METHODS FOR INTERPRETATION OF BULK TANK CULTURES AND UDDER HYGIENE TESTS FOR DIAGNOSING HIGH BACTERIA COUNTS: OR, A HIGH LPC COUNT IS NOT ALWAYS A CLEANING FAILURE

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A simple, yet powerful, method for diagnosing high bacteria counts in bulk tank milk using the relative relationships between bulk tank standard plate count (SPC), somatic cell count (SCC), laboratory pasteurized count (LPC), and coliform count was presented by Guterbok and Blackmer (1984) and is used as the basis for the NMC Guide, Troubleshooting Cleaning Problems in Milking Systems (Figure 1).

This method uses coliform bacteria as an indicator of the level of environmental contamination (organisms drawn into the milk from the environment – mainly from the skin of teats and udders) in bulk tank milk. There are, however, many other types of environmental bacteria and elevated coliform counts can occur for other reasons. The method also uses the LPC (or thermoduric count) as the primary indicator of a cleaning failure in milking and milk storage equipment. There are, however, many different types of thermoduric bacteria and elevated LPC can occur for reasons other than cleaning failures.

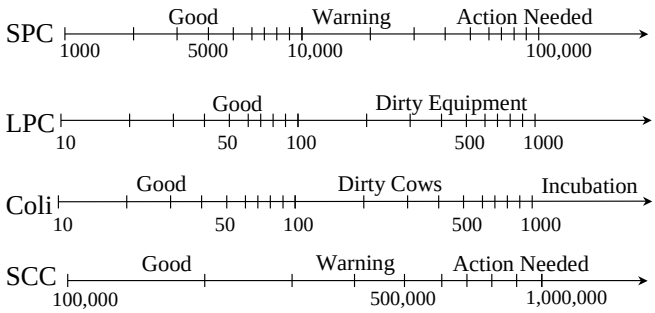


Figure 1. Diagnostic Chart for Bulk Tank Bacteria Counts.

This method relies on the RELATIVE COMPARISON between numbers to formulate a diagnosis. (Examples of this are presented in the appendix). The most common misapplication of the method is the formulation of a diagnosis based on only one of these numbers without considering the relative values of the others (a high LPC count does not always indicate a cleaning failure). As indicated in the interpretive guidelines for the use of Figure 1, LPC data is correlated with coliform data because there are thermoduric bacteria are present in the environment so that when environmental contamination occurs both numbers go up. Coliform (and other environmental organisms) make up a larger percentage of the population than thermoduric organisms so that the increase in coliform count is larger than the increase in LPC.

LPC's are correlated with coliform counts because there are thermoduric bacteria that are present in the environment. Coliform (and other environmental organisms) make up a larger percentage of the population than thermoduric organisms so that the increase in coliform count is larger than the increase in LPC. Following are some examples of the 3-part decision tree to properly

implement this diagnostic technique. Following are some examples and the decision rules for the diagnosis:

Milking wet and/or dirty cows:

- Coliform count is between 100 and 1000 cfu/ml
- AND LPC is less than Coli
- AND SPC is moderately elevated (5000 – 20,000) cfu/ml

Persistent milking machine cleaning problem

- LPC is between 100 and 1000 cfu/ml
- AND Coli less than LPC (probably because of the use of an effective sanitize cycle)
- AND SPC is moderately elevated (5000 – 20,000 cfu/ml)

Incubation in the milk handling system

- Coli is greater than 1000 (or to numerous to count TNTC)
- AND LPC is greater than 100 but less than Coli (Or TNTC)
- AND SPC is extremely elevated (greater than 50,000 to 100,000 or TNTC)

Multiple sanitation problems are likely contributing to these elevated counts and further investigation is recommended (strategic sampling from various points in the milk handling system both early and late in the milking process).

Another recommendation in using this method is that data from a single bulk tank culture is of limited valued in forming a diagnosis. Consideration of the changes in these numbers and changes in their relative proportion is also required for diagnosis. This paper will present additional methods that include using a wider variety of bacteria types and the application of some simple statistical methods such as trend and correlation analysis to improve your diagnostic skills. The application of these methods will be illustrated in several case studies.

Diagnostic Rule 1: Know Your Enemy

Acquiring more information about the specific bacterial species represented in bulk tank milk will improve the power of a diagnosis. Quantitative bulk tank cultures (QBTC) enumerate a range of specific organisms. QBTC is typically focused on types of bacteria related to the level of mastitis in the herd (environmental and contagious). Some of these organisms are also useful in diagnosing sources of environmental contamination of milk, cleaning failures and incubation in milk handling equipment. Following is a summary of sources and growth characteristics of specific bacteria types commonly found in bulk tanks from the excellent review by Murphy and Boor (2008) *(additional comments by the author in italics).* This continually updated document on the E-extension website is required reading for anyone interested in the diagnosis of bulk tank bacteria counts. Applying this research based information for a broader range of bacteria types will greatly improve your diagnostic abilities.

