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Testing for antibacterial properties of cotton/flax denim☆

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ABSTRACT

The 'AATCC Test Method 100-1999, Antibacterial Finishes on Textile Materials: Assessment of' was modified by not assaying for the initial population density and only reporting the population density at the end of incubation for comparing treatments. This seemed to be a reasonable change since the assay challenges the treatments at the start with the same population inoculum density. This permitted the AATCC Test Method 100-1999 to be flexible and easier for testing materials that may or may not actually exhibit bacteriostatic properties. A substantial savings in material and time was gained by not taking the initial population density. This change allows testing more treatments, using more replicate samples, or shortens the time to conduct the assay which can permit more assays to be conducted. However, the control treatment needs to be integral with each assay; but since this is usually done, this would be a small inconvenience compared to the savings. The assay responded well when used to test fabric containing variable amounts of material with known antibacterial properties. The population density of the challenge bacteria decreased as the concentration of the antibacterial component of the fabric increased. The results from the use of the modified AATCC Test Method 100-1999 assay did not indicate that adding flax provided any additional bacteriostatic properties to the flax denim against the two challenge bacteria, *Staphylococcus aureus* and *Klebsiella pneumoniae*. The long held assumption that flax is bacteriostatic or antibacterial was not supported by the results and probably does not contribute to its resistance to rotting as much as its physical or chemical composition.

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1. Introduction

Members collectively in the flax family have been used for just about everything, from clothing to paper (Hook and Heimlich, 2006; Karakus et al., 1999). Economically, the major interest lies in the use of flax fibre (*Linum usitatissimum*, L.) in the textile (McAlister et al., 2002) and nonwoven industries (Foulk et al., 2004). Recently, fabrics have been introduced that were modified to have bacteriostatic properties and thus odor controlling properties permanently embedded in the yarn polymer (Jiang et al., 2006; Lee et al., 1999; Lee and Jeong, 2005; Seong et al., 1999). The added feature helps to protect against microbes that can cause foul odors, discoloration, mildew formation and apparel degradation for the life of the garment. With this trend in mind, flax has often been described to be a natural fibre already having some of these same characteristics

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(Cierpucha et al., 1997, 2004) and linen is sometimes described as being antibacterial (Anonymous, 2005, 2006). Historically, bast fibre such as from hemp and flax was regarded for their antimildew and antimicrobial properties, which made them suitable for sails, tarps, awnings and floor coverings (Anonymous, 2000). Flax has been described as being more resistant to bacterial decomposition (Bose, 1952) and more resistant to fungal growth than other natural fibres (Basu and Bhattacharyya, 1951). Exactly what manifests these properties is not currently known but compounds associated with flax may exhibit antibacterial properties (Akin et al., 2003; Gamble et al., 2000). Regardless, the promotion of flax as being bacteriostatic or antibacterial would be enhanced if the quantitative or at least some qualitative measure of its bacteriostatic properties could be presented for comparison. Scant information is found in the literature, so the measurement of the general bacteriostatic nature of flax was the initial goal of this study.

A simple testing method where viable bacterial cells were placed on the flax fabric and then later the viable population remaining was determined. This would provide an estimation of the antibacterial/bacteriostatic property of the fabric. The 'AATCC (American Association of Textile Chemists and Colorists) Test Method 100-1999, Antibacterial Finishes on Textile Materials: Assessment of' (Anonymous, 1999), was chosen because it has been in use by other researchers to evaluate antibacterial properties (Seong et al., 1999) and because the method appeared to have the malleability for testing a wide-range of properties and materials for future investigations. Additionally, the assay is relatively easy to perform and requires modest resources. This paper will report on using this test method for measuring the bacteriostatic properties of flax fabric and the small changes to the method to save materials and time.

2. Materials and methods

2.1. *General protocol for assaying antibacterial property of textile materials*

The general method described in 'AATCC Test Method 100-1999, Antibacterial Finishes on Textile Materials: Assessment of' (Anonymous, 1999) with modifications was the basis for the protocol used to measure the qualitative and quantitative antibacterial tendency of textile materials. As described in the AATCC Test Method, swatches of the textile materials were 'inoculated' with a test or challenge bacteria. After a period of incubation, the bacteria were eluted from the swatches using known volumes of extraction solution. Then the number of bacteria present in this extraction solution was determined for each treated textile material for comparison. In the AATCC Test Method, the percent reduction by the treated specimen was calculated and used for comparison. The test method was found to be more flexible and more efficiently carried out when only the final population densities were compared. This was because all the test materials were initially treated with the same density of bacteria; and it was found that after incubation, the population densities instead of decreasing were often the same as the starting population density or had actually increased. The treatments were fabrics composed of increasing proportions of flax content so a change in population densities proportional to the flax content was expected if flax fibre contained antibacterial properties.

