**Materials and Methods  
Extraction and Freezing of Spirulina**

The harvested blue-green algae S. platensis was imported to Çukurova University in Adana, *Türkiye*. The prepared *Spirulina platensis* biomass was kept in a -18°C freezer for 2 h in sanitary packaging. The freeze-thaw method as described in [24] was used to extract Spirulina, with adjustments in concentrations of 0.5, 1, and 5 g of freeze-dried Spirulina, along with 100 ml of sterilized distilled water for each solution group, designated as Extract A (EA), Extract B (EB), and Extract C (EC). Macerating cells to break down proteins and extract polysaccharides has antimicrobial effects against harmful bacteria. The Hu angle was employed to assess the blue color of the Spirulina extract [25, 26].

Total coliform and Staphylococcus aureus bacterial strains were selected for their critical public health and economic significance, particularly in the context of fresh product marketing and consumption. Both total coliform and *S. aureus* are key indicators of fish and other seafoods safety and quality, water hygiene and sanitation practices, as well as the cleanliness of personnel involved in the processing of fish products.

The freeze-thaw extraction method was chosen due to its simplicity and the availability of the required technology in our laboratory. This method offers an efficient and cost-effective means of extracting antimicrobial compounds from Spirulina.

Negative control was used in this experiment; this group did not receive any treatment or procedure and was expected not to yield a positive result. Its purpose was to ensure that any positive results observed in the experimental groups were attributable solely to the treatment or procedure applied.

Afterward**,** 0.5, 1, and 5 g of Spirulina biomass was carefully measured from the frozen stock, placed into sanitized bottles with labels, and filled the volumetric flask with distilled water up to the 100 ml mark. After a brief mixing, the bottles were placed in a water bath at 25°C and covered with aluminum sheet to maintain darkness during the 24-hour extraction process. Following 24 h, the liquid at the top was removed and kept at a temperature of +4°C.

**Experimental design preparation**

Twenty-five entire Nile tilapia fish (*Oreochromis niloticus*) were bought from the Nicosia fish market, then dissected and filleted, resulting in 100 fillets that weighed 50 g each. Those 100 fillets .multiplied by 4 (three-time intervals and the control group) and 2 bacteria species (Total coliform and S. aureus) resulted a total of 800 experimental data. Out of these, 200 data points were in the experimental trial phase and not included in the analysis. Thus, 600 experimental data were analyzed.

The sterile plates held the fish fillets while extracts were added to the samples. Microbial counts were initially measured in each fresh fillet and served as a control prior to the application of the extracts. The fish fillets were exposed to Spirulina extracts in different concentrations (0.5%, 1%, and 5% w/v) named as EA, EB, and EC. During the experiment, sterile conditions were upheld, and alcohol/flame were utilized to sterilize any items that touched the samples. Microbial analysis was performed to assess the total viable count of Total Coliform bacteria and Staphylococci pre- and post-application of *Spirulina platensis* extracts. The fish fillets were kept in a fridge at 4°C, and tests were done at 1, 24, and 48 h to evaluate the antimicrobial efficiency of the extracts.

**Analysis of Total Coliform bacteria and *Staphylococcus aureus***

Tilapia fish fillets involved weighing 5g of samples before and after treatment. The samples were placed in a sterile glass jar with 45mL of Maximum Recovery Diluent (MRD) and homogenized. Serial dilutions of 1:10 were then performed. The enumeration of Total Coliform bacteria and Staphylococcus aureus was conducted on Plate Count Agar (PCA) by incubating at 37±1°C for 24±2 h and at 10°C for 7 days for enumeration. Findings were obtained stated as log CFU/g [27].

**Analysis of results by artificial intelligence**

According to Metekia et al. (2022), ANN and ANFIS were chosen over conventional statistical techniques in studying Spirulina platensis antimicrobial activity because; first, Artificial Neural Networks (ANN) and Adaptive Neuro-Fuzzy Inference Systems (ANFIS) are prioritized over conventional statistical methods in studying Spirulina platensis antimicrobial activity due to their ability to address limitations of traditional approaches. Unlike linear regression or ANOVA, which assume simplistic relationships, ANN and ANFIS model complex, non-linear interactions (e.g., pH, solvent polarity, and extraction time synergies) inherent in biological systems. This enables accurate prediction of outcomes like inhibition zone size without predefined equations, capturing dynamic variables that linear models miss.

Second, the handling complexity and uncertainty, ANN automates detection of hidden patterns in high-dimensional data (e.g., temperature, solvent ratios), avoiding manual specification of interactions required by conventional methods. ANFIS enhances this by integrating fuzzy logic to process subjective or uncertain inputs (e.g., "moderate temperature" or semi-quantitative microbial ratings), bridging qualitative observations with quantitative predictions. This is critical in biological research, where crisp numerical data and strict probabilistic frameworks often fail to reflect real-world variability [28].

Thread, adaptability and data efficiency, classical models require reconfiguration for new data, hindering iterative optimization (e.g., refining extraction protocols). ANN and ANFIS adapt dynamically through training, improving predictions as new experimental results emerge. They also outperform conventional techniques with limited or heterogeneous datasets, tolerating noisy inputs and generalizing patterns even from sparse pilot studies, where traditional methods demand large, homogenous data [28].

Fourth, balancing power and transparency, while ANN’s "black box" nature limits interpretability, ANFIS incorporates fuzzy rules (e.g., "long extraction + polar solvent → increased activity") to provide transparency. This hybrid approach retains predictive power while offering insights into variable relationships, surpassing purely statistical models. By verifying experimental data and enhancing analytical accuracy, ANN and ANFIS streamline the design of antimicrobial studies, accelerating the development of Spirulina-based natural agents [28].

