Phagocytosis and Intracellular Killing of *Staphylococcus aureus* by Normal Mouse Peritoneal Macrophages

ROBERT E. BAUGHN* AND PETER F. BONVENTRE

Department of Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

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Although *Staphylococcus aureus* is incapable of intracellular multiplication in cultured mouse peritoneal macrophages, it is killed at a much slower rate than the avirulent *Staphylococcus epidermidis*. In addition to the presence of capsular material which inhibits phagocytosis of specific strains of *S. aureus*, the data show that a number of cellular and environmental factors affect the functional capacities of mononuclear phagocytic cells. The data obtained by varying the initial level of infection indicate that the number of ingested bacteria may subsequently alter the kinetics of intracellular killing. In vitro maturation of macrophages in culture was also found to exert a pronounced effect on the kinetics of bacterial death.

A variety of in vitro methods have been devised to study the host-parasite relationship of phagocytic cells infected with microbial pathogens. In spite of considerable effort, it has not been possible in many instances to quantitate the interaction between microbes and phagocytic cells. Many reasons for this situation may be cited, but all can be reduced to technical problems associated with the task of separating extracellular and intracellular events while at the same time preserving the validity of the in vitro model. Because of these technical difficulties little is known concerning the consequences of related phagocytic events on the bactericidal activity of mononuclear leukocytes or, conversely, if the intracellular microbial burden alters the process of phagocytosis. Therefore, it is important that meaningful models be designed and methods formulated so that valid data of a quantitative nature are obtained. Developments in clinical immunology and medicine in recent years describing a variety of defects in cellular defenses (8, 14, 17, 24) and the importance of cell-mediated immunity for the outcome of infectious disease (16) have amplified the need for accurate methods to assess kinetics of phagocytosis and subsequent antibacterial activity of phagocytic cells independently of each other.

The present study was undertaken to assess phagocytosis and intracellular killing of *Staphylococcus aureus* within cultured normal mouse peritoneal macrophages by a method which circumvents the technical difficulties usually associated with such studies. The assay system employed allowed dissection of the phagocytic process from the subsequent process of intracellular killing of *S. aureus*. Although no method yet devised circumvents all potential difficulties and the generation of artificial data, a procedure utilizing the staphyloytic enzyme lysostaphin (Schwarz/Mann, Inc., Orangeburg, N.Y.) described by Hirt and Bonventre (11) and subsequently modified by Baughn and Bonventre (1) was found to offer distinct advantages. The technique made possible the evaluation of a number of factors which influence the kinetics of the *Staphylococcus*-macrophage interaction.

**MATERIALS AND METHODS**

**Animals.** The following strains of female mice (6-8 weeks old) were obtained from the Jackson Laboratory, Bar Harbor, Me.: (i) inbred-BALB/c, C57BL/6J, CBA/J and (ii) F1, hybrid-B,D2F, (C57BL6J female × DBA/2J male). Random-bred Swiss-Webster mice from a local source and CF1 mice from Carworth Farms, New City, N.Y., were used in the preliminary investigations. Mice for any given experiment were matched with respect to age. Animals were housed five per covered cage on dust-free litter and fed pellets and water ad libitum.

**Peritoneal macrophage cultures.** Murine peritoneal cells were collected by a modification of the procedure of Chang (2). Groups of 8 to 16 mice were used for each experiment. After cervical dislocation, 4 ml of a mixture composed of 75% NCTC-135 (GIBCO, Grand Island, N.Y.) and 25% heat-inactivated horse serum containing 10 U of heparin per ml was injected into the unstimulated peritoneum. After collection of peritoneal exudates, pooled cell suspensions were washed twice in the same medium and the cell concentration was adjusted to 5 x 10^6 cells/ml in complete tissue culture medium (CTCM). The latter consisted of 55% NCTC-135, 40% heat-inactivated horse serum, 5% beef embryo extract (GIBCO), 2.5 μg of Fungizone (GIBCO; amphotericin B) per ml, and 100 μg of streptomycin per ml. Two-tenths milliliter of
the cell suspension was pipetted onto sterile glass cover slips (10.5 by 22 mm) and incubated for 2 to 3 h at 37 C in a CO2 incubator. After the removal of nonadherent cells by rinsing the cover slips in sterile saline, groups of four cover slips were transferred to 60-mm petri dishes (Falcon Plastics, Los Angeles, Calif.) containing 5 ml of fresh CTCM. The cells were then reincubated until the time of assay. The spent medium was aspirated, and fresh CTCM was added back to the dishes every third day for those monolayers used in long-term experiments.