2.2. *Flax fabric*

The fabrics used for the swatches were denim cloths containing different amounts of flax in its construction. The cloths were constructed by using 100% cotton yarn in the warp direction and 100%, 75%, 50% and 25% cotton fibre in the weft direction with flax as the other fibre. These warp and weft yarns produced four fabrics that contained 0%, 9.6%, 19.6% and 27.6% flax in the fabric, respectively. Each fabric composition, 0%, 9.6%, 19.6% and 27.6% flax, was considered as a treatment. The four fabric treatments were scoured (McAlister, 1994), cut into square swatches (18.1 cm^2, 4.25 cm on a side) and sterilized in an autoclave (10 min sterilize and 30 min drying cycle; 121 °C). The swatches were autoclaved in small polypropylene bags and stored in the bags until used. Four swatches, the least number of swatches able to absorb 1.0 ml of inoculum without leaving free liquid in a jar, were used for each fabric treatment replicate sample.

2.3. *Bacterial challenge*

The two challenge bacterial species used in the 'AATCC Test Method 100-1999, Antibacterial Finishes on Textile Materials: Assessment of' (Anonymous, 1999), were used throughout: *Staphylococcus aureus*, American Type Culture Collection No. 6538 (a Gram-positive organism), and *Klebsiella pneumoniae*, American Type Culture Collection No. 4352 (a Gram-negative organism). Stock cultures of these bacteria were maintained on Difco Brain Heart Infusion Agar slants (Difco Brain Heart Infusion Agar, Man #237500). The stock cultures were transferred once every 3–4 weeks by incubating a freshly inoculated slant at 37 ± 2 °C for 2 days before storing at 5 ± 1 °C.

Before each assay, the test bacteria were incubated in either a trypticase soy broth (TSB, BBL® No. 11768 trypticase soy broth) or on trypticase soy agar slants (TSA, BBL® No. 11768 trypticase soy broth, and 2.0% agar) at 37 ± 2 °C for 1–3 days before being used to inoculate broth (TSB) cultures for testing. The inoculated broth cultures were incubated in a shake incubator (37 ± 2 °C and 300 rpm) for 24 h. At the end of incubation, the broth cultures were placed in an ice bath and held until chilled.

A standardized density of bacteria was used for the challenge inoculation. The standardized density of bacteria was (1–2) × 10^5 CFU/ml (colony forming units per milliliter) which was prepared from the chilled broth cultures. To get the standardized density of bacteria, the chilled cultures were diluted with TSB to a pre-determined turbidity to provided approximately 2 × 10^9 CFU/ml or 2 × 10^8 CFU/ml. The turbidity was measured at 500 nm on a Beckman DU-7 Spectrophotometer (Beckman Instruments, Inc., Irvine, CA 92713) using chilled TSB from the same batch, as the cultures were grown in, to zero the instrument. Then the diluted broth cultures were serially diluted with chilled diluent (Chun and Perkins, 1996) for a final approximate bacterial density of (1–2) × 10^5 CFU/ml.

The diluent used in-house normally contains both Tween®-80 (T-164, Fisher Scientific Company, Fair Lawn, New Jersey), a nonionic surfactant, and 0.01% gelatin which were omitted at this stage; however the 0.01% gelatin was retained in the diluents for the extractions and serial dilutions used elsewhere when determining bacterial population density. The bacterial suspensions were kept in an ice bath. A magnetic stirring bar and stirring plate was used to keep the bacteria suspended during inoculation.

For each fabric treatment sample replicate, 1.0 ± 0.1 ml of inoculum was dispersed over the four swatches using a Rainin EDP-Plus Electronic Pipette (RAININ Instrument Co., Inc., Woburn, MA), twenty-five 10-μl droplets/swatch. The swatches were inoculated while in pre-sterilized 237 ml (half pint) canning jars (Mason or Kerr Brand, locally obtained), one swatch at a time. The band and lid of the canning jar were screwed on the jar to prevent evaporation. After all the treatment sample replicates had been inoculated, the jars were either incubated at $37 \pm 2\,^{\circ}C$ for 24 h before being assayed for bacterial population density or were immediately assayed for bacterial population density as the zero-time population density.

The bacterial population densities were determined by first extraction of the bacteria from the fabric by adding 100 ml of diluent to each jar and shaking the jars on a tabletop shaker for 1 min. Then aliquots were removed and plated directly into petri dishes or further diluted before being plated. The pour plate method (Chun and Perkins, 1996) was used to determine the bacterial density. No antibiotics were used and incubation was at $37 \pm 2\,^{\circ}C$ for at least 24 h before the plates were counted.

