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Establishment and Validation of RNA-Based Predictive Models for Understanding Survival of *Vibrio parahaemolyticus* in Oysters Stored at Low Temperatures

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ABSTRACT This study developed RNA-based predictive models describing the survival of *Vibrio parahaemolyticus* in Eastern oysters (*Crassostrea virginica*) during storage at 0, 4, and 10°C. Postharvested oysters were inoculated with a cocktail of five *V. parahaemolyticus* strains and were then stored at 0, 4, and 10°C for 21 or 11 days. A real-time reverse transcription-PCR (RT-PCR) assay targeting expression of the *tlh* gene was used to evaluate the number of surviving *V. parahaemolyticus* cells, which was then used to establish primary molecular models (MMs). Before construction of the MMs, consistent expression levels of the *tlh* gene at 0, 4, and 10°C were confirmed, and this gene was used to monitor the survival of the total *V. parahaemolyticus* cells. In addition, the *tdh* and *trh* genes were used for monitoring the survival of virulent *V. parahaemolyticus*. Traditional models (TMs) were built based on data collected using a plate counting method. From the MMs, *V. parahaemolyticus* populations had decreased 0.493, 0.362, and 0.238 $\log_{10}$ CFU/g by the end of storage at 0, 4, and 10°C, respectively. Rates of reduction of *V. parahaemolyticus* shown in the TMs were 2.109, 1.579, and 0.894 $\log_{10}$ CFU/g for storage at 0, 4, and 10°C, respectively. Bacterial inactivation rates (IRs) estimated with the TMs (−0.245, −0.152, and −0.121 $\log_{10}$ CFU/day, respectively) were higher than those estimated with the MMs (−0.134, −0.0887, and −0.0732 $\log_{10}$ CFU/day, respectively) for storage at 0, 4, and 10°C. Higher viable *V. parahaemolyticus* numbers were predicted using the MMs than using the TMs. On the basis of this study, RNA-based predictive MMs are the more accurate and reliable models and can prevent false-negative results compared to TMs.

IMPORTANCE One important method for validating postharvest techniques and for monitoring the behavior of *V. parahaemolyticus* is to establish predictive models. Unfortunately, previous predictive models established based on plate counting methods or on DNA-based PCR can underestimate or overestimate the number of surviving cells. This study developed and validated RNA-based molecular predictive models to describe the survival of *V. parahaemolyticus* in oysters during low-temperature storage (0, 4, and 10°C). The RNA-based predictive models show the advantage of being able to count all of the culturable, nonculturable, and stressed cells. By using primers targeting the *tlh* gene and pathogenesis-associated genes (*tdh* and *trh*), real-time RT-PCR can evaluate the total surviving *V. parahaemolyticus* population as well as differentiate the pathogenic ones from the total population. Reliable and accurate predictive models are very important for conducting risk assessment and management of pathogens in food.

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Vibrio parahaemolyticus is a Gram-negative, halophilic asporogenous and curved bacterium. It is a human pathogen that grows naturally in marine environments and can be isolated from various seafoods, including oysters, fish, and shrimp (1). Oysters are a nutrient-rich seafood popular worldwide. In the United States, oyster consumption increased in recent decades. Compared with consuming 10 to 12 pounds of seafood during the 1980s, the average American now eats approximately 16.5 pounds every year (2). Unlike most other foods, oysters are often consumed raw or uncooked (3).

Oysters feed by filtering large volumes of seawater. During this process, pathogenic microorganisms can accumulate and concentrate to levels up to 100 times greater than those present naturally in seawater (2, 4). The consumption of raw oysters has the potential to cause *V. parahaemolyticus* infection, with symptoms such as watery diarrhea, abdominal cramps, nausea, vomiting, and even septicemia (5, 6). The largest raw oyster-associated outbreak happened in the United States in 1998 and caused 416 illnesses in 13 states (2). In 2006, another outbreak of *V. parahaemolyticus* caused 177 illnesses in three states in the U.S. due to the consumption of raw oysters (7). In the U.S., most (about 72.8%) of the *Vibrio* infection cases occurred during summer months, when water temperatures were warmer (2). Other research also showed that higher densities of *V. parahaemolyticus* in harvested oysters were found in spring and summer months (7–9).

All *V. parahaemolyticus* isolates contain the thermolabile hemolysin (*tlh*) gene, a marker gene that has been used to qualitatively or quantitatively detect *V. parahaemolyticus* in different food systems (1). However, clinical illnesses caused by *V. parahaemolyticus* strains are more closely associated with the expression of the thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes. The prevalence of *tdh* gene-positive isolates ranges from 3% to 70% and the prevalence of the *trh* gene ranges from 17% to 60% in all *V. parahaemolyticus* isolates (4, 10).

One reason that *V. parahaemolyticus* has been a common problem in seafood is its ability to survive and persist over a range of temperatures. It was reported that the population of *V. parahaemolyticus* in oysters increased significantly when the temperature increased from 15°C to 30°C and decreased when temperatures were lower than 10°C (4, 11). To better ensure oyster safety and to control the levels of *V. parahaemolyticus* in harvested oysters, the National Shellfish Sanitation Program (NSSP) Guide requires harvested shellfish to be cooled to below 10°C (50°F) within 12, 18, 24, and 36 h after harvesting when the average monthly maximum air temperature is ≥27°C (80°F), between 15 and 27°C (60 to 80°F), between 10 and 15°C (50 to 60°F), and below 10°C (50°F), respectively (12).

An important method for validation of postharvest techniques and monitoring the behavior of *V. parahaemolyticus* is that of establishing predictive models. These models not only help with validation and monitoring but can also contribute information in a risk assessment. Through mathematical functions, primary predictive models are developed to describe population dynamics of pathogenic and spoilage bacteria under different environmental conditions (4). Based on the primary models, secondary predictive models are constructed to evaluate the effect of temperature on growth rates (GRs) or inactivation rates (IRs) of bacteria (11). Predictive models have been developed to describe the behavior of *V. parahaemolyticus* in slurries of inoculated oysters (*Crassostrea gigas*) over a range of temperatures from 10 to 30°C and in inoculated Pacific oysters (*C. gigas*) at temperatures from 4 to 30°C, as well as the behavior of naturally occurring *V. parahaemolyticus* in Eastern oysters (*C. virginica*) at temperatures ranging from 5 to 30°C (4, 7, 11).

