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Suppressing *E. coli* activation in probiotic yogurt during fermentation by supplementing bitter melon (*Momordica charantia* L.) ethanol extract

Unterdrückung von E. coli in probiotischem Joghurt während der Fermentation durch den Zusatz von Ethanolextrakt aus Bittermelonen (Momordica charantia L.)

Selin Kalkan[1]), Selahaddin Batuhan Akben[2]), Demet Çanga[3])

Summary

In this study, the survival of *Escherichia coli* (ATCC® 25922™) in probiotic yogurt supplemented with or without *Momordica charantia* L. ethanol extract (MCE) during the fermentation period (0–300 minutes) was analysed for various inoculation levels (10^2; 10^4 and 10^6 log CFU/mL). So, the measured *E. coli* number was modelled as a function of fermentation time and the number of inoculated *E. coli*. The polynomials models were calculated separately for the probiotic yogurts with and without MCE added. Then, *E. coli* numbers predicted by the models were analysed using various scientific methods (Transforming predicted *E. coli* numbers into images, image processing, and hierarchical classification algorithms). The analysis results showed that the *E. coli* inactivation at between 2.44–3.47 log CFU/mL depending on inoculation levels was determined at the end of fermentation for probiotic yogurts supplemented with MCE. However, the determined inactivation for probiotic yogurts without MCE was between 1.84–2.99 log CFU/mL.

Keywords: Probiotic yogurt, antimicrobial, additive, hierarchical clustering, image processing

Introduction

Probiotics are living microorganisms that positively contribute to human health as they increase beneficial intestinal bacteria after antibiotic treatment, also they produce enzymes that help lactose digestion (Kalkan et al., 2019a). Furthermore, they prevent intestinal infections thanks to the production of organic acids and other antibacterial agents (Aryana and McGrew, 2007). However, the probiotics must reach the intestinal flora by exceeding stomach acid and bile salt and have a sufficient number of live microorganisms that can compete with the harmful flora when they reached the intestine, in order to show their targeted beneficial effect. So, the probiotics should be consumed together with some foods so that they can reach the intestinal flora in sufficient numbers. Since yogurt and yogurt-derived products are capable of exceeding bile salt and stomach acid, they help to overcome these problems of probiotics, and therefore the probiotic bacteria are usually sent with them to the intestinal tract. The most common probiotic microorganisms found in yogurt and yogurt-derived products are *Lactobacillus* spp., (*L. johnsonii, L. paracasei, L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. rhamnosus*), and *Bifidobacterium* spp. (*B. bifidum, B. animalis* ve *B. longum*), *S. thermophilus*) (Adolfsson et al., 2004; Wassenaar and Klein, 2008; Hill et al., 2017; Kalkan et al., 2019a).

Yogurt has also a bio protective feature as it contains lactic acid bacteria that exhibit a broad antimicrobial activity against many foods that originated pathogenic bacteria such as *Aeromonas hydrophila, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Proteus* spp., *Salmonella* spp., *Bacillus* spp., and *Streptococcus* spp. (Taş et al., 2017). In addition, the growth of acid-sensitive bacteria is prevented thanks to the decreasing pH during the production (fermentation) of yogurt, and yogurt becomes antimicrobial since the pH level is low at the end of production. In summary, yogurt is beneficial and reliable food for human health besides its carrier role for probiotics. However, some highly resistant pathogenic bacteria can still survive at the end of fermentation, despite the acidic nature of yogurt (Savran and Halkman, 2017). Many studies on pathogen development during the fermentation and storage of yogurt in the literature also support this phenomenon (Kost et al., 1990; Gulmez and Guven, 2003; Kasımoğlu and Akgün, 2004).

It has been reported in many studies in the literature that certain strains of *E. coli* are one of the highly resistant pathogenic bacteria and can still survive at the end of fermentation. For example, in a study conducted in 1993, the development of HUS (Hemolytic-uremic syndrome) was observed after commercial yogurt consumption, and the cause was determined as *E. coli* O157: H7 (Morgan et al., 1993). In the continuation of this study completed in 1993, it has also been determined by various studies that *E. coli* which is one of the important food-borne pathogens together with *L. monocytogenes* and *Y. enterocolitica,* can cause foodborne diseases through the consumption of contaminated milk and fermented milk products including yogurt (Nataro and Kaper, 1998; Gulmez and Guven, 2003). These studies also showed that it is necessary to be careful especially against *E. coli* contamination in terms of hygiene during the yoghurt production stages such as heat treatment and packaging. However, although producers are cautious at these stages, yogurt may still contain pathogens such as *E. coli* and *S.* Enteritidis due to some reasons such as the high microbial load of raw milk, automation error causing insufficient heat treatment, and secondary contamination during packaging (Savran and Halkman, 2017). On the other hand, the pasteurization process applied to destroy pathogens and other microorganisms in raw milk cause the sensory and nutritional properties of milk to change (Ozcan and Kurtuldu, 2011). Therefore, the method to be used should inactivate the pathogens and also not change the sensory and nutritional properties.

Herbal extracts are among the important alternatives that can be used for this purpose due to their natural origin, their unique flavours and aromas, and their wide bioactivity profile. Because, studies in the literature have reported that herbal extracts have a large number of positive biological activities, including potential anticarcinogenic effects, in terms of human health (Erturk and Demirkol, 2014). There are some products on the market, such as lemongrass extract or apple/green tea supplemented fermented milk, but previous academic studies have shown that other herbal extracts can also be added to these foods (Granato et al., 2018). For example, bitter melon (*Momordica charantia* L.), a member of the Cucurbitaceae family is one of them since it has therapeutic benefits such as anti-diabetic, antioxidant, antiviral, and antineoplastic and is currently produced worldwide (Gover and Yadav, 2004; Beloin et al.,2005; Semiz and Sen, 2007; Paul and Raychaudhuri, 2010). Also, it has been reported in some literature studies that *Momordica charantia* L. seed and seed oil ethanol extracts and acetic acid-water ethanol and hexane extracts are antimicrobial against *E. coli, S. aureus, Salmonella enterica* subsp. *enterica* serovar Typhi, and *A. niger* (Yaldız et al., 2015; Engin et al., 2018). However, the antimicrobial effect of *Momordica charantia* L. ethanol extract against possible *E. coli* (ATCC® 25922™) contamination that may occur during the fermentation of the prebiotic yogurt has not been investigated yet.

