Journal of Microbiological Methods 165 (2019) 105691

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Note

Morphometric parameters of foodborne related-pathogens estimated by transmission electron microscopy and their relation to optical density and colony forming units

C.J. González-Pérez[a], J. Tanori-Cordova[b], E. Aispuro-Hernández[a], I. Vargas-Arispuro[c], M.A. Martínez-Téllez[a,*]

[a] Coordinación de Tecnología de Alimentos de Origen Vegetal, Centro de Investigación en Alimentación y Desarrollo, A.C., C.I.A.D. A.C., Hermosillo, Sonora, Mexico
[b] Departamento de Investigación en Polímeros y Materiales, Universidad de Sonora, Hermosillo, Sonora, Mexico
[c] Coordinación de Ciencias de los Alimentos, C.I.A.D. A.C., Hermosillo, Sonora, Mexico

ARTICLE INFO

Keywords:
Bacterial cell morphology
CFU·mL^{-1} calculation
Escherichia coli O157:H7
Listeria monocytogenes
Salmonella Saintpaul
Salmonella Typhimurium
Staphylococcus aureus
Transmission electron microscopy

ABSTRACT

The different morphological characteristics of five bacterial pathogen strains were analyzed through transmission electron microscopy for addressing the particular relationship between optical density and colony-forming units for each strain. Generated linear equations will allow a reliable calculation of bacterial concentrations through simple optical density measurements.

Nearly a million original research papers with “bacteria” in the title, abstract or keywords were published in the last five years according to the Scopus database, mainly in the areas of Medicine, Biochemistry, Genetics and Molecular Biology, and Immunology and Microbiology. This information highlights the relevance of these microorganisms to either find control strategies or facilitate their manipulation for biotechnological purposes. In this sense, the study of bacteria will allow researchers to screen and test for antibiotic resistance, antagonistic compounds, and antimicrobial activity of novel compounds (Aisha et al., 2017; Bae and Lee, 2017; Moon et al., 2017; Severino et al., 2015). However, one problem arising in these type microbiological investigations is the need for defining an accurate number of bacterial cells used in a test.

In the field of microbiology, different methods are used to determine the number of bacteria in order to perform tests for evaluating the effect of a certain treatment. One of the methods widely used on a daily basis is the estimation of colony-forming units by measuring absorbance at 600 nm (Mytilinaios et al., 2012). Nevertheless, this estimation depends on bacterial size and shape, where different cell densities can present the same optical density value, leading to inappropriate estimations. This relation differs according to the strains' particular morphometric characteristics, which can accurately be determined by electron microscopy combined with negative staining (Golding et al., 2016). Thus, the information derived from electron micrographs can be related to the ratio of optical density and bacterial concentrations in order to make more reliable estimations. Table 1 summarizes the advantages and drawbacks of current approaches and other commonly used methods for general bacterial quantification that mainly differ but are not limited to the accuracy, access to laboratory facilities, cost of reagents and supplies, technical training, and time to get the results.

Escherichia coli O157:H7, *Salmonella* spp., *Staphylococcus aureus,* and *Listeria monocytogenes* are among the main foodborne-related pathogens. Due to their importance in food safety, these bacteria are widely used as model microorganisms in diverse research studies (Adhikari et al., 2018; Cho et al., 2016; González-Pérez et al., 2019; Moon et al., 2017). Therefore, any method or technique that facilitates the calculation of viable cells would be of great help and would decrease the time of experimentation.

In this study, transmission electron microscopy (TEM) was used to relate the morphometric characteristics of model pathogenic bacteria to the ratio of optical density and colony-forming units. The bacteria studied were *Listeria monocytogenes* ATCC7644, *Staphylococcus aureus* ATCC6538, *Escherichia coli* O157:H7, *Salmonella* Saintpaul, and

* Corresponding author at: Carretera Gustavo Enrique Astiazarán Rosas, #46, Col. La Victoria, 83304 Hermosillo, Sonora, Mexico.
E-mail address: norawa@ciad.mx (M.A. Martínez-Téllez).

https://doi.org/10.1016/j.mimet.2019.105691
Received 31 May 2019; Received in revised form 15 August 2019; Accepted 15 August 2019
Available online 19 August 2019

Table 1
Methods for estimation of bacterial concentrations.