Unfortunately, most of the current predictive models were constructed using plate counting methods. These methods are time-consuming and labor-intensive and do not

culture the bacteria in a viable but a nonculturable (VBNC) or stressed state (13, 14). VBNC pathogenic bacteria are considered a threat to public health and food safety because they retain their viability and ability to express their virulence (15). Therefore, predictive traditional models (TMs) based on plate counting methods may underestimate the populations of bacteria (15, 16). With the development of molecular quantitative methods, predictive models based on real-time PCR and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) methods have been reported to describe the survival and growth of *Listeria monocytogenes* and *V. parahaemolyticus* in food matrixes (13, 17). However, all of these molecular methods are based on DNA, which can be extracted from both live and dead bacteria (13, 18, 19).

Propidium monoazide (PMA)–real-time PCR based on DNA has been proposed to be a good way to avoid amplification of DNA isolated from dead cells (20). Unfortunately, PMA becomes increasingly toxic at higher concentrations and is very expensive; PMA–real-time PCR methods that include higher PMA concentrations are considered to be counterproductive and cost prohibitive (21). According to Nocker and Camper, PMA inhibits amplification of the DNA of the dead cells based on membrane integrity and cell wall penetration (22). Given this mechanism, Nocker et al. reported that the PMA method may not allow monitoring of the killing efficacy of UV treatment and other inactivation mechanisms (e.g., low-temperature storage) that do not directly impact cell membrane integrity. When membrane damage occurs as a secondary effect, little is known about the time span within which membranes become susceptible to PMA treatment (23). Thus, RNA, which can be extracted only from live bacteria, and RNA-based molecular methods serve as a more accurate way to quantitatively measure populations of surviving *V. parahaemolyticus* cells (24).

Pathogenic *V. parahaemolyticus* with the *tdh* or *trh* virulence gene has been recognized as a major factor causing human illnesses. Traditional plate counting methods (using, e.g., thiosulfate-citrate-bile salts-sucrose [TCBS] or TCBS agar) can detect the total *V. parahaemolyticus* population but cannot differentiate pathogenic *V. parahaemolyticus* from nonpathogenic *V. parahaemolyticus*. Real-time PCR has the advantage of being able to quantify the total *V. parahaemolyticus* population targeting the *tlh* gene and to differentiate and quantify the pathogenic *V. parahaemolyticus* population with the *tdh* or *trh* gene (25, 26).

To summarize, the objectives of this study were to establish and validate RNA-based predictive molecular models (MMs) using real-time reverse transcription-PCR (RT-PCR) to describe the survival of a *V. parahaemolyticus* cocktail in oysters during low-temperature storage.

RESULTS

***tlh* gene expression at 0, 4, and 10°C.** According to Meng et al., changes in the gene expression level that were less than 2 $\log_2$-fold were not considered to be significant (27). As shown in Fig. 1, the fold changes of *tlh* expression determined using *pvsA* and *recA* as reference genes showed no significant differences ($P > 0.05$) between different days (days 3, 6, 9, and 11) or different temperatures (0, 4, and 10°C). The transcription of the *tlh* gene was maintained at constant levels at different temperatures during storage.

The primary models of *V. parahaemolyticus* in oysters. The final *V. parahaemolyticus* inoculation level in oysters was 5.449 $\pm$ 0.218 $\log_{10}$ CFU/g. By using the plate counting method and real-time RT-PCR, the populations of surviving *V. parahaemolyticus* in oysters were enumerated and calculated at different time intervals. For real-time RT-PCR, to convert the threshold cycle (C_T) values to cell counts, standard curves based on the *tlh*, *tdh*, and *trh* genes were constructed (Fig. 2). The coefficient of determination (R^2) values were 0.999, 0.998, and 0.969 and the equations were $y = -3.849x + 43.701$, $y = -3.521x + 42.139$, and $y = -3.487x + 42.751$ for the *tlh*, *tdh*, and *trh* genes, respectively.

The survival data, obtained from the plate counting method and the real-time RT-PCR method at three storage temperatures, were then fitted by the Baranyi function

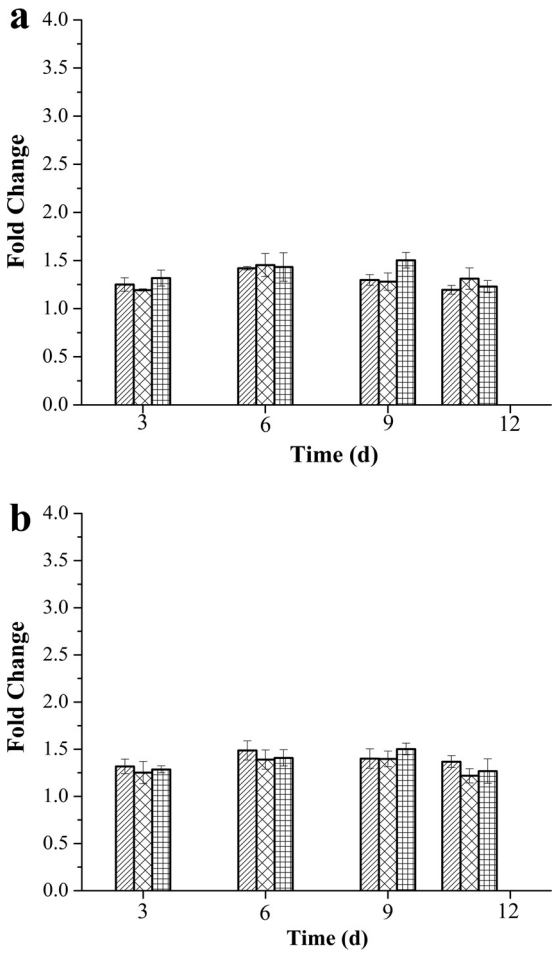

FIG 1 Fold change of *tlh* gene expression based on endogenous reference genes on days (d) 3, 6, 9, and 11 at 0, 4, and 10°C. (a) *pvsA* used as endogenous reference gene. (b) *recA* used as endogenous reference gene. Bars with diagonal lines represent fold change of *tlh* gene expression at 0°C; bars with diagonal crosshatching represent gene expression at 4°C; bars with squares represent gene expression at 10°C.

