TOWARDS THE DISCRIMINATION OF MILK (ORIGIN) APPLIED IN CHEDDAR CHEESE MANUFACTURING THROUGH THE APPLICATION OF AN ARTIFICIAL NEURAL NETWORK APPROACH ON *LACTOCOCCUS LACTIS* PROFILES

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ABSTRACT

An artificial neural network (ANN) that is able to distinguish between Cheddar cheese produced with milk from mixed and single breed sources was designed. Samples of each batch (4 pure Ayrshire/4 mixed with no Ayrshire milk) were ripened for 92 days and analysed every 14 days. A novel ANN was designed and applied which, based only on *Lactococcus lactis* counts, provided an acceptable classification of the cheeses. The ANN consisted of a multilayered network with supervised training arranged in an ordered hierarchy of layers, in which connections were allowed only between nodes in immediately adjacent layers.

Keywords: *Lactococcus*, neural network, cheddar cheese, Ayrshire milk, 16S rDNA

1.   INTRODUCTION

The deception of consumers regarding the origin of food products or the raw materials applied in the manufacturing thereof is a regular occurrence (Karoui and De Baerdemaeker, 2007; Pillonel et al., 2006; Sacco et al., 2005; Sacco et al., 2009). Consequently manufacturers and retailers rely on accurate characterization of products, frequently applying different analytical methods (Brescia et al., 2005; Luykx and van Ruth, 2008; Marilley and Casey, 2004; Sacco et al., 2005; Sacco et al., 2009). However, a product such as cheese poses unique challenges, as its character is influenced by complex biochemical and microbiological interactions that occur during production and ripening. Furthermore, the main properties and organoleptic quality of ripened cheeses such as Cheddar are largely due to differences (i.e., origin) in the raw material, milk, and key cheese manufacturing processes (Atasoy and Tnrkoglu, 2009; Hernandez et al., 2009; Hickey et al., 2006; Pappa et al., 2006). Therefore the qualification of authenticity requires a combination of selected analytical techniques combined with compound multivariate analysis, also known as chemometrics.

Several reviews on chemometrics have highlighted its application in the pattern recognition of selected product components in the battle against food adulteration

(Luykx and van Ruth, 2008; Pillonel et al., 2006a; Puerto et al., 2004). Pillonel et al. (2006a) defined chemometrics as the application of mathematical and statistical methods to maximize the chemical/biological information extracted from data. Methods commonly applied in chemometrics include discriminant analysis (DA), principal component analysis/regression (PCA/PCR), partial least square (PLS), and artificial neural network (ANN) (Barile et al., 2006; Dias et al., 2009; Pillonel et al., 2006a; Rodriguez-Nogales, 2006; Sacco et al., 2005).

Regardless of the compound/microbes analysed and the method applied to produce recognition patterns, subsequent model validation is essential and dynamic. Without validation, models applied in food authentication might result in acceptable clustering but may lack statistical significance. These models are usually overfitted or insufficiently adapted, and they may suffer from large deviations between the training (model) and validation (evaluation) sets. In many instances, these deviations occur as a result of compound/microbe concentration fluctuations due to varying production procedures or raw material consistency (Puerto et al., 2004; Rodriguez-Nogales, 2006).

Nevertheless, specific microbes and chemical compounds/parameters are commonly applied in the development of validated recognition patterns (models) of cheese and have successfully been applied in the authentication of Cheddar cheese age (Lues and Bekker, 2002). Such analyses usually include microbial cultivation followed by rRNA verification, chemical analysis through chromatography, nuclear magnetic resonance (NMR), classical methods, etc. and biochemical assays. These methods are employed mainly to yield profiles with the potential to solve selected problems in the dairy industry that are related to the assessment of Cheddar cheese quality (Karoui and De Baerdemaeker, 2007; Luykx and van Ruth, 2008; Marilley and Casey, 2004; Singh et al., 2003). For example, in cases where the denomination of cheese origin is protected, the origin of the milk used to manufacture these cheeses is verified (Barile et al., 2006; Luykx and van Ruth, 2008; Pillonel et al., 2006; Pillonel et al., 2006a; Sacco et al., 2009).