Approach	Advantages	Limitations / drawbacks	Accuracy	Reference
Standard microbiological culture methods by serial dilutions and direct agar-plate counting	– Affordable costs of reagents and supplies – Conventional and common laboratory equipment	– Time-consuming (24 to 72 h) – Not appropriate for cells in a dormant physiological state (VBNC[a])	– Good	Cabrera-Díaz et al., 2018
Microscopy-based techniques	– Able to detect VBNC[a] cells and differentiate living and dead cells – Little time to get results (minutes or hours)	– Specialized equipment and materials required – Often requires colony plate counts following cultivation – ATT[b] required	– Good	Jung and Lee, 2016
Coulter counting	– Able to detect VBNC[a] cells – Very little time to get results (minutes)	– Specialized equipment required – ATT[b] required	– Very good	Vembadi et al., 2019
Flow cytometry	– Able to detect VBNC[a] cells – Little time to get results (minutes to hours)	– Specialized equipment required – High cost of reagents – Requires variable times for sample processing prior to measurement – ATT[b] required	– Very good	Raymond and Champagne, 2015
qPCR-based techniques	– Able to detect VBNC[a] cells – Little time to get results (hours)	– Specialized equipment required – High cost of reagents – ATT[b] required	– Very good	Takahashi et al., 2017
Equations proposed in the present work	– Calculations made from direct absorbance readings of the bacterial solution – There is no need of plate inoculation nor incubation times – Very little time to get results (minutes) – No cost – Useful for bacterium with similar morphometric characteristics	– Not appropriate for VBNC[a] cells – Not appropriate for the evaluation of bacterial concentrations under non-lytic treatments.	– Good	This research

[a] Viable but not culturable = VBNC.
[b] Advanced technical training = ATT.

Salmonella Typhimurium ATCC14028. This research can provide a tool for facilitating the calculation of colony-forming units in experiments involving the tested bacteria by using easy linear equation models.

Each type of bacteria was grown in Brain Heart Infusion (BHI) broth for 18 h at 37 °C. Bacterial cells were recovered by centrifugation (10,000 × *g*, 10 min, 4 °C) and were washed twice with phosphate-buffered saline (PBS) pH 7.4. A range of optical densities (OD) (0.01 to 1.00) was measured at 600 nm (OD 600 nm) in a spectrophotometer (Shimadzu BioSpec-1601). To determine the colony-forming units per milliliter (CFU/mL) at each OD tested, serial dilutions in PBS were plated on BHI and incubated for 24 h at 37 °C. All determinations were performed in triplicate. The relation between OD and CFU/mL was analyzed through linear regression analysis in the R software version 3.5.1 (R Core Team, 2018), which generated the proposed linear equations.

The morphology and size of bacteria were determined using images obtained with a JEM 2010F transmission electron microscope (TEM) with an operating voltage of 200 kV (JEOL, Ltd., Tokyo, Japan). For TEM observations of bacterial pathogens, negative staining was used. Five microlitres of a solution of each bacterium (7×10^9 CFU/mL) was spread on silicon monoxide type-A (300 mesh) copper TEM grid and dried. After that, a drop of 1% phosphotungstic acid was spread on the TEM grid for 5 min and the excess was removed. All samples were subsequently dried in vacuum before observation. The morphometric parameters were determined using ImageJ (NIH) and DigitalMicrograph™ software. The results were expressed as the mean ± SD of 60 representative cells according to Bratbak (1993), who mentioned that for populations with similar sizes a minimum count of 50 cells is necessary.