(equation 3) (57) to obtain primary TMs and MMs (Fig. 3). The parameters of primary TMs and MMs are listed in Table 1. For primary TMs, the average R^2 value for plate counting data fitted by the Baranyi function (equation 3) of three storage temperatures was 0.925. For storage at 0, 4, and 10°C, the population of *V. parahaemolyticus* decreased from the initial values (IVs) to the final values (FVs) with reductions of 2.109, 1.579, and 0.894 $\log_{10}$ CFU/g, respectively. The inactivation rates (IRs) showed an

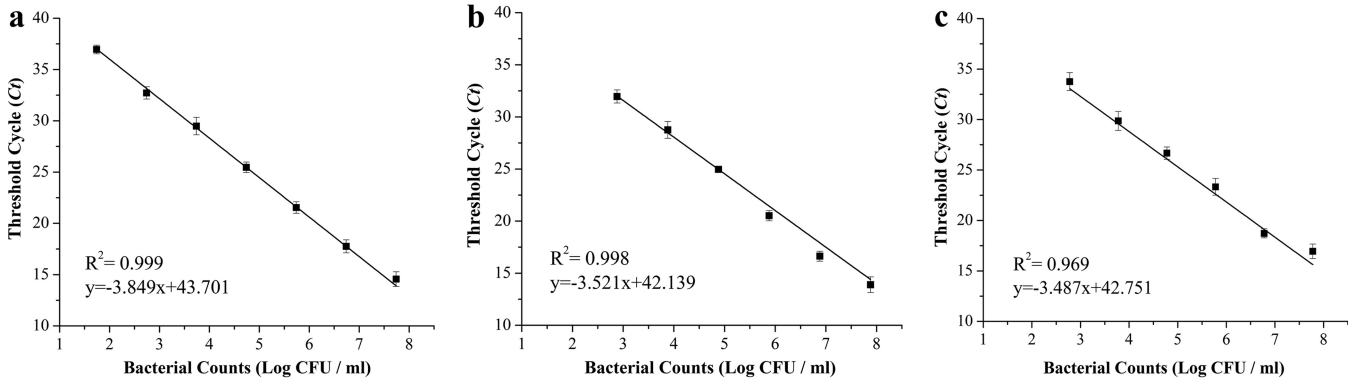

FIG 2 Quantification standard curves targeting *tlh* (a), *tdh* (b), and *trh* (c) genes in *V. parahaemolyticus*.

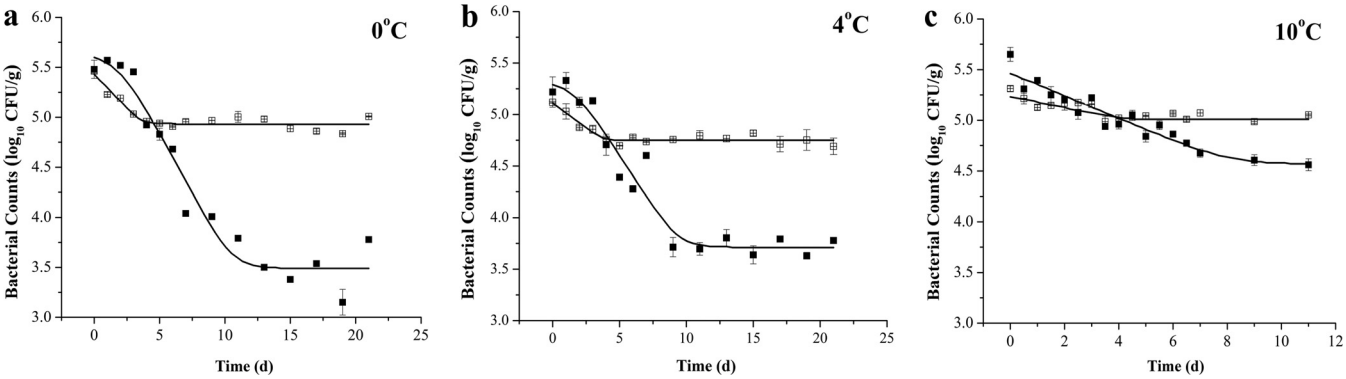

FIG 3 The primary MMs established based on the *tlh* gene and the primary TMs based on the plate counting method for *V. parahaemolyticus* after storage at 0 (a), 4 (b), and 10°C (c) for 21, 21, and 11 days, respectively. Filled squares represent plate count data; squares with boxes represent RT-PCR data; solid lines represent plate Baranyi fit data.

increasing trend as the temperature increased from 0 to 4 and 10°C. *V. parahaemolyticus* did not have lag times (LTs) when stored at 10°C. For primary MMs (Table 1), the average R^2 value for molecular data fitted by the Baranyi function (equation 3) of the three storage temperatures was 0.859. The respective reductions from IVs to FVs were 0.493, 0.362, and 0.238 $\log_{10}$ CFU/g for 0, 4, and 10°C storage. The IRs also showed an increasing trend as temperature increased from 0 to 4 and 10°C storage. All of the LTs of the primary MMs of *V. parahaemolyticus* at 0, 4, and 10°C were zero.

The survival of *V. parahaemolyticus* with the *tdh* or *trh* virulence gene during cold storage. The survival behavior of pathogenic *V. parahaemolyticus* (*tdh* gene positive) in a *V. parahaemolyticus* cocktail in oysters was monitored during cold storage at 0, 4, and 10°C for 21, 21, and 11 days, respectively (Fig. 4). The initial populations of these *V. parahaemolyticus* were 4.876, 4.704, and 4.924 $\log_{10}$ CFU/g and the final populations were 4.663, 4.297, and 4.687 $\log_{10}$ CFU/g for storage at 0, 4, and 10°C, respectively. However, monitoring the survival of pathogenic *V. parahaemolyticus* containing the *trh* gene, no amplification was detected through real-time PCR after day 0. On day 0, the C_T values for the *trh* gene were 26.737, 25.917, and 26.560 for 0, 4, and 10°C, respectively, representing 4.592, 4.827, and 4.643 $\log_{10}$ CFU/g.