To overcome this shortcoming of the literature, the survival of *E. coli* (ATCC® 25922™) in probiotic yogurt with or without *Momordica charantia* L. ethanol extract (MCE) during the fermentation period (0–300 minutes) was analysed for various contamination levels (10^2; 10^4ve 10^6 log CFU/mL) in this study.

Materials and Methods

Material

The probiotic yogurt culture used in the study was the mixed culture consisting of *Lactobacillus delburueckki* spp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium animals* ssp. *lactis*. The culture was obtained from the „Doğadan Bizim" Food and Dairy Products Industry and Trade Limited Company (Istanbul, Turkey)" as a lyophilized powder culture.

Escherichia coli (ATCC® 25922™) was obtained from Giresun University Food Engineering Department Culture Collection. *E. coli* was used after activating overnight at 37°C on the tryptic soy (CASO) broth (TSB; Merck 1.05459, Germany) medium.

The bitter melon (*Momordica charantia* L.) used in this study was obtained from Anamur county of Mersin city in Turkey. The product has been carefully cultivated for the experiment of this study, so no chemical fertilizer has been used during cultivation. The ripe fruits were harvested as fully yellow. 20 specimens were mixed in order to obtain a

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homogenate sample. It was transferred to Giresun province with a cold chain from the harvest to extraction.

Bitter melon (*Momordica charantia* L.) extraction

Ethyl alcohol (80% w/w) extraction was carried out for melon ground up and dried at 40 °C. Extraction was continued at room temperature for 24 hours using an orbital shaker. Plant extraction was carried out three times. After extraction, filtration was carried out first and then the filtrates were dried by evaporation (Engin et al., 2018).

Probiotic yogurt production and *E. coli* inoculation

Raw milk samples used in the study were obtained from the Giresun city of Turkey. Samples were brought to the laboratory with a cold chain and used in yogurt production. The chemical composition of raw milk expressed in average weight percentage was 12.16±0.18 of dry matter, 3.29±0.13 of fats, 3.15±0.08 of proteins, 0.77±0.01 of ashes, and pH value was 6.58±0.06. For the production of probiotic yogurt, a mixture of raw milk and milk powder (3%; Bağdat Baharat, Ankara, Turkey; 0.86 g fat/100 g, 34.83 g protein/100 g, 8.20 g/100 g ash, 53.43 g carbohydrate/100 g) was heat-treated for 10 minutes at 85 °C. After heat treatment, the temperature of the 20 L milk was reduced to 43 °C and a 4%probiotic yogurt culture (800 mL pasteurized milk containing 10^7–10^8 log CFU/mL lyophilized commercial culture) was added to the milk at this temperature. Simultaneously, 0.02 mL, 20 mL, and 200 mL of overnight culture of *E. coli* (~ 10^8 log CFU/mL) were added to obtain an *E. coli* inoculum of 10^2, 10^4, and 10^6 log CFU/mL approximately in the milk before fermentation. The ratio of bitter melon (*Momordica charantia* L.) ethyl alcohol extract (MCE) added to probiotic yogurt is 1% (for 0.01 g extract to 1 g yogurt sample). Milk samples to be used in the production of probiotic yogurt with and without MCE supplementation were mixed using a magnetic stirrer then 50 mL of them were distributed to 100 mL containers and incubated at 43 °C until the desired acidity level (4.2–4.5 pH) was reached. Experiments of each trial set (samples containing *E. coli* at an inoculum level of 10^2, 10^4, and 10^6 log CFU/mL and 1% MCE) were carried out at different times and the experiments were carried out with three repetitions.

Microbiological analyses

The number of *E. coli* was measured at 30-minute intervals from the start during fermentation. Before the microbiological analysis, the samples were mixed enough, and they were made homogeneous. Afterward, 10 mL samples were added to dilution liquid containing 90 mL maximum recovery diluent (MRD, Merck 1.12535, Germany) in aseptic conditions, and 10^{-1} dilution was obtained. Serial dilutions were prepared by adding 1 mL of the 10^{-1} dilution into the dilution liquid containing 9 mL of MRD.

Depending on the initial *E. coli* inoculation levels (10^2, 10^4, and 10^6 log CFU/mL), the dilution process was continued. Two successive dilutions were used. The spreading plate method was used for *E. coli* count. So, a 0.1 mL sample was added to Petri dishes containing Violet Red Bile Glucose Agar medium (VRBG Agar Merck 1.10275, Germany) then these Petri dishes were incubated at 37 °C for 24 hours. For *E. coli* counting, parallel cultivation was made to 2 Petri dishes from each dilution. The number of bacteria was calculated according to the standard count method (AOAC, 1994) and the results are expressed in log CFU/mL.

pH measurement

The pH values of samples were also measured immediately after completing the microbiological analysis. The pH measuring process was carried out using the pH meter (Ohaus Starter 2100 F, New Jersey, USA).

Transforming data into image

Analysis of the dependent variable values obtained by experiment or dependent variable values predicted using polynomial models by transforming them into images is a new method presented to food engineering literature recently to provide visual analyse (Akben et al., 2017; Akben, 2018; Gamlı et al., 2018; Kalkan et al., 2019).

The method first normalizes the data to between 0–$2^{\text{Bit Depth}-1}$ and creates the image I by the equation $I = 2^{\text{Bit Depth}-1} \times \text{Data}/\max(\text{Data})$. The bit depth value determines number of values to be matched to gray tones. In this study, the bit depth value was chosen to obtain images size of 500×500 (0.25 Megapixel). Then shades of gray (gray tones) are assigned to data proportionally to these values from 0 to $2^{\text{Bit Depth}-1}$. In this case, 0 is represented by black, 2 is represented by white and the other values between 0 and $2^{\text{Bit Depth}-1}$ are represented by other gray tones. Gray tones can also be matched to the tones of the colours are navy-blue, blue, cyan, green, yellow, orange, red, and dark red for better visuality (Çelik et al., 2019).

