Neural Network Modelling of Phenolic Content, Antioxidant Capacity and Microbial Population Dynamics of a Household Scale Spontaneous Fermentation of Carica Papaya Leaf

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ABSTRACT

Beneficial effects of spontaneous fermentation on Carica papaya leaf (CPL) have been observed in terms of enhanced total phenolic content and antioxidant capacity, as well as cultivation of lactic acid bacteria (LAB). Nonetheless, these responses were nonlinear, thus Artificial Neural network (ANN) was used as a predictive tool. The chosen ANN architecture consisted of multi-layer perceptron (MLP) with 2-7-7-1 and 2-10-10-1 topologies,

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Levenberg-Marquardt training algorithm, and hyperbolic tangent sigmoid activation function. Enhanced total phenolic content (TPC) and antioxidant capacity were recorded in final CPL extracts (day 90) of 5-L fermenter origin; 48.42±0.31 mg GAE/g dm and 467.38±4.09 mM TE/g dm, respectively, as compared to 12.13±0.39 mg GAE/g dm and 275.46±3.09 dm of respective extracts at initial (day 0). Likewise, enhanced total phenolic content (TPC) and antioxidant capacity were also observed for 50-L fermenter origin extracts. The chosen ANN topologies displayed the highest predictive ability as indicated by their correlation coefficient (R) of greater than 0.9, the marginal difference in mean square error (MSE) between training and testing data sets, and the absolute average deviation (AAD) of less than 10% between the predicted and experimental values of most responses. In conclusion, ANN was a reliable predictive tool for nonlinear responses during spontaneous fermentation of CPL.

Keywords: *Carica Papaya Leaf; Actic Acid Bacteria; Artificial Neural Network; Antioxidant Capacity; Total Phenolic Content*

Introduction

Carica papaya leaf (CPL) is a medicinal herb used in folklore medicine to treat the wound, burn, infections, and fever. Recent studies demonstrated the CPL potency in treating myriad illnesses; type-2 diabetes, cancer, autoimmune disorder, inflammation. In tropical regions where dengue epidemic affects 500 million, CPL is well known for its anti-dengue potency, as clinical evidence linked CPL to increased platelet production [1].

The medicinal properties of CPL are contributed by its polyphenol compounds such as ferulic acid, caffeic acid, rutin, quercetin, protocatechuic acid, and kaempferol. Though bioactive, these compounds are large polymer molecules which are not easily absorbed by human's digestive system in their native form, except for a very small fraction of them (5-10%) [2]. However, the digestion of most dietary polyphenols (90-95%) takes place at the colon by colonic microbiota. The metabolism of these polyphenols at the colon in anaerobic condition is akin to anaerobic fermentation [2]. Thus, various fermentation techniques such as spontaneous fermentation have been applied on phenolic-rich medicinal plants such as spider flower (*Gynandropsis gynandra*) [3], leek [4], carrot juice [5], garlic [6] and cornelian cherry [7] to break down these complex polyphenols, hence increasing the bioactivities of the original plant materials. In other instances, starter culture fermentation using a particular microorganism strain was also used such as *Enterococcus faecalis* and *Aspergillus oryze* on papaya fruit [8]. Various strains of LAB were also employed as starter culture during fermentation of Myrus communis berries [9], cactus pear (*Opuntia ficus-indica* L.) [10] and *Echinacea spp* [11].

Given the benefit of fermentation in improving the bioactivity of various plants, Penawar Farm (location: 3.009945, 101.710782) has implemented a household scale of spontaneous fermentation on CPL. In addition, industrial scale CPL fermentation has been simulated on Medic IG Biopharma company (location: 3.047676274602906, 101.5306561107789) by our previous work [12]. By imitating the exact fermentation process setup by Penawar Farm in our laboratory, our recent studies revealed the enhanced phenolic content and antioxidant capacity of the fermented CPL, as well as population dynamics and identities of the microorganism in the ecosystem of the CPL fermentation, which consisted of LAB, enterobacteria and yeasts [13].

Nevertheless, these fermentation process responses displayed a nonlinear pattern, as often the case in many biological processes. Thus, a reliable empirical modelling tool is imperative to characterize these nonlinear process responses. Meanwhile, the artificial neural network (ANN) is an increasingly known as the common tool in microbiology and bioprocessing to model highly unpredictable and nonlinear process responses. ANN is a supervised learning tool that recognized the complex pattern of input-output relationship of empirical or historical data, and later makes an intelligent generalization based on the experience learned during its training with the experimental data. This learning capability of the ANN allows the optimization and prediction of various process responses e.g., growth, yield, aging time, which are normally encountered during complex biological processes [14]-[15]. ANN was also employed to predict the effect of extraction conditions on total phenolic compounds (TPC), anthocyanin (ANT) and antioxidant activity (AOA) of beetroot [16] and strawberries [17]. It is also more advantageous than the other tools e.g., response surface method (RSM), since input-output relationship can be established by exploiting the mathematical models even without prior information of the physical relationship between them [18]. Such capability renders ANN as a reliable tool to model the process responses during spontaneous fermentation of CPL.

