarbonate plate (2.4 mm thick); D, central polycarbonate plate (0.5 mm thick); E, aluminum base plate (2.0 mm thick). Arrows indicate the direction of the flow.

surface of the bottom cover slip. A suspension of polystyrene beads (diameter, 1 μm) in distilled water was introduced into the Mariotte flask and circulated in the chamber at a fixed flow rate. The time necessary for each of 30 beads to travel a fixed distance (1,030 μm) was recorded, and the average time was used to calculate v_s. The same procedure was used to measure the fluid velocities along the surfaces of fat or tendon slices.

Organisms and cultures. *Pseudomonas fluorescens* and *Acinetobacter* strain LD2 were isolated from refrigerated beef (3). A *Lactobacillus* sp. was isolated from refrigerated vacuum-packed ham. Stock cultures were kept frozen at −80°C in reconstituted skim milk (skim milk powder in distilled water; final concentration, 20% [wt/vol]). Working cultures were prepared in brain heart infusion broth (for *Acinetobacter* strain LD2 and *P. fluorescens*) or lactobacillus MRS broth (for the *Lactobacillus* sp.) (both broths were from Difco Laboratories, Detroit, Mich.). All incubations were done at 25°C without agitation. The bacterial suspensions for the adhesion experiments were prepared by growing the organisms for three consecutive 24-h periods, with transfer in fresh medium each day. The cultures were diluted 1:50,000 in fresh medium, incubated at 25°C, and harvested during the late exponential phase of growth (after 8, 10, and 12 h of incubation for *P. fluorescens*, *Acinetobacter* strain LD2, and the *Lactobacillus* sp., respectively). Immediately before use in the adhesion experiments, viable counts of the bacterial suspensions were determined with standard dilution and plating techniques.

Meat slices. Beef fat (from the flank area) and beef heel tendon were obtained fresh from a local meat-processing plant. They were immediately vacuum packed to prevent dehydration and frozen and stored at −10°C. When required, blocks of tissue (1 by 1 by 0.5 cm) were cut from the frozen material. The blocks were subsequently sectioned into 40-μm-thick slices (1 by 1 cm) by using a Tissue-Tek II cryostat (Ames, Div. Miles Laboratories, Inc., Naperville, Ill.) adjusted to −20°C (tendon) or −30°C (fat). Slices were transferred onto glass cover slips (24 by 60 mm), stored at 4°C, and used within 24 h in the adhesion experiments.

Adhesion experiments. Adhesion experiments were performed at 25°C. A glass cover slip with its adherent meat slice was mounted in the observation chamber, and the chamber was positioned so that the meat slice was on the bottom, face up. The chamber was filled with a bacterial suspension and left to stand for 20 min. In experiments with motile *P. fluorescens*, the chamber was rinsed with distilled water (5 min; 110 μl · s^{-1}) to remove nonadherent bacteria and filled with a staining solution (basic fuschin in distilled water, 0.25% [wt/vol]). After coloration (10 min), the chamber was rinsed with distilled water again (2 min; 110 μl · s^{-1}), disconnected from the distribution line, and drained. The cover slip bearing the meat slice was removed from the chamber, dried for a few hours at 4°C, and stored at −10°C. Within 48 h, the meat slice was observed under light microscopy (160 to 205×) to determine the distribution of bacteria on the meat surface. Also, the number of adherent bacteria was counted in 30 separate fields (640×; total area, 1.14 mm^2) evenly distributed over the whole surface.

The same procedure was used for the nonmotile organisms (*Acinetobacter* strain LD2 and the *Lactobacillus* sp.), with minor modifications. Following the introduction of the bacterial suspension into the chamber, the bacteria started to sediment. After 20 min, photomicrographs of the bottom cover slip (near the meat surface) were taken and the sedimented bacteria were counted. This count was also assumed to represent the number of bacteria that sedimented on the meat slice. The chamber was turned over, and nonadherent bacteria were allowed to fall away from the surface for 10 min before the subsequent rinsing and staining steps.