Results showed that each bacterial strain exhibits different behavior in terms of optical density and bacterial concentration, parameters that can be related to the amount of light that cells can absorb depending on their size and shape. The linearity at OD 600 nm occurs when absorbance values are < 1.0, so that these variables can be related to a linear equation (Y = mX + b), obtained by means of the least squares method, where "Y" represents CFU/mL and "X" the optical density. Table 2 shows the different equations for the bacteria used in this study. In all the cases, linear models fit the experimental data (Supplementary material S1) with R^2 values higher than 0.9. Interestingly, for most of the tested pathogens, lower OD values exhibited a substantially lower variation in the bacterial concentrations. In this research, PBS washes were performed but if determinations were made in growth media, the linearity would not be affected as long as they were done in a short period of time, and with the medium as a blank.

In order to validate the proposed equations, theoretical CFU/mL values at OD of 0.05 and 0.5 were compared against experimentally obtained total viable count values (Supplementary material S2). All the comparisons showed good accuracy, except for the trial with the higher *E. coli* OD, where the mean experimental value was slightly below the 99% confidence interval. Hence, for the proper estimation of *E. coli* concentrations, it is advised to work with OD_{600nm} lower than 0.5. Also, preliminary results suggest these equations are able to predict the bacterial concentration after lytic treatments (data not shown). However, they are not recommended for evaluating treatments that irreversible damage the bacterial cells but without lysing them, because they would be non-viable cells that would still be able to absorb light.

The analyzed strains differ in shape, length, and width sizes (Table 2), which can be directly related to the particular generated equations. Morphological parameters (shape, length, and width) obtained are comparable with previous reports (Fernandez Escartin, 2000; Parra et al., 2002). The slope of the equations for determination of CFU/mL number decreased when increasing bacterial size. The smallest slope was found in the equation for *E.coli* O157:H7, which corresponds to this bacterium having the largest size. In addition, the aforementioned bacterium and *S.* Typhimurium have flagella, which may likely increase the surface area absorbing light from each colony-forming

Table 2
Morphologic characteristics of foodborne-related pathogens and equations to determine the number of colonies forming units from optical densities.

Bacteria	Shape	Length (μm)[a]	Width (μm)[a]	Equation to determine colony-forming units[b]	R^2
Listeria monocytogenes	Bacillus	1.41 ± 0.19	0.56 ± 0.06	$Y = (12.98 \times 10^8)\ X - 1.71 \times 10^6$	0.96
Staphylococcus aureus	Coccus	0.89 ± 0.07	0.89 ± 0.07	$Y = (9.164 \times 10^8)\ X + 24.1 \times 10^6$	0.92
Salmonella Saintpaul	Bacillus	1.64 ± 0.3	0.55 ± 0.04	$Y = (9.365 \times 10^8)\ X - 9.62 \times 10^6$	0.97
Salmonella Typhimurium	Bacillus	1.88 ± 0.29	0.71 ± 0.11	$Y = (6.895 \times 10^8)\ X - 0.319 \times 10^6$	0.95
Escherichia coli O157:H7	Bacillus	2.11 ± 0.41	0.97 ± 0.14	$Y = (5.841 \times 10^8)\ X - 5.04 \times 10^6$	0.98

[a] Mean ± standard deviation of 60 measured cells.
[b] Y = Colony forming units; X = Optical density at 600 nm.