In this study, ANN was used to model various process responses within the experimental constraints (volume and duration) of spontaneous fermentation of CPL (initially developed by the Penawar Farm). The robustness of the selected ANN models was evaluated in terms of the correlation coefficient (R), mean square error (MSE), and the absolute average deviation (AAD).

Methodology

Fermentation

About 500 g of fresh CPL was washed, shredded into small pieces and loaded into 5-L fermenter. Later, about 10 %w/v of brown sugar was added into the fermenter, followed by addition of purified water to make up a 5-L working

volume. The lid of the fermented was closed airtight, followed by 100-day incubation. A simple, one-way gas channel was created to vent off accumulating gas. No other control measures were implemented. Sampling was carried out at different intervals during the incubation; day 0, 2, 4, 6, 8, 15, 30, 30, 45, 60, 75, 90 and 100. The 100-day period was purposely chosen to allow growth succession of different microorganisms. The exact fermentation method was replicated in 50-L fermenter with 5 kg CPL was used.

Total phenol content and antioxidant capacity

For the measurement of total phenolic content and antioxidant capacity, 15 mL of fermented CPL suspension was collected at the aforementioned intervals. Solid debris was first removed by centrifuging the suspension at 10,000 g for 20 min at 4 °C, followed by dewatering using a rotary evaporator at 30 °C for 45 min. The concentrated sample was later re-suspended in 80% of methanol (MeOH) at 1:1 (v/v) to yield methanolic extract (ME).

For the construction of standard curve, exactly 1 %w/v (10 mg/100 mL) of standard gallic acid (Sigma-Aldrich) in 50% MeOH was prepared as stock assay and further diluted into appropriate working concentrations. Then, the assays were incubated at room temperature for 5 min, followed by the addition of 4 mL of 20% w/v Na_2CO_3 (Sigma-Aldrich). The absorbance value was measured at 765 nm using a UV-vis spectrophotometer (Shimadzu) against blank (distilled water) [19]. Similarly, ME was dissolved in 50% MeOH and its absorbance was taken. All readings were done in triplicate and calculated as average±standard deviation. Total phenolic content (TPC) of the assay was expressed as mg gallic acid equivalent per ME dry mass (mg GAE/g dm) using the following formula, i.e.:

$$TPC = \frac{C \times V \times D}{W} \tag{1}$$

where C is the concentration of gallic acid (mg/mL), V is volume of extract solution (mL) and W is mass of dried ME (g).

The antioxidant activity was estimated based on the scavenging activity of ME against DPPH (Sigma-Aldrich) as a free radical model. A standard calibration curve was prepared by diluting 0.05 %w/v of Trolox solution in MeOH as an antioxidant reference into appropriate working concentrations. For the DPPH scavenging assay, 0.15 mL of each Trolox assay was mixed with 2.85 ml of DPPH assay. The reaction of Trolox and DPPH was allowed for 24 hr of incubation under darkness followed by absorbance reading at 515 nm using UV-vis spectrophotometer. Similarly, ME was dissolved in MeOH and its absorbance was measured. All readings were done in triplicate and calculated as average±standard deviation. DPPH scavenging activity was

expressed as µM Trolox equivalent (TE) per g of dried ME dry mass (mM TE/g dm) using the following formula:

$$TE = \frac{C \times V \times D}{W} \quad (2)$$

where C is the concentration of Trolox (mM), V is volume of extract solution (mL), D is dilution factor and W is mass of dried ME (g)

Microbial population dynamics

Precisely 0.1 mL of broth collected from each sampling interval was homogenized in 0.9 mL of sterile saline-peptone water on vortex shaker, then serially diluted into appropriate dilution factors (10^1 to 10^6). Exactly 0.1 mL of each dilution was dispensed onto the following selective media in duplicate: Man Rogosa Sharpe agar (MRS) for lactic acid bacteria (LAB), MacConkey agar for enterobacteria and potato dextrose agar (PDA) for yeasts. All inocula were incubated at 30-37 ºC for 1-2 days, in oxygen-free candle jars. Afterwards, single colonies were enumerated in duplicate and expressed in terms of Log 10 colony forming unit per mL (CFU/mL).

