RESEARCH ARTICLE

Prediction and control measures of ergosterol content during the growth of tobacco leaf mold

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Received: September 29, 2025; **accepted:** January 12, 2025.

To address the challenge of inaccurate prediction and control of mold growth during the storage of tobacco strips, this study established a robust predictive model for ergosterol content and evaluated the effectiveness of different conservation management measures. The research utilized B2F grade K326 tobacco strips from six major production regions located in Southwest, Central, and East China over three years (2022-2024) as experimental materials. Mold induction experiments were conducted under nine environmental conditions, combining three temperatures (20°C, 25°C, 30°C) and three relative humidity levels (75%, 85%, 95% RH). Ergosterol content was quantified using high-performance liquid chromatography with comprehensive evaluation incorporating total mold count and sensory assessment. The results indicated that temperature, humidity, and time all significantly influenced ergosterol accumulation ($P < 0.001$) with relative humidity being identified as the primary driving factor. Under extreme conditions of 30°C and 95% RH, ergosterol content peaked at 371.0 μg/g on day 30, approximately 80 times higher than that under mild conditions of 20°C and 75% RH. A strong positive correlation existed between ergosterol content and total mold count (Spearman's rank correlation coefficient ρ = 0.9628), confirming its reliability as a core indicator of mold growth. Tests on conservation measures revealed that, after 21 days, the chemical antibacterial agent treatment group achieved a 99.70% inhibition rate, the temperature-humidity coordinated regulation group reached 88.42%, and the enhanced ventilation group attained 56.98%. This study uncovered the key driving mechanisms of mold growth in tobacco strips, providing critical evidence for advancing tobacco warehousing toward a modern model that integrated intelligent early warning with refined conservation management.

Keywords: cigarettes; mold growth; ergosterol; predictive model; maintenance management; high performance liquid chromatography.

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Introduction

As an important economic crop, the quality of tobacco largely depends on the stability of post-harvest processing and storage processes [1]. During the long-term aging and storage process, tobacco leaves are highly susceptible to factors such as temperature and humidity in the environment, which can cause mold growth. This is not only the main reason for the deterioration of tobacco quality and huge economic losses, but also the potential threat to consumer health posed by mold and its metabolic toxins such as aflatoxins [2, 3]. Therefore, how to achieve early and accurate monitoring of the mold growth process of tobacco and take effective prevention and control measures based on it has become a key technical problem that urgently needs to be

solved in the tobacco warehousing and logistics field [4, 5].

Vella *et al*. used ergosterol as a biomarker to detect restored melon juice contaminated with brewing yeast using spectrophotometry and compared it with plate counting method. The results showed that the method was linearly stable and could replace time-consuming conventional analysis, indicating that ergosterol could be used to identify mold and yeast in contaminated beverages [6]. Huang *et al*. studied the effects of six thiolases (AoErg10A-F) in *Aspergillus oryzae* on growth, fatty acid and ergosterol synthesis. Combining bioinformatics, quantitative real-time polymerase chain reaction (qRT-PCR), localization, yeast complementation and overexpression experiments, they found that there were differences in their expression and localization, and some could complement yeast mutants. Overexpression significantly affected the growth and ergosterol content of the strain [7]. Tian *et al*. analyzed the expression, localization, and metabolic effects of ergosterol synthase in *Aspergillus oryzae*. The results showed that the synthase gene was distributed on 8 chromosomes and expressed differently. Some co-overexpressing strains had higher ergosterol content than single overexpressing strains, providing a basis for subsequent research [8]. Lian and He proposed a support vector machine model for wind power prediction using wavelet denoising and improved slime mold algorithm optimization. After comparing two sets of measured data with seven models, the results showed that its prediction accuracy was higher. This strategy is analogous to the precise detection approach using ergosterol [9]. Sun *et al*. developed a hybrid prediction framework that integrates a new denoising method with multiple models to address the difficulty of predicting crude oil futures prices. By introducing confidence interval coefficients to optimize prediction accuracy and stability, their method could also provide ideas for modeling mold growth processes [10]. Slater *et al*. used a hybrid system that integrated data-driven methods with multiple prediction sources, combined with artificial intelligence (AI) and big data technology to improve mold prediction performance. The results showed that model integration improved accuracy but pointed out that model interpretability and adaptability to new data still needed to be improved [11]. Edde and Phillips explored the hazards of tobacco beetles in tobacco storage and integrated pest management strategies, summarized its prevention and control practices, and provided reference for pest control in tobacco maintenance [12]. Jin *et al*. designed an AI process detection system to optimize the tobacco shredding process, determined the optimal parameters for leaf cutting and re-roasting through data analysis, and significantly improved leaf yield and tobacco quality, providing technical support for quality maintenance in storage [13]. Tian *et al*. evaluated the impact of storage conditions on nicotine release from cigars. By utilizing Fe_3O_4@AuNRsNPs as a surface-enhanced Raman scattering substrate combined with capillary detection methods, they found that temperature and humidity significantly influenced nicotine release. This study provided a basis for optimizing tobacco storage conditions [14].