In addition to the traditional chemometrics approach to authenticating food, several reports on the application of statistical analysis in combination with ANN have appeared in recent years. Here, artificial intelligence (AI) or neural networks is applied as an engineering science whereby intelligent machines are created through the application of intelligent computer programs. The success of ANN relates to the application of AI, which simulates human intelligence without being confined to approaches that are biologically/humanly observable (Callan, 2003).

ANN is based on collections of nodes or neurons that are connected in a tree pattern to allow communication (Callan, 2003).

A single node is a simple processor, which computes by combining the input signals with an activation rule to produce an output signal (Figure 1) (Callan, 2003). These nodes are interconnected with weighted connections; where weight is a multiplying constant for the connection's input. In isolation these nodes are limited in operation; however, interconnections in a multilayered network provide them with the ability to perform complex tasks such as distinguishing between compound biological systems as they occur in cheese (Barile et al., 2006; Pillonel et al., 2006a).

The chemometrics approach to validating food authenticity relies mainly on multivariate statistical analysis (MSA) as an alternative to linear regression. This approach has been presented thoroughly in the literature (Brescia et al., 2005; Sacco et al., 2009). It has also been noted that ANN, in several cases, accomplishes food authentication to a greater and faster degree than any traditional chemometrics approach (Barile et al., 2006; Pillonel et al., 2006a).
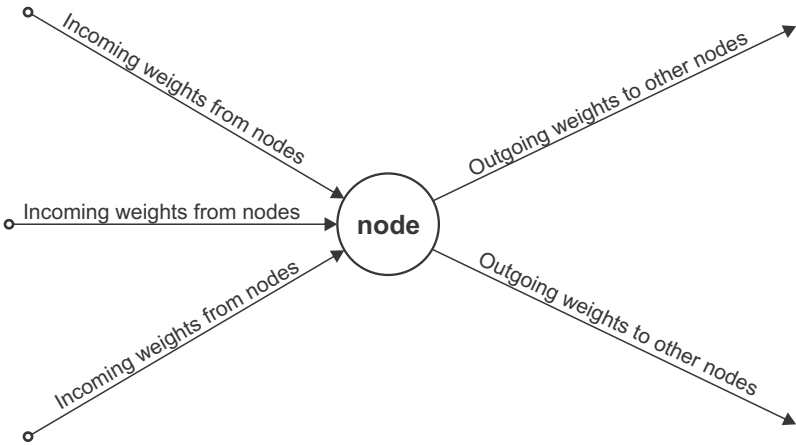
Figure 1: The layout of a single network node

Therefore, the present paper deals with the application of a custom-designed ANN to a selected relevant basic variable (assessable through standard laboratory analysis) to be applied in order to produce and validate a recognition pattern (model) that could be used to distinguish between Cheddar cheese that is produced with milk from mixed sources (i.e., different bovine breeds) and that which is produced exclusively from a single source (i.e., the Ayrshire breed). The selected variable was the biological indicator, *Lactococcus lactis*. This paper also sets the stage for retailers dealing in boutique dairy products to move towards the continuous application and validation of ANN models in their routine quality control and authentication strategies.

2.   MATERIALS AND METHODS

2.1   Cheese sampling

Sixty-four Cheddar cheese samples were supplied by a local manufacturer who specializes in the manufacturing of boutique cheeses. Thirty-two samples originated from whole Cheddar cheese produced with a mixture of bovine breeds' milk (excluding Ayrshire milk), while 32 samples originated from whole Cheddar cheese produced exclusively with Ayrshire milk. Manufacturing of both cheeses was standardized to limit product variability induced by the cheese-making process. After production the samples were repined at 10°C for 92 days and samples were collected for analysis at 14-day intervals.

