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Microbial Evaluation of Fermented Beetroot Juice Produced by Probiotic *Lacticaseibacillus paracasei*

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Abstract

Probiotic products have a significant proportion in functional food market, and research on the use of fruits and vegetables instead of dairy products in the production of probiotic products is increasing due to many factors. Red beetroot juice can be produced spontaneously or by probiotic bacteria. It is important to determine a product-specific pasteurization parameter to ensure that the product is microbially safe. Red beetroot is a very valuable plant due to its phenolic components and betalains, which have many important effects on health. The objective of this study was to develop a probiotic beverage using red beetroot, a valuable source of health-promoting compounds, with the intention of enhancing the potential health benefits for consumers. In this study, the red beetroot juice samples produced by probiotic strain *Lacticaseibacillus paracasei* 431®, 17 different runs were created with the Box Behnken experimental design in Response Surface Methodology. As independent variables; temperature (60-80℃), time (10-30 min.), and fermentation temperature (24-36℃) were selected. To demonstrate the effectiveness of the pasteurization process, total yeast and mold (TYM), and total mesophilic bacteria (TMB) were determined right after pasteurization and before fermentation. The results showed that; before fermentation, TYM and TMB counts of the samples were 0.50-2.87 log CFU/mL and 0.35-4.12 log CFU/mL, respectively. According to the ANOVA test results, models were significant, and also temperature and time were significant for both responses ($p < 0.05$). After fermentation, TYM, TMB and total lactic acid bacteria (LAB) counts of the samples ranged between 8.29-9.12 log CFU/mL, 8.50-9.25 log CFU/mL, and 8.17-9.01 CFU/mL, respectively. Although differences were determined between the microbial loads of the samples at the beginning of the fermentation, the effect of the models determined were found insignificant at the end of the fermentation ($p > 0.05$).

Keywords: *Beetroot, functional food, probiotic vegetable product, Lacticaseibacillus paracasei, Response Surface Methodology*

1.INTRODUCTION

In recent years, consumers' demands for products beneficial for health have been increasing for many reasons. Functional foods, which show the presence of multiple health-beneficial components such as antioxidants, bioactive peptides, vitamins, minerals, prebiotics and probiotics, are of great interest in these products (Mantzourani et al., 2019). Probiotic foods are the fastest growing area in functional food production, representing 70% of the functional food market (Lillo-Pérez et al., 2021). The global probiotic market is estimated to be worth 61.1 billion US dollars in 2021 and will reach 91.1 billion US dollars by 2026 (Markets and Markets, 2020). It is predicted that the demand for foods containing probiotics will remain high as awareness of the benefits of these products and consumers' desire to purchase premium products combined with probiotics (Szutowska, 2020; Terpou et al., 2019). In addition, the COVID-19 epidemic process, which has been in the whole world recently, has led to a change in the consumption patterns of consumers and this has affected the demand for diet. Fear of being infected and the adopting of healthy lifestyle have also increased demand for probiotics (Singh & Rao, 2021).

Fruits and vegetables are a good matrix for lactic acid bacteria due to their large amounts of

carbohydrates, polyphenols, vitamins, minerals, and dietary fibers (Septembre-Malaterre et al., 2018). Lactic acid fermentation is an effective biological process that ensures product safety, and longer shelf-life, provides probiotic properties, maintains or increases nutritional value, and develops new products with unique sensory quality (Szutowska, 2020).

