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Development of a functional chocolate using gamma-amino butyric acid producer *Lacticaseibacillus rhamnosus* NRRL B-442

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ABSTRACT

The human microbiota is influenced by the immune and nervous systems of the host. Gamma-aminobutyric acid (GABA) is known as bioactive compound and it has important physiological functions, such as anti-hypertensive and antidepressant activities. Lactic acid bacteria (LAB), especially *Lactobacillus* species are known as the most important GABA producers because of the food-grade nature. The purpose of this study is to develop a functional chocolate using microencapsulated GABA producer *Lacticaseibacillus rhamnosus* NRRL B-442 strain for patients having an anxiety disorder.

Water-in-oil emulsion technique was conducted for microencapsulation using whey-pullulan complex. Microencapsulated and free *L. rhamnosus* cell counts were 6.75 and 7.20 log CFU/g in chocolates, respectively, at the end of 60 days. During simulated *in vitro* digestion analysis, survival rate of microencapsulated bacteria in chocolate samples was found at higher percentage (87%) than free bacteria (75%). Furthermore, microencapsulated *L. rhamnosus* did not affect the physical, chemical, and sensory properties of chocolate.

Consequently, *L. rhamnosus* with the highest GABA producing capability may provide insight for an anxiety disorder patient, since this strain has been thought as having a therapeutic effect. A new functional food model was developed for "GutBrain Axis" phenomena since the chocolate could be accepted as a good carrier for GABA producer bacteria.

1. Introduction

In recent years, consumer perception of health has moved away from conventional chronic disease prevention and treatment toward a more holistic approach. Strengthening brain function to improve memory, regulate mood, and reduce anxiety and depression are the most important component of holistic health. Recently, many people are turning to functional foods to overcome the above mentioned disorders. Functional foods are defined as foods or food ingredients which improve health and decrease the disease risks beyond nutritional values (Ozen et al., 2012). World Health Organization (WHO) promote food industries should to contribute to improve the human nutrition by providing high quality foods in terms of nutritional and functional attributes (Valerio et al., 2021). LAB are widespread microorganisms which can be found in any environment such as plants, fermented foods, and the mucosal surfaces of humans. LAB are also a part of the normal microbiota or microflora of the human gastrointestinal system. Several LAB strains can be considered to have "Qualified Presumption of Safety-status" QPS-status according to the European Food Safety Authority (EFSA). They are used as starters, biocontrol agents or probiotics in many food applications. Recently, they have also been used in the production of functional foods because they act as vectors for nutraceuticals and synthesize biomolecules for human health (Laroute et al., 2021).

The gut-brain network serves as a protector for immune and neurological systems. This interaction plays a critical role in human health when stress alters (Maa et al., 2021). The gut microbiota in the digestive tract may affect the brain function in different ways. Anxiety is caused by dysregulation of neurobiological systems, but the mechanisms of anxiety disorders are not to be fully understood (Lydiard, 2003).

GABA is considered an inhibitory neurotransmitter because it blocks certain brain signals and decreases activity in your nervous system. The absence of GABA receptors and/or the its lower levels of GABA may be linked to anxiety in human. The relationship between diet and psychiatric disorder could be related not only because of depression risk but also anxiety disorder (Logan & Jacka, 2014). In this context, the LAB produce neurochemicals and these may be effective on anxiety disorder

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(Lyte, 2011). GABA can be produced by chemical synthesis and microbial production. Although the chemical synthesis of GABA has a faster reaction rate and high product yield, the toxic by-products of the reaction seriously hinder its application to food because of this reason chemically synthesized GABA was prohibited as a food additive (Luo et al., 2021). When comparing the two methods, microbial production seems to be more promising than chemical synthesis, due to the more safety applications in food.

Chocolate has a unique aroma, taste, structure, and bioactive compounds like polyphenols. The main ingredients of chocolates are cocoa mass, cocoa butter, and sugar. Milk powder gives a creamy taste to white and milk chocolate. Psychoactive components of chocolate, such as polyphenols, methylxanthines, and biogenic amines, have been hypothesized to cause positive effects on mood (Fusar-Poli et al., 2021). Consumption of certain amounts of chocolate may be beneficial to prevent psychiatric disorders (Parker & Crawford, 2007). Chocolate may interact with a range of neurotransmitter systems that contribute to appetite, reward, and mood regulation (Parker et al., 2006).

Viability of microorganisms is affected by storage and food processing conditions, as well as the gastrointestinal tract. Maintaining viability and functionality of the probiotic in gut system, encapsulation is used to protect microorganisms from these conditions (Burgain et al., 2011; Raddatz et al., 2020). Therefore, microencapsulation is known as one of the effective method conserving bacteria against these factors (Gadhiya et al., 2015). Also, the encapsulation process provides preservation of the cells and long-term viability of the live microorganisms (Eslami et al., 2017).

