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Application of E-nose technology combined with artificial neural network to predict total bacterial count in milk

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ABSTRACT

Total bacterial count (TBC) is a widely accepted index for assessing microbial quality of milk, and cultivation-based methods are commonly used as standard methods for its measurement. However, these methods are laborious and time-consuming. This study proposes a method combining E-nose technology and artificial neural network for rapid prediction of TBC in milk. The qualitative model generated an accuracy rate of 100% when identifying milk samples with high, medium, or low levels of TBC, on both the testing and validating subsets. Predicted TBC values generated by the quantitative model demonstrated strong coefficient of multiple determination ($R^2 > 0.99$) with reference values. Mean relative difference between predicted and reference values (mean ± standard deviation) of TBC were 1.1 ± 1.7% and 0.4 ± 0.8% on the testing and validating subsets involving 24 and 28 tested samples, respectively. Paired *t*-test implied that the difference between predicted and reference values of TBC was insignificant for both the testing and validating subsets. As low as ~1 log cfu/mL of TBC present in tested samples were precisely predicted. Results of this study indicated that combination of E-nose technology and artificial neural network generated reliable predictions of TBC in milk. The method proposed in this study was reliable, rapid, and cost efficient for assessing microbial quality milk, and thus would potentially have realistic application in dairy section.

Key words: rapid detection, microbial quality, food safety, dairy spoilage

INTRODUCTION

Milk is recommended by most nutritional societies as a valuable and nutritious food because of the contained macronutrients and bioactive constitutes; consequently, the demand for milk continues to increase due to the increase in per capita consumption and global population (Kamana et al., 2014; Paraffin et al., 2018; O'Kane et al., 2018). Milk also provides an excellent growth environment for microorganisms, and their propagation in milk leads to spoilage, decreased nutritional value, altered sensory and physicochemical properties of milk, and increased risk of foodborne diseases (Claeys et al., 2013; Boor et al., 2017; Porcellato et al., 2018). Implementing heat-treatment (e.g., pasteurization or UHT sterilization) is a common practice in the dairy industry to control spoilage organisms and potential pathogens, thus preventing spoilage and protecting the health of consumers (Weisbecker, 2007; Boor et al., 2017). However, microbial quality issues of milk continue to evolve due to failure in heat-treatment or posttreatment contamination that may occur due to improper handling (e.g., temperature abuse during transporting or storage) or inadequate sterilization of filling equipment (Salustiano et al., 2009; Martin et al., 2018).

Dairy microbiology to date has predominantly focused on bacteria, which are the main causes of spoilage, although a full range of microorganisms can be present in milk (Boor et al., 2017). Microbial propagation exceeding a certain amount (ranging 10^6–10^8 cfu/mL depending on nature of spoilage and microbial types) leads to detectable spoilage (Poghossian et al., 2019). Given that milk spoilage is a complex indefinite parameter and difficult to measure with accuracy, consumers generally check the expiration or "best if used by" dates to determine spoilage; on the other hand, producers usually use overly conservative expiration dates to avoid legal and economic consequences, leading to billions of pounds of unspoiled milk discarded (Poghossian et al., 2019). Total bacterial counts (**TBC**) has commonly been adopted as an index of microbial quality for milk over 100 yr because of its direct correlation to spoilage (Boor et al., 2017). Standard cultivation methods for TBC determination are reliable, sensitive, and capable of giving both qualitative and quantitative information on the amount and nature of microorganisms (Poghossian et al., 2019). However, they require a relatively

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long time, leaving a gap between sampling and results when assessing microbial quality (Wang and Salazar, 2016).

To reduce sample preparation and detection time for microbial analysis, intensive interest has focused on methods for rapid detection, especially PCR-based, immunological, colorimetric, and ATP-based techniques (Shama and Malik, 2013; Law et al., 2015; Poghossian et al., 2019). E-nose is an intelligent device that mimicks the mammal olfactory system, constituting a chemical sensor array system coupled with bioreceptors to sensitively identify volatiles even at very low concentrations (Wilson and Baietto, 2009; Jia et al., 2016). Because of its ability to mimic biological olfaction, E-nose has been widely accepted and adopted in many fields, including but not limited to food safety and quality, to detect a variety of compounds in complex environments (Wilson and Baietto, 2011; Jia et al., 2016; Dung et al., 2018). Given that each food product has a characteristic odor profile, E-nose has been studied for its application in the dairy sector for milk adulteration, classification, and spoilage caused by microbial growth (Kalit et al., 2014; Poghossian et al., 2019), and could be thus potentially used to assess microbial quality of milk by estimating TBC in test samples.