Many experts have conducted in-depth research on the biomarker role of ergosterol in mold and yeast detection, as well as its metabolic regulation mechanism and related enzyme gene expression regulation during mold growth. However, most existing research still focuses on qualitative analysis of single strains or conditions, lacking dynamic modeling and prediction models for ergosterol content changes in complex environments with limited accuracy, and failing to fully integrate multi-source data and advanced algorithms to achieve efficient prediction. There is a lack of systematic research on the linkage mechanism between multi factor regulation and biochemical indicators in tobacco storage and maintenance. Therefore, researching and constructing an ergosterol driven mold prediction and control system can promote the transformation of tobacco mold management from traditional passive detection to modern

active prediction and precise control. This research proposed a dynamic prediction model based on the random forest (RF) algorithm to facilitate early warning in tobacco warehousing. Furthermore, the study quantified and compared the inhibitory effects of different maintenance measures in simulated high-risk environments, thereby offering a basis for precise management. The study carried out large-scale artificial induction experiments and gathered data on mold growth in tobacco from multiple zones under the three-dimensional influence of temperature, humidity, and time. The research endeavored to drive the transformation of tobacco mold management from conventional passive detection to modern active prediction and precise control.

Materials and methods

Tobacco leaf resources

Sliced *Nicotiana tabacum* L. cultivar K326 tobacco that had completed the process of leaf beating and re-baking was collected from Yuxi, Yunnan, China (24°21′N, 102°32′E), Zunyi, Guizhou, China (27°41′N, 106°54′E), Liangshan, Sichuan, China (27°53′N, 102°15′E), Chenzhou, Hunan, China (25°48′N, 113°01′E), Sanming, Fujian, China (26°15′N, 117°38′E), and Xuchang, Henan, China (34°02′N, 113°51′E). A total of 50 kg of tobacco strips were obtained from each region to ensure statistical robustness [15].

Induction of mold growth

B2F tobacco referred to the second-grade orange leaf from the upper stalk position according to the standard grading system. To eliminate initial differences, B2F tobacco samples were ground using an IKA-Werke A11 basic analytical mill (IKA-Werke, Staufen, Germany) and weighed with a Mettler Toledo AE240 analytical balance (Mettler Toledo, Greifensee, Switzerland). The samples were equilibrated for a short period at 40°C and then equilibrated for ≥ 48 hours in a saturated $MgCl_2$ dryer at 25°C to accurately control the moisture content at 13.0 ± 0.3%. Subsequently, mold growth was induced in 9 static combinations in a KBF 720 constant climate chamber (Binder GmbH, Tuttlingen, Germany) at 20/25/30°C × 75/85/95% RH.

Evaluation of maintenance measures

To quantify the inhibitory effects of conservation strategies, a high-risk environment (30°C, 95% RH) was selected as the baseline condition. Four experimental groups were established for a 21-day trial, which included blank control, enhanced ventilation, coordinated temperature and humidity regulation, and chemical antifungal treatment. Tobacco samples in blank control group were stored continuously at 30°C and 95% RH without intervention. Samples in enhanced ventilation group were maintained at 30°C and 95% RH for 23 hours daily, then transferred to a standard environment (30°C, 65% RH) for 1 hour to simulate intensive ventilation. In coordinated temperature and humidity regulation group, samples were stored at 30°C and 95% RH for 22 hours followed by shifting to a low-temperature, low-humidity condition (18°C, 70% RH) for 2 hours daily, simulating automated warehouse climate control. Chemical antifungal treatment used a 0.5 g/L natamycin aqueous solution (purity ≥ 95%) (Sigma-Aldrich, St. Louis, MO, USA) to treat samples using a 402AI ultrasonic nebulizer (Yuwell, Danyang, Jiangsu, China) for 15 minutes daily to ensure uniform surface coverage. Samples from all groups were collected at days 0, 7, 14, and 21 for ergosterol quantification, mold counting, and sensory evaluation.