Then, resulting image models can be analysed more easily by applying image processing methods. For example, in this study, histogram stretching was applied to the data predicted by polynomial models since the dependent variable values ranged from 0–6.5 log CFU/mL, thus the number of colour-tones was reduced to show statistically significant changes of data. However, how many colour-tones the images should consist of is an important issue. In the previous studies, the value of 8 was proposed heuristically to solve the problem. Although this value is a guaranteed choice, another problem is that the data with a wide range are represented as a single colour-tone. To solve this problem, in this study, the number of colour tones was determined by the agglomerative hierarchical clustering method which is frequently used in other fields but will be used for the first time in food engineering literature (Contreras, 2015).

Agglomerative hierarchical clustering

Agglomerative Hierarchical Clustering is a method that groups (clusters) the elements of the data set according to their similarities. It's also known as AGNES (Agglomerative Nesting). In this study, it was used to represent the predicted dependent variable values without statistically significant difference between them as single colour-tone in image models. In other words, it was used to determine how many colour-tones the images should consist of. Thus, the colour variations in the images and the variation of corresponding dependent variable values were easily analysed.

The method accepts each element in the data set as a cluster initially and creates in the first step a new cluster by combining the two existing clusters having the maximum similarity. Thus, the number of clusters in the data set decreases by one in the first step. In the further steps, the same process is repeated for new clusters until one cluster is obtained (Kaufman and Rousseeuw, 2018).

The similarity values used to obtain a new cluster from two clusters are recorded for each clustering step while performing the method. The recorded similarity values can also be visualized by a dendrogram plot to analyse the

clustering (Espinoza et al., 2012). The method can be terminated if the similarity value to be used to obtain a new cluster is higher than the selected threshold (cut-off) value. Then the number of clusters obtained until the termination is determined as the number of clusters in which the data set should be divided. As a result, it can be said that there is no statistical difference between the elements of the clusters obtained until the termination of steps because their similarity measure is less than the threshold value and they are similar to each other statistically. However, the selected threshold value must depend on statistical rules. The measure of similarity used in this study is the Euclidean distance between cluster centers because the aim is to include the data set elements in the nearest cluster according to the difference of their values and the value difference of elements is determined by the Euclidean distance. The threshold value selected in this study is the sum of the mean and standard deviation of the recorded Euclidean distances until a single cluster is obtained. Because if a cluster's distance to other clusters is greater than this threshold value, there is a statistically significant difference between that cluster and other clusters and that cluster should not be included in another cluster.

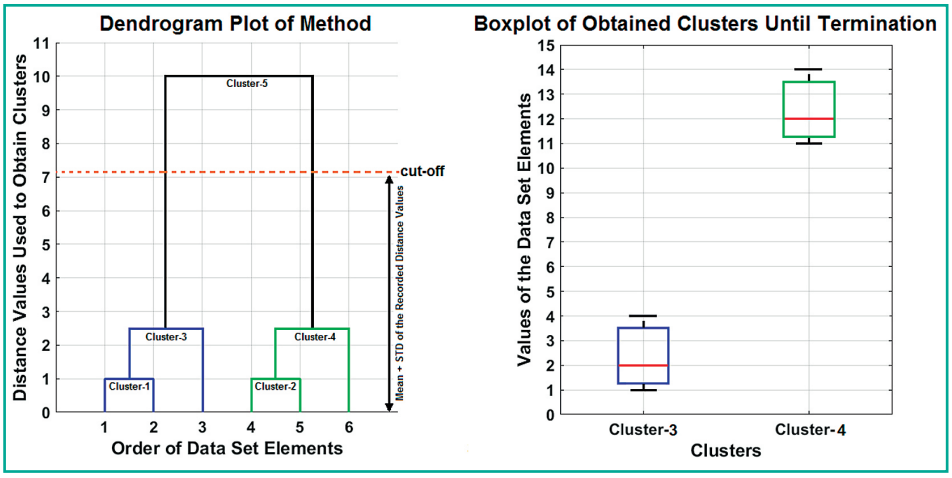

FIGURE 1: *Clusters and dendrogram plot obtained by applying the hierarchical clustering method to the sample data set for a better understanding of the methods and aim.*

For better understanding of the AGNES, the sample data set X = x_1, x_2, x_3, x_4, x_5, x_6 = 1, 2, 4, 11, 12, 14 was produced synthetically, then the result of the method was described on the dendrogram plot shown in Figure 1. In this study, AGNES will be applied to dependent variable values predicted by polynomial models instead of sample data set X.

Results and discussion

The data measured in the experiment can be seen in Table 1. In the study, the number of *E. coli* in probiotic yogurts with and without MCE was modelled based on fermentation time and the number of inoculated *E. coli*. Thus, two surface equations (quadratic polynomial surface equations) were created for probiotic yogurts with and without MCE as in Table 2.

Although the fit coefficients of the surface equations are high, it is difficult to examine the *E. coli* change during the fermentation process using equations. Therefore, *E. coli* in probiotic yogurt predicted by equations have been transformed into images and a visual evaluation facility has been provided. Images consisting of 256 colours in Figure 2 represent the number of *E. coli* predicted using the equations in Table 2.