Development of Artificial neural network (ANN)

MATLAB® (Math Works Inc. version: R2018a) software was employed for coding the ANN algorithm and analysis of the neural network. The neural network architecture consists of four layers: The input layer consist two input nodes , i.e. the fermentation day and fermenter's volume, two hidden layers and ended by a single output layer (fourth layer) to predict the phenolic content, antioxidant capacity, microbial population and pH at its node, as illustrated in the Figure 1.

The experimental database, which was used as input during ANN model development for spontaneous fermentation of CPL pericarp is shown in Table 1. The relationship of each process response (or output) with inputs was based on the hypothesis:

Process response= f(fermentation day, fermenter's volume) (3)

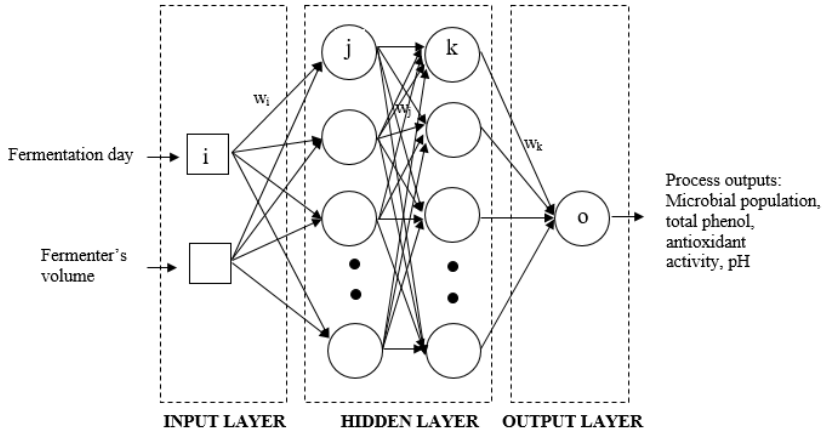


Figure 1: Multilayer ANN topology

Table 1: Database used for the development of ANN model for the fermentation of CPL

Responses	Unit	Range
Fermenter's volume	L	0-50
Fermentation day	No unit	0-90
LAB population	log 10 CFU/mL	0-8.73
Enterobacteria population	log 10 CFU/mL	0-4.8
Yeast population	log 10 CFU/mL	0-7.98
pH	No unit	2.71-6.39
Total phenol content	mg GAE/g dry mass	5.7-79.8
Antioxidant capacity	mM TE/g dry mass)	86.9-574.7

As the raw values of input and output variables have significant difference between their maximum and minimum values, data normalisation was carried out by transforming both input and response values in a range of -1 to 1 i.e. [-1, 1] for a fair weighted effect. After the ANN simulation ended, the normalised data were reverted to their original values.

ANN is considered a black box since there is no universal ANN model topology which is representative to every input-output correlation. Here, trial and error computation was carried out using different topologies by manipulating the number of neurons of hidden layer (s) and the number of hidden layers, and selecting the one that provided the best performance based on the error between observed and predicted response values. Eventually, 2-7-7-1 and 2-10-10-1 ANN architectures were chosen as the optimum networks

(Figure 1). The whole network was trained based on random biases and weighted values. Starting from the input neuron x_i, a single neuron of the first hidden layer, n_j produces its own output by summing the weighted inputs ($w_{ij}x_i$) together with linked bias, θ_j as well as changing the input data to non-linear form, as follows [18], [20]:

$$n_j = \sum_{i=1}^{n} w_{ij} x_i \tag{4}$$

In the context of this study, the ANN model learned the correlation between each of the process response (microbial population, phenolic content and antioxidant capacity) with inputs (fermentation day and fermenter's volume) based on the training algorithm assigned. Later, each activation of each neuron was calculated according to hyperbolic tangent sigmoid activation function (*f*), as follows:

$$y_j = f(n_j + \theta_j) = \frac{2}{1 + e^{-2(n_j + \theta_j)}} - 1 \tag{5}$$

Equations (4) and (5) were applied from the input layer neurons (inputs) which transmitted signal to first hidden layer. Similar process were applied to the second hidden layer, leading up to the predicted responses at the final output node (y_o) at the fourth layer.

In this paper, the original input-output data set was randomly divided into three different data sets; of which 70% of the data set was randomly selected for the development of the statistical model and training of the network, another 15% of the data set was excluded for the validation and the final remaining 15% of the dataset was used as the independent data set to check the robustness of the network architecture. Later, the best ANN model was selected and used to interpolate the new predicted response values allied with the new input data set.