**Bacteria.** *S. aureus* 502A, a coagulase-positive, non-encapsulated strain, was used as the test organism for the majority of the experiments. For comparative purposes, several of *S. aureus* strains, i.e., 18Z, Smith, and M240 Welwood, and an avirulent *S. epidermidis* were also studied. The non-encapsulated strain designated 18Z was obtained through the courtesy of F. A. Kapral. The Smith and M240 Welwood strains are classified as encapsulated.

A streptomycin-resistant mutant of *Listeria monocytogenes* 19115 (American Type Culture Collection, Rockville, Md.) was obtained by selection on brain heart infusion agar plates (BBL, Cockeysville, Md.) containing 0.1 mg of streptomycin per ml. This strain, designated *L. monocytogenes* 19115-SM, was used for assessing the behavior of a facultative intracellular parasite within normal peritoneal macrophages.

Suspensions of bacteria were prepared from 3-h (early log) broth cultures to obtain a maximum number of viable bacteria in the inoculum used for infection of macrophages. Trypticase soy broth (BBL) was used to culture all staphylococci and brain heart infusion broth for the growth of *L. monocytogenes*. Cell pellets were washed twice in 0.2% gelatin-phosphate-buffered saline, and the number of organisms was estimated by nephelometry prior to dilution in tissue culture medium for infection of monolayers.

**Phagocytosis of *S. aureus* by mouse peritoneal macrophages.** One hour prior to the start of the experiment, spent medium was aspirated and replaced with 5 ml of CTCM containing no antibiotics. Monolayers were examined microscopically and representative fields were counted. Macrophages were also counted prior to each successive sampling. The trypan blue exclusion method showed that the percentage of viable adherent leukocytes, even after long-term maturation, was greater than 90%.

Cultures of staphylococci in gelatin-phosphate-buffered saline were resuspended to 0.2 optical density unit at 660 nm and subjected to sonic vibration for 6 min in a Cole-Parmer ultrasonic cleaner. Earlier studies in our laboratory, designed to correlate viable bacterial counts as determined by the pour-plate technique with those counts obtained using a Petroff-Hauser counting chamber (A. H. Thomas Co., Philadelphia, Pa.), had shown that a 6-min period of sonic treatment was necessary for optimal dispersion of clusters of staphylococci into single cells. Nonsonicated suspensions of *S. aureus*, as well as suspensions treated for shorter periods of time, uniformly yielded lower counts. Treatment for periods in excess of 10 min, on the other hand, affected viability presumably via damage and/or lysis.

Bacteria were diluted in CTCM without antibiotics to concentrations consistent with the desired multiplicity of infection (MOI). After addition of the bacterial inoculum in 4 ml of CTCM, dishes were reincubated for 20, 40, or 60 min at 37 C and 5% CO2. The fluids were then aspirated and replaced with 4 ml of CTCM (without antibiotics) containing 5 U of lysozyme per ml. After a 10-min incubation at 37 C with the enzyme, fluids were again aspirated and 5 ml of NCTC-135 was added to each dish for 5 min. At this point each cover glass was removed and gently washed in two sterile saline baths. Three of the four cover glasses were dropped into separate tubes containing 5 ml of 0.05% Saponin and subjected to sonication for 6 min to enhance disruption of infected macrophages. Viable staphylococci were enumerated by the pour-plate procedure using blood agar base (BBL) immediately after sonication. The fourth cover glass from each dish was used for protein determination (20).

In a number of earlier experiments phagocytosis was assessed in the presence of heat-inactivated horse serum which had been adsorbed with heat-killed staphylococci. The adsorption of heat-inactivated horse serum did not appreciably alter the phagocytic index and was interpreted as evidence that heat-stable opsonins in horse serum were incapable of enhancing phagocytosis.