1) Mastitis organisms
    a) Mastitis organisms that most often influence bulk milk count are *Streptococcus spp.*, most notably *S. agalactiae* and *S. uberis.*
    b) *Staphylococcus aureus* is not a frequent contributor to total bulk tank bacteria count.

c) Detection of *(environmental)* mastitis pathogens does not necessarily indicate that they originated from cows with mastitis as environmental mastitis pathogens occur in milk as a result factors other than mastitis infection.
d) Correlation of somatic cell responses and bulk tank environmental mastitis organisms is poor.

2) Environmental Contamination
   a) Organisms associated with bedding materials that contaminate the surface of teats and udders include streptococci, staphylococci, spore-formers *(or thermodurics, djr)* coliform, and other Gram-negative bacteria.
   b) Both thermoduric (bacteria that survive pasteurization) and psychrotrophic (bacteria that grow under refrigeration) strains of bacteria are commonly found on teat surfaces. Contamination from the exterior of the udder can influence Lab Pasteurization Counts (LPC) and Preliminary Incubation Counts (PIC).
   c) Milking heavily soiled cows could potentially result in bulk milk bacteria counts exceeding 10^4 (or 10,000) cfu/ml, although higher coliform *(or other environmental bacteria, djr)* counts are more likely to occur due to incubation in milk handling equipment. Elevated bulk tank coliform counts can also result from coliform mastitis in the herd.
3) Cleaning and Sanitation
   a) Significant buildup of (thermoduric) organisms in milk residue to a point where they influence the total bulk tank count may take several days to weeks *(and are therefore an indication of a persistent cleaning failure, djr).* Old cracked rubber parts are also associated with higher levels of thermoduric bacteria.
   b) Some types of cleaning failures can also select for faster growing, less resistant organisms, principally Gram-negative rods (coliforms and Pseudomonads) and lactic streptococci and can result in high PIC.
   c) Effective use of chlorine or iodine sanitizers has been associated with reduced levels of psychrotrophic bacteria that cause high PIC.
4) Refrigeration
   a) Elevated psychrotrophic bacteria counts are often associated with poorly cleaned refrigerated bulk tanks.
   b) In milk produced with low initial psychrotrophic populations, psychrotrophic bacteria can quickly become dominant after incubation at 4.4°C (40°F) resulting high PIC.

<u>Case Study 1: Distribution of Data, Log Transformation and the Moving Average</u>

Bacteria of all types grow at an exponential rate and therefore produce highly skewed distributions. The same is true for the increase in somatic cell counts in cows infected with mastitis. The linear mastitis score was developed to adjust these highly skewed SCC indicators into a linear effect on milk production. Linear scores use a log transformation to convert SCC into a linear relationship between the level of SCC and the resulting biological effect of reduced milk production. A similar scheme is very useful when analyzing bacteria counts.

One problem with the highly skewed distributions is that many common statistical methods cannot be used on data that is not normally distributed. A log transformation will convert bacteria count data into a more normally distributed population and give a better estimate of the resulting milk quality effects of increased bacteria counts. Furthermore, when plate counts are enumerated, a series of dilutions is used. Each dilution is accurate for a narrow range of accuracy. Log transforms can help illustrate limitations in bacteria count data and allow appropriate adjustments to be made when analyzing these data.

It is common in natural systems to use the natural log transform (denoted as ln(x) or base 'e'). While this is an elegant method, it is difficult to interpret the transformed numbers. The base 10 transform (denoted as $\log_{10}(x)$ or simply log) makes interpretation of numbers much easier. For example, the $\log_{10}$ transform of 1000 is 3 (just count the number of zeros behind the one) and the $\log_{10}$ transform of 100,000 is 5. So if you have a $\log_{10}$ value of 4.4 you know that it is between 10,000 and 99,999. As an interpretation aid, if the first decimal place is 5 you are about 1/3 of way between the $\log_{10}$ values (30,000 expressed as a $\log_{10}$ value is about 4.5). The halfway point between $\log_{10}$ values occurs when the first decimal place is 7 (50,000 expressed as a $\log_{10}$ value is about 4.7).