Tansig function which represented the hyperbolic tangent sigmoid function was chosen as the activation function in the multi-layer perceptron (MLP), feedforward with backpropagation network as shown in Figure 2 to connect the inputs to intermediate layers (or hidden layer). Due to the nonlinearity of each response, the same activation function i.e. *tansig* (rather than *radbas, satlin, poslin* etc.) was used between the two hidden layers and finally between the second hidden layer to the output (response) neuron. Furthermore, hyperbolic tangent sigmoid function (*tansig*) was often used during ANN modelling of several biological processes such as *Aspergillus flavus* growth [21], bioethanol [22] and biogas yields [23]. The networks were further trained with the Lavenberg-Marquardt algorithm (trainlm). During this

training, the weight values between neurons was adjusted to minimize the error between the predicted and experimental responses by a number of iteration cycle (epoch) until the best performance goal i.e. mean square error (MSE). These were fixed at 1000 and 0.001, respectively at learning rate of 0.01. After the training was completed, the neural network was tested using the remaining 15% of unused data set.

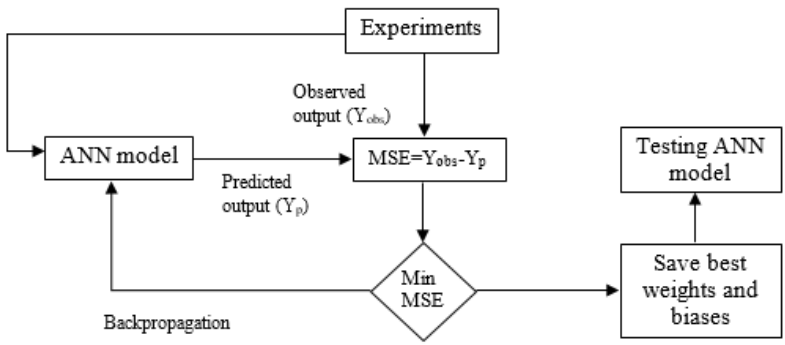


Figure 2: The feedforward training flowchart for ANN

Statistical evaluation of Artificial Neural Network (ANN) prediction

The performance of the ANN models were evaluated using the correlation coefficient (R), the mean square error (MSE) and the absolute average deviation (AAD), which are defined as follows [14]:

$$R = \sqrt{1 - \sum_{i=1}^{n} \left(\frac{(y_i - y_{di})^2}{(y_{di} - y_m)^2} \right)} \tag{6}$$

where n is the number of points, y_i is the predicted value obtained from the neural network model, y_{di} is the experimental value and y_m is the average of the experimental values. The mean square error (MSE) was calculated as follows:

$$MSE = \frac{1}{n} \sum_{i=1}^{n} (y_i - y_{di})^2 \tag{7}$$

where n is the number of points, y_i is predicted value obtained from ANN and y_{di} is the experimental values.

Another performance index of an ANN model is its output error due to the difference between predicted and experimental response values, which was measured by absolute average deviation (ADD), as follows [22]:

$$AAD(\%) = \left\{ \left[\sum_{i=1}^{n} \left(\left| y_i - y_{di} \right| / y_{di} \right) / n \right] \right\} \times 100 \quad (8)$$

where y_i and y_{di} are the predicted and experimental response values, respectively, and n is the number of data points. A good network performance is obtained if the AAD is minimum i.e. less than 10% [23].

Results and Discussion

Total phenolic content (TPC)

The TPC of final (day 90) fermented CPL extract collected from 5 L fermenter was 48.42±0.31 mg GAE/g dm, i.e. four-time higher than the initial (day 0) extract, i.e. 12.13±0.39 mg GAE/g dm as shown in Figure 3. Likewise, the TPC of final fermented CPL extract collected from 50 L fermenter displayed higher TPC value (31.14 mg GAE/g dm) as compared to its initial extract (5.71 mg GAE/g dm).

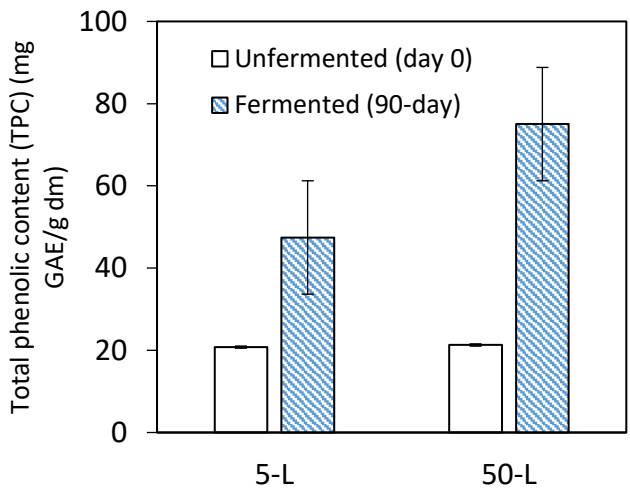


Figure 3: TPC of unfermented (day 0) and fermented CPL (day 90) extracts from 5 L and 50 L fermenter