This study involves researching microencapsulation of GABA producer LAB by using water-in-oil emulsion technique and an investigation of viability levels of microencapsulated and non-microencapsulating GABA producer bacteria in milk chocolate and determination of main quality parameters such as texture, color, and sensorial properties during the storage period. Additionally, the survivability of GABA producer LAB was evaluated under simulated *in vitro* gastro-intestinal tract.

2. Materials and methods

Lactobacillus helveticus NRRL B-4526, *Lacticaseibacillus rhamnosus* NRRL B-442 and, *Lactobacillus delbrueckii* subsp. *bulgaricus* NRRL B-548 cultures were provided from Northern Regional Research Laboratory (NRRL) culture collection and *Lactococcus lactis* subsp. *lactis* CECT-4432 was provided from Spanish Type Culture Collection (CECT). They were preserved in glycerol stocks at −80 °C. They were inoculated on 1% to MRS broth (de Man Rogosa and Sharpe, Merck, Germany) and incubated at 37 °C for 24 h for experiments.

Milk chocolate was provided from ETİ Food Industrials and Commerce Inc, Eskişehir, Turkey. It contains 8.3 g protein, 50.7 g carbohydrate, 34.5 fat, and 3.4 g dietary fiber per 100 g of milk chocolate.

2.1. Screening of GABA producer bacteria

L. helveticus, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. lactis* subsp. *lactis* were tested to find their GABA producing capability by reversed phase HPLC (RP-HPLC) using Pico. Tag Column (3.9 × 300 mm, 4 μm), and Shimadzu LC-20AD series HPLC system (Shimadzu LC-20AD HPLC, Japan). GABA concentration was measured by amino acid analysis using HPLC method described in Hayaloğlu et al. (2011).

2.2. Microencapsulation of GABA producer bacteria

Water-in-oil emulsion method was conducted for the microencapsulation of GABA producer bacteria according to the method of Cabuk and Harsa (2015). After microencapsulation, bacteria were freeze-dried in a Labconco freeze-dryer using a standard lyophilization program (Freezone 18, Kansas, USA). The conditions were at −55 °C and under 0.050 mbar vacuum for 48 h. After that, they were kept at 4 °C in the screw-capped glass bottle for further analysis.

2.3. Enumeration of microencapsulated bacteria

Microcapsules were subjected to homogenization with an Ultra Turrax homogenizer (Ultra Turrax, model T25, Janke and Kunkel, IKA Labortechnik, Staufen, Germany) at 11.000 rpm for 5 min before enumeration. MRS agar was used for incubation at 37 °C for 24 h under anaerobic conditions with anaerobic kit (Thermo Scientific, Oxoid AnaeroGen, England). Viable cell number was determined as colony forming units per gram (CFU/g) and microencapsulation yield were calculated by using following equation:

$$Microencapsulation\ yield\ (\%) = 100\ x\ (N_1/N_0) \quad (1)$$

N_0 was the viable count of bacteria before microencapsulation and N_1 was the viable count of bacteria after microencapsulation.

Microencapsulated and non-microencapsulated bacteria in chocolate samples were preserved at 4 °C for 60 days of storage. Bacterial viability and survival rates of both forms of bacteria were counted using MRS agar by pour plate method in triplicates.

2.4. Preparation of functional chocolate

Chocolate samples were prepared as follows, Control chocolate without any bacteria (CNB), chocolate including microencapsulated bacteria (CMB) and chocolate containing non-microencapsulated bacteria (CFB). Firstly, tempering procedure was applied to chocolate masses. For these purposes, to melt all fats, they were heated at 45 °C, then they slowly cooled at 25 °C with stirring. After that, they were reheated at 29–31 °C for melting out of unstable crystals. After tempering step, chocolate was molded in the forms of 11.67 ± 0.5 g cubes and these samples were cooled. Microencapsulated and non-microencapsulated bacteria were added before molding step. *L. rhamnosus* cells powder dose was weighed and diluted 1:100 in chocolate. All chocolate samples were stored at room (25 °C) and refrigerated (4 °C) temperatures to determine probiotic viability during different storage conditions (Klindt-Toldam et al., 2016).

2.5. Physicochemical analysis of chocolates

The moisture content of the chocolate was determined gravimetrically by oven drying at 105 ± 2 °C for 24 h to reach weight equilibration (AOAC, 1990).

Ash content of chocolate samples were also determined according to AOAC (1990).