**Intracellular killing of *S. aureus* by mouse peritoneal macrophages.** Monolayer cultures were processed as for the phagocytic assays except that phagocytosis was allowed to proceed for 60 min. After lysozyme treatment for 10 min and the wash in NCTC-135, a dish of four cover slips was processed as described before and designated as the “zero” time sample. Five milliliters of CTCM, containing 2.5 µg of Fungizone and 20 µg of kanamycin per ml, was added to the other cover slip cultures. Preliminary studies showed that kanamycin at this concentration did not significantly alter the fate of intracellular staphylococci during a 6-h period after phagocytosis. Incorporation of the antibiotic into the medium was necessary to prevent contamination and to eliminate staphylococci released from lysed infected cells. Samples were processed at 1, 3, 6, 24, and 48 h after infection for enumeration of surviving staphylococci within the macrophages. In comparative assays using *S. epidermidis* as the test organism, horse serum, which is bactericidal for the organism (10), as well as the antibiotics were omitted from the CTCM for 1 h prior to and during phagocytosis.

**Studies with *L. monocytogenes*.** For comparative purposes, the fate of *L. monocytogenes* within normal mouse macrophages was also determined. A modification of the technique of Jones and Youmans (12), described in detail elsewhere (1), was used to monitor the intracellular growth of this facultative intracellular parasite.

**Analyses of data.** The MOI was determined by calculating the number of bacteria in the small fluid volume directly over the area of each cover slip. This was deemed necessary because the surface area of each cover slip constituted only 11.3% of the total inside surface area of the dish. Values, expressed as the number of viable staphylococci per 100 macrophages, were calculated from duplicate plate counts
of samples processed in triplicate. Microscopic quanti-
tation of the numbers of macrophages per monolayer
was also done in triplicate at each time interval using
phase optics. The data for samples between zero and 6
h and between 0 and 48 h post-phagocytosis for
each experiment were analyzed by polynomial regres-
sion. Data were also analyzed by analysis of covari-
ance and interpreted according to standard proce-
dures outlines by Dixon and Massey (9).

RESULTS

Phagocytic and staphylocidal properties of
peritoneal macrophages from outbred mice. Ini-
tial experiments were designed to assess the fate of S. aureus 502A within normal peritoneal
macrophages obtained from outbred CF1 and
Swiss-Webster mice after in vitro tissue culture
for 24 h. The results of these studies were
difficult to interpret. In most instances, cells
clumped, and 24 h after seeding a significant
loss (35 to 65%) of the macrophages initially
adherent to cover slips was observed. Loss of
viability was probably attributable to mixed
lymphocyte reactions since rinsing of the mono-
layers after establishment of the adherent cell
population did not remove all lymphocytes.
Thus, pooling of lymphocytes from genetically
heterogenous animals best explains the exten-
sive cell damage observed and is consistent with
the report of Neiburger and Youmans (19). In
view of this situation all subsequent experi-
ments were conducted with peritoneal exudate
cells from inbred or hybrid mice. The prelimi-
nary experiments with the random-bred mice
also established that some loss of macrophages
during extended periods of culture in vitro was
inevitable. Thus it became clear that data
should be expressed in terms of viable bacteria
found within cells at any given time. In all
experiments thereafter, results were expressed
as the number of viable bacterial units per 100
peritoneal macrophages in monolayer culture.

Phagocytosis of S. aureus by peritoneal
macrophages from inbred and F1 hybrid mice. Table 1 shows the results of experiments
designed to assess the phagocytic potential of
BDF1 female mice and 16:1 11.3