As defined by FAO/WHO (2001), probiotics; are live microorganisms (mainly bacteria and a few yeasts, strains that provide a beneficial health effect on the host) that positively affect health when taken into the body in sufficient quantities. We can align many positive effects on health for probiotic bacteria. Among them; a) increasing the nutritional value of food products, b) controlling and lowering serum cholesterol, c) improving the immune system, d) preventing intestinal infections and suppressing antibiotic-associated diarrhea, e) reducing symptoms of lactose intolerance, f) reducing the risk of colon cancer, and g) depending on the type of probiotic strain, there is improvement of gliadin digestion in gluten-containing foods against celiac disease (Zendeboodi et al., 2020). Because of these functionalities, people tend to consume products containing probiotics. As a result of this increasing trend, marketing efforts aiming to produce new functional food products are emerging. For this purpose, many different food products containing probiotics can be designed and commercialized. Traditionally, fermented milk products have been considered the most excellent carriers for probiotics, but milk-based products may be limited in use due to lactose intolerance, allergies, dyslipidemia, and vegetarianism. In addition, dairy products contain high cholesterol, 75% of the world population suffers from lactose intolerance (Silanikove et al., 2015) and for economic reasons for developing countries, they do not contain cholesterol but contain protein, starch, minerals, fiber, vitamins and antioxidants that prevent diseases. fruits, vegetables, cereals and legumes, etc., from products rich in its content. can be good alternatives for designing probiotic products (Panghal et al., 2017, 2018).

Red beetroot (*Beta vulgaris L.*) is a flowering plant belonging to the *Amaranthaceae* family. Although its homeland is the Mediterranean Region, it is produced in a wide area, recently extending to America, Europe and India. There are many researches on the effects of red beet on human health. In recent years, there has been an increasing interest in the biological activities of red beet, including its positive effects on gastrointestinal health (Chhikara et al., 2019; Clifford et al., 2015; Ninfali et al., 2017). Metabolism of the oligo and polysaccharides in red beetroot by the bacteria that make up the gut microbiota has demonstrated the ability of these components to modulate positive gut microbial communities and stimulate the production of specific metabolites indicative of potential prebiotic properties (Gómez et al., 2016; Holck et al., 2011; Leijdekkers et al., 2014; Malik et al., 2019). For this reason, it is thought that red beet will be a suitable matrix for the development of probiotics and many studies have been carried out on the growth status of different probiotic bacteria in red beetroot juice.

In this study, it was aimed to investigate the effect of different pasteurization treatments and ambient temperature on quality in red beetroot juice production using a probiotic strain *Lacticaseibacillus paracasei* 431.

2. MATERIALS AND METHOD

2.1. Materials

Red beetroot and the other ingredients (garlic, water, salt and bay leaf) used in the red beetroot production were purchased from a local store in Sivas, Turkey. *Lacticaseibacillus paracasei* 431® *(Lc. paracasei)* strain was supplied from CHR Hansen® (Denmark). Strain was kept at -20°C until they use.

2.2. Method

2.2.1. Preparation of Red Beetroot Juices

Inoculum was prepared from overnight cultivation of *Lcb. paracasei* 431 at 37°C in a 250-rpm shaking incubator (ISS-3075, Jeio, Tech Lab Companion).

At the end of the incubation period, 1 mL of this suspension cultivated in sterile red beetroot juice, and the inoculated under the same incubation conditions. After incubation suspension of *Lc. paracasei* used in fermentation process.

The beetroot juices were prepared by slicing the fresh vegetables, followed by passing through a juice extractor (Philips, HR1861). A volume of 160 mL of a mixture comprising beet juice (62.5%), garlic (1.25%), water (37.5%), salt (2%), and bay leaf was prepared in 300 mL glass bottles. The bottles were subsequently sealed and subjected to the process of pasteurization. The temperature was controlled with a thermometer in a control flask containing the same amount of components and closed with cotton. After the pasteurization period is completed, the beetroot juices cooled at average 30℃ and 2% suspension of *Lc. paracasei* inoculated into red beetroot juices. The inoculum density was average 6-7 log CFU/mL. The samples fermented in the temperatures which are given in Table 1 and 2.