Color of the chocolate samples was measured after tempering process and the 7 days of storage period at room temperatures by using Chroma Meter (Model CR-400 Konica Minolta Sensing Inc., Japan). Color measurements were performed at 20 °C to determine the L* (bright), a* (green to red) and b* (blue to yellow) values of the chocolate samples.

The hardness and fracturability of chocolate samples were measured by using a Texture Analyzer Model (TA-XT PLUS, Stable Micro Systems, Godalming, UK) with a load cell of 50 N. The hardness measurements of chocolate samples were done by needle probe at the temperature value of 25 °C (Cikrikci et al., 2016).

2.6. Viability analysis of chocolate samples

The viability of bacteria in chocolate samples were evaluated after the chocolate preparation (initial count) and 7, 14, 30, and 60 days of storage at 4 °C and 25 °C. Total cell count of microencapsulated bacteria was also evaluated immediately after freeze-drying and 7, 14, 30, and 60 days of storage at 4 °C.

2.7. Sensory analysis

Sensorial evaluation of functional chocolate samples was performed by 20 panelists. A 5 point-hedonic scale (1 is the lowest and 5 is the highest) was used for sensory analysis (Granato et al., 2010). Panelists were asked to evaluate chocolate samples for their sweetness, appearance, greasiness, color, odor, texture, taste/flavor, and general acceptance.

2.8. Simulated in vitro digestion

Simulated *in vitro* digestion of microencapsulated and non-microencapsulated bacteria in chocolate samples were conducted according to the method proposed by Paz-Yepez et al. (2019). To evaluate *in vitro* digestion, salivary, gastric, and intestinal fluids were prepared freshly.

Simulated salivary fluid (SSF) was prepared for oral digestion containing 15.1 mmol/L, KCl, 3.7 mmol/L KH_2PO_4, 13.6 mmol/L of $NaHCO_3$, 0.15 mmol/L of $MgCl_2(H2O)_6$, 0.06 mmol/L of $(NH_4)_2CO_3$ and 1.5 mmol/L $CaCl_2$. α-Amylase was added as part of the saliva stock solution to achieve a concentration in the saliva mixture of 75 Unit/ml.

Simulated gastric fluid (SGF) was prepared for gastric digestion containing 6.91 mmol/L KCl, 0.9 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 47.2 mmol/L of NaCl, 0.1 mmol/L $MgCl_2(H_2O)_6$, 0.5 mmol/L $(NH_4)_2CO_3$ and 0.15 mmol/L of $CaCl_2$ (pH 3.0) and 2000 U/ml pepsin.

Simulated intestinal fluid (SIF) were prepared for intestinal digestion including 6.8 mmol/L of KCl, 0.8 mmol/L KH_2PO_4, 85 mmol/L $NaHCO_3$, 38.4 mmol/L NaCl, 0.33 mmol/L $MgCl_2(H2O)_6$, 0.6 mmol/L of $CaCl_2$ (pH 7.0), bile salts (1 mM) and pancreatin (2000 LU/g of fat).

2.9. Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were used to determine the significant differences ($P < 0.05$). Minitab 18.0 (Minitab Inc., State College, PA, USA) program was used to evaluate the data obtained. All experiments were carried out in duplicate.

3. Results and discussion

3.1. Screening of GABA producer bacteria

L. helveticus, L. rhamnosus, L. delbrueckii subsp. *bulgaricus* and *L. lactis* subsp. *lactis* were screened to determine their GABA-producing performance.

It was found that the separation of the phenytfhiocarbamyl derivatives of the amino acids takes 12 min using sodium-based buffers. The optimum pH of all strains for GABA production was 3.65 and 4.88. *L. rhamnosus* had the lowest pH throughout the fermentation period among other reference strains.

The calibration curve was prepared using five different concentrations (20, 50, 100, 200, 500 mg/L) of GABA standard by RP-HPLC. Chromatogram pike of retention time of GABA standard was found at 6.08 min. *L. rhamnosus* produced the highest GABA concentration (approximately 58800 mg/L) at pH 4.07 at 37 °C for 24 h at the end of fermentation period (Table 1).

Song and Yu (2018) demonstrated that *L. rhamnosus* GG produced 0.44 mg/mL of GABA at the end of 36 h fermentation. Sun et al. (2009) reported that two different *L. helveticus* strains (E2303 and ND01) were isolated from koumiss produced GABA between 56.44 and 113.35 mg/L. GABA production by *L. lactis* NCDO 2118 was investigated in the different osmotic stress conditions, and they were found 413 mM GABA (Laroute et al., 2021).