The evolution of TPC apparently displayed continuous increasing trend with fermentation time (as shown by Figure 5a in later section). The enhanced TPC found in this study was in line with favourable effect of fermentation

reported by other studies involving phenolic rich plant materials such as five-fold increase of TPC of fermented Myrtus communis [9] as well as higher and fermented Echinacea spp. [11].

Antioxidant capacity

The antioxidant property of fermented CPL extract was measured in terms of its ability to scavenge DPPH radical which reduced the coloured DPPH to DPPH-H [18]. Antioxidant capacity is the hallmark of various health-promoting functionalities against cancer, obesity, cardiovascular disease and other chronic diseases. The antioxidant capacity of final (day 90) fermented CPL extract from 5 L fermenter was higher than its initial (day 0), i.e. 467.38±4.09 mM TE/g dm and 275.46±3.09 mM TE/g dm, respectively as shown on Figure 4. Likewise, antioxidant capacity of final fermented CPL extract from 50 L fermenter was also higher than its initial, i.e. 405.8 mM TE/g dm vs 130.5 mM TE/g dm, respectively.

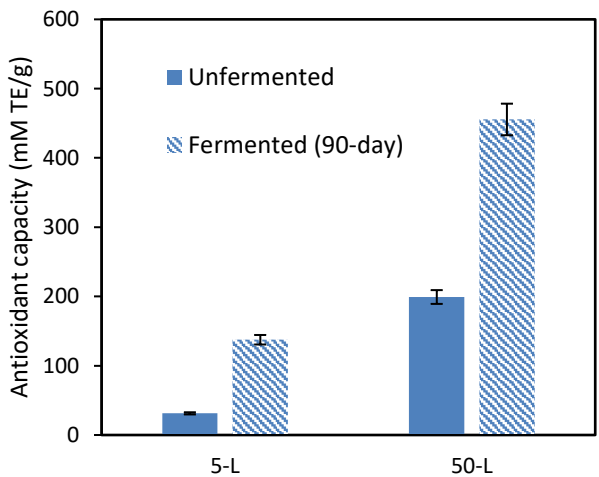


Figure 4: Antioxidant capacity of initial (day 0) and final (day 90) fermented CPL extracts from 5 L and 50 L fermenter

The increase in phenolic content, as mentioned earlier, was the suspected cause for the enhanced antioxidant capacity of the fermented CPL. Similar effect was observed in earlier studies on fermented *Myrtus communis* [9], cactus cladodes (*Opuntia ficus-indica L.*) [10] and *Echinacea spp* [11]. The breakdown of polyphenol matrix by the enzymatic action of microorgaisms caused the accumulation of compounds with antioxidant properties which contributed hydrogen atom for reduction of DPPH radical [24][4].