RESULTS

Characteristics of the observation chamber. The observation chamber could be rinsed at flow rates of up to 200 μl · s^{-1}. At these flow rates, the Reynolds number did not exceed 22, indicative of a laminar flow (14). The laminar nature of the flow was verified experimentally by using a 24-h-old culture of the *Lactobacillus* sp. (ca. 10^8 CFU · ml^{-1}) grown in MRS broth at 25°C. A 1-ml portion of the culture was mixed with the rinsing water in the Mariotte flask. The suspension was circulated through the chamber at various flow rates (up to 200 μl · s^{-1}), and the movement of bacteria within the chamber window was observed under the microscope. Bacterial cells always travelled in parallel and
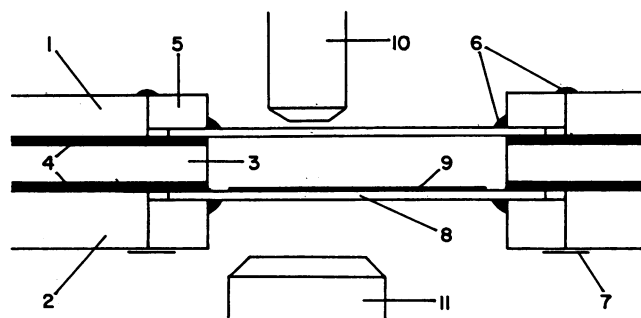

FIG. 2. Schematic representation of the observation chamber on the microscope stage. 1, Aluminum plate; 2, external polycarbonate plate; 3, central polycarbonate plate; 4, Parafilm gaskets; 5, window frame; 6, silicone sealant; 7, electrician's tape; 8, glass cover slip; 9, meat slice; 10, objective; 11, condenser.
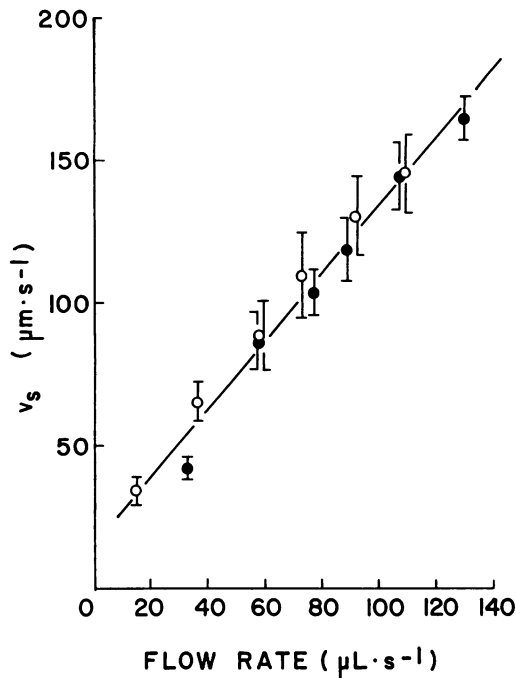
FIG. 3. v_s along the surface of the observation chamber (cover slip) as a function of the fluid flow rate. Closed and open circles represent the results of two separate experiments with six replicates each.

only in the direction of the flow, confirming that the flow was laminar.

The intensity of the disruptive force exerted on the bacteria during rinsing was characterized in two ways. Firstly, the shear stress exerted on the surface of the observation chamber by the fluid in motion (r_w) was calculated from the general equation of a laminar flow between parallel plates (14). The stress was directly proportional to the fluid flow rate and varied from 0 to 0.08 N · m^{-2} when the flow rate was increased from 0 to 200 µl · s^{-1}. Secondly, the fluid velocity along the surface of the cover slip (v_s) was measured with 1-µm-diameter polystyrene beads. v_s was found to be directly proportional to the fluid flow rate (Fig. 3). Velocities between 40 and 170 µm · s^{-1} could be selected by adjusting the fluid flow rate to the appropriate value in the range from 20 to 140 µl · s^{-1}. At flow rates higher than 140 µl · s^{-1}, precise measurements of v_s were impossible, owing to the fast movement of the beads across the microscope field.

A rinsing flow rate of 110 µl · s^{-1} was selected for the adhesion experiments and corresponded to a shear stress of 0.05 N · m^{-2} and to a v_s value along the surface of the cover slip of 150 µm · s^{-1}. At this flow rate, the v_s values along the surface of fat and tendon slices were found to be 133 ± 13 µm · s^{-1} (five replicates) and 131 ± 10 µm · s^{-1} (five replicates), respectively.

To verify that rinsing conditions were uniform across the observation chamber, we measured v_s values along the surface of the cover slip in 12 different locations situated 1.42 mm apart on a line perpendicular to the direction of the fluid motion. Identical v_s values were found in the 12 locations (coefficient of variation, 6.6%).