The secondary models of *V. parahaemolyticus* in oysters. The secondary models were developed to describe the parameter changes of the primary models as a function of temperature. The IRs obtained from the primary TMs and MMs were used to produce the secondary TMs and MMs. The Arrhenius model (equation 4) (56) was utilized to describe the change of *V. parahaemolyticus* ln (−IRs) from 0 to 10°C (Fig. 5). For the secondary TMs, the coefficients A (collision factor) and E_a/R (activation energy/universal gas constant) were 9.156×10^{-10} and −5,280.115, respectively (equation 1). The R^2 value of goodness of fit was 0.818 after Arrhenius function fitting. The values for B_f (bias factor [relative average deviation of predicted and observed values]) and A_f (accuracy factor [spread of the results around the predicted values]) were 1.003 and 1.048, respectively.

$$\ln r = \ln 9.156 \times 10^{-10} + 5{,}280.115/\left(273.150 + T\right) \quad (1)$$

For the secondary MMs, the A and E_a/R values were 7.503×10^{-9} and −4,543.456 (equation 2). The R^2 value corresponding to the goodness of fit was 0.812 with Arrhenius equation fitting. The B_f and A_f values were 1.001 and 1.031, respectively.

$$\ln r = \ln 7.503 \times 10^{-9} + 4{,}543.456/\left(273.150 + T\right) \quad (2)$$

The secondary TM and MM were compared using one-way analysis of variance (ANOVA). Significant differences ($P < 0.05$) between them were found.

Validation of the secondary MMs in oysters. In order to validate the secondary models of *V. parahaemolyticus* in oysters, the secondary TM was compared with a previously reported secondary predictive model (4). The secondary model published by

TABLE 1 Inactivation parameters of the primary molecular and traditional models

	Value(s)[a]														
	0°C					4°C					10°C				
Parameter	R^2	Initial value (log_{10} CFU/g)	Final value (log_{10} CFU/g)	Lag time (day)	Inactivation rate (log_{10} CFU/day)	R^2	Initial value (log_{10} CFU/g)	Final value (log_{10} CFU/g)	Lag time (day)	Inactivation rate (log_{10} CFU/day)	R^2	Initial value (log_{10} CFU/g)	Final value (log_{10} CFU/g)	Lag time (day)	Inactivation rate (log_{10} CFU/day)
Primary MMs	0.881	5.428 ± 0.0477^{A}	4.935 ± 0.0177^{A}	0^{A}	-0.134 ± 0.0263^{A}	0.891	5.114 ± 0.0335^{A}	4.752 ± 0.0125^{A}	0^{A}	-0.0886 ± 0.0184^{A}	0.806	5.261 ± 0.0345^{A}	5.023 ± 0.0205^{A}	0^{A}	-0.073 ± 0.0168^{A}
Primary TMs	0.943	5.601 ± 0.167^{A}	3.492 ± 0.0955^{B}	1.931 ± 0.528^{B}	-0.245 ± 0.05^{B}	0.931	5.293 ± 0.138^{A}	3.714 ± 0.0718^{B}	1.605 ± 0.559^{B}	-0.152 ± 0.0434^{B}	0.902	5.463 ± 0.0467^{A}	4.569 ± 0.0783^{B}	0^{A}	-0.121 ± 0.0635^{B}

[a]Different uppercase superscript roman letters indicate that a significant difference ($P < 0.05$) exists between the values determined for the parameters from the primary MMs and TMs.

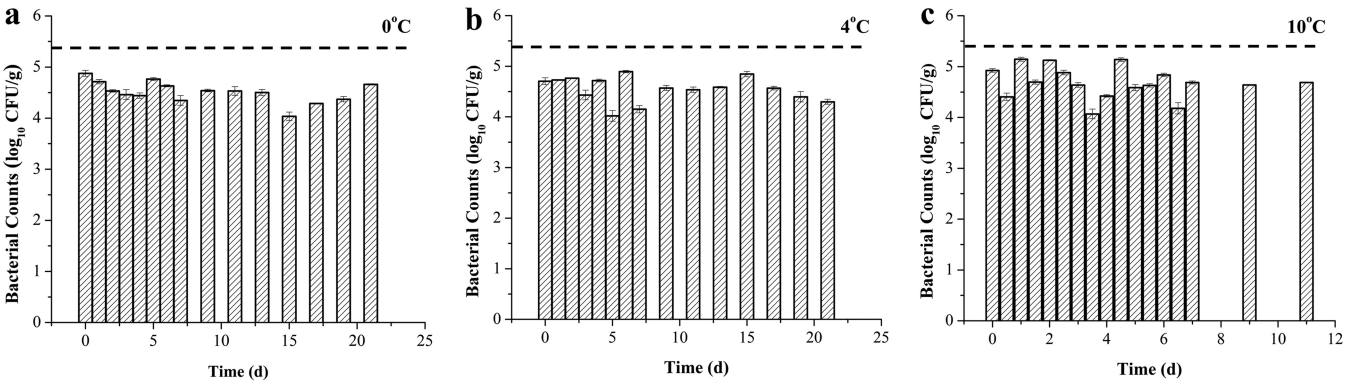

FIG 4 Survival of pathogenic *V. parahaemolyticus* (with the *tdh* virulence gene) in oysters during cold storage at 0°C (a), 4°C (b), and 10°C (c). The dotted line represents the initial total population of *V. parahaemolyticus*.

Fernandez-Piquer et al. described the effects of storage temperatures from 3.6 to 12.6°C on the survival of *V. parahaemolyticus* in inoculated Pacific oysters (*C. gigas*) (4). The secondary MM in oysters was compared with a secondary validation model built with oysters harvested in different months.

As shown in Fig. 6a, the secondary TM and the reported model had the same decreasing trend: as the temperature increased, the IRs increased. The B_f and A_f values, calculated based on the comparison of IRs in the secondary TM and the reported model, were 0.887 and 1.127, respectively. The predicted IRs in the secondary TM were significant lower at every given temperature ($P = 0.034 < 0.05$) than the IRs predicted in the model reported by Fernandez-Piquer et al. (4). In Fig. 6b, the secondary MM and the validation model nearly overlapped. The B_f and A_f values, calculated on the basis of the comparison of IRs in the secondary MM and validation model, were 1.004 and 1.015, respectively. No significant difference was seen between the IRs of the two models ($P = 0.519 > 0.05$). Differences between predictions of models were analyzed by one-way ANOVA, and the results indicated that the secondary MM in this study is a reliable predictor for IR changes as a result of temperature change.