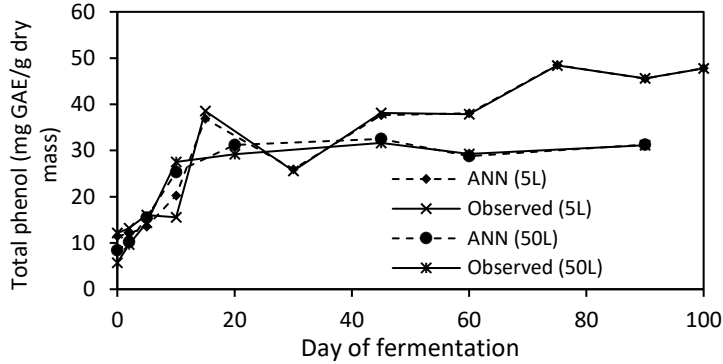
Microbial population dynamics

The population dynamics of different microorganism classifications displayed peculiar trends (as later observed in Figure 5 which compared the ANN predicted data and the experimental ones). The CPL fermentation was characterized by steep growth of presumptive lactic acid bacteria (LAB) as displayed by its viable colonies on MRS medium. The identities of LAB which predominantly *Lactobacillus plantarum* was confirmed by our earlier work [25]-[26]. In both 5-L and 50-L fermenters, LAB grew rapidly within a week of fermentation, then declined after day 10 and maintained its presence throughout the remaining fermentation period, as shown in Figure 5c. The presumptive *enterobacteriaceae*, of which mostly belong to Enterobacter and Cronobacter genera as reported by our earlier study, was only present at the early stage of fermentation before completely inhibited, as shown in Figure 5d. The prevalence of LAB was reported in some spontaneously fermented foods such as *sauerkraut* [27], *kimchi* [25], pickles [3], carrots [5], pineapple [28], *tempoyak* [29], *doklu* [30], cocoa bean [31] and leek [4]. LAB species were known as health-promoting probiotics which prevent diarrhea and colorectal cancer, stimulate immune system and hypocholesterolemia effect [32]. Their presence also conferred the microbiological safety of fermented products by inhibiting food pathogens [30], and potentially linked to total inhibition of *enterobacteriaceae* since the early stage of fermentation.

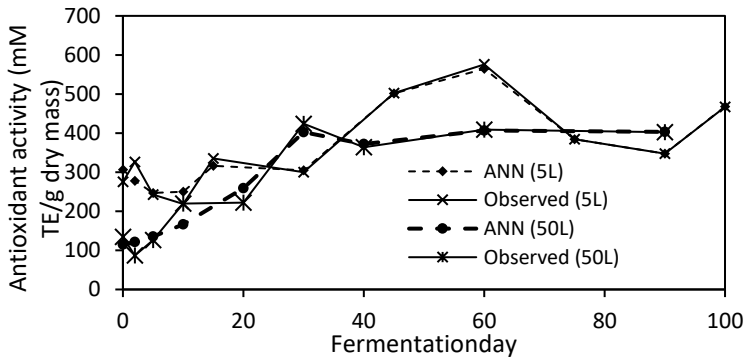
Yeast was absent at the onset of fermentation, but its population increased exponentially to its maximum within a week. Thereafter, the yeasts displayed steady presence throughout the fermentation period as shown in Figure 5e. Yeasts were known for their contribution to the sensory quality of fermented cocoa bean, which later impacted chocolate flavor [33] and soy sauce [34] favorably. The pleasant sensory quality was derived from the presence of alcohol and higher alcohols which were converted from sugar and primary metabolites such organic acids by yeasts metabolisms. It was also believed that yeasts modulated the pH value which otherwise would became too acidic and spoiled the taste due to accumulation lactic acid by LAB dominance [35].

The pH dropped rapidly from pH 6.0 at the onset of the fermentation to pH 3.0 at day 4 as shown in Figure 5f, and later persisted at pH 3.0 throughout the remaining fermentation.

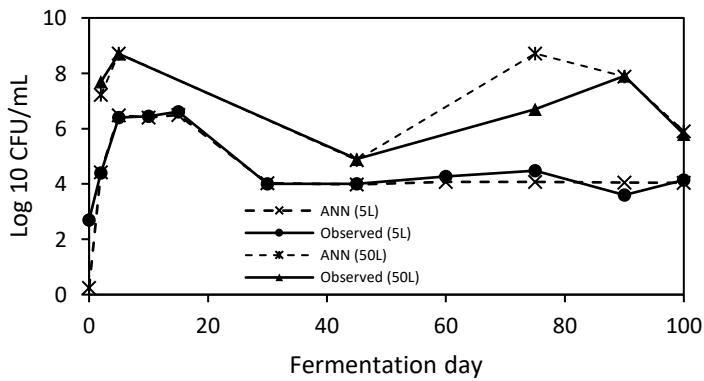
Other study surmised the coincidence of pH drop with the accumulation of lactic acid due to the rapid growth of presumptive LAB population [29].


Total phenol (mg GAE/g dry mass)
Day of fermentation
ANN (5L)
Observed (5L)
ANN (50L)
Observed (50L)

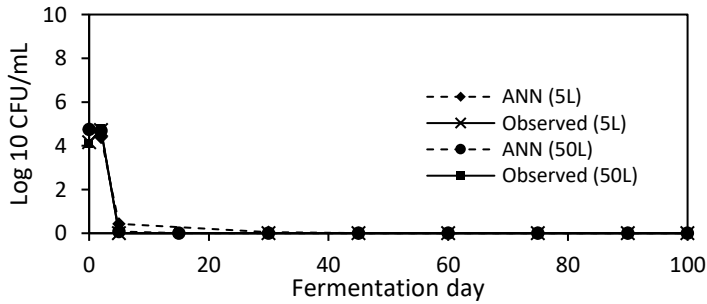
(a)


Antioxidant activity (mM TE/g dry mass)
Fermentationday
ANN (5L)
Observed (5L)
ANN (50L)
Observed (50L)