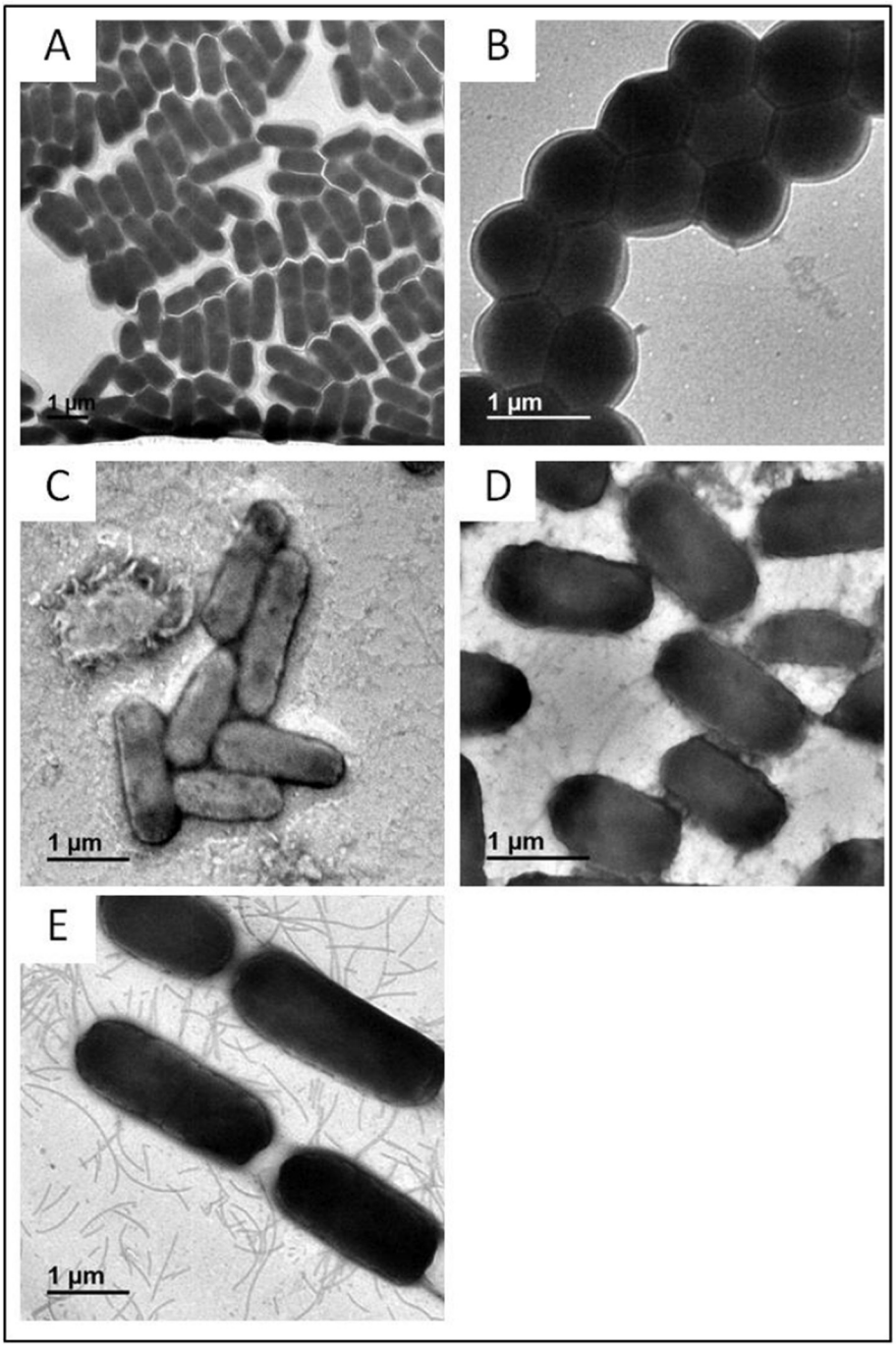

Fig. 1. Electron micrographs of foodborne bacterial pathogens. A) *Listeria monocytogenes*; B) *Staphylococcus aureus*; C) *Salmonella* Saintpaul; D) *Salmonella* Typhimurium; E) *Escherichia coli* O157:H7.

unit, in part explaining why equations for these two strains showed the smallest slopes. On the other hand, the similarity among *L. monocytogenes*, *S. aureus*, and *S.* Saintpaul cell sizes is reflected in the similar generated equations.