Table 1. *Range and levels of parameters in Box-Behnken experimental design*

Parameters	**Levels**		
	-1	0	1
Pasteurization Temperature (°C)	60	70	80
Pasteurization time (min.)	10	20	30
Fermentation temperature (°C)	24	30	36

2.2.2. Microbiological Analysis

Microbiological analysis of the samples was applied before and after fermentation. With the microbiological analysis, the effect of pasteurization on the microbial quality of the samples just after pasteurization, and also before fermentation were determined. Then, effect of fermentation on microbial quality of the samples was determined after different fermentation temperatures. The following groups of microorganisms were identified using selective media and incubation conditions: counts of total yeast and mold (TYM) on PDA at 25°C for 7 days; counts of mesophilic bacteria (TMB) on PCA at 35°C for 2 days; and total count of lactic acid bacteria (LAB) on MRS at 35°C for 3 days. Microbiological quality of juices was investigated using the standard plate method.

2.2.3. Experimental Design

In the study, it was aimed to produce a probiotic drink from red beetroot juice, which is so important for health, and to enable consumers to benefit more from red beetroot. The effects of different process parameters on red beetroot juice production were investigated. The influencing factors on determing the parameters can be listed as follows. In conventional fruit and vegetable juice production, the pasteurization temperature and time are applied generally between 60-80℃ and 10-30 minutes, respectively (Alcántara-Zavala et al., 2021; Atter et al., 2015; González-Aguilar et al., 2004). The intervals were determined in accordance with these studies. Traditional red beetroot juice is made at room temperature (24–25°C), while LAB bacteria often grow optimally at 30-37°C, hence the temperature range is 24-37°C.

A trial design with the three-factor, and three-level was created as 17 experiments with 5 repetitions at the center point by Box-Behnken experimental design (BBD) in the Response Surface Methodology (RSM) (Table 1). As independent variables; pasteurization temperature (60, 70, 80°C), pasteurization time (10, 20, 30 min.) and fermentation temperature (24, 30, 36°C) were determined. In BBD design, in response TYM, TMB and total count of LAB as microbiological analysis were examined. Fermentation studies were carried out in duplicate.

2.2.4. Statistical analysis

One-way ANOVA test was used to assess the results of the microbial analysis of 17 run obtained through the BBD using MINITAB 20.0 (State College, PA). After fermentation, the effect of 3 independent variables (pasteurization temperature, pasteurization time and fermentation temperature) on the responses was investigated using ANOVA results in BBD at 95% confidence interval. Before fermentation of 17 different run,

designed with BBD, samples were pasteurized at the temperatures and times given in the trial design, and after inoculation, the samples were fermented. Therefore, the one-way ANOVA test with a 95% confidence interval was used to examine the impact of just two independent factors (pasteurization temperature and pasteurization time) on the samples before fermentation.

3. RESULTS AND DISCUSSION

The microbiological quality of red beetroot juice before and after fermentation was investigated. For this purpose, counts of TYM and also TMB of the samples were determined after pasteurization (just before inoculation). *Lc. paracasei* 431 was inoculated into the samples immediately after the pasteurization. TYM, TMB and total LAB counts of the samples were determined end of fermentation.

3.1. Microbial quality before fermentation

It was determined that counts of TYM of the samples changed at the levels of 0.50-2.87 log CFU/mL after pasteurization (Table 2). The effect of independent variables was examined by the ANOVA, and the results are given in Table 3. According to the results of the ANOVA, the pasteurization temperature in linear terms, all square terms and interactions were effective on the counts of TYM ($p<0.05$). Figure 1A showed that highest average count of TYM was obtained at 60℃, and the lowest count was obtained with pasteurization applications at 70℃ and 80℃. A similar study was carried out by Tamme et al. (2010), in their study, after 10 s heat treatment at 65℃, the red beetroot juice samples fermented at 20-22℃. They found average 2.94 log CFU/mL of TYM after pasteurization. In our study, the count of TYM in the samples coded 15 which is pasteurized 60℃ for 10 min., had the highest value among the samples, as 2.87 log CFU/mL (Table 2).