Distributions of daily bulk tank SPC data from a farm using a robotic milking machine (Helgren and Reinemann, 2006) are illustrated in Figure 2. The left hand figure is the raw SPC data while the right hand figure is the same data after converted to $\log_{10}$ values. Common descriptive statistics of these data are presented in the Table 1. The raw SPC data is highly skewed and has a very long 'tail' with a few very high values. These high values exert undue 'leverage' on the calculation of both the average value and the standard deviation

The average values of these two data sets are quite different with the raw SPC data almost twice the average of the $\log_{10}$ values. The medians values are the same by definition (½ values below and ½ values above). A large difference between the median and average value is an indication of a highly skewed data set. Many common statistical methods are only valid under the assumption of a normally distributed data set. For example, statistical process control uses the standard deviation as a yardstick to measure if changes in data are within the normal range of variation or a data pint is abnormally high or low. Because of the artificially high standard deviation from skewed data, the SPC series would not flag a value as abnormal until it exceeded 75,000 while the $\log_{10}$ SPC series would flag an abnormal value as being above 38,000.

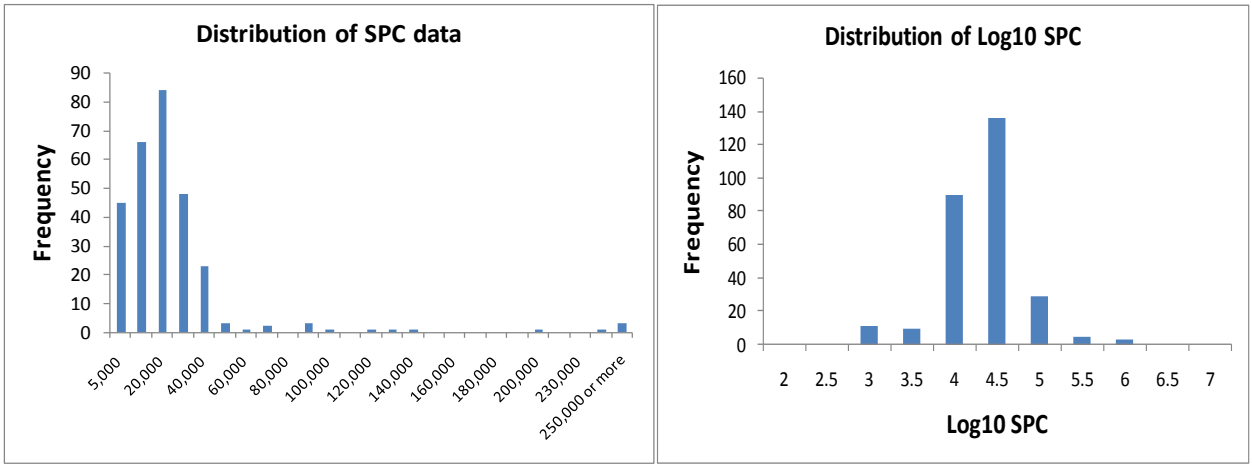


Figure 2. Distribution of SPC and $\log_{10}$ SPC data.

Table 1. Descriptive statistics of raw SPC data and log10 transformed SPC data.

	SPC (cfu/ml)	*log_{10} SPC (cfu/ml)*	*log_{10} values converted back to SPC (cfu/ml)*
Average	24,000	4.10	12,500
Median	14,000	4.15	14,000
Standard Deviation	51,100	0.49	
Skewness	7.8	-1.4	
Average – 1 SD	-27,400	3.61	4,000
Average + 1 SD	75,100	4.59	38,000

Log transformations therefore offer a better yardstick for true deviations in bacteria count data and provide a more accurate assessment of deviations over time.

The Moving Average (or Rolling Average)

Statistical process control uses a combination of a moving average (an average of the last x data points, where x may be from 3 days to many months) and the deviation from this moving average based on the standard deviation of the data. We saw in the previous example how a skewed distribution distorts both the average value and the standard deviation of a data set. For data sets with the long tail problem, the standard deviation will be artificially large. This reduces the power to find real deviations in the data stream as well as trends in the data.

A time series plot (one data point per day) of the raw and log10 SPC data presented above is shown in Figure 3. A 5 day moving average trend line has been added to the figure. A moving average trend line in an Excel graph is an excellent way to visually separate real short term (shorter than the averaging interval) responses from the 'noise' in the data and also helps to visualize longer term (longer than the averaging interval) trends in data.