The activities of Spirulina algae extracts' antibacterial effects on Total Coliform bacteria and Staphylococcus aureus of fresh tilapia fish fillets were compared using Artificial Neural Network (ANN), Adaptive-Neuro Fuzzy Inference System (ANFIS), and descriptive statistics. The ANFIS is employed in AI to predict different problems. The ANFIS consists of two primary layers: forward propogation networks and adaptive multi-layer networks. Once more, feed forward networks utilize fuzzy Takagi-Sugeno instructions to incorporate input-output variables. The key elements of the design in the fuzzy database system include the fuzzyer and defuzzifier.

The fuzzy logic membership functions involve transforming input values into fuzzy information. Nodes functioning as membership functions help in representing the relationship between inputs and outputs. As a result, the node acts as a link feature that allows for the modeling of the relationship between the input and output structures. Various connection functions, including triangular, sigmoid, Gaussian, and trapezoidal are available [28, 29]. Two primary factors in the method must be considered in terms of input and output configurations: the FIS involves two variables, 'x' and 'y', as input data, and one output 'f', which is a first-order Sugeno fuzzy and typically follows this formula.

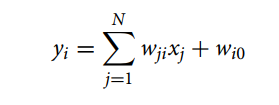
Method 1: if µ(x) is A1 and µ­y‑ B1 then f1 = p1x + q1y + r1 (1)  
 Method 2: if µ(x) is A2 and µ­y‑ is B2 then f2 = p2x + q2y + r2 (2)

The parameters p1, q1, r1, p2, q2, r2 are outlet function parameters, with constraints A1, B1, A2, B2 being membership functions for x and y. ANFIS is structured with a neural network arrangement consisting of five layers.

The multilayer perceptron (MLP) neural network is a commonly used example of an artificial neural network (ANN) that helps in operating and solving non-linear systems. Many scholars think that this specific estimator is widely acknowledged in comparison to other forms of ANNs. The structure of the Multilayer Perceptron (MLP) neural network is comparable to that of traditional Artificial Neural Network (ANN) models, including an input layer, a hidden layer, and an output layer. The Levenberg–Marquardt algorithm is frequently utilized as a learning algorithm to correct and reduce the discrepancy between observed and predicted values. The training procedures are done repeatedly until the desired results are evident. The Multi-Layer Perceptron consists of an input, multiple hidden layers, and output layers, like a traditional Artificial Neural Network. Additionally, the input data included the concentrations of Spirulina extracts EA, EB, and EC (0.5, 1, and 5) w/v, as well as the initial microbial load measured as log CFU/g. The output data focused on the antimicrobial activity of Spirulina extracts and microbial reduction at 1, 24, and 48 h (log CFU/g). The study's flowchart can be seen in Figure 1.



**Figure 1.** Flow chart for the experimental study of antimicrobial activity of *S. platensis* extracts on fresh tilapia fish fillets.

**** (3)

Where N is the total number of nodes in the top layer of the node, i; wji is the weight between the nodes i and j in the upper layer; xj defines the output derived from node j; wi0 is the bias in node i, and yi describes the input signal of node i which crosses via the transfer function. The data was from the laboratory experimental study findings at Food Hygiene and Technology Department, Veterinary Medicine Faculty of Near East University, Nicosia, Cyprus. In this analysis the initial microbial load before treatment as the control microbial load before treatment (CFU log10/g) and the results from each treated samples were taken as an input variable. Total bacterial load reduction after treatment at the specified time interval i.e., 1, 24 and 48 h (CFU log10/g) were taken as output variables in the analysis and modeling of this study.

**Evaluation criteria for data—driven models.** The performance accuracy of any type of data driven study is usually determined by comparing projected values to measured values. To estimate the models, the determination coefficient (DC) as a goodness of fit, correlation coefficient (CC), and two statistical errors, root mean-squared error (RMSE) and mean-squared error (MSE), were used [28].

(4)

(5)

(6)

MSE = (7)

Where N, Yobsi, Y and Ycomi are data number, observed data, average value of the observed data and computed values, respectively.

**Description and validation of the models for the dataset**. The main objective of a data-driven approach is to organize data for models using functional markers for a particular value range, ensuring precise and reliable predictions for unfamiliar data sets. In this approach, over-fitting values and acceptable working activities are typically disregarded. In the endorsement phase, various verification methods like k-fold cross-validation, holdout, leave-one-out were employed. The main advantage of the k fold proof tool is that both the verification and working sets are automatically determined at each point. As mentioned before, the data is split into two separate groups, with 75% used for training and 25% for testing, while also utilizing k-fold cross-validation. Another important aspect to note regarding this procedure is the data validation techniques that were utilized. There are 25 instances for each of the variables within the data set.

**Patient and public involvement.** The study was conducted on fresh fish fillets from a food hygiene and safety perspective, hence there was no patient or public involvement in this study.

Code Availability

Code Availability

The custom code and mathematical algorithms developed for the AI-driven analysis in this study, titled "AI Verification for Spirulina's Antimicrobial Power in Total Coliform and Staphylococcus aureus Isolated from Tilapia Fillet," are publicly available to ensure reproducibility and transparency. The code repository is hosted on <https://github.com/WAM2785/ai-verification-spirulina-antimicrobial-tilapia/new/main>.