Compared with colorimetric techniques and PCR-based methods, which require 7 to 18 and 1 to 3 h, respectively, to generate results (Poghossian et al., 2019), E-nose generates results almost immediately. Immunological techniques are unreliable to determine TBC in milk due to the presence of various microbial species (Law et al., 2015); furthermore, it is difficult to use ATP bioluminescence measurements to determine microbial load of milk because of nonmicrobial ATP, as well as variations in ATP content of microbial cells (Shama and Malik, 2013). In contrast, E-nose is rapid and capable of determining changes of volatile compounds caused by the totality of microorganisms, and thus could be used for rapid prediction of TBC by testing volatiles. In addition, E-nose technology requires no chemical or biological reagent and are thus cost-effective and environment-friendly.

However, considering the diversity of bacteria potentially present in milk, as well as the complexity of volatile compounds in milk, a reliable pattern-recognition approach is needed to correlate tested E-nose signals with microbial load in milk samples. This study aimed at developing a rapid and convenient method to predict TBC, combining E-nose with artificial neural network (**ANN**) modeling, for assessing microbial quality of milk. The TBC values determined with plate counting were used as references for model training, testing, and validation.

MATERIALS AND METHODS

Preparation of Milk Samples

Considering the variations in their components, we collected as many brands of milk products as possible from local markets. Raw, HTST, and ultrapasteurized (**UP**) milk were kept in an iced box before determination of baseline TBC, which was performed within 2 h after sample collection. For UHT, HTST, and UP milk, collected products were poured into flasks (washed with distilled water but unsterilized) and exposed to the surrounding environment in the laboratory, office, or passageway in the building for a period of 10 min to 5 h, in consideration of the diversity of microorganisms potentially present in milk and their growth characteristics. For raw milk, sterile flasks were used for sample collection without following exposure to the surrounding environment. The prepared milk samples were stored at 4°C, room temperature (25 ± 2°C), or a mixture of the 2 conditions with different temperature-time combinations, considering the diversity in the conditions of temperature abuse that might occur in the realistic conditions. Profiles of collected milk products and conditions used to prepare test samples are summarized in Table 1. For each test sample, the reference value of TBC was determined with a standard plate counting method, and its odor profile was tested with E-nose at the same time.

Determination of TBC with Plate Counting

The reference value of TBC in milk was determined with the plate counting method, according to procedures described in China food safety standard GB 4789.2 (CFDA and NHFPC, 2016). Briefly, 1-mL aliquots of milk, or appropriate dilutions (diluted with PBS buffer), were plated onto Plate Counting Agar (Hope Bio-Technology Co.), followed with incubation at 37°C for 48 ± 2 h. For a specific milk sample, each dilution was enumerated in triplicate, and the average value for an appropriate dilution was used to calculate the reference value of TBC in that sample.

Given that regulatory authorities in some countries or regions require a strict criterion of TBC ($<10^4$ cfu/mL) in raw or pasteurized milk and other countries or regions permit higher values of TBC (10^5–10^6 cfu/mL) in raw or pasteurized milk (Boor et al., 2017; Wang, 2018), test samples were classified into 3 grades (with TBC values $<10^4$, 10^4–10^6, or $>10^6$ cfu/mL), representing high, medium, and low microbial quality, respectively. High microbial quality implies limited risk of spoilage and safe for consumption, medium microbial

quality implies the necessity of processing measures before consumption, and low microbial quality implies unsuitability for consumption purpose.