Some clinical studies showed that different probiotic bacteria decrease anxiety symptoms (Pirbaglou et al., 2016; Wallace & Milev, 2017). Bravo et al. (2011) suggested that consumption of *L. rhamnosus* could decrease anxiety disorder because of the effect of GABA neurotransmitter in central nervous system. Other clinical studies focused on taking probiotic bacteria instead of various drugs such as lorazepam and diazepam with pharmacological properties acts on GABA receptors (Cryan & Kaupmann, 2005; Rudolph & Möhler, 2004; Singewald et al., 2015). Nakamura et al. (2009) found that chocolate including GABA reduce the acute psychological stress level after 15 min ingestion.

Table 1
The concentration of GABA produced by bacteria at different times.

Bacteria	GABA (mg/L)			
	24 h	48 h	72 h	96 h
L. helveticus	48993 ± 286[a]	50277 ± 211[a]	50680 ± 258[a]	–
L. rhamnosus	58881 ± 985[a]	57524 ± 269[ab]	54893 ± 837[bc]	52950 ± 105[c]
L. delbrueckii subsp. *bulgaricus*	55147 ± 263[a]	48160 ± 151[c]	50117 ± 162[b]	51288 ± 414[b]
L. lactis subsp. *lactis*	44947 ± 232[c]	48082 ± 320[b]	49166 ± 162[ab]	50177 ± 101[a]

Averages (± standard deviation) followed by different lower-case letters (a-c) in the same line differ statistically ($P < 0.05$).

L. rhamnosus was chosen as the highest GABA-producer strain and it was microencapsulated by using water-in-oil emulsion technique.

3.2. Viability of L. rhamnosus after microencapsulation

The effect of microencapsulation by emulsion method using pullulan as a coating material on the viability of *L. rhamnosus* after freeze-drying was shown in Fig. 1.

The approximate count of *L. rhamnosus* in emulsion was 1.2×10^{11} CFU/mL. During freeze-drying process 2-log decrease in bacterial count was obtained; therefore, microencapsulation efficiency of *L. rhamnosus* were found to be as 81.81%.

In Fig. 1, viable cell counts of microencapsulated *L. rhamnosus* were decreased from 9.01 log CFU/g to 8.41 log CFU/g after 60 days of storage at 4 °C. The survival of *L. rhamnosus* was found as 93.34% after 60 days, likewise, Cabuk and Harsa (2015) found survival rate of *Lactobacillus acidophilus* was 92% after 4-week storage period.

3.3. Viability of L. rhamnosus in chocolate samples

In functional chocolate formulation, the initial microencapsulated and non-microencapsulated bacteria counts were 10^9 CFU/g. However, the final concentration was found as 10^7 CFU/g because of the dilution effect. Decrease in the cell counts immediately after chocolate production was reported in previous studies, as well (Lalicic- Petronijevic et al.,

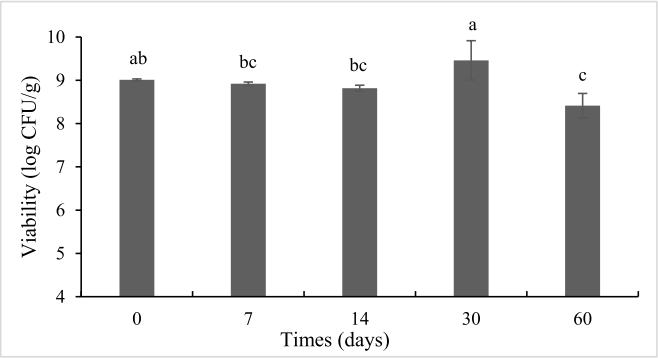

Fig. 1. Viability of microencapsulated L. rhamnosus during storage.
Mean values in the certain group of columns, labelled with the different lower-case letters (a–
c) are significantly different ($P < 0.05$).

2017; Erginkaya et al., 2019).

All chocolate samples (CNB, CFB, and CMB) were stored both at 4 °C and 25 °C for 2 months. The survival rate of *L. rhamnosus* after 60 days in CFB and CMB at 25 °C was decreased. It was observed that the survivability of bacteria was higher at 4 °C during storage (Table 2). Keeping CFB and CMB samples at 4 °C could be preferred since it is the best storage condition for preserving the viability of the bacteria as observed by other researchers (Khanafari et al., 2012; Konar et al., 2018). Likewise, survivability of *L. rhamnosus* was reported as high in chocolate at 4 °C for 25 days (Khanafari et al., 2012). Bulatovic et al. (2016) demonstrated that the survival rate of *Lactobacillus* strains in chocolate was 90% after 6 months of storage. Survival rate of *Bacillus indicus* was found in the range of 88–91% in chocolate (Erdem et al., 2011).