When v_s values were repeatedly measured in the same chamber, with removal and reinsertion of the window between each measurement, the coefficient of variation was
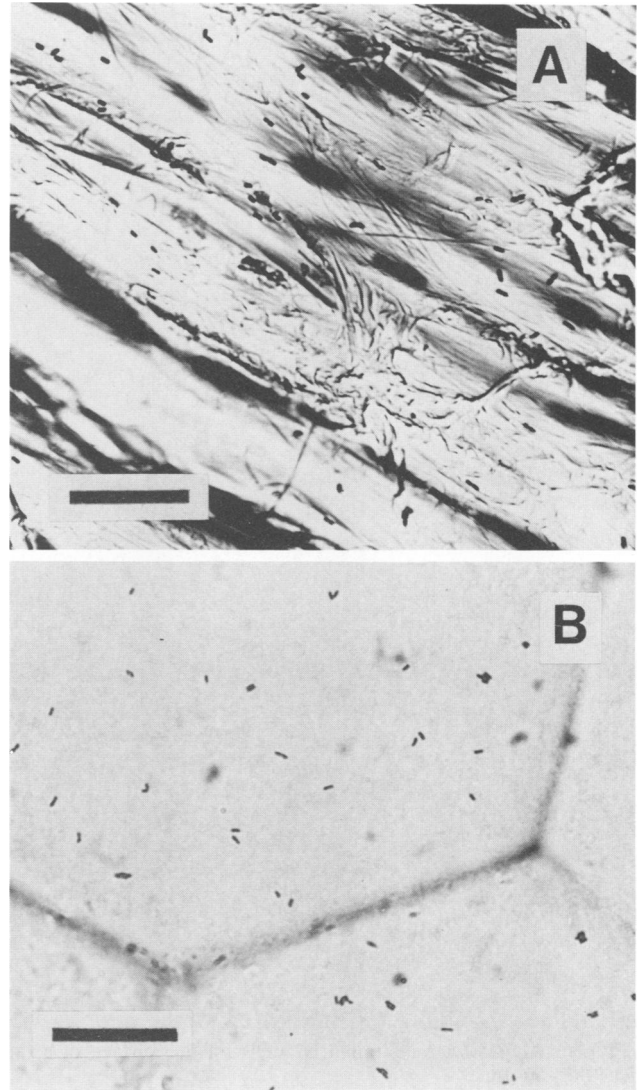

FIG. 4. *P. fluorescens* cells on surfaces of tendon (A) and fat (B) slices. Bars, 20 µm.

found to be 8.3% or lower (three experiments of four, six, and six replicates), indicating that the rinsing conditions were constant from sample to sample.

To accelerate the accumulation of data, we used six identical observation chambers. In two replicate experiments, the v_s values in the six chambers were found to be very similar (coefficient of variation, ≤6.1%).

Adhesion experiments. The bacteria remaining on fat and tendon slices after rinsing could be observed unstained at a 640× magnification. However, a much sharper image was obtained after coloration with basic fuschin. Experiments in which the sample was continuously observed during staining indicated that staining did not affect the number and distribution of adherent bacteria. After coloration, even the smallest bacterium used in the adhesion experiment, *P. fluorescens*, was clearly discernible on both fat and tendon slices (Fig. 4) and could therefore be enumerated precisely.

The distribution of bacterial cells over the meat surface was evaluated by observing the meat slices at low magnifications (160 to 205×). There was no obvious change in the

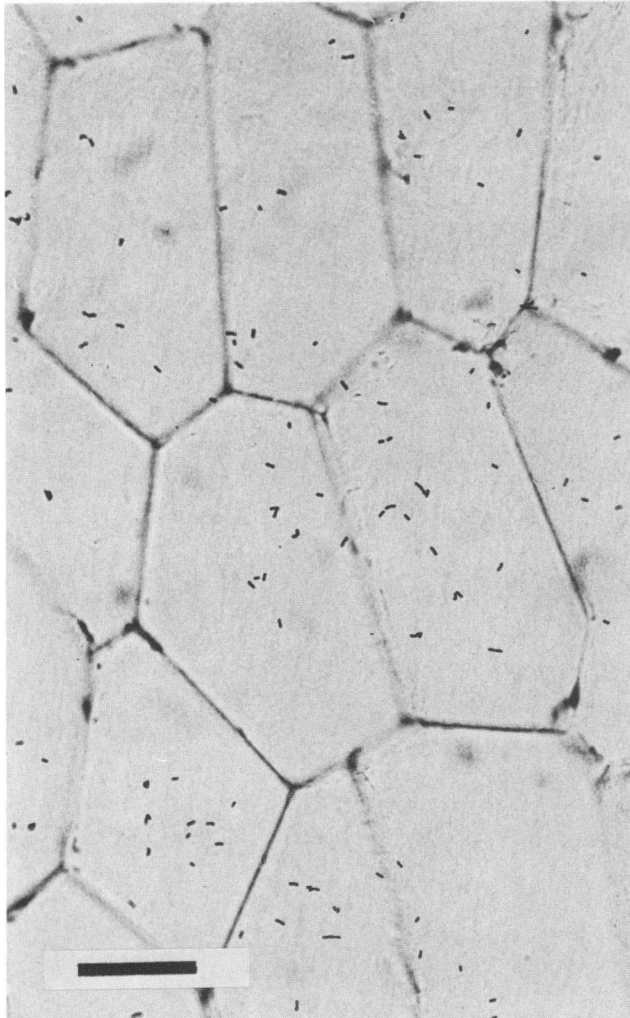FIG. 5. *Acinetobacter* strain LD2 cells on the surface of a fat slice. Bar, 50 μm.