DISCUSSION

The current study developed RNA-based predictive models to describe the survival of a *V. parahaemolyticus* cocktail in Eastern oysters (*C. virginica*) at low storage tem-

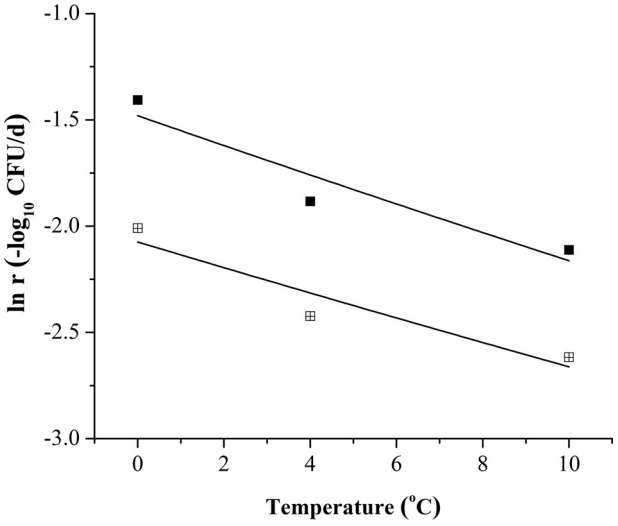

FIG 5 Data from the secondary TM established based on IR data from the primary TMs are represented by filled squares. Data from the secondary MM established based on IR data from the primary MMs are represented by squares with boxes.

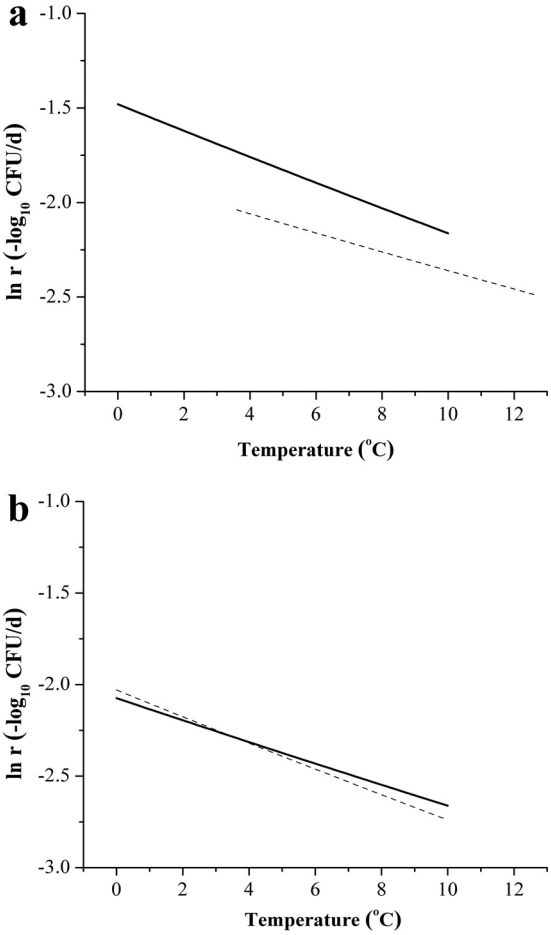

FIG 6 Validation of the secondary models. (a) Comparison of the secondary TM (solid line) in this study and the inactivation model reported by Fernandez-Piquer et al. (the secondary validation model [VM]) (4) (dashed line). (b) Comparison of the secondary MM (solid line) in this study and the validation model (the secondary VM) conducted in this study (dashed line).

peratures (0, 4, and 10°C). The predictive MMs showed great potential to predict *V. parahaemolyticus* activities in oysters during postharvest cold storage. During the past decade, predictive models have been established to describe the growth and inactivation of *V. parahaemolyticus* in different broth and seafood matrices (6, 11, 28, 29). In a recent report, the population of *V. parahaemolyticus* in a Korean oyster slurry decreased under conditions of storage at 10 and 15°C for 80 h and 70 h, respectively, with approximate IRs of −0.900 and −1.371 $\log_{10}$ CFU/day. Those authors also found that the *V. parahaemolyticus* numbers increased in nutrition broth in storage at 10 and 15°C (7). Both findings indicated that both the storage temperature and storage conditions such as nutrient concentrations impacted the survival of *V. parahaemolyticus*. According to Burnham et al., the minimum temperature for *V. parahaemolyticus* to grow in tryptic soy broth (TSB) was 5°C (30).

Traditionally, conventional plate counting methods have been used to collect data to construct predictive models (26, 31). For instance, TCBS agar is widely used to enumerate *V. parahaemolyticus* colonies and to monitor their growth and survival without enrichment (7). These methods do not count cells in VBNC and stressed states (32) and may underestimate bacterial levels (4, 13). In addition, the presence of background bacteria may negatively impact the enumeration efficiency of the plating media (17). Compared with traditional plate counting methods, molecular quantitative methods have the advantage of quantifying all of the viable bacteria, including cells in VBNC and stressed states (33, 34). In this study, *V. parahaemolyticus* RNA was extracted

and the real-time RT-PCR method was used to construct predictive MMs. RNA is extracted only from live cells, which may give more accurate results when using real-time PCR (35). The *tlh* gene is used to establish the prediction models as it has been widely used as a species-specific marker for *V. parahaemolyticus* (36). In the genome of *V. parahaemolyticus*, the *tlh* gene has one copy, the *tdh* gene has two copies, and the *trh* gene has one copy (37). Before it was used to establish RNA-based models, *tlh* gene expression was evaluated first. As shown in this study, *tlh* gene expression is maintained at a constant level at different temperatures and can be used as a reliable target for monitoring survival of *V. parahaemolyticus*.

Three storage temperatures (0, 4, and 10°C) were tested in this study. According to Fernandez-Piquer et al. (4), *V. parahaemolyticus* was inactivated when the storage temperature of the oysters was below 12.6°C. *V. parahaemolyticus* stored at low temperature may enter into the viable but nonculturable (VBNC) or stressed state (38), and this may explain why *V. parahaemolyticus* was detected in winter months when water temperatures were below 10°C (8). Therefore, to better predict the survival or persistence of *V. parahaemolyticus*, the Baranyi model was utilized in this study to fit the collected data for establishing the primary TMs and MMs for *V. parahaemolyticus* in oysters during low-temperature storage (0, 4, and 10°C). The average R^2 of the primary TMs was 0.925 and that of the primary MMs was 0.859, indicating that the Baranyi model was appropriate to fit data for the primary models in this study. The Baranyi model has been used to model the survival of high hydrostatic pressure (HHP)-treated *Listeria innocua* cells (39) and *Enterobacter sakazakii* in infant formula milk (40) in previous studies. To construct the secondary models, the Arrhenius model was used, as it is a common mathematical model to fit bacterial IR data. Fernandez-Piquer et al. applied the Arrhenius function to fit a secondary model of *V. parahaemolyticus* in oysters with an R^2 value of 0.78 (4). In our study, the R^2 values obtained from TM and MM were greater than 0.8, and B_f and A_f were also within the satisfactory limit ($1.0 < B_f < A_f < 1.1$), which indicated that the two secondary models have high goodness of fit (41).