E-nose Test

The odor profiles of milk samples were determined with a PEN3 E-nose (AIRSENSE Analytics GmbH), composed of a sensory array unit (consisting of 10 metal oxide semiconductor sensors), a sampling apparatus, and a pattern-recognition system. For E-nose testing, 10 mL of milk were transferred into a 30-mL glass bottle, which was then sealed with a plastic cap and incubated at room temperature (25 ± 2°C) for 30 min to generate headspace volatiles (which were pumped into the sensor chamber of E-nose). A 30-min incubation also allowed temperature of the testing sample to reach to the ambient level. After headspace generation, samples were immediately tested with E-nose. A dry run (only air, instead of milk contained in the bottle) was performed before testing the first milk sample. Sample gas was pumped into the sensor chamber at a flow rate of 400 mL/min, and signal data were collected at a 1-s interval during the sampling process. The duration of measurement was set at 60 s, associated with 5 s for zero-point trim time, 5 s for presampling time, and 60 s for flush time. During the flushing stage, filtered air (acted as cleaning gas) was injected into the sample gas path to normalize sensor signals. Responses of sensors to testing samples were expressed as G/G_0 (for sensors 2, 4, and 6–10) or G_0/G (for sensors 1, 3, and 5), where G_0 and G represented the resistance of a specific sensor in clean air and that in the testing gas, respectively.

Principal Component Analysis and ANN Modeling

In contrast to previous studies in which signals of sensors at a single point in the stable phase were used for analysis on E-nose data (Qiu et al., 2015), in this study, E-nose signals at different points of time (starting from the sixth second at an interval of 6 s, 90 variables in total) during measurement were selected as original input characteristics for developing ANN models. To avoid the phenomenon of saturation in modeling caused by a specific input characteristic with relatively large values compared with others, E-nose signals of test samples were scaled down to 0 to 1. To avoid overfitting, principal component analysis (**PCA**) was performed for dimensionality reduction (Shlens, 2014), and generated principal components (**PC**) constituting 95% variance of the original input characteristics were used for model construction.

The ANN, which came from AI and was initially proposed for machine learning and function approximation, was adopted for qualitative microbial analysis of and quantitative prediction of TBC in milk based on its odor characteristics tested with E-nose. A multilayer perceptron feedforward algorithm was used for ANN model construction because of its universal approximation and compactness of representation (Gosukonda et al., 2015). The multilayer perceptron ANN model was trained with backpropagation algorithm, which aimed

Table 1. Profiles of collected milk products used to prepare test samples[1]

Product	Brand	Milk origin	Fat (g/100 mL)	Protein (g/100 mL)	Treatment	Days to expiration	Shelf life (d)	Total bacteria count baseline (cfu/mL)
Milk_1	A	Cow	3.7	3	UHT	165	180	<1
Milk_2	A	Cow	4.4	3.6	UHT	133	180	<1
Milk_3	B	Cow	3.8	3.2	UHT	149	180	<1
Milk_4	C	Cow	3.8	3	UHT	168	180	<1
Milk_5	D	Cow	3.6	3.1	UHT	136	180	<1
Milk_6	B	Cow	3.6	2.9	UHT	127	180	<1
Milk_7	E	Cow	3.5	3	UHT	154	180	<1
Milk_8	A	Cow	3.7	3	Ultrapasteurized	19	21	<1
Milk_9	B	Cow	3.5	3	HTST	6	7	<1
Milk_10	C	Cow	3.6	3.1	HTST	6	7	<1
Milk_11	NA[2]	Cow	NA	NA	Raw	NA	NA	13,800
Milk_12	NA	Cow	NA	NA	Raw	NA	NA	18,400
Milk_13	NA	Cow	NA	NA	Raw	NA	NA	4,700
Milk_14	NA	Cow	NA	NA	Raw	NA	NA	2,600

[1]Milk_11 and Milk_12 were collected from a vendor on 2 separate days; Milk_13 and Milk_14 were collected from a small local yogurt producer within 1 h after its supplier arrived for raw milk delivery, on 2 separate days. After artificial recontamination, milk was stored at 4°C for 0.5 to 7 d, room temperature for up to 1 to 48 h, or alternatively at the 2 temperatures for different combinations of time lengths. From the 14 collected milk samples, 84 samples with varying combinations of time-temperature abuse were prepared. Some of the 84 prepared samples were tested in duplicate (51) or triplicate (33 samples) to simulate the realistic condition that 2 or more samples may come from the same lot, resulting in a total of 201 test samples.