In Table 2, counts of TMB of the red beetroot juice samples were given. It was found that counts of TMB of the samples were at the level of 0.35-4.12 log CFU/mL. The effect of independent variables after pasteurization on the counts of TMB of the samples is given in the ANOVA table (Table 3). It was determined that the linear effects of pasteurization temperature and pasteurization time, as well as all square effects, were significant ($p\leq0.05$). Interactions were not found to be significant ($p>0.05$). Figure 1B shows that results were the same as TYM, with the highest average values at 60℃ and the lowest values with 70℃ and 80℃ pasteurization applications. Also, it was observed that the highest number was obtained in 10 min., as expected. *3.2. Microbial quality after fermentation*

Probiotic *Lc.paracasei* 431 was inoculated into to red beetroot juice samples under aseptic conditions just after pasteurization. Samples fermented at the temperatures which are given Table 4. Approximately 6 log CFU/mL of LAB were found at the beginning of the fermentation process.

At the end of fermentation, counts of TYM of red beetroot juice samples which are fermented with *Lc. paracasei 431* were found to be in the range of8.06-9.01 log CFU/mL, as indicated in Table 4. As can be seen in Table 5, the established model was determined to be insignificant ($P>0.05$). Although TYM counts of the samples at the beginning of fermentation were determined at the level of 0.50-2.87 log CFU/mL, the effect of pasteurization temperature, all square terms and interaction on the TYM was significant ($P<0,05$) (Table 3), all effects were found to be insignificant as a result of fermentation ($P>0.05$) (Table 4).

Table 2. *Counts of TYM and TMB of the samples before fermentation*

Order	Sample code	Pasteurization temperature (°C)	Pasteurization time (min.)	TYM (log CFU/mL)	TMB (log CFU/mL)
1	12	60	20	2.00±1.00	2.29±0.11
2	1	70	10	1.00±0.85	1.74±0.19
3	4	60	30	1.18±2.13	2.09±0.09
4	3	80	30	1.18±0.59	1.06±0.37
5	7	60	20	1.54±1.62	2.97±0.07
6	6	70	20	1.11± 0.01	1.09±0.01
7	14	80	20	1.33±0.33	1.35±0.35
8	2	70	10	1.05±0.35	1.50±0.20
9	13	70	30	1.27±0.57	2.00±0.52
10	5	80	20	1.00±0.00	1.24±0.06
11	15	60	10	2.87±0.22	4.12±0.01
12	8	70	20	1.00±0.01	1.00±1.09
13	11	80	10	1.00±0.01	1.73±0.01
14	9	70	20	0.51± 0.01	1.00±1.09
15	16	70	20	1.45±0.01	0.85±0.15
16	17	70	20	0.50±0.01	0.35±0.01
17	10	70	30	0.85±0.15	1.24±0.06

± Standart Deviation (N=2). Abbreviations of the responses; Total Yeast and Mold (TYM), and Total Mesophilic Bacteria (TMB)

Table 3. *ANOVA summary of counts of TYM and TMB of the samples before fermentation*

Variables	TYM				TMB		
	DF	Adj. MS	F	% contrubition	Adj. MS	F	% contrubition
Model	5	0.72571	6.15**	77.82	2.14394	11.1***	77.82
Linear	2	0.72773	6.17**	29.84	2.76555	14.32***	29.84
Heat treatment temperature (°C) (X_1)	1	1.19359	10.12**	24.23	4.62731	23.96***	24.23
Heat treatment time (min.) (X_2)	1	0.26187	2.22	5.32	0.90379	4.68**	5.32
Square	2	0.64821	5.5**	26.36	2.36101	12.22**	26.36
X_1 x X_1	1	1.21276	10.28**	25.27	3.26802	16.92**	25.27
X_2 x X_2	1	0.0518	0.44**	1.05	1.21777	6.3**	1.05
2-Way Interaction	1	0.87669	7.43**	21.62	0.46657	2.42	21.62
X1 x X2	1	0.87669	7.43**	17.80	0.46657	2.42	17.80
Residual error	11	0.11792		22.18	0.19316		22.18
Lack-of-Fit	7	0.08867	0.52	8.45	0.25327	2.88	8.45
Pure error	4	0.16911		13.73	0.08798		13.73
Total	16			100.00			100.00

Significance levels: *P < 0.05, **P < 0.01, ***P < 0.00. Abbreviations of the responses; Total Yeast and Mold (TYM), and Total Mesophilic Bacteria (TMB).