Viable cell counts of microencapsulated and non-microencapsulated bacteria in chocolate samples increased after 30 days, while viable cells count after 7, 14 or 60-days' in the samples decreased when stored at 4 °C (Table 2). The highest bacterial count was found at 30 day of storage, while lowest was at 60 days, under 4 °C in functional chocolate samples. Furthermore, microencapsulated bacteria in chocolate reduced significantly at the end of 60 days at 25 °C (Table 2). Viability of L. *rhamnosus* in CMB reduced 0.329 log CFU/g and in CFB decreased 0.253 log CFU/g at the end of storage at 4 °C. Klindt-Toldam et al. (2016) examined the viability of freeze-dried *L. acidophilus* NCFM and *Bifidobacterium lactis* in chocolate samples and they observed 1.1–1.6 log CFU/g reduction at the end of 14 months. Succi et al. (2017) studied with the combination of *Levilactobacillus brevis* and *L. rhamnosus* GG in dark chocolate they found approximately 1.0 and 0.4 log CFU/dose decrease during 90-days of storage.

The viable cell count in CFB was not found to be significantly different at both 4 °C and 25 °C ($P > 0.05$). Chocolate with microencapsulated and non-microencapsulated bacteria was found statistically different from each other at 25 °C ($P < 0.05$). Nebesny et al. (2007) studied with *Lactobacillus* in dark chocolate, and they demonstrated that the total bacterial count was 6–7 log CFU/g after 12 months at 4 °C. Ozturk et al. (2021) also demonstrated that microencapsulated *Streptococcus thermophilus* CCM4757 exhibited an excellent survivability in milk and dark chocolate samples during 180-day storage period at 4 °C.

Previous research demonstrated that chocolate has protective properties for bacteria because of its lipid fraction of cocoa butter and high phenolic content which is affected on reduced oxidative stress (Burgain et al., 2011; Lahtinen et al., 2007; Yonejima et al., 2015; Pedroso et al., 2013). Thus, chocolate structure can represent a good media to carry probiotic or GABA-producing bacteria.

3.4. Physicochemical analysis of functional chocolate

3.4.1. Moisture and ash analysis of functional chocolate

The moisture content is an important criterion for chocolate products because it increases the viscosity by affecting the agglomeration and particle structure of the sugar. Moisture content of CNB and CMB were not different, while CFB was significantly different from other chocolate samples ($P < 0.05$) (Table 3).

Table 3
Color properties, moisture, and ash contents of chocolate samples.

	CNB	CFB	CMB
COLOR ANALYSIS			
L*	32.05 ± 0.28^{a}	31.69 ± 0.98^{a}	31.47 ± 0.84^{a}
A*	10.14 ± 0.10^{b}	10.86 ± 0.41^{a}	9.83 ± 0.15^{c}
B*	11.51 ± 0.17^{b}	12.31 ± 0.72^{a}	11.07 ± 0.29^{c}
ΔE*	–	2.329 ± 0.96^{a}	1.575 ± 1.16^{a}
MOISTURE CONTENT (%)	1.664 ± 0.03^{b}	1.809 ± 0.07^{a}	1.678 ± 0.03^{b}
ASH CONTENT (%)	3.766 ± 1.396^{a}	3.058 ± 0.68^{a}	3.70 ± 2.24^{a}

Means shown with different letters in the same row are different ($P < 0.05$).
CNB was control chocolate without any bacteria, CMB was chocolate including microencapsulated bacteria, and CFB was chocolate containing non-microencapsulated bacteria.
L* = brightness, black - white.
a* = green - red.
b* = blue - yellow.

The chocolate samples (CNB, CMB, and CFB) were not statistically different from each other in terms of ash content ($P > 0.05$). The addition of microencapsulated and non-microencapsulated bacteria did not adversely affect the moisture and ash content of the chocolate.

3.4.2. Color analysis

L* values were found not significantly different in all milk chocolate samples ($P > 0.05$). However, the highest L* value was belonged to the CNB and the lowest L* value was observed in the CMB. *L. rhamnosus* addition decreased L* value, but not significantly. Likewise, in a recent study, functional chocolate was prepared using *S. thermophilus*, and no significant reduction was observed in L* values of control, non-microencapsulated bacteria containing, and microencapsulated-bacteria containing chocolates (Ozturk et al., 2021). This means that L* values are not affected by microencapsulation of bacteria and will not a problem in terms of consumer acceptance. a* and b* values were found to be significantly different for all chocolate samples, CFB had the highest a* and b* values, while CMB had the lowest values ($P < 0.05$). However, total color difference (ΔE*) was calculated by using the color parameters. The existence of microencapsulated bacteria in chocolate did not show a significant effect ($P > 0.05$) (Table 3).