TABLE 1. Adhesion of three meat spoilage bacteria to the surfaces of tendon or fat slices

| Organism | No. of bacteria ($\log_{10}$ CFU · cm^{-2}) | | | |
| | Introduced in the chamber[a] | Sedimented after 20 min[b] | Adherent to the surface of[c]: | |
			Tendon[b]	Fat[b]
Acinetobacter strain LD2	5.53	5.14 ± 0.06	5.12 ± 0.12	
	5.28	4.94 ± 0.06	4.75 ± 0.05	
	5.61	5.12 ± 0.03		4.87 ± 0.09
	5.52	5.14 ± 0.03		4.89 ± 0.10
Lactobacillus sp.	6.63	6.12 ± 0.03	2.52 ± 0.22	
	6.43	5.91 ± 0.06	2.99 ± 0.22	
	6.61	6.11 ± 0.05		2.92 ± 0.17
	6.56	6.01 ± 0.04		2.43 ± 0.30
P. fluorescens	5.88	NA[d]	5.26 ± 0.20	
	5.90	NA	5.78 ± 0.03	
	6.15	NA		5.27 ± 0.24
	6.00	NA		5.44 ± 0.09

[a] Expressed as the number of bacteria poised over a unit area.
[b] Mean ± standard deviation (six replicates).
[c] Remaining on the surface after rinsing.
[d] NA, Not applicable (motile *P. fluorescens* does not sediment).

microtopography of the meat slices during the adhesion experiments, and no crevices were formed. Bacteria were uniformly distributed over the whole surface of fat (Fig. 5), and no difference in the concentration of adherent bacteria was observed between adipocytes and the interlobular strands of connective tissue. Similarly, bacteria were evenly distributed on the surfaces of tendon slices and showed no preference for the collagenous tendon bundles or the peritendineum.

Regardless of the organism, the nature of the meat tissue had no apparent effect on the number of bacteria remaining on the meat surface after rinsing (Table 1). In contrast, adhesion to meat slices varied with the organism. Almost all ($\geq$99.9%) of the deposited cells of the *Lactobacillus* sp. were washed away during rinsing, while a large number of *Acinetobacter* strain LD2 and *P. fluorescens* cells remained adherent to the meat surface.

DISCUSSION

In earlier studies of bacterial adhesion to meat, pieces of meat were immersed in bacterial suspensions constantly kept agitated (3, 5, 6, 11–13, 17, 18, 21, 24). During immer-

sion, bacteria collided with the meat, and a fraction of them became adherent to the meat surface. The pieces of meat were taken from the bacterial suspension and rinsed to remove nonadherent or weakly adherent bacteria before enumeration of the remaining adherent bacteria. Rinsing was done in a flow of tap water or saline (11–13) or by gently moving the pieces of meat in sterile water baths (3, 5, 6, 17). The number of bacteria which came in contact with the meat during immersion and the number of bacteria remaining on the meat after rinsing depended on the conditions of flow (bacterial suspension or rinsing fluid) in the immediate surroundings of the meat surface. These conditions were undefined, difficult to replicate, and likely not uniform over the whole surface, especially when large samples with irregular shapes, such as whole chicken carcasses (18) or teats of cows (17), were used. Also, the disruptive force exerted on the bacteria during rinsing was impossible to quantify.

The number of bacteria remaining on the meat after rinsing was generally determined by classical microbiological techniques (homogenization, dilutions, and plating). The number obtained included "attached" (i.e., adherent) bacteria but also the bacteria physically entrapped in crevices formed at the meat surface during immersion. These entrapped bacteria in fact might have constituted the majority of the bacteria associated with the meat surface (13, 24). Therefore, a viable count of the bacteria remaining on a meat sample after immersion in a bacterial suspension and rinsing does not truly represent the number of bacteria adhering to the meat surface.