Table 4. *Counts of TYM and TMB of the samples after fermentation*

Order	Sample code	Pasteurization temperature (°C)	Pasteurization Time (min.)	Fermentation temperature (°C)	TYM (log CFU/mL)	TMB (log CFUmL)	LAB (log CFU/mL)
1	12	60	20	36	8.52±0.10	8.50±0.23	8.56±0.10
2	1	70	10	36	8.60±0.10	8.71±0.06	8.58±0.03
3	4	60	30	30	8.06±0.30	9.25±0.30	9.06±0.11
4	3	80	30	30	8.17±0.47	8.28±0.43	8.29±0.48
5	7	60	20	24	8.81±0.01	8.87±0.15	8.79±0.01
6	6	70	20	30	8.60±0.10	8.52±0.80	8.59±0.32
7	14	80	20	24	9.01±0.10	9.26±0.14	8.85±0.07
8	2	70	10	24	8.81±0.04	8.74±0.05	8.63±0.07
9	13	70	30	24	8.75±0.02	8.84±0.05	8.62±0.01
10	5	80	20	36	8.77±0.11	8.60±0.24	8.78±0.19
11	15	60	10	30	8.79±0.10	8.91±0.07	8.85±0.11
12	8	70	20	30	8.86±0.12	8.63±0.23	8.78±0.21
13	11	80	10	30	8.33±0.13	8.54±0.38	8.37±0.16
14	9	70	20	30	8.98±0.10	8.83±0.34	8.78±0.12
15	16	70	20	30	8.37±0.10	9.10±0.15	8.82±0.32
16	17	70	20	30	8.80±0.05	8.80±0.32	9.12±0.16
17	10	70	30	36	8.77±0.10	8.63±0.02	8.45±0.30

As given in Table 4, it was determined that total LAB of the samples after fermentation varied between 8.29 and 9.12 log CFU/ml. As the results of ANOVA, the model built on the total LAB was determined to be insignificant ($p>0.05$) (Table 5). The effective factor here is that the counts of LAB of all samples are almost the same. Despite the internal and external factors expected from a probiotic beverage, probiotic microorganisms must remain alive in the product until consumed. In the probiotic product, probiotic microorganisms should be at least 6 log CFU/mL and acceptable levels should be 7-8 log CFU/mL All red beet juices produced meet this criteria (Ozcan et al., 2021). Similar results also reported in beverages produced by probiotic *Lc. paracasei* (Mantzourani et al., 2019; Marnpae et al., 2022; Pimentel et al., 2015; Silva & Ferrari, 2016) and *Lc. paracasei* (Bartkiene et al., 2022; Demarinis, 2022; Mesquita et al., 2020). Figure 1 shows the distribution of TYM, TMB and total LAB numbers of the samples before and after fermentation. Although the TYM and TMB values of the samples were determined in a wide range depending on the change in the temperature and time of pasteurization treatments before fermentation, this distribution is limited after fermentation. The effective factor is red beetroot juice is an excellent medium for microorganisms. All samples have good amount of lactic acid in the medium due to fermentation of probiotic *Lc. paracasei* and also there are other acids in the medium, these cannot prevent the growth of yeast and moulds.

The fact that the the higher counts TYM of the samples does not mean that the samples were spoiled. Although the results were not shared here, the samples, whose TYM numbers did not differ from each other at the end of the fermentation, were generally appreciated. We can also say that they are stable in storage (data not shown).