As seen in Figure 2-a and Figure 2-b, the vertical order of colours is the same at the start of fermentation, but different at the end of the fermentation. For example, Figure 2-b has only navy blue and blue colours at the end of fermentation while Figure 2-a has navy blue, cyan, and green colours. This colour difference occurred at the end of fermentation means that the number of *E. coli* represented by colours is also different. That is, the addition of MCE to probiotic yogurt before the fermentation has changed the number of survived *E. coli* at the end of the fermentation process. How-

TABLE 1: *Data measured in experiment (Data are average of repeated measurements).*

Measurement time (Minutes)	Inoculated *E. coli* (Log CFU/mL)	Measured *E. coli* (Log CFU/mL)		Measured pH	
		Without MCE	With MCE	Without MCE	With MCE
0	2	2.36	2.40	6.42	6.54
30	2	2.40	2.44	6.23	6.32
60	2	2.51	2.55	6.12	6.20
90	2	2.53	2.53	5.71	5.89
120	2	2.47	2.44	5.55	5.52
150	2	2.27	2.23	5.32	5.20
180	2	2.21	2.09	5.12	5.10
210	2	1.94	1.79	4.82	4.80
240	2	1.30	1.14	4.79	4.66
270	2	0.90	0.65	4.63	4.52
300	2	0.52	0.22	4.52	4.49
0	4	4.66	4.52	6.48	6.50
30	4	4.68	4.70	6.32	6.30
60	4	4.84	4.87	6.10	6.17
90	4	4.94	4.91	5.75	5.89
120	4	4.50	4.41	5.62	5.63
150	4	4.08	3.92	5.23	5.30
180	4	3.62	3.39	5.05	5.00
210	4	3.00	2.69	4.50	4.75
240	4	2.89	2.53	4.65	4.65
270	4	2.42	2.02	4.52	4.40
300	4	1.98	1.51	4.38	4.30
0	6	6.07	6.10	6.45	6.50
30	6	6.17	6.19	6.38	6.33
60	6	6.25	6.28	6.15	6.10
90	6	6.32	6.29	5.82	5.92
120	6	5.39	5.32	5.60	5.72
150	6	5.09	4.93	5.15	5.12
180	6	4.68	4.41	5.00	5.00
210	6	3.99	3.67	4.75	4.78
240	6	3.71	3.24	4.50	4.52
270	6	3.18	2.66	4.40	4.49
300	6	3.08	2.55	4.35	4.38

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TABLE 2: *Surface equations predicting the change of E. coli counts during the fermentation time.*

Predicted *E. coli* value in probiotic yogurt without MCE (*P*=Predicted *E. coli* ; *t*=Fernentation Time ; *i*=Inoculated *E. coli*)
R^2: 0.9881 **; Adj-R^2:** 0.9865 **; SSE:** 2.1988 **; RMSE:** 0.2405
$P(t,i) = -0.26682 + 0.00725 \times t + 1.14208 \times i - 2.65932 \times 10^{-5} \times t^2 - 0.00196 \times t \times i - 0.00694 \times i^2$
Predicted *E. coli* value in probiotic yogurt with MCE (*P*=Predicted *E. coli* ; *t*=Fernentation Time ; *i*=Inoculated *E. coli*)
R^2: 0.9827 **; Adj-R^2:** 0.9804 **; SSE:** 3.2407 **; RMSE:** 0.2920
$P(t,i) = -0.25628 + 0.00775 \times t + 1.12321 \times i - 3.01229 \times 10^{-5} \times t^2 - 0.00223 \times t \times i - 0.00091 \times i^2$

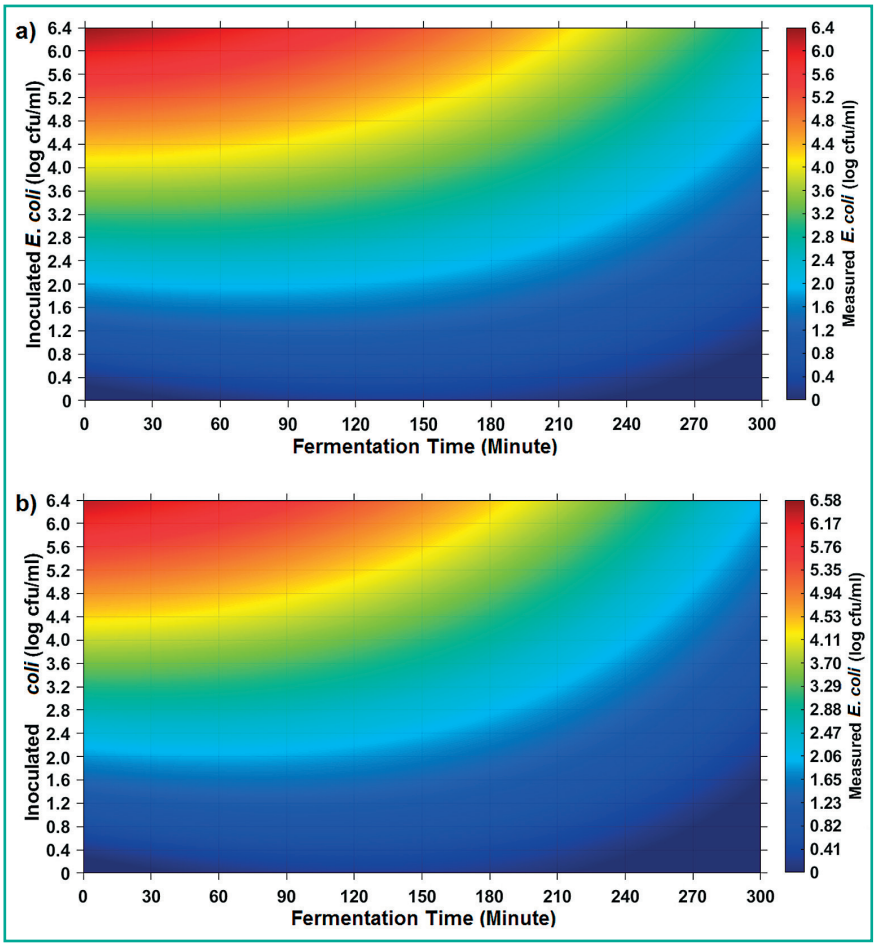

FIGURE 2: *Predicted E. coli in probiotic yogurts with and without MCE depending on fermentation time and inoculated E. coli a) Probiotic yogurt without MCE b) Probiotic yogurt with MCE.*

ever, the number of colours in the images has been reduced by using fewer bits to obtain more evident analysis and to determine whether the *E. coli* difference that occurred at the end of fermentation is meaningful. How many colours should the images consist of was determined using hierarchical clustering. Therefore, the hierarchical clustering was applied to the predicted *E. coli* values, and it was determined that both images should be consisting of 16 colours. In Figure 3, images are consisting of 16 colours. In the images in Figure 3, the values of two or more *E. coli* are not statistically different if they are represented by single colour or shades of the single colour.