Indicator determination

A comprehensive evaluation system integrating chemical analysis, microbial counting, and sensory evaluation was developed in this study to ensure that the judgment results had both quantitative accuracy and practical user experience. The core indicator was ergosterol content, which is a unique component of fungi and is recognized as the "gold standard" for reflecting fungal contamination. The study utilized an internal standard method combined with Agilent 1260 Infinity II high-performance liquid chromatography (Agilent Technologies,

Santa Clara, CA, USA) for quantitative analysis. Ergosterol standard (purity ≥ 99.0%) (Sigma-Aldrich, St. Louis, MO, USA) and 7-dehydrocholesterol internal standard (purity ≥ 98.0%) (Sigma-Aldrich, St. Louis, MO, USA) were used for calibration. Samples were saponified with potassium hydroxide in methanol using a Hei-VAP thermostatic magnetic-stirring water bath (Heidolph Instruments, Schwabach, Germany). The extracts were concentrated using a BÜCHI R-300 rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) and filtered prior to analysis. The tobacco sample was spiked with the 7-dehydrocholesterol internal standard and subjected to saponification with 6% KOH-methanol solution at 80°C. The unsaponifiable fraction was extracted with n-hexane, evaporated to dryness, and reconstituted in chromatographic grade methanol. Separation was performed using a C18 reverse-phase column with methanol as the mobile phase, and the absorbance was monitored at 282 nm to calculate the ergosterol content based on the internal standard ratio. Confirmatory analysis was conducted using a Agilent 7890B–5977B gas chromatograph–mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The method had high sensitivity (LOD: 0.1 μg/g) and good accuracy (recovery rate of 92 - 105%). Meanwhile, the traditional fungal colony counting method was employed to evaluate the number of fungi with reproductive ability (CFU/g) according to the national standard GB 4789.15-2016 (National Health and Family Planning Commission of the People's Republic of China, Beijing, China) using potato glucose agar medium (Beijing Luqiao Technology Co., Ltd., Beijing, China) in SW-CJ-2FD laminar flow cabinet (Suzhou Antai Air Tech Co., Ltd., Suzhou, Jiangsu, China). Samples were homogenized using a BagMixer 400 CC Stomacher (Interscience, Saint Nom la Bretèche, France) and cultured in LRH-250 incubator (Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China). At the sensory level, a team of five experts from Zhejiang University (Hangzhou, Zhejiang, China) was employed to score the samples on a scale of 0 - 4 based on color, aroma, mold, and texture, converting subjective experience into quantitative data to complement the physicochemical test results.

Data processing and model construction

Data processing and modeling were implemented using the Python 3.8 programming environment (Python Software Foundation, Wilmington, DE, USA). The box plot analysis was performed using the Matplotlib library (NumFOCUS, Austin, TX, USA), and the RF model was constructed using the Scikit-learn package (Inria, Le Chesnay-Rocquencourt, France). The study first used a box plot to eliminate outliers followed by using Z-score to unify dimensions, and then constructed a prediction model based on temperature, humidity, time, and ergosterol content to ensure accurate and reliable results. This method converted each column of feature data into a standard normal distribution with a mean of 0 and a standard deviation of 1 as shown below.

$$x' = \frac{x-\mu}{\sigma} \quad (1)$$

where x' was the standardized new data. x was the original data point. μ was the average of all data in the feature column. σ was the standard deviation of all data in the feature column. To verify the effectiveness of ergosterol as a core indicator of mold growth, the Pearson correlation coefficient between its content and the total number of fungal colonies and sensory evaluation values was calculated [16]. This coefficient measured the strength and direction of the linear relationship between variables and was calculated as follows.

$$r = \frac{\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i-\bar{y})^2}} \quad (2)$$

where *n* was the sample size. x_i and y_i were the n-th observation values of the two variables. i and $\bar{x}$ were the average values of the two variables. If ergosterol content exhibited a

Table 1. Dynamic changes in ergosterol content in tobacco under different temperature and humidity conditions (μg/g).