Table 5*. Estimated regression coefficients and ANOVA summary TYM, TMB and Total LAB of the samples after fermentation*

	TYM				TMB			Total LAB		
	DF	**Adj. MS**	**F**	**% contrubition**	**Adj. MS**	**F**	**% contrubition**	**Adj. MS**	**F**	**% contrubition**
Model	9	0.032892	0.39	33.11	0.046744	0.47	37.69	0.037113	0.58	42.54
Linear	3	0.048272	0.57	16.20	0.095873	0.97	25.77	0.049392	0.77	18.87
Heat treatment temperature (°C) (X_1)	1	0.079	0.92	8.84	0.089572	0.9	8.03	0.115777	1.8	14.74
Heat treatment time (min.) (X_2)	1	0.001425	0.02	0.16	0.00113	0.01	0.10	0.00008	0	0.01
Incubation temperature (°C) (X_3)	1	0.064391	0.75	7.20	0.196918	0.98	17.64	0.032318	0.5	4.12
Square	3	0.037005	0.43	12.42	0.00507	0.05	1.36	0.051551	0.8	19.70
$X_1 \times X_1$	1	0.013296	0.16	1.46	0.002115	0.02	0.15	0.000002	0	0.11
$X_2 \times X_2$	1	0.045759	0.54	4.55	0.013302	0.13	1.17	0.125582	1.95	16.76
$X_3 \times X_3$	1	0.057327	0.67	6.41	0.000458	0	0.04	0.02221	0.34	2.83
2-Way Interaction	3	0.013398	0.16	4.50	0.03929	0.4	10.56	0.010396	0.16	3.97
$X_1 \times X_2$	1	0.026176	0.31	2.93	0.090133	0.91	8.08	0.021358	0.33	2.72
X1 x X_3	1	0.000568	0.01	0.06	0.020095	0.2	1.80	0.006154	0.1	0.78
$X_2 \times X_3$	1	0.013452	0.16	1.50	0.007641	0.08	0.68	0.003676	0.06	0.47
Residual error	3	0.123787		66.89	0.099341		62.31	0.064455		57.46
Lack-of-Fit	44	0.056649	2.19	41.54	0.166704	0.41	44.81	0.100467	2.68	38.39
Pure error	16			25.35	0.048818		17.50	0.037445	0.58	19.08
Total				100.00			100			100

Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations of the responses; Total Yeast and Mold (TYM), and Total Mesophilic Bacteria (TMB)