2.2   Microbiological analysis

All media and reagents were obtained from Merck. One gram of cheese sample was finely ground using sterile equipment. The sample was blended in 9 ml Peptone buffer after which serial dilutions were prepared. Sterile M17 media were use for the cultivation of *Lactococcus* spp. Dilutions were plated on a solidified agar medium and these plates were incubated at 30°C for 48 hours after which the colonies were enumerated using a colony counter. Saline solution (1 ml) was added to one dilution plate from each sample containing 250 or more colonies and genomic DNA was extracted from the suspension using the MagPrep® Bacterial Genomic DNA kit (Novagen). The same kit was used to extract DNA from reference strains *Lactococcus lactis* subsp. *lactis* (ATCC 19435) and *L. lactis* subsp. *cremoris* (ATCC 19257), as well as lyophilized starter culture (0.16 g/ml saline). A region of the histidine biosysthesis operon of *Lactococci* was amplified using primer set LI-2F (5'-CTT CGT TAT GAT TTT ACA-3') and LI-2R (5'-CAA TAT CAA CAA TTC CAT-3') allowing differentiation between the two *L. lactis* subspecies based on different PCR product lengths (Corroler et al., 1998; Giraffa and Rossetti, 2004). PCRs were performed in a total volume of 25 l in a C1000 thermal cycler (BioRad) according to the protocols and reaction setups reported in the referenced literature. PCR amplicons were resolved by electrophoresis on 1% agarose gels in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) stained with 0.005% (v/v) GoldView (SBS Genetech).

2.3   Statistical analysis

Every analysis was conducted at least in duplicate, and the values reported are the means. Cheese samples were classified according to the milk source by designing and applying a novel ANN.

2.4   Artificial neural network design

A multilayered network with supervised training capable of learning a required function was designed. This was accomplished by calculating the error at each net or node followed by the adjustment of weights accordingly to produce all the required outputs. This process can be mathematically simulated with the formula of the neuron as follows:

$$net_j := \sum_{i=1}^{N} x_{i,j} w_{i,j}$$

where:
N is the amount of inputs
i is the node number for a specific input
j is the number of the net
x is the input value w is weights or constants

This is commonly put through a sigmoid function as follows:

$$f_j := \frac{1}{1 + \left[e^{(-net_j)} \right]}$$

where:
net is the output of the net
j is the number of the net

To calculate the error, the network applies a generalization of the delta rule by starting at the last layer with (Callan, 2003; Chauvin and Rumelhart, 1995).

$$\delta_j := \left(t_j - o_j \right) o_j \left(1 - o_j \right)$$

where:
t is the required output
o is the net output
j is the number of the net

Subsequently, the error at the hidden layers is calculated

$$\delta_j := o_j\left(1 - o_j\right)\sum_k \delta_k\, w_{j,k}$$

where :
o is the net output
j is the number of the net
k is the number of the net from where the error originates dk is the error from the previous layer
l is the number of that specific path

The weight change for each node is then calculated with

$$\Delta w_{i,j} := \eta \cdot \left(x_{i,j} \cdot \delta_j\right)$$

where:
h is the learning rate
i is the node number for a specific input
j is the number of the net
x is the input value d is the error from the each layer

Thereafter, the weights are adjusted as follows:

$$W_{i,j} := w_{i,j} + \Delta w_{i,j}$$

where:

Δw is the weight change
w is the old weights

A training data set that simulates a real-world problem was mapped. This training data set consisted of inputs with the corresponding outputs that were fed to the neural network for weight adaptation. Callan (2003) and Gurney (2003) posit that it is beneficial to randomize the order of the presentation for each training sample.

Finally a test data set, similar to a real-world problem, was given to the adapted neural network to verify whether sufficient generalization was possible (i.e., whether the network produced the correct output for the majority of input samples of the test data set). This implies that the network should produce smooth, non-linear mapping with the ability to interpolate non-exact samples. If the neural network were over-trained it would be like a memory looking up an output for input, with interpolation or prediction becoming impossible(Callan, 2003; Gurney, 2003). Therefore, similar to the requirement for traditional chemometrics approaches, accurate validation was fundamental to the success of ANN.

3. RESULTS AND DISCUSSION

The artificial neural network (ANN) has been successfully applied in food authentication. The application of ANN to sensory and chemical data for the classification of selected food products, such as wine (Cichelli et al., 2000; Perez-Magarino et al., 2004) and honey (Cordella et al., 2003) has been reported. In addition, there have been reports on the application of ANN to qualify dairy products based on chemical data, but with little reference to the successful application of ANN to biological indicators to authenticate Cheddar cheese manufactured from milk originating from cattle herds in an African environment.