[2]NA = not tested or not applicable.

at optimizing the weights of each neuron so that a predicted output vector as close as possible to the known target vector was generated based on the input vector from the train data set (Silva et al., 2015). A data set containing 177 test samples randomly selected from the 195 test samples prepared from collected milks (except Milk_10) was used for model training and testing. According to TBC values, the data set was randomly divided into 85% and 15%, which were used for model training and testing, respectively. Architecture of the ANN model was designed by increasing the number of neurons and changing the learning rate until model performance reached to an acceptable level for both the training and testing subsets, followed by validation using a validating data set.

For qualitative microbial analysis, a single hidden layer ANN model was designed, with the 6 generated PC as input characteristics and the grade (with a value of high, medium, or low for a specific test sample) of microbial quality as output. An increasing number of neurons contained in the hidden layer starting from 4 neurons was tested; model performance was regarded as acceptable when the accuracy rate was no less than 95%.

For quantitative prediction, generated PC were used as input variables and logarithm values of TBC were regarded as target output. An increasing number of neurons contained in a single hidden layer starting from 4 neurons was tested as in the qualitative model. Performance of the quantitative model was tested with paired *t*-test, coefficient of multiple determination (R^2), as well as relative difference, between predicted and reference TBC values. Model performance was regarded as acceptable when the difference between predicted and reference values was insignificant ($P > 0.05$) and the mean absolute value of relative differences between them was significantly less than 5%. An increasing number of hidden layers was tested if the model with a single hidden layer didn't generate acceptable performance.

Statistical software R 3.5.3 (https://www.r-project.org/) was used for data scaling down, PCA, training, testing and validation of ANN model, as well as model performance test. Codes for performing the established ANN models are available upon request.

Model Validation

To confirm the robustness and reliability of the ANN model, model validation was performed on another 24 test samples, including all the 6 test samples prepared from a pasteurized milk (Milk_10), as well as 18 test samples randomly selected from the 195 test samples prepared from the other 13 collected milks. Among the 18 randomly selected test samples, 5 were prepared from raw milk (2 from Milk_11 and 3 from Milk_14). All the 24 test samples in the validating data set were exclusively involved for model validation.

RESULTS AND DISCUSSION

TBC Tested with Plate Counting

The TBC is an important index for the microbial quality of milk, and is determined with methods based on cultivation. Results of plate counting indicted that values of TBC for the prepared milk samples involved in this study were in the range of 0.91 to 8.92 log cfu/mL, representing a range reasonably large enough to cover the level of bacterial loads in milk that might occur in the real world (Kamana et al., 2014; Kalmus et al., 2015). Considering that TBC is an important and widely accepted criterion for grading milk (Boor et al., 2017), the prepared milk samples involved in this study were classified into high, mediate, and low microbial quality according to the determined values of aerobic plate count, which were <4, 4 to 6, and ≥ 6 log cfu/mL, respectively. Unsurprisingly, diverse bacterial species were involved in the artificially contaminated milk samples, as demonstrated by the different morphologies of colonies that appeared on the agar plates, even though morphologies of bacterial colonies were incapable of providing detailed information on their biological taxonomy.

Results of TBC determined with cultivation-based methods provide reliable information on the microbial quality of milk (Kamana et al., 2014; Kalmus et al., 2015; Boor et al., 2017). However, these methods are usually laborious (requiring a complex process for preparing sample and experimental materials), costly, time-consuming, and incapable of providing immediate information on the microbial quality of milk (Kalmus et al., 2015; Porcellato et al., 2018); therefore, alternative methods capable of reliably assessing microbial quality of milk in a rapid manner are expected.