2.4. Testing flax for antibacterial properties

The four fabric treatments were tested with the *S. aureus* and *K. pneumoniae* bacteria, first one and then the other, using 4 sample replicates per test for a total of 64 samples for each test. The fabric treatment applications were completely randomized. Usually 2 tests were conducted in the same day, one in the morning and the second in the afternoon; or the second test was conducted the following day after the 24-h extraction of the previous test was completed. In the set of testing for antibacterial properties, 4 tests were done.

2.5. Testing the effect of gelatin on the assay

The in-house diluent normally contains a surfactant and gelatin in its composition. During the early stages of this assay, check tests were conducted on the possible effect of the 0.01% gelatin when the diluent was used to extract the viable bacteria from the textile fabrics and was used as the diluent to dilute the extracted bacteria for population density measurements. The four fabric treatments were assayed first with *K. pneumoniae* bacteria as the challenge and then in a second test, *S. aureus* was used as the challenge bacteria. In both tests, the assay used diluent/extraction solutions with and without 0.01% gelatin, 4 sample replicates per test for a total of 64 samples for each test. The fabric treatment applications were completely randomized.

2.6. Assay check, using fabric known to have antimicrobial properties

To check that the modified assay could detect antibacterial properties of a fabric, the assay was used on a fabric known to have antimicrobial properties due to the presence of silver in its composition. A knit sock of varying amounts of Fosshield® (Foss Manufacturing Company, Inc., Hampton, NH) fibre, a polyester fibre impregnated with silver, was made in-house and used for this test. Each fabric composition, containing a different proportion of Fosshield® fibre, was considered a treatment. But because of the limited availability of this knit sock fabric, only the 100%, 50%, and 10% Fosshield® fibre, the *S. aureus* bacteria and the 24-h incubation time, was used. Also, while four swatches per sample replicate were used, only 3 replicate samples per fabric composition treatment were tested.

3. Results and discussion

The original intent was to use the AATCC Test Method 100-1999 to get a handle on the level of bacteriostatic property acquired by cloth when flax becomes incorporated in its composition. But during the testing, the observed population density after the 24-h incubation at 37 °C rarely showed a reduction in bacterial density. Instead the actual population density usually increased during incubation. The results from the four flax assays were averaged. Instead of decreasing, the population density increased by about two or more orders of magnitude after 24 h of incubation (Table 1) for both challenge bacteria. The actual average initial population density was reasonably close to the calculated population and about the same for both challenge bacteria. After 24 h on the test fabrics, *K. pneumoniae* density was higher than the *S. aureus* density by almost 1.6 orders of magnitude indicating different bacterial growth responses to the fabric treatments. Even when the individual fabric treatments were looked at (Table 2), the individual fabric treatment averages did not differ much from the overall average of 4.8 at the start of incubation. No significant differences were observed by the *K. pneumoniae* challenge at the start of incubation or at the end of incubation between the fabric treatments. Significant differences were observed when

Table 1 – Average population density at start and end of 24 h of incubation

Incubation time (h)	*Staphylococcus aureus*[a,b], $\log_{10}(CFU + 1)$	*Klebsiella pneumoniae*[a,b], $\log_{10}(CFU + 1)$
0	4.804[B]	4.808[B]
24	6.430[A]	8.058[A]

[a] Starting inoculum made from broth cultures diluted to provide $(1–2) \times 10^9$ CFU/ml or $(1–2) \times 10^8$ CFU/ml, based on turbidity at OD_{500}, which were then serially diluted to a final $(1–2) \times 10^5$ CFU/ml density.

[b] Mean separation within column by Duncan's multiple range test, 5% level. Means with the same letter are not significantly different.

Table 2 – Average population density at start and end of 24 h of incubation for flax fabric treatments

Flax fabric blend (%)	Zero time[a,b], $\log_{10}$(CFU + 1)	24-h[a,b], $\log_{10}$(CFU + 1)
S. aureus		
27.6	4.706^{AB}	7.199^{A}
19.6	4.975^{A}	7.328^{A}
9.6	4.590^{B}	6.214^{B}
0.0	4.943^{A}	4.977^{C}
K. pneumoniae		
27.6	4.767^{A}	8.190^{A}
19.6	4.865^{A}	8.245^{A}
9.6	4.747^{A}	7.925^{A}
0.0	4.852^{A}	7.871^{A}

[a] Starting inoculums made from broth cultures diluted to provide (1–2) × 10^9 CFU/ml or (1–2) × 10^8 CFU/ml, based on turbidity at OD_{500}, which were then serially diluted to a final (1–2) × 10^5 CFU/ml densities.