Through the application of software, a functional neural network was designed that was able to associate the proliferation pattern of *Lactococcus lactis* (Table 1) in the cheese samples with the corresponding milk origin (pure Ayrshire/mixed with no Ayrshire). PCR analysis indicated *L. lactis* subsp. *lactis* as the prevalent *Lactococci* in all 64 cheese samples while *L. lactis* subsp. *cremoris* was detected only in the starter culture, but not during the cheese ripening process (Figure 2). Training phases were applied to optimize the ANN through adjustment of the parameters involved in the learning process. This was accomplished by dividing the data into a training set and an evaluation set. The data set would adjust the parameters and the evaluation set would evaluate whether a realistic prediction could be achieved. The results of the ANN analysis are presented in Table 2. The ANN design (Figure 3) and the related parameters involved in the learning process were selected exclusively on the basis of their ability to recognize and predict. The best results were obtained by sequentially optimizing the values of the learning rate and the momentum followed by the values of nodes in the hidden layer and the epoch quantity. Two hidden nodes with ca. 1×10^4 epochs yielded the best results (Figure 4). The authors sufficed with the network design, as it resulted in root-mean-square errors ranging from 0.2%–2.76% for the test sets and an overall (including the training sets) root-mean-squares error ranging from 0.92%–1.59%.
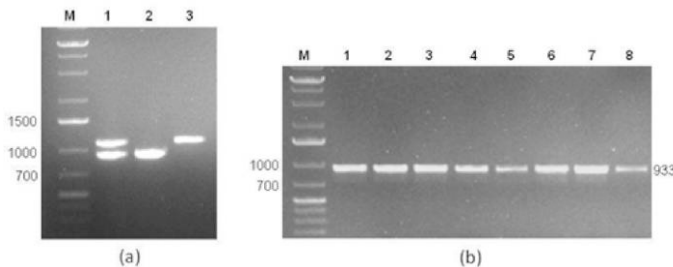

Figure 2: PCR products representing amplified regions of the histidine biosynthesis operon of *Lactococcus lactis* subsp. *lactis* (933 bp) and *Lactococcus lactis* subsp. *cremoris* (1100-1150 bp) for the starter culture (lane 1), *L. lactis* subsp. *lactis* (ATCC 19435) (lane 2) and *L. lactis* subsp. *cremoris*

(ATCC 19257) (lane 3) (a). PCR profiles indicating the presence of only *L. lactis* subsp. *lactis* (933 bp) for cheese batch A1 manufactured from Ayrshire milk during ripening days 2 (lane 1), 10 (lane 2), 22 (lane 3), 36 (lane 4), 50 (lane 5), 64 (lane 6), 78 (lane 7) and 92 (lane 8) (b). Lane M = molecular weight marker 1 kb plus DNA ladder (Fermentas).

Table 1: Values obtained from the quantification of *Lactococcus lactis* during the ripening of Cheddar cheese manufactured with milk originating from the Ayrshire breed only ($A_{(batch)}$) and with milk originating from a mixture of sources that excluded the Ayrshire breed ($G_{(batch)}$).

| Cheese batches	Input values for *L. lactis* (CFU/1x10^7)							
	Ripening (Days)							
	2	10	22	36	50	64	78	92
A$_1$	0.085	1.250	2.980	0.100	0.383	0.461	0.289	0.520
A$_2$	0.100	1.300	1.405	0.234	0.200	0.308	0.437	0.520
A$_3$	0.070	0.518	0.160	0.240	0.135	0.096	1.610	0.110
A$_4$	0.113	0.670	1.600	0.190	1.125	0.200	1.130	0.135
G$_1$	0.082	0.084	0.063	0.022	0.095	0.053	0.028	0.037
G$_2$	0.051	0.011	0.065	0.027	0.048	0.006	0.017	0.052
G$_3$	0.050	0.019	0.060	0.068	0.045	0.005	0.068	0.019
G$_4$	0.087	0.150	0.047	0.031	0.003	0.002	0.017	0.018
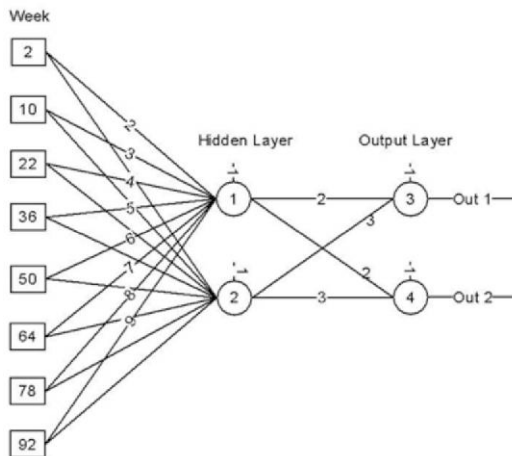
Figure 3: The multilayered network for the five analogue inputs to two nodes to the two outputs; designed to distinguish between the proliferation patterns of *Lactococcus lactis* during the ripening (week) of Cheddar cheese manufactured with milk originating from the Ayrshire breed only (Out 1) and with milk originating from a mixture of sources that excluded the Ayrshire breed (Out 2).