Response Curve of E-nose

Typical response curves of E-nose sensors were shown in Figure 1A and 1B, which, respectively, illustrate signal changes of the 10 E-nose sensors during the measurement stage when testing milk samples with relatively low and high values of TBC. Each curve represented the transient changes of a specific sensor during the measurement process. E-nose response curves implied that the 10 sensors differed in their sensitivity and that the signal intensity of specific sensors changed along with time and finally reached a relatively stable

phase (Figure 1). Thus, in addition to E-nose signals of the 10 sensors at a signal point of time, change patterns of E-nose signals during measurement might provide useful information on volatile compounds in test samples. Growth and propagation of microorganisms in milk lead to the production of various volatile organic compounds (e.g., acetic acid, propionic acid, valeric acid and acetone; Nalepa et al., 2018), which are responsible for signal changes of E-nose sensors. The capability of E-nose for rapid detection and discrimination of a wide diversity of chemical species or their mixtures (including those produced by living microbes during their growth) promises potential application for rapid microbial analysis, as encouraged by successful detection and identification in various circumstances in the field of food quality and safety (Qiu et al., 2015; Jia et al., 2016; Wang et al., 2019).

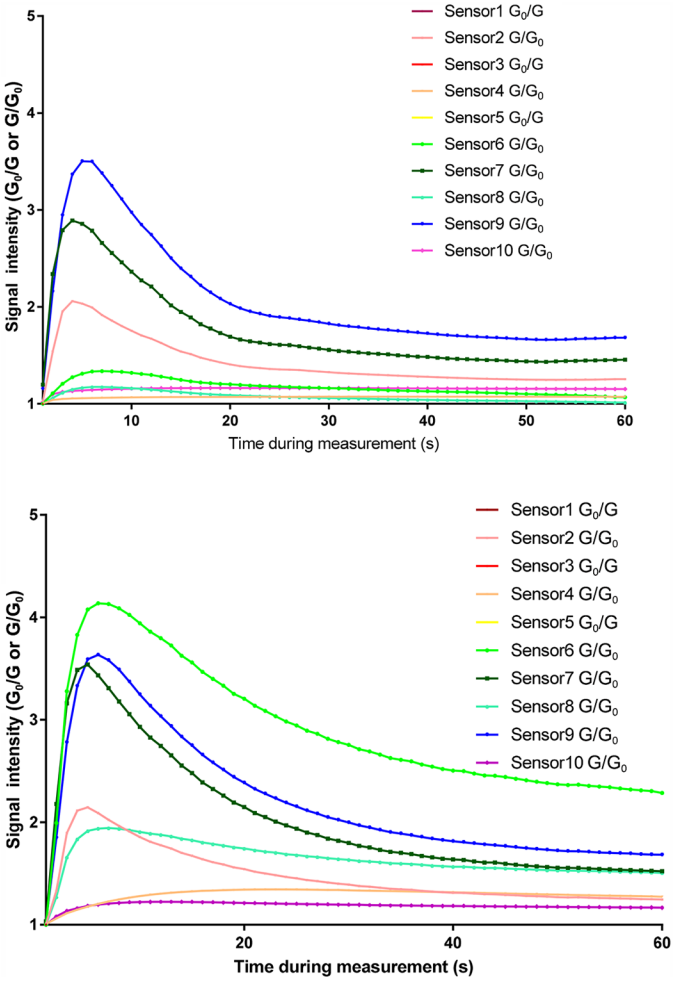

Figure 1. E-nose response curves for testing milk samples with 3.3 log cfu/mL (A) and 8.1 log cfu/mL (B) of aerobic plate count determined with plate counting. G_0 and G represent the resistance of a specific sensor in clean air and in the testing gas, respectively.

Given the diversity of microorganisms potentially present in naturally contaminated milk, as well as the relative consistence in nutrient composition, bacterial compositions and their proportions in milk were regarded as relatively stable (Giannino et al., 2009; Mallet et al., 2012; Liang et al., 2016). Thus, changes in bacterial loads might be a major factor affecting smell changes of milk, despite that the species of microorganisms present in milk, as well as their proportions, might differ between samples. Therefore, the odor features of a specific milk sample could be used to assess its microbial quality once the odor features of milk samples with various known values of TBC are "memorized." We adopted ANN modeling, which had been commonly accepted and widely used as a useful tool in food safety and quality research, in this study to assess microbial quality of milk based on its odor features tested with E-nose because of its predictive power and ability to analyze nonlinear relationships (Gevrey et al., 2003; Gosukonda et al., 2015; Tanajura da Silva et al., 2015; Behkami et al., 2019; Shi et al., 2019).