Time (days)	20°C, 75% RH	20°C, 85% RH	20°C, 95% RH	25°C, 75% RH	25°C, 85% RH	25°C, 95% RH	30°C, 75% RH	30°C, 85% RH	30°C, 95% RH
0	1.8824	1.9017	1.8933	1.8769	1.9103	1.8856	1.9088	1.8992	1.9114
3	2.0136	2.4311	3.1128	2.1094	2.9872	4.6731	2.2437	3.8916	8.7642
7	2.2349	3.9842	8.7654	2.4116	6.8819	19.3427	2.7891	14.7623	48.9183
11	2.5018	7.1126	24.6719	2.8341	18.4328	65.8814	3.4677	49.3371	135.2246
15	2.8163	15.8933	58.9921	3.3782	45.1193	142.7638	4.6129	115.8924	248.1179
20	3.2457	38.6781	115.3486	4.1023	98.7642	231.5543	6.2384	201.4468	321.6732
25	3.8991	72.4319	178.2247	5.0338	154.3381	289.1027	8.1946	268.3217	358.4398
30	4.6722	105.7894	211.5633	6.1249	191.6724	315.4892	10.7831	295.1186	371.0315

significant and highly positive correlation with CFU and sensory deterioration scores with an r value close to 1 and $P < 0.01$, it fully demonstrated the reliability of ergosterol as an objective quantitative indicator of mold growth severity. To construct a model that could accurately predict ergosterol content, RF was chosen for the study. A large number of decision trees were constructed, and their prediction results were integrated for regression analysis. Specifically, each tree was trained on a randomly selected subset of samples (bootstrap sampling) and feature subsets, which significantly enhanced stability and resistance to overfitting of the model. The final prediction result was obtained by calculating the average of the predicted values from all component decision trees. The proposed model was named Ergosterol based Prediction and Control of Tobacco Lamina Mildew (EPC-TLM). The study selected the convolutional block attention module-based deep learning prediction model (CBAM-DL), the vision transformer-based mildew identification model (ViT-mildew), and the multi-source information fusion model for early-stage mildew detection (MIF-EMD) for comparative analysis with proposed EPC-TLM. Hyperspectral imagery of Fusarium-infected corn Kernels (HSI-Corn-Fusarium) and the publicly available PlantVillage datasets were used for different models' comparison.

Statistical analysis

SPSS 26.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Multi-way Analysis of Variance (ANOVA) was applied to evaluate the effects of environmental factors, and Spearman's rank correlation test was utilized to analyze the relationship between indicators. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

Changes in ergosterol content during the mold growth process of tobacco under different temperatures and humidity

The dynamic changes (μg/g) of ergosterol content in tobacco under different temperature and humidity conditions demonstrated that, in all 9 temperature and humidity combinations, the ergosterol content increased monotonically with prolonged storage time, and the increase became steeper with higher temperature and humidity (Table 1). On day 0, the baseline values for each group were only 1.88 - 1.91 μg/g with a difference of less than 0.04 μg/g. On the 7th day, the 30°C/95% RH group first jumped to 48.9 μg/g, which was about 17.5 times more than that of the 75% RH group (2.79 μg/g) at the same temperature. However, the 20°C/75% RH group still maintained a low level of 2.23 μg/g. On the 15th day, the 25°C/95% RH and 30°C/95% RH reached 142.8 and 248.1 μg/g, respectively, indicating that the high temperature of 30°C pushed the concentration of mold markers to about 1.7 times more than that of 25°C and the same humidity. On the 30th day, the peak value of the 30°C/95% RH group was 371.0 μg/g, which was nearly 80 times higher than that of the 20°C/75% RH group (4.67 μg/g). Under 75% RH