3.4.3. Texture profile analysis

Texture analysis was evaluated for hardness and fracturability properties for 7 days after chocolate was prepared at the end of storage time. CNB, CFB, and CMB samples did not show noticeable significance in 7 days. However, all chocolate samples were significantly different after 60 days of storage ($P < 0.05$). CNB and CFB samples had higher hardness value than CMB sample for 7 days of storage. Hardness of CMB chocolate samples slightly reduced at the end of 60 days of storage (Fig. 2). This case can be attributed to the emulsion properties of microcapsules which can be easily integrated to chocolate fatty structure. On the other hand, hardness of CFB increased during storage period. The milk chocolate that had optimal hardness was demonstrated as a good carrier to produce chocolate containing probiotics experimentally. It

Table 2
Viability of L. *rhamnosus* in milk chocolate samples.

Chocolate Sample	Storage time					Storage Temperature
	0^{th} day	7th day	14th day	30th day	60th day	
CFB	7.457 ± 0.07^{aA}	7.318 ± 0.09^{aAB}	7.132 ± 0.14^{aB}	7.284 ± 0.11^{aAB}	7.204 ± 0.17^{aB}	4 °C
	7.457 ± 0.07^{aA}	7.307 ± 0.22^{aA}	7.288 ± 0.22^{aA}	7.252 ± 0.10^{aA}	6.953 ± 0.19^{aB}	25 °C
CMB	7.076 ± 0.13^{bABC}	7.096 ± 0.12^{aAB}	6.795 ± 0.11^{bBC}	7.248 ± 0.28^{aA}	6.747 ± 0.32^{Ac}	4 °C
	7.076 ± 0.13^{bA}	6.708 ± 0.14^{bA}	6.706 ± 0.16^{bA}	6.677 ± 0.13^{bA}	5.879 ± 0.65^{Bb}	25 °C

a-c: Means indicated with different small letters represent significant differences on the same column ($P < 0.05$). A-C: Means indicated with different capital letters present significant differences on the same line ($p < 0.05$).
CNB was control chocolate without any bacteria, CMB was chocolate including microencapsulated bacteria, and CFB was chocolate containing non-microencapsulated bacteria.

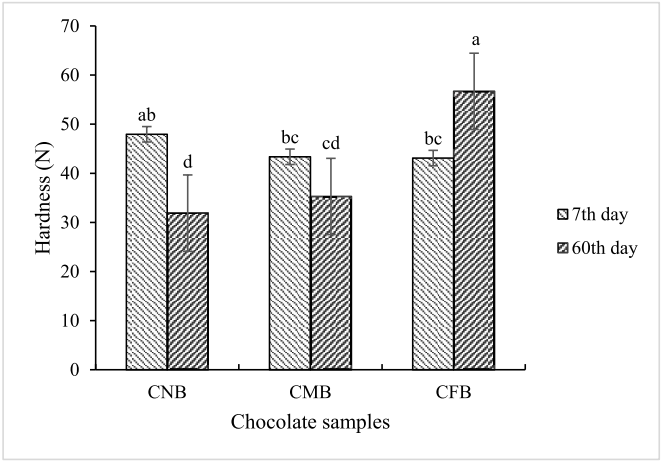

Fig. 2. The hardness of chocolate samples after 7 and 60 days of storage.
Mean values in the certain group of columns, labelled with the different lowercase letters (a, b, c, d) are significantly different ($P < 0.05$).
CNB was control chocolate without any bacteria, CMB was chocolate including microencapsulated bacteria, and CFB was chocolate containing non-microencapsulated bacteria.

was shown that free bacteria influenced chocolate hardness because of migration of fat (Bulatovic et al., 2016).

Fracturability represents a low and high degree of cohesiveness and the high degree of the hardness in the product, respectively. Therefore, it refers to force needed to compress product and compress before it breaks between teeth (De Clercq et al., 2012; Szczesniak, 2002). All chocolate samples were statistically notable different from each other between samples ($P < 0.05$). Fracturability values did not change significantly 7th days' storage time for CFB and CMB samples. While CFB sample was remained unchanged with higher fracturability, CNB and CMB samples kept the same fracturability value until the end of storage period (Fig. 3).