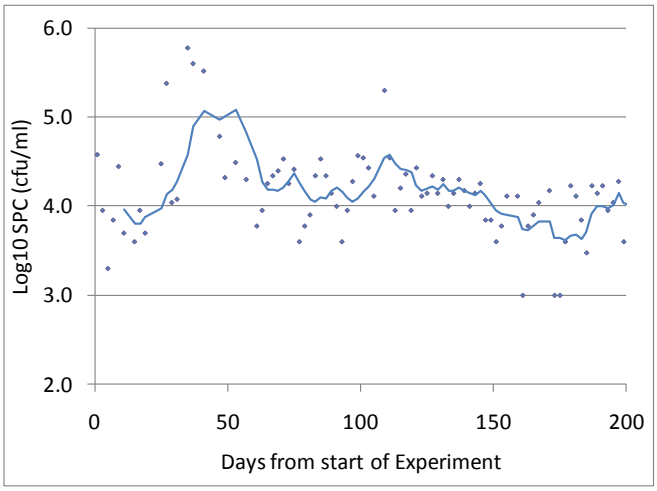


Figure 3. Time series plot of log10 PSC data with a 5 day moving average trend line added.

Case Study 2: Extreme Incubation

In an experiment reported by Reinemann, et al., (2006) milk was inoculated with 28 strains of bacteria representing a broad spectrum of thermoduric, psychrotrophic, environmental and contagious mastitis organisms that had been isolated from raw milk samples taken from Wisconsin dairy farms. Bacteria colonies were added to 250 ml of milk which was allowed to incubate at room temperature for 12 hours. This 250 ml inoculant was then added to 20 liters of raw milk that was incubated for an additional 6 hours at room temperature. Plate counts from the inoculant (12 hr. incubation) and the incubated raw milk samples (18 hr incubation) are presented in Figure 4.

The growth rate of thermoduric bacteria was much slower than other bacteria types and accounted for their small percentage (<0.01%) of the final SPC. Thermoduric bacteria include species of micrococcus, streptococcus, lactobacillus, bacillus, and occasionally gram-negative rods. Thermoduric bacteria are also referred to as spore formers because they can create a protective form called a spore that makes the resistant to both heat and other lethal agents such as sanitizers (more on this later).

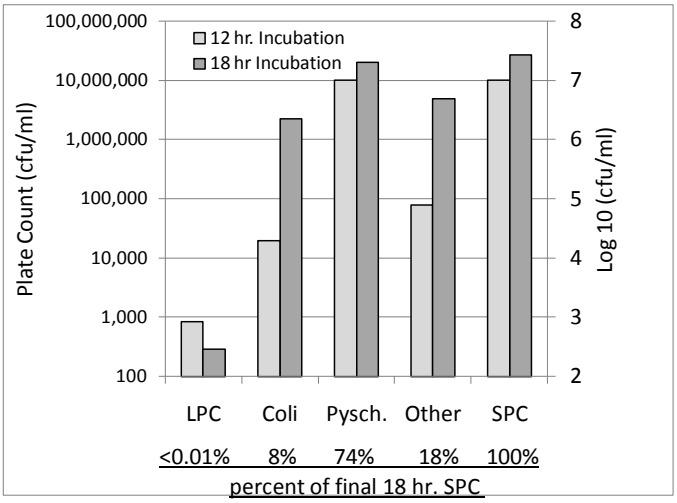


Figure 4. Bacteria plate counts from inoculated milk allowed to incubate at room temperature for 12 and 18 hours.

The coliform population increased much faster than the thermodurics, made up 8% of the total SPC, and ranged from 20,000 cfu/ml after 12 hours to 2,000,000 cfu/ml after 18 hours of incubation. This rapid growth rate is the basis for the diagnosis in the simple method that if coliform counts are in excess of several thousand cfu/ml it is likely that incubation is occurring somewhere in the milk handling system. The number of coliform or other environmental organisms directly harvested from the skin of teats and udders is usually less than $\log_{10}$ values of 4 (10^4 or 10,000) cfu/ml in bulk milk when some form of pre-milking sanitation is practiced and milk is harvested in a sanitary milking machine and cooled quickly. Moderate udder and milking hygiene will reduce bulk tank numbers to the range of log 3 to log 4 (1,000 to 10,000). Excellent udder hygiene (clean stalls) and excellent pre-milking sanitation can reduce these counts to below log 1, or 10 cfu/ml.

The growth rate of the psychrotrophs exceeded that of the coliforms and became the dominant organism, making up 74% of the total. Common psychrotrophic bacteria include species of pseudomonas, and bacillus. There are many 'other' bacteria types (18%) not identified by the use of these 4 tests.