[b] Mean separation within column by Duncan's multiple range test, 5% level. Means with the same letter are not significantly different.

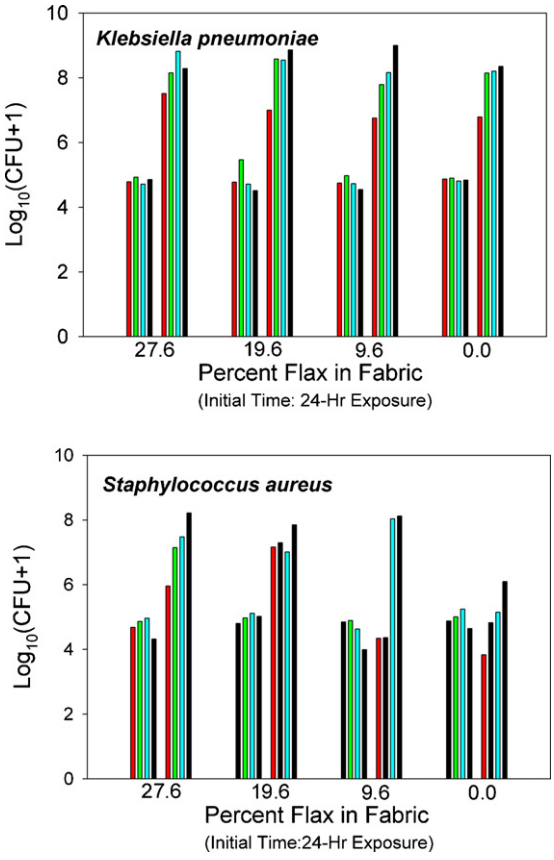

Fig. 1 – The average of each of the four assays runs. The bacterial population density at the zero time (first four bars on the left) and after 24-h incubation (first four bars from the right) on the 27.61%, 19.64%, 9.56% and 0.00% flax denim fabric.

the fabric treatments were challenged by *S. aureus* (Table 2). All of the treatments containing flax had significantly higher densities than the control and the two high flax fabrics had higher densities than the low flax and the control fabric treatments. When the averages of the individual four tests were compared (Fig. 1) instead of the overall averages (Table 2), the variation in results between individual test assays appeared to be small at the start of the assay (time = 0) and at the end of the assay (time = 24 h) the variation were still relatively small; with the variation between individual tests greater after the 24 h incubation compared to the initial density averages at the start of the assay, as would be expected.

The actual average initial population densities at the beginning of the assay showed no significant differences and varied very little, so modifying the AATCC Test Method 100-1999 by not assaying for the initial population density, the population density at zero incubation time, and only reporting the population density at the end of incubation seemed to be a reasonable change especially since the assay challenges the treatments at the start with the same population inoculum density. This also made reporting results for comparison simpler since the AATCC Test Method 100-1999 method reports percent reduction which result in 0 or negative percent reduction in situations where the test material do not have bacteriostatic properties. This permits the AATCC Test Method 100-1999 to be more flexible for testing materials that may or may not actually exhibit bacteriostatic properties. In addition, by not taking the initial population density, a substantial savings in material and time is gained which could be put into testing more treatments or using more replication or shorten the time to conduct the assay to permit more assays to be conducted. The disadvantage is that a control treatment needs to be integral with each assay; but since this is usually done, this would be a small inconvenience compared to the savings.

The population densities after exposure to the fabric after the 24-h incubation showed that the population densities underwent the most change, usually as an increase in density. However, as with the overall average, the individual assay averages for population densities for the two challenge bacteria did not decrease with increasing flax content which did not support the notion that flax denim harbor bacteriostatic tendencies. On the contrary, with *S. aureus* there is a trend for the population density to increase with flax content after the 24-h incubation (Table 2 and Fig. 1); although this may really be a reflection of the greater influence of the cotton content in the denim. The *K. pneumoniae* challenge while not showing significant differences, weakly shows the same trend. The different response of the two challenge bacteria to the increasing flax content may be to possible differences in surface makeup such as waxes, sugars, heavy metals, differences in the fibre surface of the flax and cotton (Akin et al., 2003; Brushwood, 2005; Himmelsbach et al., 2003). However, this is probably unlikely and remains a mystery since most of these factors would be minimized or removed during the scouring process before the bacterial challenge was applied.