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain of S. aureus</th>
<th>Treatment</th>
<th>Staphylococci added/100 macrophages</th>
<th>Cell-associated* staphylococci/100 macrophages after 60 min</th>
<th>Approximate MOI</th>
<th>% Phagocytosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>502A</td>
<td>LST*</td>
<td>179</td>
<td>18</td>
<td>2:1</td>
<td>10.1</td>
</tr>
<tr>
<td>2</td>
<td>502A</td>
<td>Rinsec</td>
<td>200</td>
<td>43</td>
<td>2:1</td>
<td>21.5</td>
</tr>
<tr>
<td>3</td>
<td>502A</td>
<td>LST</td>
<td>1,562</td>
<td>153</td>
<td>16:1</td>
<td>9.8</td>
</tr>
<tr>
<td>4</td>
<td>502A</td>
<td>Rinse</td>
<td>1,893</td>
<td>451</td>
<td>19:1</td>
<td>23.8</td>
</tr>
<tr>
<td>5</td>
<td>502A</td>
<td>LST</td>
<td>16,389</td>
<td>1,722</td>
<td>16:1</td>
<td>10.5</td>
</tr>
<tr>
<td>6</td>
<td>502A</td>
<td>Rinse</td>
<td>17,445</td>
<td>4,790</td>
<td>17:1</td>
<td>27.5</td>
</tr>
<tr>
<td>7</td>
<td>18Z</td>
<td>LST</td>
<td>183</td>
<td>19</td>
<td>2:1</td>
<td>10.4</td>
</tr>
<tr>
<td>8</td>
<td>18Z</td>
<td>LST</td>
<td>1,604</td>
<td>181</td>
<td>16:1</td>
<td>11.3</td>
</tr>
<tr>
<td>9</td>
<td>18Z</td>
<td>LST</td>
<td>192</td>
<td>3</td>
<td>2:1</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>18Z</td>
<td>LST</td>
<td>1,702</td>
<td>31</td>
<td>17:1</td>
<td>1.8</td>
</tr>
<tr>
<td>11</td>
<td>M240-Welwoodc</td>
<td>LST</td>
<td>171</td>
<td>5</td>
<td>2:1</td>
<td>2.9</td>
</tr>
<tr>
<td>12</td>
<td>M240-Welwoodc</td>
<td>LST</td>
<td>1,548</td>
<td>52</td>
<td>16:1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* Cell-associated, adherent and phagocytized.
* LST, Lysostaphin, 5 U/ml for 10 min.
* Monolayers were rinsed four times in sterile saline baths.
* Encapsulated.
substantial increases in viable staphylococci in the extracellular fluids during a 3-h period (data not shown). Substantial increases in viable bacteria in the extracellular medium were also seen when monolayers were treated with 10 U of lysozyme, but not after exposure to 20 U for 10 min.

**Intracellular killing of *S. aureus* by peritoneal macrophages.** Figure 1 compares the intracellular fate of *S. aureus* with *S. epidermidis* and *L. monocytophages*. Predictably, *L. monocytogenes* was not killed by normal (unstimulated) macrophages (12). On the other hand, avirulent *S. epidermidis* was killed very rapidly. Greater than 99% reduction in viability of phagocytized *S. epidermidis* was noted after 6 h and complete sterilization was effected by 24 h. The fate of intracellular *S. aureus* was quite different than its avirulent counterpart. The kinetics of killing by peritoneal macrophages were considerably slower, and complete elimination of intracellular *S. aureus* was never achieved. The latter observation appeared to be independent of strain of *S. aureus*, MOI, or age of mice used as the source of peritoneal macrophages.

The data in Table 2 show the relationship between MOI and the rate of bacterial killing subsequent to phagocytosis. Whereas normal macrophages appear to handle intracellular *S. aureus* without difficulty after extended periods (i.e., 24 to 48 h), it appears that the intracellular bacterial load modulates the kinetics of killing at least during the initial 6-h period. Covariance analysis on the observed and predicted regression lines on grouped data from several experiments indicated significant differences, depending on the number of staphylococci per cell initially ingested. For any one MOI, however, covariance analysis failed to reveal significant differences in bacterial killing by macrophages obtained from different strains of mice or from mice of different ages.

**Effect of in vitro maturation on phagocytosis and staphylocidal activities of peritoneal macrophages.** Table 3 shows that in vitro maturation of mouse macrophages obtained from the unstimulated peritoneum also has a significant effect on bactericidal potential. The phagocytic capacity increases as the cultures mature during a 2-week period, but the staphylocidal potential appears to diminish significantly after 3 days in culture. Microscopic examination of monolayers maintained in culture for either 6 or 14 days indicated that less than 5% of the total adherent cell population at either time period was fibroblasts.