PCA

The PCA of the 90 original input characteristics generated 6 PC, which covered 95% variance of the original input characteristics. Weights of the original input characteristics to generate PC are listed in Supplemental Table S1 (https://data.mendeley.com/datasets/57969sbmzb/1; Yang, 2021). Inclusion of sensor signals at multiple time points during measurement for analysis was based on the perception that in addition to signal intensity at a specific point attributed to compounds present in sample gas and their concentrations, the change pattern of signal intensities was also influenced by the composition of compounds and their concentrations, due to differences in their physicochemical properties (e.g., polarity, affinity to the sensors).

ANN Model for Qualitative Microbial Analysis

For qualitative microbial analysis, improved model performance was generally observed with an increasing number of neurons in the hidden layer. Accuracy rate reached to the acceptable level ($\geq$95%) on the training subset when the number of neurons increased to 6, whereas on the testing subset accuracy rate reach to the acceptable level until the number of neurons increased to 10.

Thus, a neural network with 10 neurons contained in the hidden layer was finally adopted for qualitative microbial analysis. This model converged after 80 iterations in the training stage. The trained qualitative model generated an accuracy rate of 100% on

the testing subset for qualitative microbial analysis. Incorrect prediction occurred in a preliminary study with a smaller data set (91 test samples involved) used for model training, on a single sample with 4.31 log cfu/mL of TBC in the testing subset (20 test sampled involved), where medium microbial quality was incorrectly predicted as low microbial quality. This might be attributed to the closeness of microbial load in specific samples to the boundary between 2 grades because there isn't a clear boundary to grade microbial quality of milk even though the involved samples were artificially divided into different grades according to their TBC.

ANN Model for Quantitative Prediction of TBC

For the qualitative ANN model, improved model performance was observed with an increasing number of neurons in a single hidden layer, as indicated by R^2 and relative difference between predicted and reference values of TBC, as well as results of paired *t*-test (Table 2). Finally, a model containing 14 neurons in a single hidden layer was selected for quantitative prediction of TBC in milk samples.

The predicted TBC values generated by the quantitative model exhibited strong linear correlation with reference values for samples in the testing subset (Figure 2), as supported by a value of >0.99 for R^2. Paired *t*-test implied that the difference between predicted and reference values were insignificant ($P > 0.05$) for samples in the testing subset. Mean relative difference of predicted values compared with reference values (mean ± SD) was 1.1 ± 1.7% for samples in the testing subset. As low as 0.91 log cfu/mL of TBC in test samples was precisely predicted, with a predicted value of 0.90 log cfu/mL generated in the testing subset. Because of the high performance of the selected model in predicting TBC on the testing subset, as well as the nonexistence of a general rule for determining the number of neurons in hidden layers (Guiné et al., 2015), models with more hidden layers or neurons in a single hidden layer were not further tested.

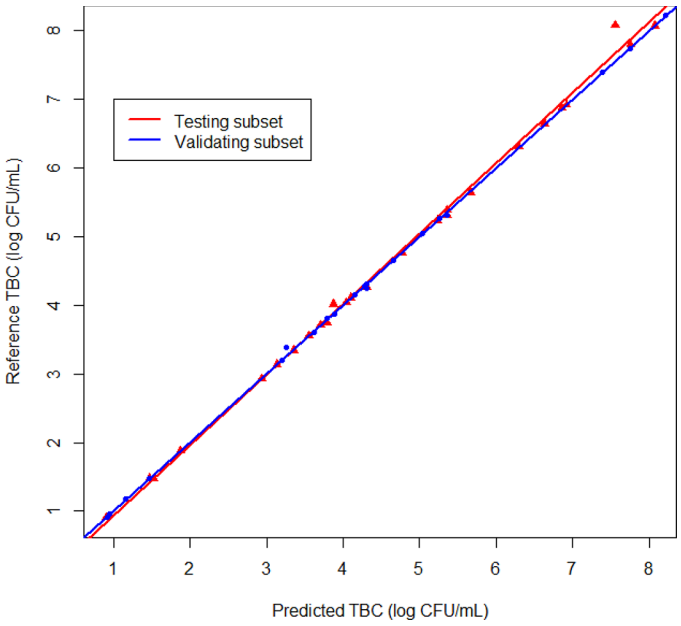

Figure 2. Plot of predicted values against reference values of total bacteria count (TBC) in milk for 28 test samples involved in testing subset (colored in red) and 24 test samples involved in validating subset (colored in blue).