Case Study 3: Correlation

Correlation between different data sets tells us which data deviate from their average values in the same manner. Data that is collected over time are positively correlated when both increase at the same time. For data that is negatively correlated, one data point tends to deviate in a positive direction while the other deviates in a negative direction. If two data sets have no significant correlation the deviations have no relationship to each other over time

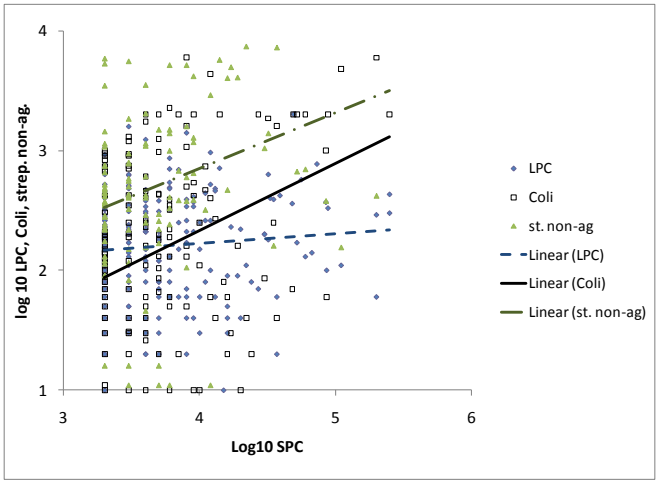


Figure 5. Correlation between $\log_{10}$ SPC count and $\log_{10}$ LPC, $\log_{10}$ Coli and $\log_{10}$ *Strep. non-ag*. bacteria counts

(sometimes one is up and the other down and sometimes the other way). Correlation is a powerful tool to help diagnose which bacteria types are the major contributor to short term elevations in SPC and therefore the likely sources to be investigated.

Figure 5 is an example of using correlation to diagnose daily bulk tank milk bacteria data. This farm performed the minimal tests (SPC, LPC, Coli) on every tank of milk and also did periodic quantitative bulk tank cultures to further identify bacteria types. Both coliform and *Strep. non ag.* were positively correlated with SPC (log_{10} values for all data). LPC had no significant correlation with SPC. The high correlation between SPC and environmental bacteria and lack of correlation between SPC and LPC is an indication that environmental contamination is a more likely cause for the high SPC than was a persistent cleaning failure. It would be very difficult to visualize these relationships without performing a correlation analysis.

Case study 4: Dealing with false positives and false negatives:

Bulk tank bacteria plate counts exhibit a high degree of variably over time. Part of this variability is due to the rapid growth rate in some bacteria types. Variability is also introduced because of the test methods. False negatives (no growth, or no detection when bacteria actually are present) and false positives (a much higher count than actual, possibly due to incubation of the sample) do occur.

The dilutions used for SPC, plate loop count (PLC) and Total Bacteria Count (TBC) are commonly performed to give accurate results down to 1000 cfu/ml but no lower. A SPC of 1000 cfu/ml reported from a laboratory may mean that there was actually 1000 cfu/ml; it may sometimes mean that the test indicated a count of less than 1000 cfu/ml; finally, it could indicate that there was no growth on the plate. These sources of variability in the test methods and reporting can cause an unusual distribution in the data as illustrated in figure 6 where no growth is reported as $log_{10}TBC = 0$.

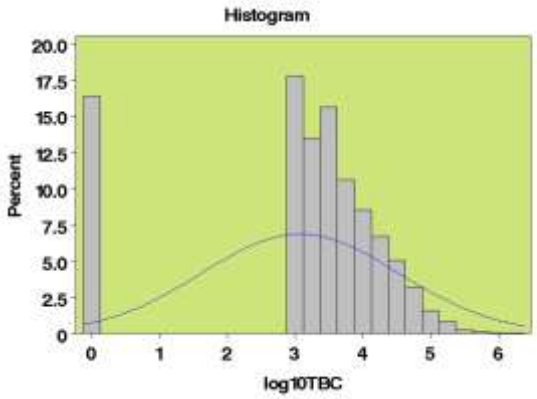


Figure 6. Distribution of log10 Total Bacteria Count (TBC) field data from J. Pantoja (pers. comm.).

Data is conspicuously absent between log 0 and log 3 values, implying no TBC counts between 1 cfu/ml and 1000 cfu/ml for this rather large data set. This is implausible from a biological perspective. Some of the data in the 'zero' category (no growth) are very likely clearly false negatives that were not detected because either the dilution was not appropriate to determine the actual count or bacteria did not grow on the plate for other reasons. Some of the data in the 3 category (TBC = 1000) are likely overstated. This unusual distribution can also cause errors in statistical analysis but are not likely to be as severe as those introduced by bacteria data that has not been log transformed.