According to the primary TMs, the total numbers of viable *V. parahaemolyticus* cells no longer changed after days 13.861, 13.219, and 10.123 at storage temperatures of 0, 4, and 10°C, respectively. However, according to the primary MMs, the numbers of viable *V. parahaemolyticus* cells did not change 5.881, 4.237, and 4.425 days after storage at the three respective temperatures (Fig. 3). The significant differences seen here indicate that many cells might have gradually entered the viable but nonculturable (VBNC) state or become stressed cells such that they did not grow on selective agar. Real-time RT-PCR is able to more sensitively and accurately quantify all of the viable cells (33) and provides a more accurate and reliable prediction than TMs.

A five-strain *V. parahaemolyticus* cocktail, including three nonpathogenic strains with the *tlh* gene, one pathogenic strain with the *tdh* gene, and one pathogenic strain with the *trh* gene, was used in this study. *V. parahaemolyticus* infections are mainly caused by *tdh* and *trh* genes (42–44). In reality, more-complicated *V. parahaemolyticus* strain mixtures and microfloras exist. Thus, models established by using cocktails made with both nonpathogenic and pathogenic strains mimic real contamination situations. The kinetic changes of the pathogenic *V. parahaemolyticus* strains in the cocktail were monitored by real-time RT-PCR. The population of pathogenic *V. parahaemolyticus* with the *tdh* gene did not decrease significantly, while the population of pathogenic *V. parahaemolyticus* with the *trh* gene could not be detected after day 0. This result showed that the *V. parahaemolyticus* strain with the *trh* gene had a poor survival capability during cold storage. This could also be the reason why the detection rate of pathogenic *V. parahaemolyticus* with the *trh* gene in the environment is low. Ward and Bej used a multiplexed real-time PCR to detect *V. parahaemolyticus* in oysters, with results that were 51% positive for the *tlh* gene and 12.1% for the *tdh* gene but negative for the *trh* gene (45). In the study conducted by Rizvi and Bej, their results showed that 58% of the 24 oysters tested were positive for the *tlh* gene, 21% for the *tdh* gene, and

0.7% for the *trh* gene (46). The prevalence of the *trh* gene was always the lowest among the three genes tested in those two studies.

A report by Burnham et al. showed that the levels of pathogenic (*tdh*-positive) *V. parahaemolyticus* decreased by 2.880 and 1.490 $\log_{10}$ CFU/ml in TSB during storage at 5°C and 8°C, respectively, for 10 days using the plate count method (30). According to Yang et al., pathogenic (*tdh*-positive) *V. parahaemolyticus* in salmon had reductions of 3.50, 2.50, and 1.00 $\log_{10}$ CFU/g during storage at 0°C, 3°C, and 9°C, respectively, for 10 days as shown using the plate count method (6). Although a lower reduction was observed for the *tdh* strain in our study, there are two potential reasons for the disparity. First of all, the methods used were different. This study used real-time RT-PCR to monitor the pathogenic strains in a cocktail, while the previous studies used plate counting methods. Second, previous studies were done using a single strain and not a cocktail. The interaction between pathogenic strains and nonpathogenic strains used in this study may have also impacted the final results. Regardless of these differences, all of the results and reports showed that pathogenic *V. parahaemolyticus* can survive for a long period of time in low-temperature storage.

The secondary TM and MM were established based on IRs generated from the primary TMs and MMs (Fig. 5). Due to the different methods (real-time RT-PCR versus TCBS analysis) used for enumerating surviving *V. parahaemolyticus* cells, the secondary MM is significantly different ($P < 0.05$) from the secondary TM. In order to validate the predictive models, the secondary TM was evaluated by comparing it to a reported secondary model of *V. parahaemolyticus* in Pacific oysters (4). The calculated B_f and A_f values were not within the satisfactory limit ($1.0 \leq B_f \leq A_f \leq 1.1$), which suggests that the predicted IRs in the secondary TM are different from those of the reported model. In Fig. 6, the TM curve was significantly higher than that of the reported model. The IRs in the TM were significantly lower than those of the reported model at every given temperature ($P < 0.05$). Differences between the two studies may be due to the different oyster species, the bacterial strains used, bacterial strain variability, and/or the oyster host defenses. For the MM, oysters harvested in summer months were used to validate the model. The calculated B_f and A_f values were within the satisfactory limits ($1.0 \leq B_f \leq A_f \leq 1.1$), which indicates that the predicted IRs in the secondary MM are close to those in the validation model in this study. The results from the secondary MM and the validation model were not significantly different ($P > 0.05$).

In summary, this study developed and validated RNA-based molecular predictive models to describe the survival of *V. parahaemolyticus* in oysters during low-temperature storage (0, 4, and 10°C). The primary MMs, as they can count all viable *V. parahaemolyticus* cells, give a more accurate evaluation of the surviving cells than the primary TMs, which were built based on plate counting methods. Among the *V. parahaemolyticus* strains studied, the level of pathogenic *V. parahaemolyticus* with the *tdh* gene showed no significant reduction ($P > 0.05$). These results reveal that it is important to have a more reliable and accurate predictive model system to better assist with *V. parahaemolyticus* risk assessment and management in seafood.

MATERIALS AND METHODS

Oysters. One-year-old Eastern oysters (*C. virginica*) were harvested from Mobile Bay, AL, USA, and depurated under a flowthrough depuration system for 7 days at the Auburn University Marine Extension and Research Center (AUMERC) located at Dauphin Island, AL, USA. The oysters were then shipped overnight to the Food Microbiology laboratory at Auburn University, Auburn, AL. Upon arrival at the laboratory, oysters were washed to remove excess mud following protocols described by the American Public Health Association for the bacteriological examination of shellfish (47). Oysters were then stored at 0°C before inoculation.