An internal criticism of the modified AATCC Test Method 100-1999 was that the in-house diluent used in the extraction of the bacteria from the fabric and for making serial dilutions (except when diluting the broth culture for inoculation) contained 0.01% gelatin. The concern was that this small amount of gelatin might stimulate bacterial growth so much as to overcome whatever weak bacteriostatic properties that the flax

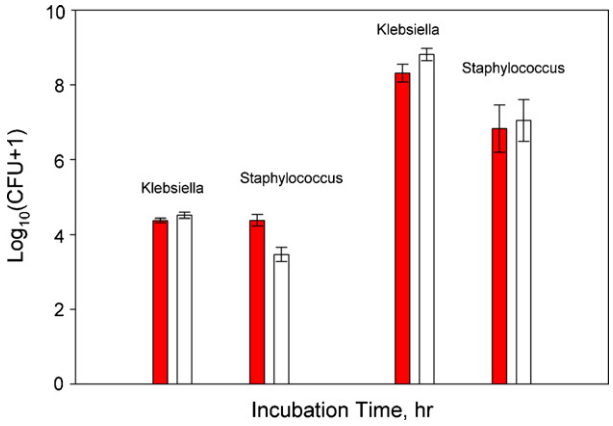

Fig. 2 – Testing the effect of 0.01% gelatin on the antibacterial assay. The overall average population densities, ignoring fabric type, at the zero time and after 24-h incubation are shown (colored bars, with gelatin; clear bars, no gelatin; each half error bar represents 2 s.e.).

may posses or that it might neutralize the compounds that might be the cause of flax being bacteriostatic; even though at this stage of the assay, the effect of gelatin was expected to be minimal. So a simple test was conducted where both challenge bacteria were placed on the flax fabrics as before but the extraction and diluents used were with gelatin and lacking gelatin as additional treatments. Irrespective of flax content, no significant stimulation was detected whether gelatin was in the diluent or not after incubation (Fig. 2). Neither the *K. pneumoniae* nor *S. aureus* challenge bacteria seemed to be advantaged or disadvantaged by the presence of gelatin. Since gelatin was generally added to aid the survival of sensitive bacterial species during plate counting assays, the use of gelatin in the diluent appears to be optional and will be omitted in future use for this assay.

Early on, the presumption was made that flax possessed bacteriostatic properties. However, the results did not indicate that the addition of flax to denim fabric added any bacteriostatic properties. An alternative explanation was that the modified AATCC Test Method 100-1999 assay as used in this laboratory might not be responsive to fabric having antibacterial properties. To verify this possibility, the assay was run using a knit sock of varying amounts of Fosshield®. The assay results showed that even though a smaller replicate size was used, the assay was sensitive enough to indicate amounts of Fosshield® fibre as low as 10% could significantly reduce the bacterial density (Fig. 3). In addition, the population density decreased as the amount of Fosshield® fibre increased as would be expected of fibre possessing antibacterial properties.

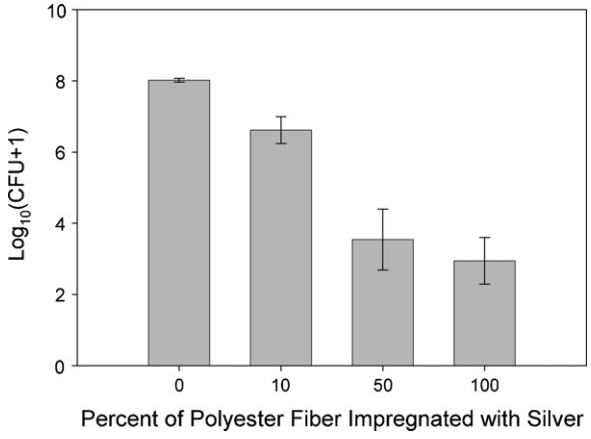

Fig. 3 – The antibacterial assay results using a known antimicrobial fabric, each half of the error bar represents ±2 s.e.

4. Conclusions

In conclusion, the use of the modified AATCC Test Method 100-1999 assay did not indicate that adding flax to denim provided any bacteriostatic properties against *S. aureus* or *K. pneumoniae*, the two challenge bacteria used in the assay. The long held assumption that flax is bacteriostatic or antibacterial was not supported by the results obtained in this work and probably does not contribute to its resistance to rotting as much as its physical or chemical composition (Gamble et al., 2000). In modifying the assay, the final bacterial densities were reported rather than the percent reduction, since the incubation results were higher than the initial challenge bacterial densities; which permitted the assay to be further simplified which saved materials and time by omitting assaying for the initial population density since the challenge inoculum was the same for all treatments and so little variation was observed anyway for the starting population densities. The assay responded well to fabric containing variable amounts of material with antibacterial properties.

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