Fig. 1 shows electron micrographs of the bacteria used in this work. *Staphylococcus aureus* is cocci-shaped while the other bacteria are rod-shaped. The different lengths and widths among the bacterial strains can be related to the given equations in Table 2, suggesting the greater the bacterial size is, the lower the slope of the equation. Additionally, among the studied strains, the equation generated for *S. aureus* resulted with a positive "b" value in the linear equation. It would be interesting to address if the cocci-shaped morphology of these bacteria is related to this observation. The morphometric results agree with previous reports showing the characteristics of the studied bacteria through electron micrographs with negative staining (Golding et al., 2016; Mahmoudi et al., 2016; Nataro and Kaper, 1998). Overall, results showed that the slope in the equations has a direct relationship with the CFU but is also inversely proportional to bacterial cell size; therefore, besides the optical density values, an adequate estimation of CFU should also consider the slope and intercept value.

In conclusion, this research provides important tools to calculate the number of colony-forming units by only measuring optical densities. These tools are linear models that relate optical density at 600 nm with bacterial concentrations, which will allow an easy and reliable calculation of CFU/mL by measuring optical densities at this wavelength. Furthermore, this relationship was addressed through transmission electron microscopy, which revealed that the slope in the equations defined in this work is a relevant indicator of cell size in terms of length and width.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105691.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by FOINS-CONACYT [grant number FON.INST/30/2017, Project Number 4325]. We are grateful to CONACYT for the scholarships granted to González-Pérez. We gratefully acknowledge the use of TEM facilities at the TEM Laboratory of Universidad de Sonora. We thank Olivia Briceño and Francisco Soto for technical support in microbiology and Sarai Rochín Wong for technical support in microscopy.

References

Adhikari, A., Yemmireddy, V.K., Costello, M.J., Gray, P.M., Salvadalena, R., Rasco, B., Killinger, K., 2018. Effect of storage time and temperature on the viability of *E. coli* O157: H7, *Salmonella* spp., *Listeria innocua*, *Staphylococcus aureus*, and *Clostridium sporogenes* vegetative cells and spores in vacuum-packed canned pasteurized milk cheese. Int. J. Food Microbiol. 286, 148–154. https://doi.org/10.1016/j.ijfoodmicro.2018.07.027.

Aisha, A., Hind, A.-R., Sufian, A.-K., Mutamed, A., 2017. Growth inhibition of foodborne pathogens in camel milk: *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp. and *E. coli* O157: H7. Czech J. Food Sci. 35 (4), 311–320. https://doi.org/10.17221/84/2017-CJFS.

Bae, Y.-M., Lee, S.-Y., 2017. Antagonism by salt addition on treatment with short chain fatty acids with one carboxylic acid against *Escherichia coli* O157: H7. Food Control 73, 1327–1333. https://doi.org/10.1016/j.foodcont.2016.10.055.

Bratbak, G., 1993. Microscope methods for measuring bacterial biovolume: epi-fluorescence microscopy, scanning electron microscopy, and transmission electron microscopy. In: Kemp, P.F., Sherr, B.F., Sherr, E.B., Cole, J.J. (Eds.), Handbook of Methods in Aquatic Microbial Ecology. CRC Press, Florida, U.S.A., pp. 309–317.

Cabrera-Díaz, E., Martínez-Chávez, L., Sánchez-Camarena, J., Muñiz-Flores, J.A., Castillo, A., Gutiérrez-González, P., Arvizu-Medrano, S.M., González-Aguilar, D.G., Martínez-Gonzáles, N.E., 2018. Simultaneous and individual quantitative estimation of *Salmonella*, *Shigella* and *Listeria monocytogenes* on inoculated Roma tomatoes (*Lycopersicon esculentum* var. Pyriforme) and Serrano peppers (*Capsicum annuum*) using an MPN technique. Food Microbiol. 73, 282–287. https://doi.org/10.1016/j.fm.2018.02.009.

Cho, T., Kim, N., Kim, S., Song, J., Rhee, M., 2016. Survival of foodborne pathogens (*Escherichia coli* O157: H7, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus*) in raw ready-to-eat crab marinated in soy sauce. Int. J. Food Microbiol. 238, 50–55. https://doi.org/10.1016/j.ijfoodmicro.2016.08.041.