Three uninoculated oysters from each shipment were randomly selected, and the presence of *V. parahaemolyticus* was checked by both a traditional plating method and real-time RT-PCR. The details of the protocols were the same as those of the procedures used for evaluating inoculated oysters, which are described below. Results from both the plate counting method and the real-time RT-PCR showed that the amounts of *V. parahaemolyticus* naturally present in the oysters (2 $\log_{10}$ CFU/g for the plate counting method and 2.74 $\log_{10}$ CFU/g for the real-time RT-PCR method) were below the limit of detection.

Bacterial strains. A total of five *V. parahaemolyticus* strains (Table 2) were used in this study. Three strains were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), and the

TABLE 2 *Vibrio parahaemolyticus* strains used in this study

V. parahaemolyticus strain	Virulence gene(s)	Isolate source
ATCC 17802	*trh* and *tlh*	Shirasu food poisoning, Japan (48)
ATCC 43996	*tdh* and *tlh*	Cockles causing fatal food poisoning, England (49)
ATCC 27969	*tlh*	Crustacean, USA (50)
12	*tlh*	Oysters, USA
21	*tlh*	Oysters, USA

other two strains were isolates from previous oyster studies. A liquid *V. parahaemolyticus* cocktail was prepared by growing individual strains in 10 ml of tryptone soy broth (TSB) (BBL/Difco Laboratories, Sparks, MD, USA) supplemented with 3% NaCl at 37°C for 18 h. The overnight fresh cultures were washed by centrifugation (Eppendorf, Hauppauge, NY, USA) at 3,000 × *g* for 3 min and resuspended with phosphate-buffered saline (PBS; pH 7.4). The optical density at 600 nm (OD_{600}) of each washed culture was adjusted to 1.7 ± 0.1 by using an Ultrospec 10-cell density meter (Amersham BioSciences, Piscataway, NJ, USA). To mix the cocktail, equal volumes of each washed culture were taken and mixed in a 15-ml Falcon sterile tube (VWR, Atlanta, GA, USA). The concentration of the *V. parahaemolyticus* cocktail was 1.12×10^8 CFU/ml.

Oyster inoculation and storage. To inoculate the oysters, a 5-mm notch was drilled in the shell of each oyster approximately 50 mm from the hinge. After that, 100 μl of inoculum cocktail was injected into the muscle of the oyster using a sterile 1-ml syringe with a 23-gauge needle (Terumo, Somerset, NJ, USA) following the protocol described in previous reports (4, 51). The inoculated oysters were kept flat on the benchtop for 5 min to allow the liquid cultures to be absorbed by the oyster muscle before being stored at different temperatures.

Three storage temperatures and formats were chosen: on ice (~0°C), in a 4°C refrigerator, and in a 10°C refrigerator. For the 0°C and 4°C storage conditions, three subsamples (three oysters/sample) of inoculated oysters were removed and analyzed once every day for the first week and then once every 2 days for two more weeks. For the 10°C storage condition, oysters were removed and analyzed every 12 h for the first week and then on days 9 and 11.

***V. parahaemolyticus* enumeration using the plate count method.** TCBS plates (BD, Sparks, MD, USA) were used to plate and enumerate the surviving *V. parahaemolyticus* bacteria. At each sampling point, three sets of three oysters were taken from each storage condition. The oysters were shucked, and the meat was removed from the shell. Approximately 25 g of oyster meat from each storage condition was then placed in a filtered Whirl-Pak bag (Nasco, Fort Atkinson, WI, USA) with 225 ml of PBS. The mixture was homogenized by the use of a Smasher laboratory blender (AES Chemunex, bioMérieux, France) at the normal speed for 2 min. The homogenized samples were diluted and plated. Colonies were enumerated after the plates were incubated at 37°C for 18 h. In addition, 1 ml of every homogenized sample was removed, transferred into a 15-ml Falcon sterile tube (VWR, Atlanta, GA, USA), and stored at −80°C with 2 ml of RNA Protect reagent (Qiagen, Hilden, Germany) for RNA extraction.

RNA extraction. In order to quantify the number of *V. parahaemolyticus* bacteria containing *tlh*, *tdh*, and *trh* genes using real-time RT-PCR, standard curves for the three target genes were established. To extract the RNA for establishment of the *tlh*, *tdh*, and *trh* gene-based standard curves, 10-fold serial dilutions of the *Vibrio parahaemolyticus* cocktail, *V. parahaemolyticus* ATCC 43996, and *V. parahaemolyticus* ATCC 17082 were used, starting at cell densities of 5.5×10^7, 7.6×10^7, and 6×10^7 CFU/ml. RNA was extracted from 1 ml of each dilution using an RNeasy minikit (Qiagen, Valencia, CA, USA). During the RNA extraction, 80 μl of DNase I stock solution (Qiagen, Valencia, CA, USA) (a total of 30 units) was added to clean up any potential DNA contamination.

To extract RNA from inoculated oyster samples, 1 ml of each stored homogenized sample solution (from step 4) was used. The same RNeasy minikit was used to extract the RNA, and all of the RNA samples were stored immediately and maintained at −80°C until they were analyzed.

The concentration and purity of all RNA samples were analyzed using a NanoVue Plus spectrophotometer (GE Healthcare, Piscataway, NJ, USA). cDNA was generated by reverse transcription of 10 μl of RNA from each sample using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and then used for real-time PCR. The reverse transcription program consisted of 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min.

Real-time PCR. The primers used to target the *tlh* gene are F-*tlh* (5′-ACTCAACACAAGAAGAGATCG ACAA-3′) and R-*tlh* (5′-GATGAGCGGTTGATGTCCAA-3′); the primers used for *tdh* are F-*tdh* (5′-TCCCTTT TCCTGCCCCC-3′) and R-*tdh* (5′-CGCTGCCATTGTATAGTCTTTATC-3′); and the primers used for *trh* are F-*trh* (5′-TTGCTTTCAGTTTGCTATTGGCT-3′) and R-*trh* (5′-TGTTTACCGTCATATAGGCGCTT-3′) (52). All of the primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

SYBR green real-time PCR was carried out on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). Each reaction system consisted of 10 μl of PerfeCTa SYBR green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), 0.5 μl each of 10 μM forward and reverse primers, 6 μl of RNase-free water, and 3 μl of cDNA templates. Real-time PCR assays were conducted following a program with an initial denaturing period at 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Standard curves were built by constructing regression lines, with the *x* axis representing C_T values and *y* axis

representing $\log_{10}$ CFU per milliliter. The standard curves were used to quantitatively calculate the levels of surviving *V. parahaemolyticus* cells in inoculated oyster samples.