One might think that bacteria adherent to the surface of meat could easily be counted from scanning electron photomicrographs of the surface. This has proved difficult, however, because many bacteria are hidden in crevices (13, 16) or in a mesh of swollen collagen fibers (21). In addition, one might question altogether whether a scanning electron photomicrograph actually represents the state of the meat surface prior to fixation and dehydration. The polymer fibrils linking adherent bacteria to the meat surface (2, 5, 16) and the troughs or pits around adherent bacteria (2, 22) observed in earlier studies have been shown to be artifacts of sample

preparation (7, 15). Substantial loss of bacteria from the meat surface during fixation has also been reported (16).

Compared with previous methods, the method presented here offers many advantages. With the observation chamber, the conditions under which bacteria were put in contact with meat could be better standardized. In replicate trials, constant numbers of nonmotile bacteria sedimented on the meat surface during the 20 min that the bacterial suspension was left in the chamber. Rinsing was also done in a well-defined manner. The geometry of the chamber was such that the flow of circulating fluid was always laminar. The flow was therefore uniform in direction and intensity over the whole meat surface and constant with time. Also, the flow was quantifiable. At each flow rate, the disruptive force exerted on the bacteria could be characterized by the shear stress (r_w) or by the fluid velocity (v_s) along the chamber wall. In addition, the intensity of the rinsing flow could be easily adjusted by changing the fluid flow rate through the chamber. Therefore, the flow characteristics in the chamber were reproducible, and similar rinsing conditions were achieved in replicate trials.

In previous studies, bacteria adherent to human epithelial cells cultured in monolayers could be observed directly by light microscopy (8–10). Similarly, the use of thin slices of meat (this study) allowed the direct observation and enumeration of bacteria on the surface of meat by simple light microscopy. The use of meat slices for the study of bacterial adhesion has some limitations. Clearly, meat slices would be inappropriate in a study of bacterial adhesion to chicken skin, which has a very complex structure (20). In other cases, however, meat slices can provide a useful model system. For example, the meat surfaces created during dressing of a beef carcass are mostly fat or perimysium. The surface of a fat slice adequately represents the surface of the fat layer left around the muscle during dressing. Both surfaces result from the sectioning of adipose tissue with knives. Also, since all the separate sheets of perimysium from one muscle fuse together in the tendon, tendon and perimysium are similar in structure and composition. The proportions of the various elements may vary in each tissue, but all the major components of perimysium are present in tendon. If one of these plays a major role in bacterial adhesion, its effect is expected to be detectable in tendon slices.

Acinetobacter strain LD2 and *P. fluorescens* were previously reported to adhere to pieces of beef longissimus dorsi muscle (3). Also, *P. fluorescens* and other *Pseudomonas* species were reported to adhere to the skin of beef (1, 6), chicken (6, 11, 18), lamb (1), and pork (1), as well as to beef and chicken muscles (6) and to teats of cows (5, 17). In all these studies, however, the extent of bacterial adhesion could not be determined, since adherent and entrapped bacteria were counted as one. In contrast, adherent bacteria were precisely counted in this study, and the results indicated that both *Acinetobacter* strain LD2 and *P. fluorescens* adhered to meat surfaces (tendon or fat) in large numbers (ca. 10^5 CFU $\cdot$ cm^{-2}).

Our strain of *Lactobacillus* sp., isolated from cooked ham, did not adhere substantially to fat and tendon slices. Less than 1 of 1,000 *Lactobacillus* sp. cells which were in contact with the meat remained on the surface after rinsing, whereas more than 56% of the deposited *Acinetobacter* strain LD2 cells adhered to the meat surface. In a previous study, a *Lactobacillus* sp. was also found to adhere to a meat surface (pork skin) in much lower numbers than was *Pseudomonas putrefaciens* (2). Differences between the ability of various

organisms to adhere to meat are well documented but still unexplained.

While the adhesion of bacteria to skin and meat surfaces has been reported (for a review, see reference 4), this study demonstrates that bacteria can also adhere in large numbers to fat surfaces. This is important, since fat surfaces represent a large percentage of the exposed surfaces of prime cuts of beef and other meats. Some bacteria, such as pseudomonads, can metabolize adipose tissue. Others cannot, but they may still be transferred by physical contact from fat surfaces to other meat surfaces, where they can grow.

ADDENDUM IN PROOF

Chung et al. (J. Food Prot. **52**:173–177, 1989) have also observed no significant differences in the numbers of cells attached to lean muscle and fat tissues.

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