As seen in Figure 3, the addition of MCE caused a statistically significant decrease in the number of *E. coli* at the end of fermentation. For example, *E. coli* between 2–2.4 log CFU/mL represented by cyan were inoculated at the start of fermentation as can be seen in Figure 3-a. At the end of the fermentation, the number of *E. coli* was reduced to between 0.4–0.8 log CFU/mL represented by shades of blue. However, the situation is different in Figure 3-b. Although *E. coli* between 2.06–2.47 log CFU/mL represented by cyan was inoculated to probiotic yogurt at the start of fermentation, the number of *E. coli* was reduced to between 0–0.41 log CFU/mL represented by navy blue at the end of fermentation. In other words, although the number of *E. coli* inoculated at the start of fermentation is almost the same, MCE provided an additional reduction of *E. coli* by about 0.4–0.5 log CFU/mL at the end of fermentation. If the same analysis is made by comparing the colours at the start of fermentation and the colours at the end of fermentation, it can be seen that the addition of MCE has an additional inactivation effect between 0–0.55 log CFU/mL. This inactivation effect varies according to the amount of inoculated *E. coli.*

To better see the relationship between this inactivation effect and the duration of fermentation and the inoculated *E. coli,* the difference of *E. coli* values measured from probiotic yogurts with and without MCE addition was modelled as in Equation-1 depending on the fermentation time and inoculation (R^2=0.99; Adj-R^2=0.99; RMSE=0.0110; SSE=0.5311). Then, the values predicted by the model were transformed into an image that can be seen in Figure 4-a. The image in Figure 4-b represents the same values as the image in Figure 4-a, but it has 16 colours since the number of colours should be in Figure4-b was determined as 16 using the hierarchical clustering method.

$$E(t,i) = 3.9690 \times 10^{-13} - 2.6690 \times 10^{-13} \times t + 0.0126 \times i + 1.5340 \times 10^{-15} \times t^2 + 3.4370 \times 10^{-4} \quad (1)$$

As seen in Figure 4, the addition of MCE reduces the number of inoculated *E. coli* at the end of fermentation up to 0.52 log CFU/mL. This reducing effect starts at the 75th minute of fermentation since from this minute there has been a visible change in colours. In addition, if the number of inoculated *E. coli* at the start of fermentation is between 0–4 log CFU/mL, the surviving *E. coli* numbers at the end of fermentation are linearly proportional to the inoculated *E. coli* numbers. However, if the number of *E. coli* inoculated at the beginning of fermentation is 4 log CFU/mL or above, the surviving *E. coli* numbers at the end of fermentation are almost the same. This antimicrobial effect determined is due to many reasons and these results are consistent with the results of the studies in the literature.

It is known that probiotics have many features used for pathogenic microorganism inhibition. Some of these are

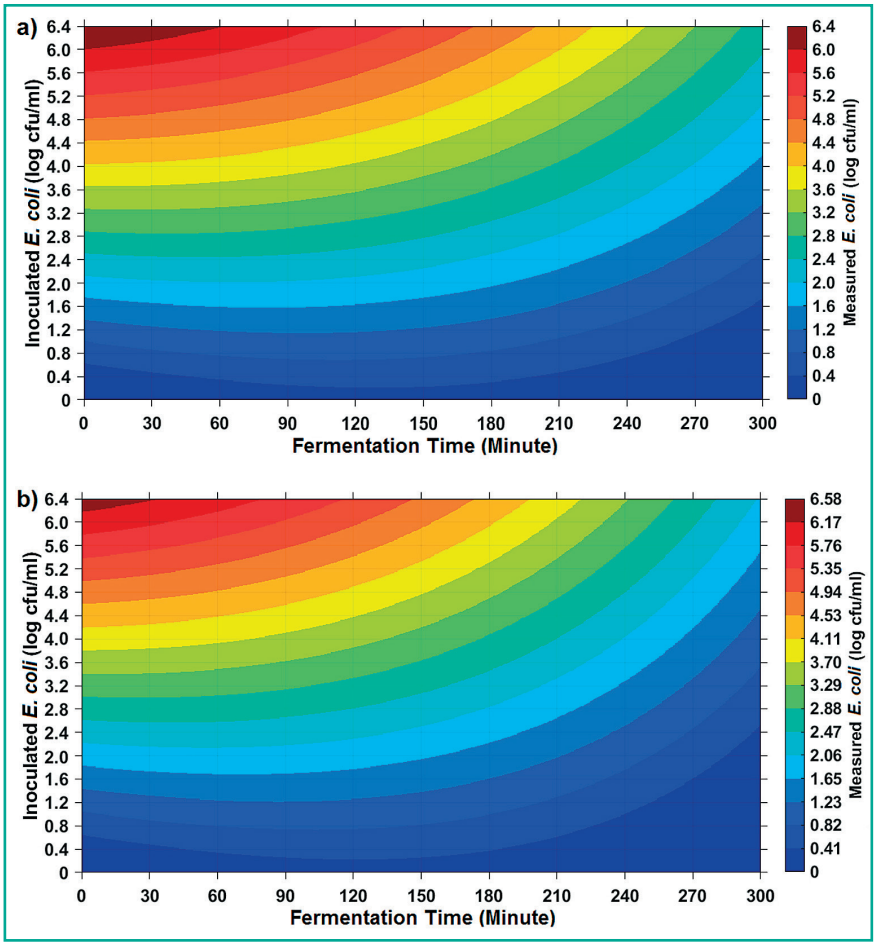

FIGURE 3: *4-bit image models representing the change in the number of E. coli in yogurt during the fermentation time a) Probiotic yogurt without MCE b) Probiotic yogurt with MCE.*