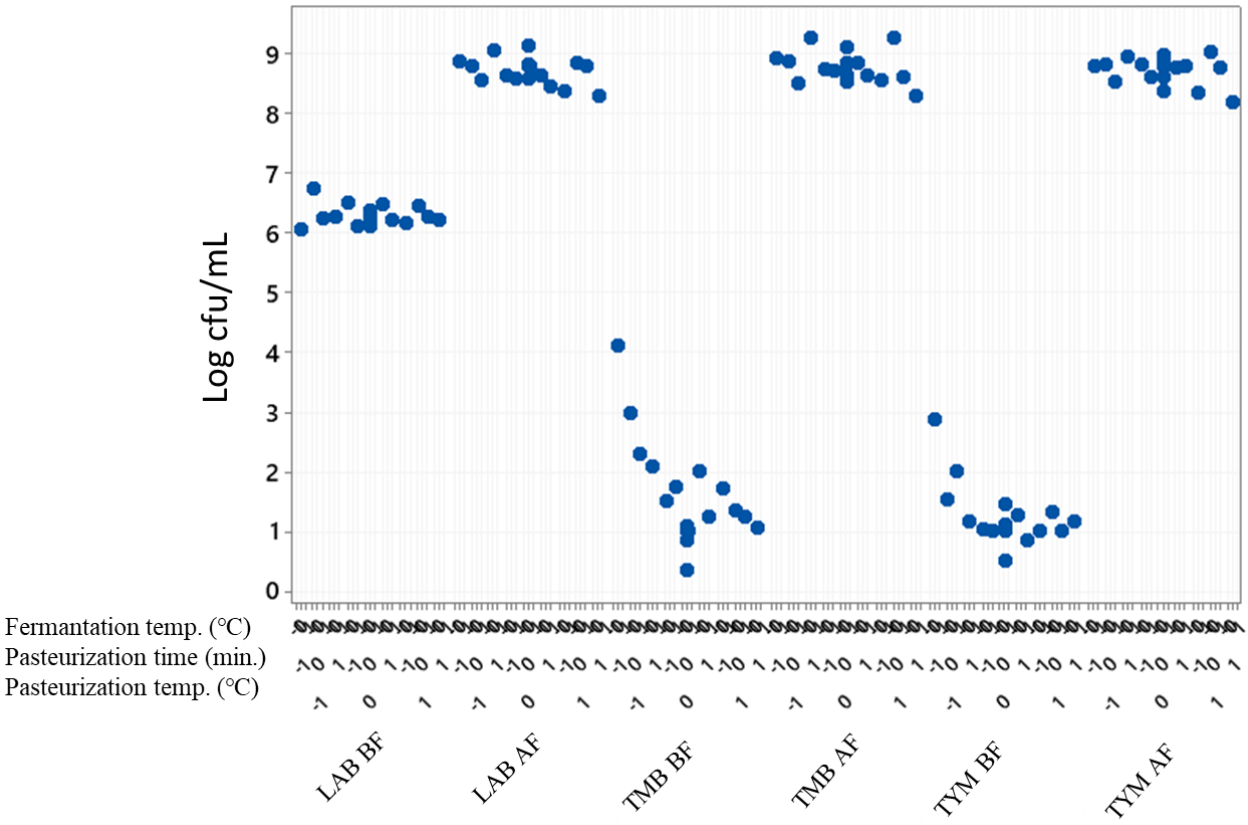

Figure 1. Change of microbial load of samples before and after fermentation

**LAB BF: Lactic acid bacteria before fermentation, LAB AF: Lactic acid bacteria after fermentation, TMB BF: Total mesophilic bacteria before fermentation, TMB AF: total mesophilic aerobic bacteria after fermentation; TYM BF: total yeast and moulds before fermentation.*

4. CONCLUSION

As a result of the study, it has been proven that vegetable juices such as red beet juice are a very good environment for the development of *Lc. paracasei* 431. Additionally, when a fermentation is performed in red beetroot juice, at the beginning of the fermentation TYM and TMB are below 2.87 log CFU/mL and 4.12 log CFU/mL, respectively, the difference between the final TYM and TMB values of the samples is insignificant regardless of fermentation temperature. Red beetroot juice is also a good growth medium for yeast and moulds, mesophilic bacteria and LAB due to its nutritional components.

It is understood from the results that the lactic acid developing in the medium due to fermentation and other acids in the medium do not prevent the growth of yeast and moulds. Lactic acid bacteria, yeast and molds and aerobic mesophilic bacteria grew together during fermentation period of samples, contributing to the characteristics of the final product, presumably by producing organic acids, carbon dioxide and other unpredictable flavour compounds (Zvauya et al., 1997). The concurrence and free proliferation of lactic acid bacteria and yeasts, as was observed in this study, is a common circumstance in fermentation of food and beverages.

Therefore, there is no need to make extreme applications when choosing the pasteurization temperature and duration in vegetable juice producing. Choosing softer treatments will be

more beneficial in terms of the chemical components of the product. Microbial analysis alone will not be sufficient when choosing spontaneous or controlled fermentation in vegetable juice production. The effects of these production techniques on chemical components should also be investigated. These analysis results should also be taken into account in the selection of the production method in fruit/vegetable juice making. Since red beetroot contains components that have many positive properties on health, the change of these components should not be ignored in the production of a probiotic beverage.

CONFLICT OF INTEREST STATEMENT

No potential conflict of interest was reported by the authors.

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