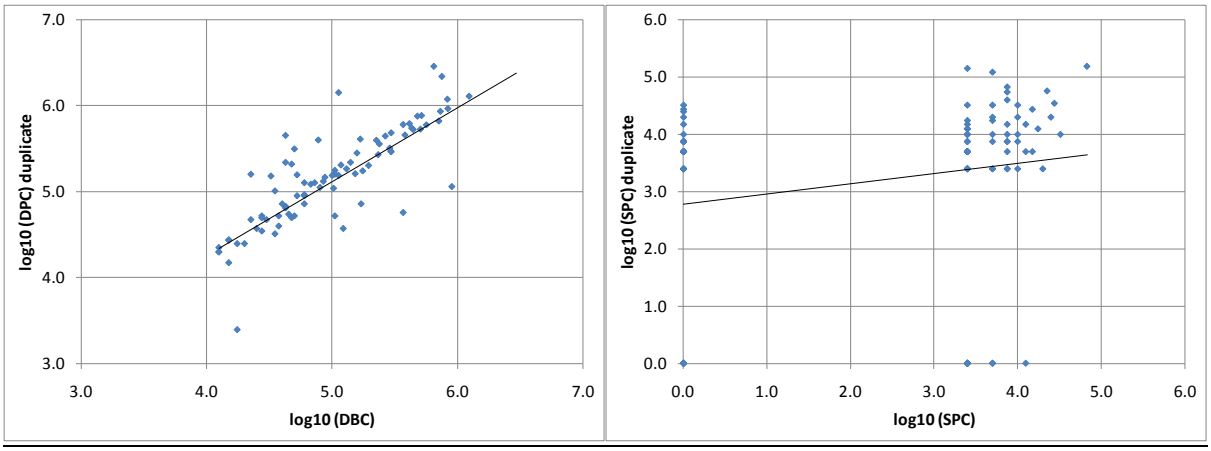


Figure 7. Correlation between bacteria counts and duplicate samples.

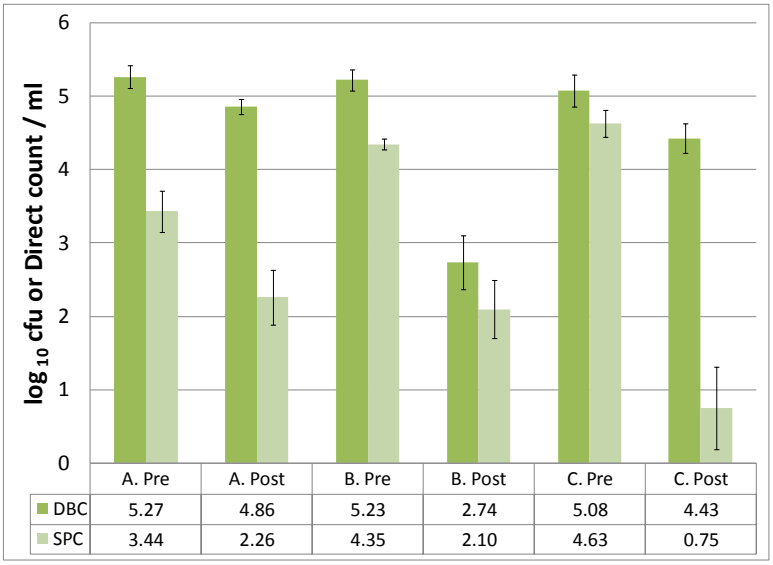
Figure 7 illustrates a use of correlation analysis to estimate the false positive and false negative rate for both SPC and Direct microscopic Bacteria Count (DBC) data of duplicate samples from the same 30 ml vial of milk (Reinemann et al., 2008). Reasonable correlations (greater than 75%) were observed between duplicate DBC samples and there did not appear to be any obvious false positive or false negative readings. The correlation between duplicate SPC counts was quite low (less than 25%). In addition there were a number of false positive and false negative readings (one sample had no detectible bacteria while its duplicate had a substantial population).

These data illustrate that the false positive and negative errors as a result of the DBC test method are greatly reduced or eliminated as compared to plate count test methods. The DBC method also had much better repeatability than the SPC test. This reduction in variability substantially improves the accuracy of both statistical and practical detection of real changes in the data. A combination of DBC to enumerate total bacteria populations combined with plate count methods to estimate the relative magnitude of different bacteria species (if needed) maximizes the strengths and minimizes the weakness each method.