A multilayered network with supervised training arranged into an ordered hierarchy of layers, in which connections were allowed only between nodes in immediately adjacent layers, was coded for the evaluations (tests). Since there were two outputs (milk origin purely Ayrshire [A] or mixed [G]), it was decided to have two output nodes, connected to an input layer consisting of two nodes to which the inputs were connected. The network was designed with two layers of weights, as this kind of network is capable of approximating any continuous functional mapping (Bishop, 2005).

The inputs consisted of the corresponding data for *Lactococcus lactis* of either sample A or sample G as measured at weeks 2, 10, 22, 36, 50, 64, 78 and 92 of ripening of the Cheddar cheese (Table 1). The network was trained to yield as output one, a percentage of probability for G, and output two, a percentage of probability for A. The network was trained with a training set that consisted of A1, A2 and A3 (batches manufactured) and G1, G2 and G3 (batches manufactured) with their corresponding outputs. As stated, this was randomized. A4 and G4 were retained for an evaluation set. After evaluation the network was reset and trained with a training set that consisted of A2, A3 and A4 and G2, G3 and G4 with their corresponding outputs, which were also randomized. In this case, A1 and G1 were retained for an evaluation set. After this evaluation the network was reset and trained again with a training set that now consisted of A3, A4 and A1 and G3, G4 and G1 with their corresponding outputs. This was also randomized. Finally, A2 and G2 were retained for an evaluation set (Table 2).

The input range was of some concern, as the microbial counts in the cheese were relatively high. Since the network had a sigmoid transfer function that would work best between 0 and 1, the decision was made to divide the input values by 1x107. This resulted in input values <1. As the best resolution for a sigmoid output is obtained between 0.9 and 0.1, it was trained with the output ranges of 0.1 indicating a 0% probability and 0.9 indicating a 100% probability. To ease interpretation, the output was stepped through a function that would give the probability as a percentage. This function was as follows:

$$y = \frac{(x - 0.1)}{0.8} \times 100$$

where:
y was the output probability as a percentage
x was the output of the network in the range of 0.1 for 0% to 0.9 for 100%

Table 2: ANN results of the eight datasets considered individually (A$_{(batch)}$ – Cheddar cheese manufactured with milk originating from the Ayrshire breed only; G$_{(batch)}$ – Cheddar cheese manufactured with milk originating from a mixture of sources that excluded the Ayrshire breed). 1x10^4 epochs were maintained with = 0.5 throughout the study.