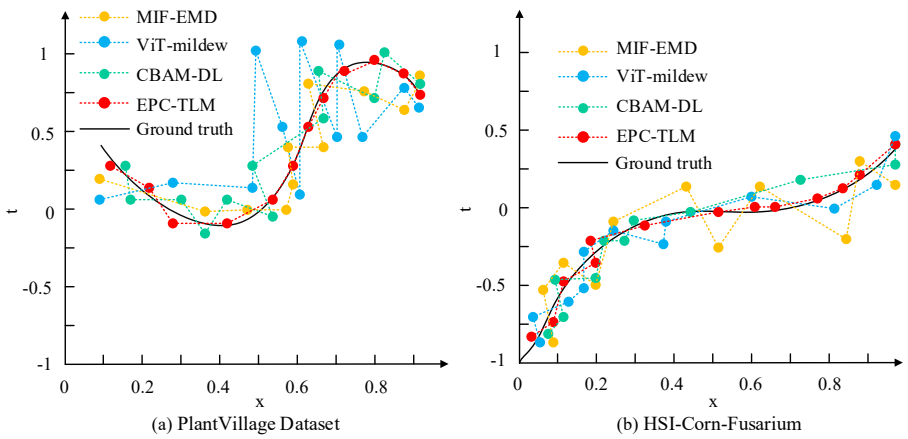

Figure 1. Comparison of prediction accuracy of different prediction algorithms. The horizontal axis (x) represented the normalized sample index (Dimensionless). The vertical axis (t) represented the normalized target value (Dimensionless).

conditions, increasing the temperature from 20°C to 30°C only increased the final content from 4.67 μg/g to 10.78 μg/g, indicating that humidity was the dominant factor driving the rapid accumulation of ergosterol. The analysis of variance results indicated that temperature, relative humidity, and storage time all significantly influenced ergosterol content (P < 0.001). Specifically, relative humidity was the most dominant factor followed by storage time and temperature. Furthermore, all second-order and third-order interactions among these three variables were statistically significant with the interaction between relative humidity and time exhibiting the most prominent effect (P < 0.001). The results clearly revealed the key synergistic effect of temperature and relative humidity in driving the process of tobacco mold growth. Water activity was a prerequisite for mold colonization and germination, while temperature played the role of an amplifier for growth rate. This model was consistent with previous research on other agricultural products, but differences in the mold rate of tobacco leaves from different origins indicated that the inherent properties of tobacco leaves were equally important as the report of Hoag *et al*. that the pH value of tobacco products could significantly affect their chemical properties [17]. It could be inferred that the pH value of the microenvironment on the surface of tobacco leaves might also affect the growth of dominant molds.

Correlation analysis between ergosterol content and total mold count

To confirm the ergosterol content used in the experiment as an objective, quantitative, and reliable alternative indicator for evaluating the degree of mold growth in tobacco, a systematic correlation analysis was conducted between it and the classical microbiological detection indicator - total fungal colony count (CFU/g). The results indicated that, with the gradual increase of ergosterol content in tobacco samples, the total number of fungi also showed systematic and synchronous growth. The Spearman correlation analysis revealed a coefficient (ρ) of 0.9628 (P < 0.001) between the two indicators, demonstrating a strong and positive monotonic relationship.

Construction and validation of ergosterol content prediction model

The comparison of prediction accuracy between different prediction algorithms demonstrated that, in the PlantVillage dataset test, the predicted data points of the three comparison models MIF-EMD, ViT mildew, and CBAM-DL had a discrete and significantly deviated distribution from the true function curve, while EPC-TLM model proposed in the study had a tightly and smoothly distributed predicted data points around the true function curve (Figure 1a). Furthermore, on the HSI-Corn-Fusarium