Case Study 5: Investigating the Influence of Udder Hygiene and Pre-Milking Sanitation

It is evident that a wide variety of bacteria species can be harvested from the skin of teats and udders during milking. Pre-milking sanitation is intended to reduce the bacteria population on teat skin before the milking unit is applied. The 'wipe test' method described in Reinemann et al. (2008) was used to recover bacteria from teat skin both before and after pre milking sanitation to assess the effectiveness of pre-milking sanitation for human workers and robotic milking units. In all cases a premilking sanitizing solution was used on teats. Both DBC and plate culture methods were used to enumerate bacteria. DBC technology typically enumerates many more bacteria than plate count methods. Plate count methods rely on the recovery of viable bacteria which form colonies on growth media, whereas DBC technology can enumerate both viable and killed bacteria. In this study the reduction in DBC were used as an estimate of the effectiveness removing solids from the teat skin in a similar way to the previous studies which used various types of tracer materials. The comparison of viable to viable+unviable bacteria reductions also allowed for an estimate of the killing action of pre-milking teat disinfection.

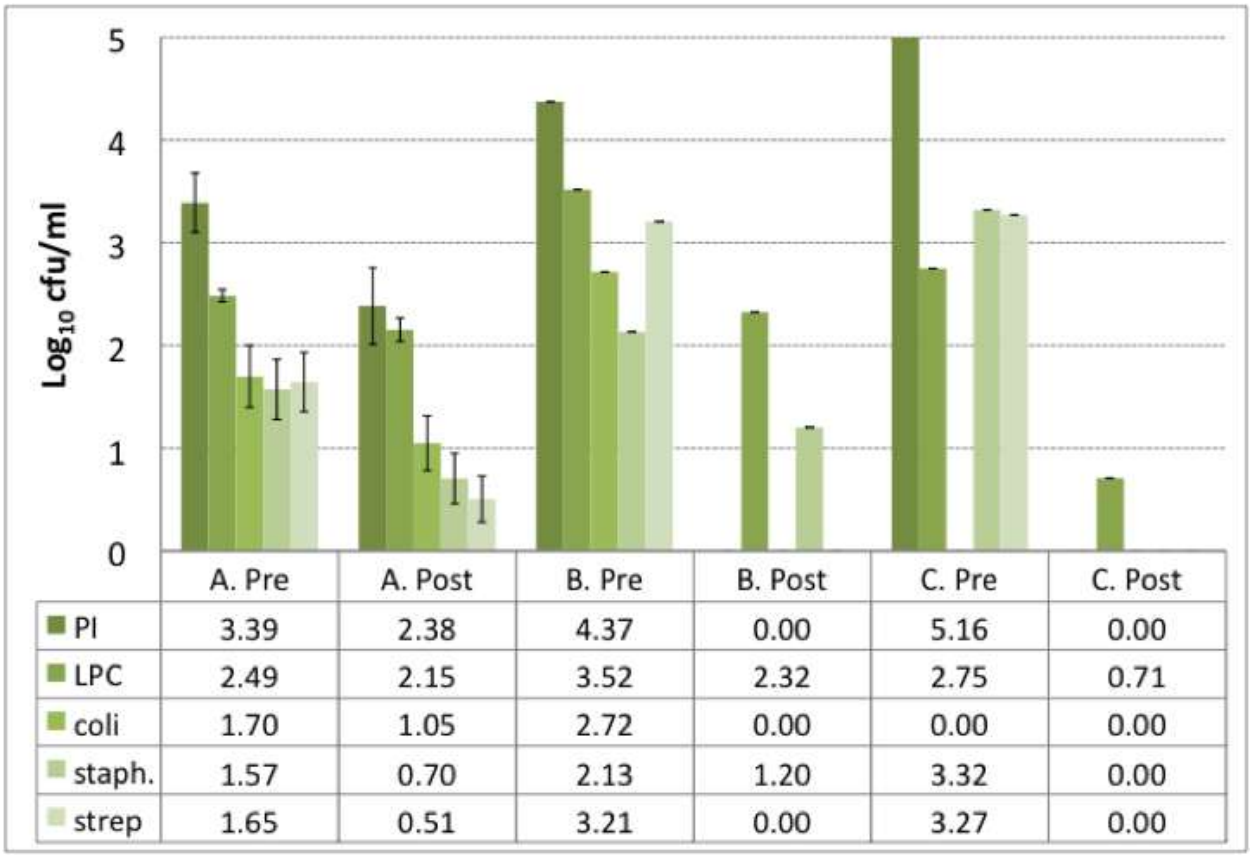
Farm A used a robotic milking machine and sand bedded free-stalls. Farm B used a conventional double-20 milking parlor with human cow prep and a free-stall barn bedded with dried manure solids from and anaerobic digester. The pre-milking preparation procedures at this farm were considered by the investigators to be excellent and above norm for Wisconsin dairy farms. Farm C used a conventional double-16 milking parlor with human cow prep and a free-stall barn bedded with sand and was also considered by the investigators to have excellent pre-milking cow preparation procedures.