Fernandez Escartin, E., 2000. Microbiología e Inocuidad de los Alimentos, 1st edition. (Querétaro, Querétaro, México).

Golding, C.G., Lamboo, L.L., Beniac, D.R., Booth, T.F., 2016. The scanning electron microscope in microbiology and diagnosis of infectious disease. Sci. Rep. 6, 26516. https://doi.org/10.1038/srep26516.

González-Pérez, C.J., Vargas-Arispuro, I., Aispuro-Hernández, E., Aguilar-Gil, C.L., Aguirre-Guzmán, Y.E., Castillo, A., Hernández-Mendoza, A., Ayala-Zavala, J.F., Martínez-Téllez, M.A., 2019. Potential control of foodborne pathogenic bacteria by *Pediococcus pentosaceus* and *Lactobacillus graminis* isolated from fresh vegetables. Microbiol. Biotechnol. Lett. 47 (2), 1–12. https://doi.org/10.4014/mbl.1808.08014.

Jung, J.H., Lee, J.E., 2016. Real-time bacterial microcolony counting using on-chip microscopy. Sci. Rep. 6, 21473. https://doi.org/10.1038/srep21473.

Mahmoudi, R., Katiraee, F., Tajik, H., Abbas, A., 2016. Inhibitory effect of *Mentha Longifolia* L. essential oil against *Listeria monocytogenes* using transmission electron microscopy. Int. J. Vet. Sci. Res. 2, 14–17. https://doi.org/10.17352/ijvsr.000008.

Moon, H., Kim, N.H., Kim, S.H., Kim, Y., Ryu, J.H., Rhee, M.S., 2017. Teriyaki sauce with carvacrol or thymol effectively controls *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, and indigenous flora in marinated beef and marinade. Meat Sci. 129, 147–152. https://doi.org/10.1016/j.meatsci.2017.03.001.

Mytilinaios, I., Salih, M., Schofield, H.K., Lambert, R.J., 2012. Growth curve prediction from optical density data. Int. J. Food Microbiol. 154 (3), 169–176. https://doi.org/10.1016/j.ijfoodmicro.2011.12.035.

Nataro, J.P., Kaper, J.B., 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11 (1), 142–201.

Parra, M., Durango, J., Máttar, S., 2002. Microbiología, patogénesis, epidemiología, clínica y diagnóstico de las infecciones producidas por *Salmonella*. Rev. MVZ Córdoba. 7 (2), 187–200.

R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (URL). https://www.R-project.org/.

Raymond, Y., Champagne, C.P., 2015. The use of flow cytometry to accurately ascertain total and viable counts of *Lactobacillus rhamnosus* in chocolate. Food Microbiol. 46, 176–183. https://doi.org/10.1016/j.fm.2014.07.002.

Severino, R., Ferrari, G., Vu, K.D., Donsì, F., Salmieri, S., Lacroix, M., 2015. Antimicrobial effects of modified chitosan based coating containing nanoemulsion of essential oils, modified atmosphere packaging and gamma irradiation against *Escherichia coli* O157: H7 and *Salmonella* Typhimurium on green beans. Food Control 50, 215–222. https://doi.org/10.1016/j.foodcont.2014.08.029.

Takahashi, H., Saito, R., Miya, S., Tanaka, Y., Miyamura, N., Kuda, T., Kimura, B., 2017. Development of quantitative real-time PCR for detection and enumeration of Enterobacteriaceae. Int. J. Food Microbiol. 246, 92–97. https://doi.org/10.1016/j.ijfoodmicro.2016.12.015.

Vembadi, A., Menachery, A., Qasaimeh, M.A., 2019. Cell cytometry: review and perspective on biotechnological advances. Front. Bioeng. Biotechnol. 7, 147. https://doi.org/10.3389/fbioe.2019.00147.