Chocolate samples with non-microencapsulated bacteria, a hydrophilic phase (difficult to interact with the hydrophobic structure of chocolate) caused high fracturability and hardness. Since chocolate fracturability can be related to hardness and correlations between the

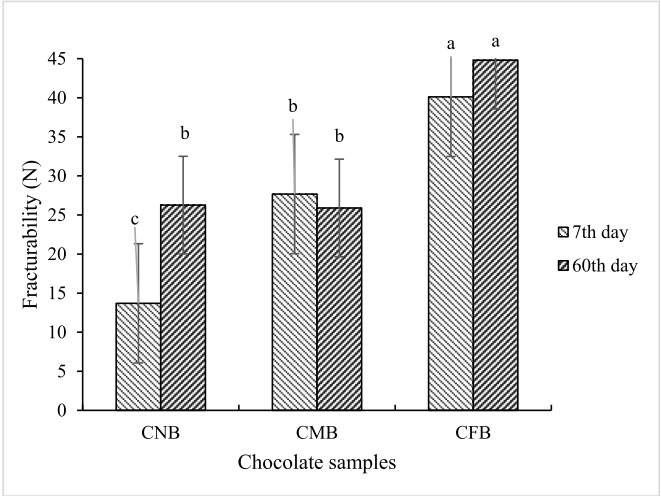

Fig. 3. The fracturabilitiy of chocolate samples after 7 and 60 days of storage.
Mean values in the certain group of columns, labelled with the different lowercase letters (a–
c) are significantly different ($P < 0.05$).
CNB was control chocolate without any bacteria, CMB was chocolate including microencapsulated bacteria, and CFB was chocolate containing non-microencapsulated bacteria.

two texture parameters were tested by Pearson correlation. There was deduced a significant correlation ($P < 0.05$) between fracturability, hardness and bacterial count. The obtaining of sufficient correlation between these parameters of the chocolate is very difficult because of the quality parameters of chocolate is affected by various uncontrollable factors during production, standardization challenging. In this study, the correlation between these parameters were found higher than 71%.

3.5. Sensory analysis

Sensory analysis was performed after microbiology analysis to ensure the safety of consumption of chocolate samples. Color, texture, taste, and general acceptance parameters were found mostly similar for all chocolate samples, it was no significant differences ($P > 0.05$). However, control chocolate was preferred as the best in terms of odor property by the panelists (Table 4).

It was observed that GABA producer bacteria usage in chocolate did not generally change the sensory characteristics of chocolate except odor property. According to sensory preferences of consumers, all the samples had high scores between 3.8 and 5.0 (Table 4). General acceptability of chocolate with GABA producer bacteria (microencapsulated and free) obtained by sensory evaluation were found as the same with CNB. Lalicic-Petronijevic et al. (2017) demonstrated that free or microencapsulated bacteria were not significantly affect chocolate sensory features. Another most important quality parameters of chocolate are the particle size of chocolate (Afoakwa, 2010). Addition of microcapsules did not affect the textural structure of the chocolate. As a result of sensory analysis, no difference found between CMB and CNB by panelists.

3.6. Simulated in vitro digestion of L. rhamnosus

Chocolate with/without microencapsulated bacteria and free bacteria were tested in simulated digestion system (SSF, SGF, and SIF) to find the viability of the bacteria, as duplicates. Initially, viable cell count of free bacteria was 9.0 log CFU/mL, while in chocolate samples, microencapsulated and non-microencapsulated bacteria counts were approximately 7.0 log CFU/g.

Viability and survivability of bacteria in CMB and CFB samples were not significantly different in SGF and SIF ($P > 0.05$). The survival rate of bacteria was found as approximately 90% for CMB sample and 83% for CFB sample (Fig. 4).

Many of the lactic acid bacteria currently used in commercial products belong to the *Lactobacillus* or *Bifidobacterium* genera (Cook et al., 2012), which are particularly sensitive to the harsh conditions found in many foods and in the human gut (Yao et al., 2020). *L. brevis* was integrated into different commercial products such as chocolate, yogurt, and beverages. Research has shown that chocolate provides protective effect against bacteria from gastric conditions when compared to other commercial products (Yonejima et al., 2015;

Table 4
The result of sensory analysis.

CHOCOLATE SAMPLES	COLOR	ODOR	TEXTURE	TASTE	GENERAL ACCEPTANCE
CNB	4.28 ± 0.60^{a}	4.28 ± 0.68^{a}	4.10 ± 0.63^{a}	4.45 ± 0.60^{a}	4.38 ± 0.59^{a}
CFB	4.225 ± 0.62^{a}	3.80 ± 0.65^{ab}	4.03 ± 0.70^{a}	4.18 ± 0.84^{a}	4.18 ± 0.71^{a}
CMB	4.28 ± 0.55^{a}	3.95 ± 0.64^{b}	3.98 ± 0.73^{a}	4.15 ± 0.89^{a}	4.15 ± 0.70^{a}

Means indicated with different letters in the same column are different ($P < 0.05$).
CNB was control chocolate without any bacteria, CMB was chocolate including microencapsulated bacteria, and CFB was chocolate containing non-microencapsulated bacteria.