	A. Pre	A. Post	B. Pre	B. Post	C. Pre	C. Post
DBC	5.27	4.86	5.23	2.74	5.08	4.43
SPC	3.44	2.26	4.35	2.10	4.63	0.75

Figure 8. DBC and SPC data for Farms A, B, and C. pre and post teat sanitation. Error bars indicate 95% confidence intervals of the mean values

There was no significant difference in the pre-sanitation DBC between any of the farms (Figure 8). The pre-sanitation SPC levels were lower for farm A than for farms B and C implying a lower number of viable bacteria on the teat skin in farm A. Assessment of pre-milking SPC levels is a good indicator of the relative bacteria challenge in the cow housing area and can be used to evaluate different bedding materials and stall maintenance practices.



	A. Pre	A. Post	B. Pre	B. Post	C. Pre	C. Post
PI	3.39	2.38	4.37	0.00	5.16	0.00
LPC	2.49	2.15	3.52	2.32	2.75	0.71
coli	1.70	1.05	2.72	0.00	0.00	0.00
staph.	1.57	0.70	2.13	1.20	3.32	0.00
strep	1.65	0.51	3.21	0.00	3.27	0.00

Figure 9. Results of bacteria speciation for farms A, B, and C. pre and post teat sanitation. Error bars indicate 95% confidence intervals of the mean values

All farms showed a significant reduction in SPC and DBC, but neither number was reduced to zero. Post-sanitation DBC values were lower for farm B than for farms A and C, while post-sanitation SPC values were lower for farm C than for farms A and B. The reduction in SPC was greater than the reduction in DBC for the farms using sand bedding (A and C). This would be expected because of the reduction of viable organisms killed by pre-milking sanitizing solution. The reduction of SPC and DBC were of similar magnitude on the farm using dried manure solids as bedding.

There was a significant reduction in all bacteria types on farm A (Figure 9). The data from farms B and C are composite samples with only 2 or 3 bacteria counts. Claims of significance cannot be substantiated, but a reduction in all bacteria types seems apparent these farms. The reduction in LPC was smaller than for other bacteria types on farm A and was the most prevalent type of bacteria after teat sanitation on farms B and C, probably because pre-milking teat sanitizing solutions are less effective at killing thermoduric organisms than common environmentals such as coliform, strep and staph organisms. Thermoduric organisms were also among the most prevalent type of bacteria in the pre-sanitation samples that were not pre-incubated. This indicates that thermoduric organisms recovered from teat skin could be a contributor to LPC in bulk tank milk especially when poor pre-milking teat sanitation is used and may still be a contributor when good pre-milking teat sanitation is used; and that failure of the milking machine cleaning and sanitation systems is not the only contributor to LPC in bulk tank milk.

The DBC and LPC had much better repeatability across duplicate samples than did the other plate culture methods (SPC, coliform, strep, staph, mold bacteria). Although DBC has much better repeatability and requires a substantially smaller sample size to detect biologically important differences, it does not indicate the type of bacteria present in the sample. A combination of DBC of individual samples and bacteria speciation using fewer composite samples as well as visual assessment methods is recommended to provide the best benefit-cost ratio for assessing teat and udder hygiene as a means of assessing the hygienic quality of cow bedding materials and management as well as methods of pre-milking teat sanitation. The authors are exploring techniques to make this test more practical for field application.

Summary Points

- Know your enemy: Acquiring information about the specific bacterial species represented in bulk tank milk will improve the power of a diagnosis.

- Bacteria count data should be converted into log values so that statistical analysis and process control algorithms are valid and more sensitive.

- The moving average is a useful tool to assess long term trends in bacteria populations in bulk milk.

- Correlation is a useful tool to aid in the diagnosis of the types of bacteria contributing to short term changes in bulk tank bacteria counts.

- Incubation causes dramatic increases in some bacteria types but not in others. Knowledge of the growth rates of different bacteria types under different conditions will improve diagnostic methods.

- Premilking sanitation will reduce bacteria population on teat skin but not eliminate bacteria. Cows entering the milking with higher degree of teat contamination will still have a higher degree of teat contamination for the same method of pre-milking teat sanitation than cows with lower initial teat contamination.

- Pre-milking sanitizers are more effective at reducing the population of some bacteria types (environmental) than others (thermoduric).

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