To monitor the survival of the pathogenic *V. parahaemolyticus* strains in the cocktail, real-time RT-PCR targeting the *tdh* and *trh* genes was conducted. The setup of PCRs was the same as described above.

Evaluation of *tlh* gene expression at low temperatures. Real-time RT-PCR was used to monitor expression of the *tlh* gene in *V. parahaemolyticus* during storage at 0, 4, and 10°C. The relative quantification data of gene expression were calculated by a $2^{-\Delta\Delta CT}$ method. Using this method, the data were presented as the fold change in gene expression normalized to the endogenous reference gene and relative to that of the calibrator as follows: $\Delta\Delta C_T = (C_{T\text{target}} - C_{T\text{reference}})_{\text{target}} - (C_{T\text{target}} - C_{T\text{reference}})_{\text{calibrator}}$ (53). Two housekeeping genes (*pvsA* and *recA*) were used as the endogenous reference genes for *tlh* gene expression monitoring. The primers for *pvsA* are F-*pvsA* (5′-CTCCTTC ATCCAACACGAT-3′) and R-*pvsA* (5′-GGGCGAGATAATCCTTGT-3′) (54); the primers for *recA* are F-*recA* (5′-GCTAGTAGAAAAAGCGGGTG-3′) and R-*recA* (5′-GCAGGTGCTTCTGGTTGAG-3′) (58). The expression level of the *tlh* gene on day 0 was used as the calibrator.

RNA was extracted from inoculated oyster samples at 0, 4, and 10°C on days 0, 3, 6, 9, and 11 using an RNeasy minikit (Qiagen, Hilden, Germany). First, 10 μl of RNA from each sample was transcribed into cDNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and real-time PCR was then conducted. The reaction system was the same as described above. Triplicates were conducted.

Mathematical modeling. The raw data obtained from the plate counting method and real-time RT-PCR method were transformed to a natural logarithm format. For the survival model, the Baranyi equation (see equation 3) was chosen to fit the data, and the calculations were performed using the DMfit tool of the Combase website (available at http://www.ifr.ac.uk/safety/dmfit/). The output data were then converted into the $\log_{10}$ format. The means and standard deviations were calculated. The fitted model based on the plate counting method was defined as the primary TM, and the model based on the real-time RT-PCR method was defined as the primary MM. Parameters of coefficient determination (R^2), lag time (LT), minimum inactivation rate (IR), initial value (IV), and final value (FV) were obtained for the models. The Baranyi equation is as follows:

$$y(t) = y_0 + \mu_{\min} A(t) - \frac{1}{m}\ln\left(1 + \frac{e^{m\mu_{\min} A(t)} - 1}{e^{m(y_{\text{end}} - y_0)}}\right)$$
$$A(t) = t + \frac{1}{\nu}\ln\left(\frac{e^{-\nu t} + q_0}{1 + q_0}\right) \quad (3)$$

where y is the natural logarithm of the bacteria concentration at any given time (ln CFU per milliliter), y_0 and y_{end} are the initial value and the end value of y, $A(t)$ is the equation governing the duration of the period (informally called the "shoulder") preceding the log linear inactivation phase, t is time (day), m determines the smoothness of the transition from the exponential inactivation phase to the survival tail, $\mu_{\min}$ is the minimum value of the inactivation rate, ν is the rate at which the bacteria lose the ability to survive during the shoulder, and q_0 is the initial physiological state of bacterial cells.

The Arrhenius equation (equation 4) was applied for the establishment of the secondary model to describe the IR of *V. parahaemolyticus* in oysters as a function of temperature. The equation is as follows:

$$\ln r = \ln A - E_a/R(273.15 + T) \quad (4)$$

where r is the inactivation rate (IR), T is the absolute temperature, E_a is the activation energy, R is the universal gas constant, and A is the collision factor. The equation was input into OriginPro 8.0 software (OriginLab Corporation, Northampton, MA, USA), which was then used to fit the IR data to obtain a plot of ln r against T and the values of ln A and E_a/R.

Evaluation and validation of models. In order to evaluate the goodness of fit of the TMs and MMs, the coefficient of determination (R^2), bias factor (B_f), and accuracy factor (A_f) were calculated by using equations 5, 6, and 7 (17). The primary TMs were compared to the primary MMs through analysis of R^2, LT, IR, IV, and FV values using one-way analysis of variance (ANOVA). To validate the MMs, the secondary MM was compared with a secondary validation model that was built by conducting a replication experiment with a new batch of harvested Eastern oysters using the same inoculation and storage processes. To validate the TMs, the fitted secondary TM of *V. parahaemolyticus* was compared with a model previously published by Fernandez-Piquer et al. (4). Differences between predicted IR data in the secondary models and validation models were evaluated by using one-way ANOVA, B_f, and A_f as follows:

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(\text{predicted} - \text{observed})^2}{\sum_{i=1}^{n}(\text{observed} - \text{mean})^2} \quad (5)$$

$$\text{Bias factor} = 10^{\left(\frac{\Sigma\log_{10}(\text{predicted/observed})}{n}\right)} \quad (6)$$

$$\text{Accuracy factor} = 10^{\left(\frac{\Sigma|\log_{10}(\text{predicted/observed})|}{n}\right)} \quad (7)$$

where n represents the number of observations and "predicted," "observed," and "mean" represent the predicted values, observed values, and average values, respectively.

In this study, R^2 greater than 0.8 for the predictive models was considered representative of a good fitting performance (55). B_f and A_f were calculated to evaluate the goodness of fit of the primary models

and to validate the secondary models. B_f measures the relative average deviation of predicted and observed values. A_f indicates the spread of the results around the predicted values. The $1.0 \leq B_f \leq A_f \leq 1.1$ range was defined as a satisfactory limit (17).

Statistical analysis. One-way ANOVA was applied using OriginPro 8.0 software (OriginLab Corporation, Northampton, MA, USA) to compare the different predictive models. $P < 0.05$ was regarded as representative of a statistically significant difference between models (4, 13, 29).

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