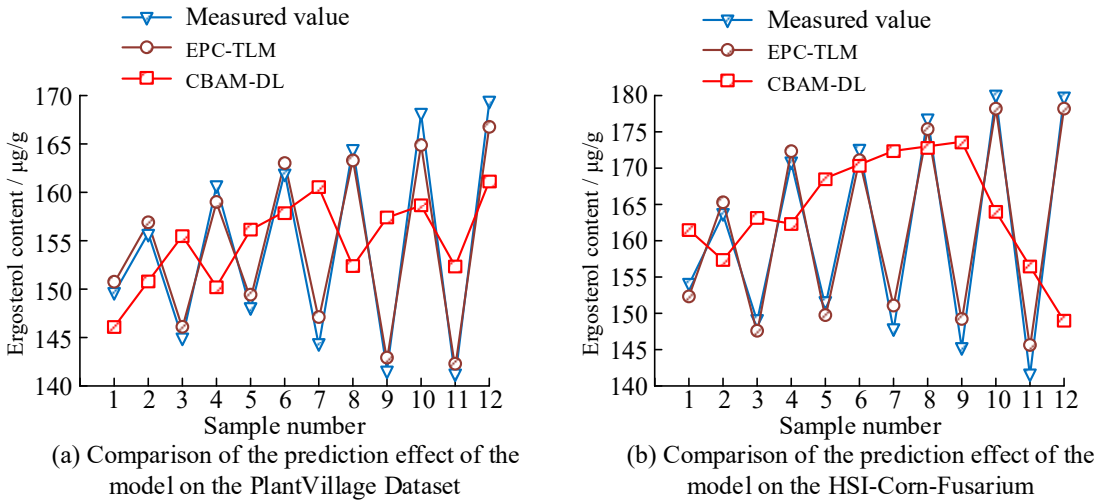

Figure 2. Comparison of predictive performance of different models on public datasets.

hyperspectral dataset, despite changes in data modality and task complexity, the prediction results of the three comparison models still exhibited significant oscillations and biases, while EPC-TLM model predicted point trajectories that coincided with the true curve height, demonstrating excellent generalization ability and robustness across datasets (Figure 1b). The comparison of predictive performance of different models on public datasets showed that, in the PlantVillage test, CBAM-DL deviated significantly from the true values at samples 2, 5, 9, and 11, while EPC-TLM almost overlapped with the measured curve (Figure 2a). On the more complex HSI-Corn-Fusarium dataset, CBAM-DL exhibited systematic distortion, while EPC-TLM still accurately tracked all fluctuations, verifying its superior fitting and generalization abilities (Figure 2b). The core advantage of the EPC-TLM prediction model was to transform the complex nonlinear mold growth process into a quantifiable and predictable mathematical problem based on key environmental factors. However, the limitations of the model should not be ignored, which included that its training data came from specific experimental conditions, and its universality under complex and variable conditions in real storage environments needed further verification. The factors that affected mold growth were multidimensional and needed to analyze complex industrial processes through multiple sensors [18]. However, the model only included three core variables of temperature, humidity, and time. Therefore, the existing form of the model should be regarded as an accurate representation of mold growth behavior under specific conditions, which was similar to the research of Gebrehiwot and Espinosa-Leal in characterization of specific material behavior [19], but continuous optimization was still needed to apply it to a wider range of real-world scenarios.

The impact of different maintenance management measures on the mold growth of tobacco flakes

This study identified the environmental conditions that were most likely to cause rapid and severe mold growth in tobacco leaves with the temperature of 30°C and relative humidity of 95% RH as the basic background for the experiment, simulating a high-risk storage scenario for effective intervention. During the 21-day trial, the quantitative analysis of the inhibitory effect of different maintenance management measures on ergosterol accumulation on the 21st day showed that the final ergosterol content of the blank control group increased drastically from a baseline of approximately 1.93 μg/g to 364.82 μg/g with an

increment of 362.9 μg/g, setting as the baseline with 0% inhibition. In comparison, the applied maintenance measures demonstrated varying degrees of effectiveness. By day 21, the enhanced ventilation group reduced the final value to 158.3 μg/g with an incremental reduction of 157 μg/g and an inhibition rate of 56.98%. The coordinated temperature and humidity regulation group proved more effective by reducing the final ergosterol value to 43.9 μg/g with an increase of only 42.0 μg/g and the inhibition rate of 88.42%, which was 31.4 percentage points higher than the ventilation group. The chemical antifungal treatment showed the most significant treatment effect with a final ergosterol value of only 3.02 μg/g and an increment of only 1.09 μg/g. The inhibition rate was 99.70%, almost completely blocking the accumulation of ergosterol. The different maintenance management measures validated by this research had clear practical significance, quantifying the significant advantages of the "temperature and humidity coordinated regulation" strategy. Looking ahead, the ultimate goal is to embed the EPC-TLM prediction model as the core algorithm into modern intelligent warehousing systems, which coincides with the concept in advanced manufacturing such as Araújo *et al*. using sensors for real-time fault diagnosis and process control in industrial production [20]. Similarly, an intelligent warehousing system that integrates environmental sensor networks and EPC-TLM models can conduct real-time mold risk assessment and coordinate with relevant equipment for precise intervention, thereby achieving a shift from "passive response" to "active prevention".

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