lowering the pH of food by producing lactic acid, competing for food sources by clinging to the receptors, producing antimicrobial compounds such as microsine, hydrogen peroxide, and free radicals (Kalkan, 2016; Wan et al., 2016). In particular, organic acids such as acetic acid and lactic acid are thought to have a strong inhibitory effect against gram-negative bacteria and are the main antimicrobial compounds responsible for the inhibitory activity of probiotics against pathogens (Bermudez-Brito et al., 2012). Therefore, the *E. coli* inactivation obtained at the end of the fermentation process is mainly due to the features of probiotics to inhibit pathogenic microorganisms. Also, the microorganism inhibition (up to 4 log CFU/mL depending on inoculation, as shown in Figure 1-a and Figure 2-a.) obtained by only probiotics in yogurt is as expected, because it is consistent with the results of the studies in the literature. For example, it was determined in a study completed by El-Kholy et al. (El-Kholy et al., 2014)that traditional yogurt containing *L. acidophilus* and *B. longum* had an inhibitory effect against *E. coli* O157:H7 and *S. aureus* during fermentation and storage. Similar to our study, researchers found a decrease of approximately 5 log unit in the number of *E. coli* O157:H7 during the 15-day fermentation process. In another similar study, it was observed that *L. casei* used as a probiotic strain could inhibit the growth of *E. coli* O157:H7 and *S. aureus* during fermentation of milk about 1–5 log unit depending on inoculation (Kamal et al., 2016).

An additional inhibition (up to 0.5 log CFU/mL depending on inoculation, as shown in Figure 4-a and Figure 4-b) was achieved by using MCE in this study. This additional inhibition is due to the bioactive compound content of the MCE. This finding is new since there is no *in vivo* study using the *Momordica charantia* L. plant in the literature. On the other hand, there are *in vitro* studies using the *Momordica charantia* L. plant in the literature. In these *in vitro* studies, antimicrobial and antioxidant activities were generally determined after extraction of the plant with solvents (Roopashree et al., 2008; Braca et al., 2008; Coutinho et al., 2010; Abalaka et al., 2011; Islam et al., 2011; Shan et al., 2012; Saengsai et al., 2015; Svobodova et al., 2017; Acet et al., 2019; Ozcan, 2019; Ozcan, 2020). For example, it was determined in a study completed by Engin et al. that the hexane, ethyl alcohol, and acetic acid-water extracts of the *Momordica charantia* L. plant showed antimicrobial effects against 12 pathogenic microorganisms including *E. coli* (Engin et al., 2018). In other studies, in the literature, it was reported that these extracts also have antibacterial effects against *E. coli, S. aureus, Salmonella* Typhi, and *P. aeruginosa* (Jabeen and Khanum, 2017) and against *A. niger,* and *C. albicans* (Yaldız et al., 2015). As seen from the *in vitro* studies in the literature, *M. charantia* has antibacterial activity and our results are supported by the previously accumulated knowledge of these studies in literature that. In addition, it can be thought that *M. charantia* may have antimicrobial effects against *Pseudomonas aeruginosa, Sta-*

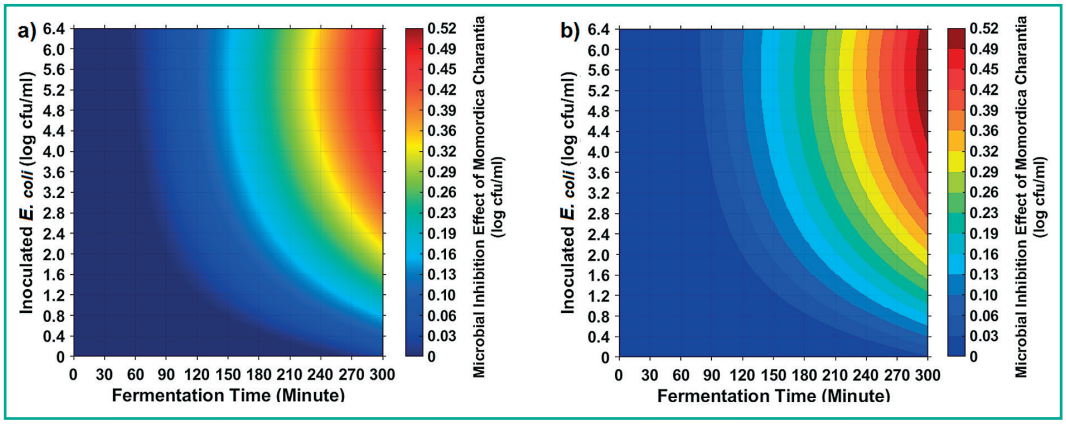

FIGURE 4: *Reduction amount of E. coli caused by the supplementation of MCE a) 16-bit image model b) 4-bit image model.*