**DISCUSSION**

In view of the fact that staphylococcal infections have a tendency to become chronic, the fate of *S. aureus* within mononuclear phagocytes is an important consideration. Experimental models developed by others to study this bacterial-host cell interaction have been of limited usefulness primarily because of technical difficulties. Techniques which do not dissect phagocytosis from subsequent intracellular killing may lead to spurious conclusions since they cannot account for staphylococci associated with phagocytic cell surfaces, to which they adhere tenaciously. These surface-associated bacteria which would be scored incorrectly as intracellular by conventional assays at times may represent as much as 90% of the viable staphylococci associated with the cells after thorough rinsing (11). Another complicating factor is the extracellular multiplication of the surface-associated bacteria or those released from lysed infected cells, since these organisms would be subject to repeated endocytic events. Several studies have stressed that unless this situation is carefully controlled results may be misconstrued as intracellular multiplication of *S. aureus* within phagocytic cells (7, 11, 13). Efforts to obviate this difficulty by adding appropriate antibiotics to the tissue culture medium are not entirely suitable since the antibiotics require significant time to sterilize the extracellular fluids. In addition, there is
some evidence that certain antibiotics may modify the physiology and viability of phagocytized bacteria (2, 21, 23). The assay utilized in these studies would appear to circumvent these difficulties, and we found that it could be used to advantage to assess accurately and quantitatively the postphagocytic fate of *S. aureus* within mouse peritoneal macrophages under a variety of circumstances. Unfortunately the methods used by other investigators (13, 15) are so different from the lysostaphin assay that similarities and contradictions in the results are

### Table 2. Effect of the MOI on phagocytosis and intracellular killing of *S. aureus* 502A by normal mouse peritoneal macrophages obtained from 18-week-old B6D2F1/J female mice and maintained in tissue culture for 24 h

<table>
<thead>
<tr>
<th>Determination</th>
<th>Approximate MOI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2:1</td>
</tr>
<tr>
<td>Number/100 macrophages</td>
<td></td>
</tr>
<tr>
<td>Added at T = 0</td>
<td>197</td>
</tr>
<tr>
<td>Phagocytosed at T + 60 h</td>
<td>17.9 (10)*</td>
</tr>
<tr>
<td>Number/100 macrophages surviving intracellularly after</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>9.4 (48)*</td>
</tr>
<tr>
<td>3 h</td>
<td>7.9 (56)</td>
</tr>
<tr>
<td>6 h</td>
<td>5.5 (69)</td>
</tr>
<tr>
<td>24 h</td>
<td>0.4 (98)</td>
</tr>
<tr>
<td>48 h</td>
<td>0.2 (99)</td>
</tr>
<tr>
<td>Regression analysis on log_{10}y^c (first 6 h)</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.1529</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.0748</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>-0.9118</td>
</tr>
</tbody>
</table>

* Percentage phagocytosed after 1 h.
* Percentage of intracellular staphylococci killed at the end of specified times.
^c, Viable bacteria/100 macrophages.

### Table 3. Effect of in vitro maturation on phagocytosis and intracellular killing of *S. aureus* 502A by normal mouse peritoneal macrophages obtained from 18-week-old B6D2F1/J female mice

<table>
<thead>
<tr>
<th>Determination</th>
<th>Length of maturation before start of assay (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 (12:1)*</td>
</tr>
<tr>
<td>Number/100 macrophages</td>
<td></td>
</tr>
<tr>
<td>Added at T = 0</td>
<td>1,225</td>
</tr>
<tr>
<td>Phagocytosed at T + 60 h</td>
<td>52.5 (4)*</td>
</tr>
<tr>
<td>Number/100 macrophages surviving intracellularly after</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>35.0 (33)*</td>
</tr>
<tr>
<td>3 h</td>
<td>27.5 (48)</td>
</tr>
<tr>
<td>6 h</td>
<td>20.0 (62)</td>
</tr>
<tr>
<td>24 h</td>
<td>1.4 (97)</td>
</tr>
<tr>
<td>48 h</td>
<td>0.1 (&gt;99)</td>
</tr>
<tr>
<td>Regression analysis on log_{10}y^c (first 6 h)</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.6612</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.0640</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>-0.9578</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are approximate MOIs.
* Percentage phagocytosed after 1 h.
* Percentage of intracellular staphylococci killed at the end of specified times.
^c, Viable bacteria/100 macrophages.
PHAGOCYTOSIS AND KILLING OF S. AUREUS