Model Validation

When validated on another 24 test samples that were not involved in model training or model testing, the qualitative ANN model generated an accuracy rate of 100%, and the quantitative ANN model generated comparable TBC values compared with reference values. Strong linear correlation ($R^2 > 0.99$) between predicted and reference TBC values for test samples in the validating data set was also observed, as shown in Figure 2. Paired *t*-test implied that the difference between predicted and reference TBC values were insignificant

Table 2. Coefficient of multiple determination (R^2), paired *t*-test, and relative difference between predicted and reference values of total bacteria count (TBC), generated by models with different numbers of neurons

	Training subset			Testing subset			Validation subset		
Number of neurons	R^2	*P*-value	Mean[1] ± SD (%)	R^2	*P*-value	Mean ± SD (%)	R^2	*P*-value	Mean[1] ± SD (%)
4	0.93	1.00	18.0 ± 31.0	0.93	0.90	17.0 ± 22.0	Not tested	Not tested	Not tested
6	0.99	1.00	7.9 ± 17.0	0.96	0.70	9.8 ± 11.0	Not tested	Not tested	Not tested
8	0.97	1.00	10.0 ± 22.0	0.99	0.80	10.0 ± 20.0	Not tested	Not tested	Not tested
10	0.99	1.00	5.2 ± 12.0	0.99	0.10	4.4 ± 4.8	Not tested	Not tested	Not tested
12	>0.99	1.00	0.6 ± 1.1	0.98	0.30	2.7 ± 8.9	Not tested	Not tested	Not tested
14	>0.99	1.00	0.5 ± 0.9	>0.99	0.20	1.1 ± 1.7	>0.99	0.60	0.4 ± 0.8

[1]Mean relative difference between predicted and reference values of TBC.

($P > 0.05$) for samples in the validating data set. Mean relative difference of predicted values compared with reference values (mean ± SD) was 0.4 ± 0.8% for samples in the validating data set. A comparable predicted TBC value (0.92 log cfu/mL) was generated for a test sample with 0.91 log cfu/mL of TBC in the validating data set. Given the successful prediction of TBC in test samples with low levels (~1 log cfu/mL) of bacterial load, and only about 2 min required to complete E-nose test for a single sample, combination of E-nose and ANN modeling demonstrated a promising potential for rapid and sensitive prediction of TBC in milk.

Results of this study implied that E-nose combined with ANN modeling was reliable and reasonably accurate to qualitatively and quantitatively assess microbial quality of milk based on E-nose data, consistent with findings in previous studies that used ANN modeling for food classification and discrimination (Anjos et al., 2015; Tanajura da Silva et al., 2015; Behkami et al., 2019). Compared with the results of quality classification based on E-nose where data analysis was based on sensor signals at a specific point (Qiu et al., 2015), it was suggested that the change pattern of sensor signals provided important information for discriminating compounds in the sample gas, and thus considering the dynamic of E-nose signals during measurement led to increased performance in qualitative microbial analysis.

CONCLUSIONS

Results of this study indicated that change patterns of E-nose signals during measurement, in addition to signals at a specific point, provided important information for detecting and discriminating specific characteristics of test samples. The E-nose ANN method is reliable for prediction of TBC in milk, similar to humans differentiating spoilage through the olfactory system despite having no exact information on the volatiles or microbial species responsible for spoilage. Successful prediction of TBC on both the testing and validating subsets implied that E-nose technology could potentially be used for rapid and even online prediction of microbial quality in the dairy section, such as assessing the microbial quality of raw milk in transportation or storage, assessing microbial quality of finished milk products where improper handling may potentially occur (e.g., temperature abuse during storage), or assessing microbial quality when recycling expired milk products for other purposes (e.g., producing feed or animal food). Considering the complex changes of volatile compounds and variances in bacterial microbiota leading to spoilage, further studies are needed for E-nose technology to be used to predict the nature of milk spoilage characterized by the presence of specific volatiles or caused by specific microbial species.

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