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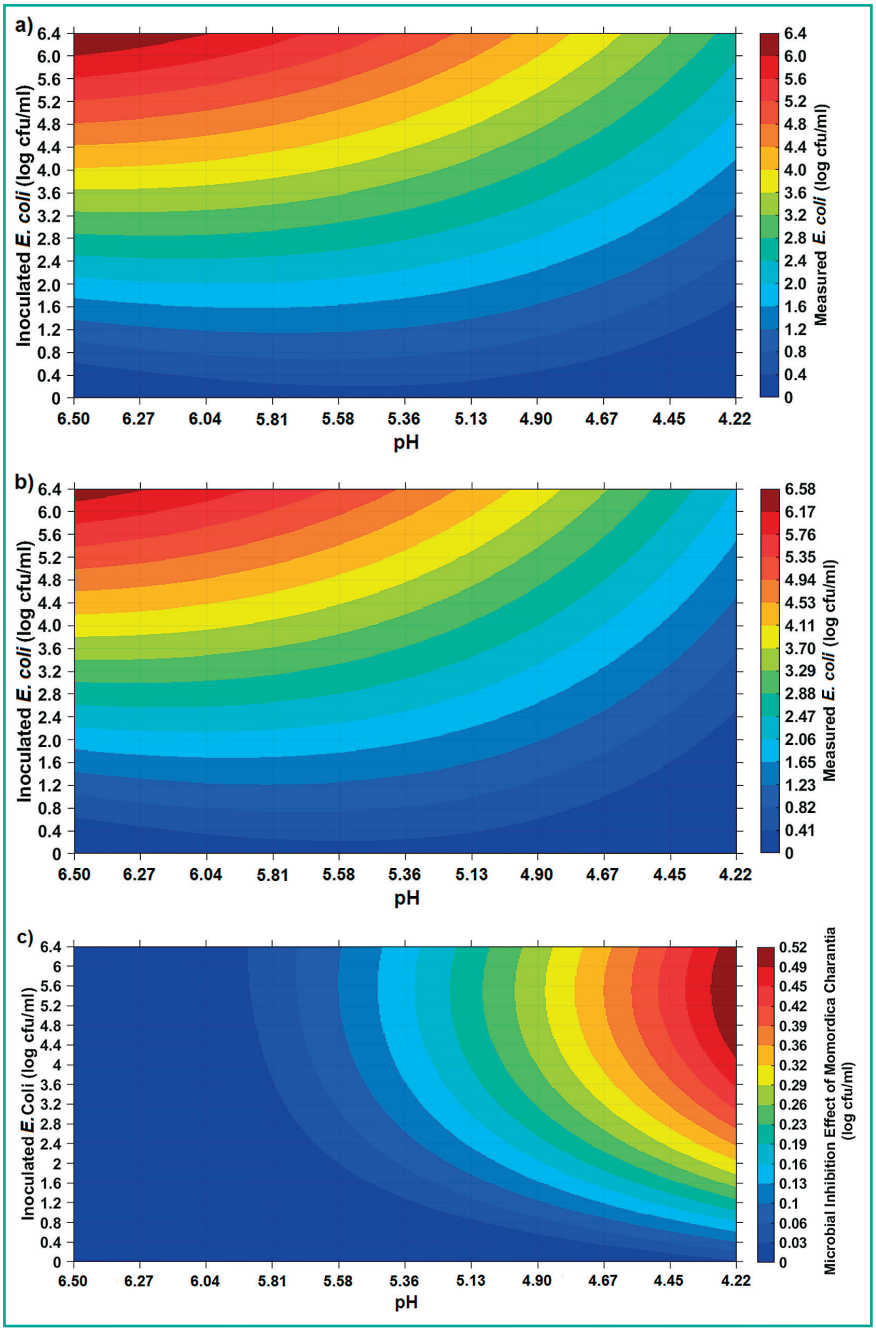

FIGURE 5: *a) Change of E. coli values in yogurt without MCE depending on pH and inoculated E. coli b) Change of E. coli values in yogurt with MCE depending on pH and inoculated E. coli c) E. coli reduction caused by the supplementation of MCE.*

phylococcus aureus, and *Streptococcus mutans* if the results of mentioned *in vitro* studies in the literature and the result of the current study are considered together. So, this study can be a good source for future studies.

In the second stage of the study, the relationship between the addition of MCE, *E. coli* inactivation effect in probiotic yogurt, and pH change were analysed. The pH values measured in the study have decreased linearly dependent on only the fermentation time and they were not affected by the number of *E. coli* inoculated. So, the pH has acted as an independent variable. Therefore, modelling was done by substituting the pH values instead of the measurement times during fermentation because the pH values corresponding to each measurement time are constant regardless of the inoculated *E. coli.* The surface equations obtained by modelling are as in Table 3.

In addition, the difference of the *E. coli* values measured from probiotic yogurts with and without MCE addition was modelled as a function of pH and inoculated *E. coli* as in Equation 2. (R^2=0.99; Adj-R^2=0.99; RMSE=0.0114; SSE=0.5747).

$$E(a, i) = 8.24 \times 10^{-5} - 2.906 \times 10^{-5} \times a + 0.2811 \times i + 2.529 \times 10^{-6} \times a^2 - 0.04136 \times a \times i - 0 \quad (2)$$

The mathematical models in Table 3 and Equation 2 were transformed into images to visually analyse the *E. coli* change during the fermentation process. Then, it was determined using the hierarchical clustering method that the images obtained should be reduced to 16 colours. Figure 5 shows these 4-bit images having 16 colours.

The most striking difference between Figure 5-a and Figure 5-b is that the *E. coli* values in probiotic yogurt are started to differentiate after pH decreased to a certain value (threshold value). This differentiation can be seen more easily in Figure 5-c. However, to determine this threshold value and to better understand the relationship between the number of *E. coli* in probiotic yogurt and pH, it is necessary to include the statistically non-different pH values into a single class. Therefore, the pH values measured between 6.5–4.22 were classified using the hierarchical clustering method. In Figure 6, there is

TABLE 3: *Surface equations predicting the change of pH-dependent E. coli during the fermentation time.*

Predicted *E. coli* value in probiotic yogurt without MCE (P=Predicted *E. coli* ; a=pH ; i=Inoculated *E. coli*)
R^2: 0.9881 **; Adj-R^2:** 0.9865 **; SSE:** 2.1988 **; RMSE:** 0.2405
$P(a,i) = -13.46247 + 5.01382 \times a - 0.53073 \times i - 0.45908 \times a^2 + 0.25743 \times a \times i - 0.00694 \times i^2$
Predicted *E. coli* value in probiotic yogurt with MCE (P=Predicted *E. coli* ; a=pH ; i=Inoculated *E. coli*)
R^2: 0.9827 **; Adj-R^2:** 0.9804 **; SSE:** 3.2407 **; RMSE:** 0.2920
$P(a,i) = -15.59895 + 5.74018 \times a - 0.78352 \times i - 0.52001 \times a^2 + 0.29343 \times a \times i - 0.00091 \times i^2$