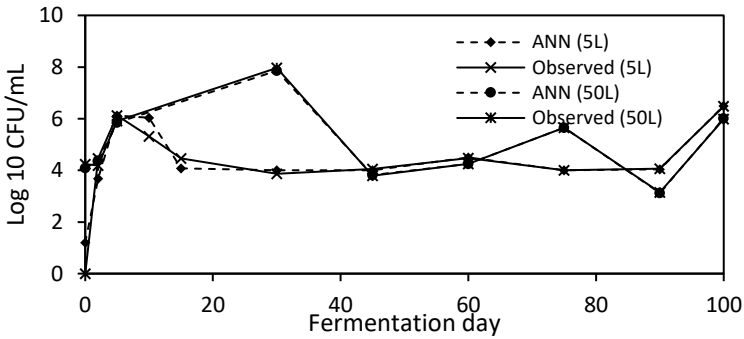
(b)


Log 10 CFU/mL
Fermentation day
ANN (5L)
Observed (5L)
ANN (50L)
Observed (50L)

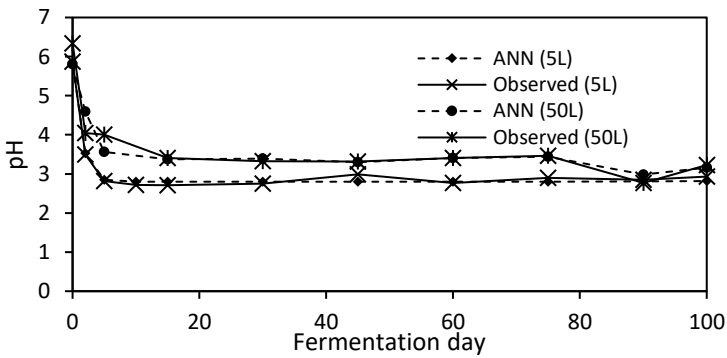
(c)



(d)



(e)



(f)

Figure 5: Comparison between experimental (solid line) and predicted (dashed-Line) of; a) total phenol b) antioxidant capacity c) LAB d) *enterobacteria* e) yeasts, and f) pH during spontaneous fermentation of CPL in 5-L and 50-L fermenters

Modeling of spontaneous fermentation responses with ANN

The ANN architecture which trained by of the first and second hidden layers of j = 7 and k = 7 as its number of neurons; respectively was selected as the best model to represent all process responses during spontaneous fermentation of CPL, except for presumptive *enterobacteriaceae* population where 10 neurons were used in each hidden layer).

During the training with 70% data set of each response, the network performance based on mean squared error selected as the best model. Generally, all selected ANN models displayed a very close proximity between the observed and predicted response data as shown in Figure 6. Accordingly, a statistical evaluation of each model in terms of R value (of greater than 0.9), low MSE and AAD (of less than 10%, which is acceptable for highly nonlinear responses) values as shown in Table 2, with the exception of TPC and antioxidant capacity. Furthermore, marginal difference of error between the observed and predicted values of testing data set and its training data set of each response, indicate the reliability of all the selected ANN models. In short these indications suggest the robustness of the selected ANN model to make prediction from known data (training data sets) as well as generalization of independent data (testing data sets) of the process responses during spontaneous fermentation of CPL.

The random distribution of the residual plots at zero horizontal line as shown in Figure 6 is indicative of the model robustness and impartiality, thus further confirming the predictive ability of the model.

The effect of fermentation on enhancing the TPC can be attributed to enzymatic action of the microorganisms during the fermentation. The role of microorganisms such as *Lactobacillus plantarum* during fermentation as reported in our previous work [13] may have caused the breakdown of glycosidic and ester bonds of polymeric phenols into free monomers, hence enriching the phenolic contents and antioxidant capacity [9]. This view was coherent with the predominance of phenolic acids and other flavonoids in spontaneously fermented Cornelian cherry as a result of metabolic activities during fermentation [7]. It was also believed that simpler compounds in fermented papaya were more bioavailable hence resulted in improved reductions in peripheral blood mononuclear cell cytolytic activity in tube-fed patients [8].