The technique enabled us to obtain exclusively intracellular populations of staphylococci for the purpose of studying their intracellular fate. Used in this manner, we found that older cells appeared to be less capable than young cells in clearing ingested staphylococci. The data show that mouse macrophages after more than 1 week in culture kill S. aureus at a slower rate and to a less degree than do their younger counterparts. Cohn and his colleagues (4-6) studied the maturation of mouse peritoneal macrophages in tissue culture and showed that intracellular inclusions, granules, and lysosomal enzymes all increased during in vitro cultivation. Our data suggest that bactericidal ability of the cells does not increase during this maturation process when profound morphological and biochemical changes are occurring. It would be instructive to determine the bactericidal capacity of immature and mature macrophages for a wide spectrum of bacterial species, since one would intuitively suspect that bactericidal capacity would be augmented upon maturation of the mononuclear phagocyte. The loss of bactericidal activity exhibited by cells in culture for extended periods may be due to senescence and might be exploited in studies attempting to identify the mechanisms of bacterial killing by mononuclear leukocytes.

An interesting relationship between the multiplicity of infection and the kinetics of bacterial killing was also observed. Although the percentage of the initial inoculum phagocytized during a 60-min period remained constant between MOIs of 2:1 to 164:1 (i.e., ca. 10%) the rate and extent of bacterial killing during the initial 6 h of intracellular residence varied considerably. If these data are considered solely in terms of the percentage of bacteria surviving after 1, 3, or 6 h of intracellular residence (or the slope of the regression line), the rate and extent of killing appears greater at the lower MOI. As a result, one may conclude that an increased number of intracellular organisms depletes the cell of its bactericidal capabilities. This might occur via elaboration of toxic products concomitantly with digestion of a large number of intracellular bacteria or some other unknown mechanism. This depletion could then foreseeably result in a decrease in the percentage of the inoculum cleared.

Alternatively, if one compares the data for the low and high MOIs in terms of absolute numbers per 100 macrophages, a different conclusion may be drawn. The intracellular load of 1-day-old cells is approximately 18 staphylococci/100 macrophages for the lower infective dose and more than 1,700 bacteria/100 cells in the case of the high MOI. At the end of 6 h, the
former cultures killed approximately 12 of the 18 staphylococci/100 cells, whereas the latter killed more than 800/100 macrophages. One must ask why the lightly infected cultures were not quickly sterilized. The macrophages have the capacity to destroy almost $10^8$ staphylococci/100 cells during a 6-h period according to the data and yet fail to completely clear an infection two orders of magnitude less than that. This raises the interesting possibility that mononuclear phagocytes must be activated by a critical threshold of ingested particles before their full bactericidal potential is realized. Melly et al. (18) suggested such a mechanism to account for an apparent heightened capacity of human leukocytes to destroy intracellular staphylococci. They postulated that ingestion of small numbers of staphylococci is well tolerated by leukocytes and does not effectively mobilize the bactericidal capabilities of the phagocyte. In contrast, they suggested that multiple phagocytic events may fully activate bactericidal mechanisms of the blood leukocytes.

Further experiments are necessary to determine if augmented bactericidal activity does exist at the higher multiplicities and, if so, if it is related in a positive manner to the ingestion of viable microbes or, alternatively, to a nonspecific phagocytic stimulus. In an attempt to resolve this question we are currently measuring the kinetics of bacterial killing by mouse macrophages after the ingestion of viable and nonviable S. aureus mixed in different ratios.

ACKNOWLEDGMENTS

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