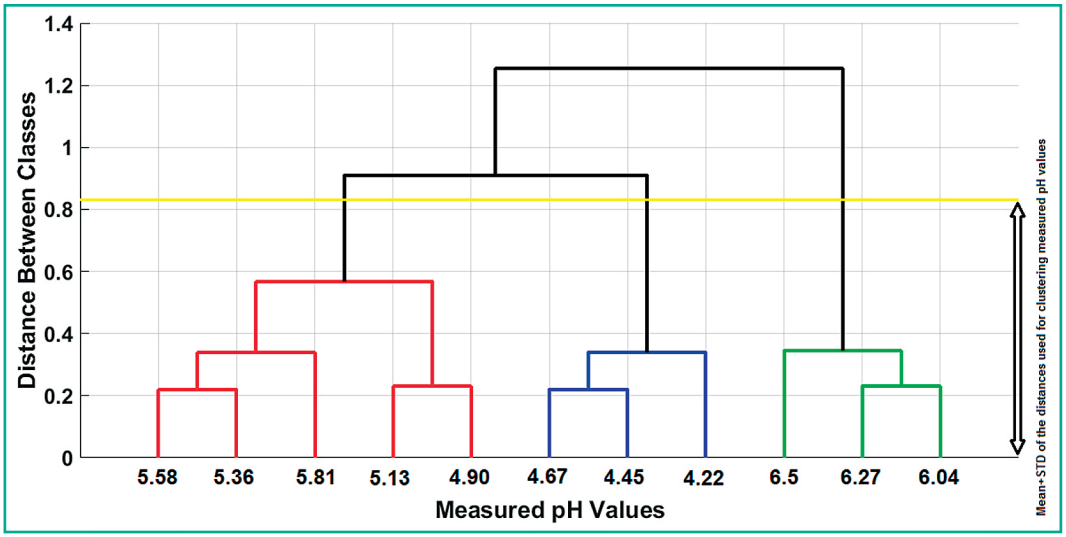

FIGURE 6: *Dendrogram plot showing the number of classes that the measured pH values need to be separated.*

a dendrogram plot showing the measured pH values that should be included to how many classes.

As can be seen from Figure 6, the measured pH values should be divided into 3 classes. The first of these classes include the pH values between 6.04–6.5. In other words, pH started to statistically significant change after decreasing to 6.04 and the pH values between 6.5–6.04 are statistically not different. If paying attention to Figure 5, the number of *E. coli* is starting to decrease after the pH value decreased to 6.04 (The colours are starting to change when the pH value decreases to 6.04). So, the statistically significant change in the number of *E. coli* is depending on the statistically significant change in the pH value. In other words, the number of *E. coli* has started to statistically significant decrease after the 75th minute of fermentation time because the pH has started to statistically significant decrease after this minute. Therefore, the number of *E. coli* is represented with single colour until the 75th minute of fermentation (pH decreases below 6.04 at the same time) time as in Figure 5.

On the other hand, pH values between 5.58–4.90 do not have a statistically significant difference from each other, according to Figure 6. However, as seen in Figure 5-c, *E. coli* values corresponding to this pH value interval decreased statistically significant (In this pH value interval, the colours representing *E. coli* values have changed). So, it can be said that the antimicrobial effect caused by the addition of MCE was not linearly related to the pH reduction. In other words, the addition of MCE together with changes in pH has a significant effect compared to pH alone. The same conclusion is available when the pH is between 4.67–4.22, as can be seen in Figure 6 and Figure 5-c. These results are supported by the results of the studies in the literature.

Studies in the literature have indicated that the number of pathogenic microorganisms (*Staphylococcus aureus, E. coli* O157:H7) is slightly decreased due to the decreasing pH value during the fermentation aiming to produce yogurt (Massa et al., 1997; Bachrouri et al., 2006; Jabeen and Khanum, 2017). The reason for this slight antimicrobial effect due to pH decrease is that the compounds produced by lactic acid bacteria, such as bacteriocins, hydrogen peroxide, ethanol, diacetyl have antimicrobial effects on the pathogen development in fermented products (Pazakova et al., 1997; Suskovic et al., 1997). These results are similar to the findings of the relationship between pathogen behaviour and pH change that occurred during the fermentation of yogurt in the current study.

In addition, pathogenic microorganisms can still survive at the end of fermentation as seen in Figure 5-a and these microorganisms can maintain their viability for a long time despite the low pH environment they are in. There are also studies in the literature supporting this problem. For example, in studies completed in 1997, researchers inoculated 10^5 CFU g^{-1} of *E. coli* O157: H7 has inoculated to traditional and acidophilus yogurts that pH level of them was in the range of 4.17–4.65. At the end of the studies, researchers reported that the microorganism survived for 7–35 days even in this acidic environment. In these studies, it was also determined that the microorganism survives until 6–14 days in case of the pH value is 4.0 (Guraya et al., 1998; Shen et al., 1997). However, the number of microorganisms that survived after fermentation was further reduced by the addition of MCE in the current study. In other words, a supportive solution has been produced to the problem that even the low pH environment cannot fully solve it completely.

Conclusions

In the period from the start to the end of fermentation, probiotic yogurt without MCE can inactivate the *E. coli* between 1.84–2.99 log CFU/mL depending on different inoculation levels, whereas the probiotic yogurt supplemented with MCE can inactivate the *E. coli* between 2.44–3.47 log CFU/mL. That is, up to 0.5 logarithmic inactivation of *E. coli* can be achieved at the end of fermentation in probiotic yogurts using MCE supplementation. However, it should be remembered that the additional inactivation varies between 0–0.5 logarithmic units according to the initial inoculation level. Although many studies in the literature investigated there the development of pathogens during the fermentation and storage stages of yogurt, there are limited studies that investigated the development of *E. coli* in probiotic yogurts supplemented with plant extracts. In particular, any food product produced and examined by adding *Momordica charantia* L. extract is not included in the literature. For this reason, these findings could be used in predictive microbiology studies and quantitative risk assessments by risk managers, as well as could be a good reference for future studies. Moreover, it can guide the studies that will investigate the inactivation effect of *Momordica charantia* L. in other foods.

On the other hand, the hierarchical clustering method has been used for the first time to evaluate the food engineering experimental data in this study. This method has been successful in distinguishing statistically different data

and has determined how many colours should be used in images representing the experimental data consist of in this study. In other words, this study is also a good resource for using the hierarchical clustering method in future studies.

Conflict of interest

The authors declare that they have no conflict of interest.

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