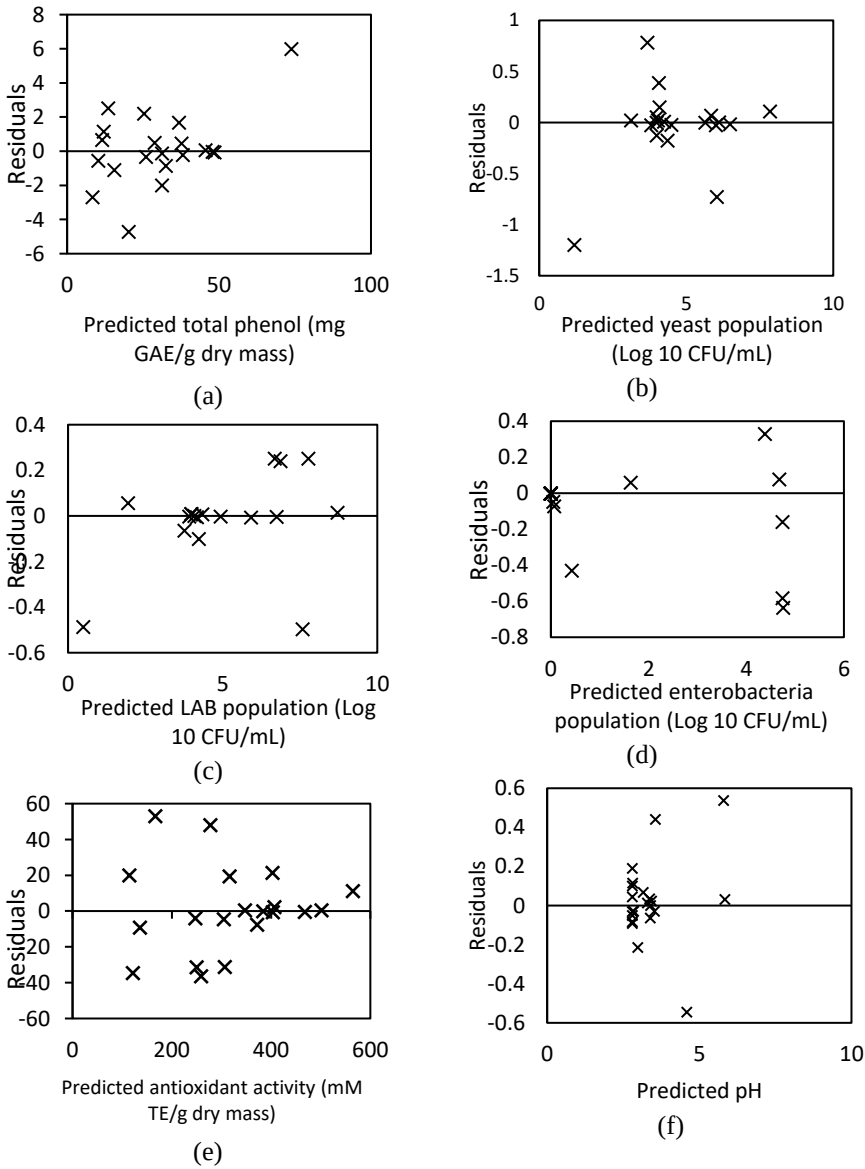


Figure 6: (a)-(f) predicted vs residual plots of responses

Table 2. Statistical measures and performance of feedforward algorithm for training, validation and testing data sets for process responses during spontaneous fermentation in terms of correlation coefficient (R), mean square error (MSE) and average absolute deviation (AAD).

Process response	ANN topology	R			MSE			AAD (%)		
		Training	Validation	Testing	Training	Validation	Testing	Training	Validation	Testing
Total phenol	2-7-7-1	0.992	0.948	0.985	4.132	16.740	9.781	6.88	14.2	12.5
Antioxidant capacity	2-7-7-1	0.998	0.962	0.993	59.8	$2.7x10^3$	499.8	1.4	14.4	8.4
Lactic acid bacteria	2-7-7-1	0.968	0.957	0.997	0.321	1.632	0.182	4.17	5.06	7.25
Enterobacteria	2-10-10-1	0.941	0.999	0.997	0.284	0.016	0.041	6.4	1.54	2.06
Yeasts	2-7-7-1	0.996	0.960	0.966	0.020	0.240	0.078	1.74	9.1	4.56
pH	2-7-7-1	0.999	0.994	0.996	0.003	0.0126	0.031	1.04	2.96	3.71

Conclusion

Spontaneous fermentation has benefited the CPL in terms of enhancing its phenolic contents and antioxidant capacity. Analysis on microbial population dynamic indicated the cultivation of presumptive LAB species, while the undesirable presumptive enterobacteria was completely inhibited since early stage of fermentation. The best network architectures which were trained by Levenberg-Marquardt training algorithm turned out to be 2-7-7-1 for all responses (except 2-10-10-1 for enterobacteria population). Their reliability to predict highly nonlinear responses of the fermentation process was demonstrated by correlation coefficient (R) of greater than 0.9, low MSE and AAD values.

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