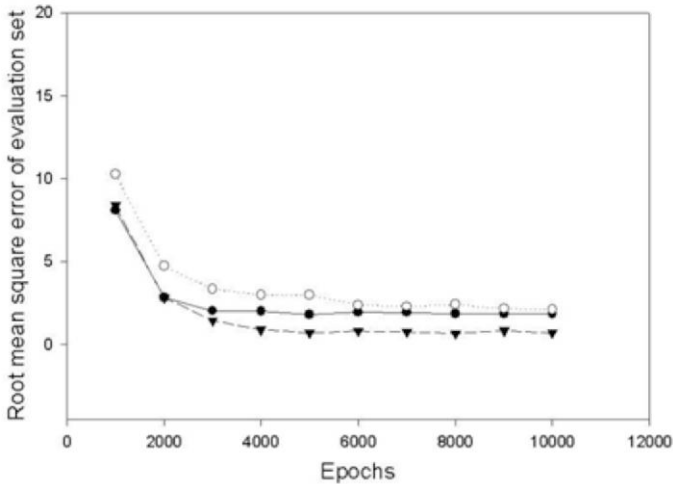| Input (training dataset)	Cheese batches	Input (testing dataset)	Recognition ability (%) (training set)		Recognition ability (%) (test set)	
			A	G	A	G
1$_a$	A$_1$		102	-2		
	A$_2$	A$_4$	98	2	99.8	0.2
	A		100	0.02		
1$_b$	G$_1$		0.3	100		
	G$_2$	G$_4$	-0.5	100	0.2	99.8
	G$_3$		-0.1	100		
2$_a$	A$_2$		99	0.5		
	A$_3$	A$_1$	101	-0.9	103	-3
	A$_4$		100	-0.04		
2$_b$	G$_2$		0.04	100		
	G$_3$	G$_1$	0.5	100	1	99
	G$_4$		-0.3	100		
3$_a$	A$_1$		102	-2		
	A$_3$	A$_2$	100	0.02	96	4
	A$_4$		99	1.24		
3$_b$	G$_1$		0.5	100		
	G$_3$	G$_2$	0.3	100	-0.2	100.2
	G$_4$		-0.5	101		

Figure 4. Performance of the training sets with *Lactococcus lactis* (●, ○, ▼ = training set 1, 2, 3). It should be noted that the recognition and prediction ability remained stable after 1x10^4 epochs. Satisfactory generalization without overtraining is also noted.

As mentioned, a network should not be over trained, as this would result in an inability to produce smooth non-linear mapping and to interpolate non-exact samples. It was noted that 1×10^4 training cycles (epochs) would give good generalization at a learning rate of 0.5 (Figure 4). Establishing the 1×10^4 training cycles took 0.6 seconds on a Pentium® 4 HT, 3.40 Ghz with 992 Mb of RAM.

Due to the inherent fluctuation of biological and chemical parameters between manufactured cheese batches, each batch was individually considered as a data set. As indicated in Table 1, the prediction ability of the network for the datasets was assessed by varying the data sets applied for training and tests. The noted number of epochs was sufficient to facilitate the desired classification of the cheese samples with no overtraining (also referred to as overfitting or overlearning, Figure 4). For the randomized training sets A_1-A_3 and G_1-G_3, A_4 and G4 were used as the evaluation set. The probability values of the training sets after 1×10^4 epochs ranged between 98.37–101.94 %. Thereafter the ANN was applied to recognize the test sets (A_4 = 99.79%; G_4 = 99.81%), successfully predicting the milk origin based only on the *L. lactis* counts.

Subsequent to the former evaluation, the ANN was reset and trained again with a training set that comprised A_2-A_4 and G_2-G_4 with their corresponding outputs. A_1 and G_1 were selected as test sets. The resulting probabilities for these training sets ranged from 99.50–100.88 %, which was acceptable. The resulting evaluation sets yielded 102.78% for A_1 and 99.02% for G_1; again with successful prediction parameters similar to those of the previous set. Finally, to confirm stability, the ANN was reset again and trained with data sets A_3-A_1 and G_3-G_1 (randomized) with their corresponding outputs. In this case A_2 and G_2 were applied as the evaluation sets. The prediction results were again within an acceptable range, thus confirming good generalization.

The ANN approach followed in this study was sufficient for the recognition/authentication of Cheddar cheeses manufactured from milk originating solely from the Ayrshire breed versus milk from a mixture of sources excluding the Ayrshire breed. ANN provided an acceptable classification of the cheeses based only on the proliferation pattern of the biological indicator *L. lactis* during cheese ripening. It is therefore argued that the superiority of the ANN-based approach is due to high prediction accuracy and the ability to compute the non-linear patterns produced during biological growth. The neural network created, trained and tested during this study ensured an objective and reliable authentication of the cheese samples. This approach sets the stage for retailers that deal in boutique dairy products to move towards the continuous application and validation of ANN models based on biological markers. However, the ANN presented in this paper will not be suitable for endpoint sampling, as growth was followed over 92 days. Therefore a network designed to incorporate more than one biological indicator in addition to chemical indicators should be considered.

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