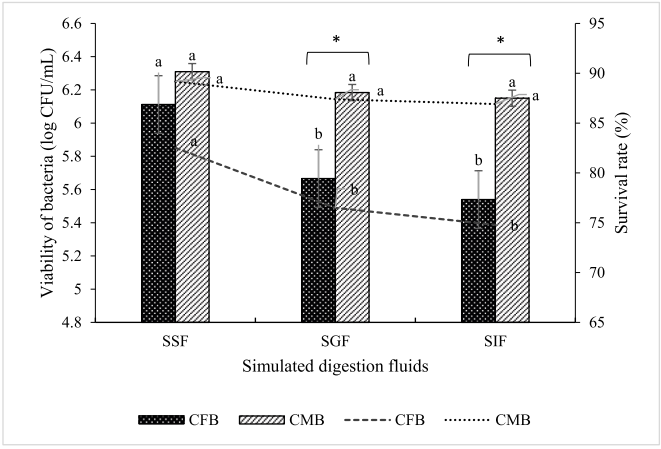

Fig. 4. Viability and survival rate of non-microencapsulated and microencapsulated bacteria in chocolate samples at the end of the simulated *in vitro* digestion (SSF, SGF, and SIF).
* shows a significant difference between the viability and survival rate of free and microencapsulated bacteria in chocolate samples within the same digestion fluid ($P < 0.05$). Different letters show a significant difference among different digestion fluids within the bacterial form ($P < 0.05$).
CNB was control chocolate without any bacteria, CMB was chocolate including microencapsulated bacteria, and CFB was chocolate containing non-microencapsulated bacteria.

Klindt-Toldam et al., 2016; Succi et al., 2017). This can be explained that chocolate butter has a protective effect for bacteria under stress factors such as bile, digestive enzymes, and acid (Lahtinen et al., 2007). An important factor that affects the survival of bacterial strains is pH; survival is constrained at low pH values. Bacteria have high mortality rates below pH 3.0 (Mainville et al., 2005). Encapsulation provides higher survival rate under pH and bile conditions (Serrano-Casas et al., 2017). However, survival of GABA producer *L. rhamnosus* were found at high rates below pH 3.0 in this study.

All samples were tested in SGF for 120 min after adjusting pH 3.0 at 37 °C. The survival rate of bacteria and the viable cell count in CMB samples was found at higher values than CFB samples (Fig. 4). In SIF, survival rate of *L. rhamnosus* in CMB sample was found as 87%.

Viable cell count of *L. rhamnosus* in CFB sample decreased approximately 2 log cycles during simulated *in vitro* analysis, whereas bacteria count in CMB sample decreased about 1 log cycle. Significantly different reduction was found between initial and final counts of CFB sample ($P < 0.05$). Viability in CMB sample was found to be more stable than CFB during simulated digestion. The viability of bacteria in these samples were found over 6.0 CFU/mL (Fig. 4).

Succi et al. (2017) investigated probiotic integration into milk and dark chocolates. Viability of lyophilized forms of *Lacticaseibacillus paracasei* and *L. rhamnosus* GG decreased about 5.5 log CFU/g and 6.0 log CFU/g under gastric conditions, respectively. However, approximately 4.0 log CFU/g decrease were found for the same strains integrated in dark chocolate at the end of SGF experiments. Besides, *L. rhamnosus* and *L. paracasei* were increased between 1.0 log and 0.7 log CFU/g in SIF. In another study, survival rates of *L. helveticus* and B*ifidobacterium longum* in milk chocolate were found 91% and 80% after the simulated intestinal conditions, respectively (Possemiers et al., 2010).

4. Conclusion

Different reference LAB strains were screened for their GABA-production abilities by RP-HPLC method using Pico. Tag column. Among the other LAB strains, *L. rhamnosus* produced the highest GABA concentration as almost 59000 mg/L at 37 °C for 24 h. Hence, the functional chocolate product was prepared by using microencapsulated *L. rhamnosus*. Survival rates of *L. rhamnosus* (microencapsulated and non-microencapsulated) in chocolate samples were 83% and 79%, respectively at 4 °C for 60 days of storage. Chocolate samples with microencapsulated bacteria showed better textural properties and higher survivability during simulated digestion system. Therefore, chocolate products can be a good carrier to protect the viability of bacteria against stress conditions. This study offers future opportunities to alleviate anxiety disorder using GABA-producer bacteria in chocolate by clinical studies.

Credit author statement

Merve OZER: Conceptualization, investigation, methodology, writing original draft, writing-review & editing, Burcu OZTURK: Investigation, methodology, writing-review & editing, Ali Adnan HAYALOGLU: Methodology, writing-review & editing, Sebnem TELLIOGLU HARSA: Conceptualization, investigation, methodology, supervision, writing-review & editing.

Declaration of competing interest

There are no conflicts of interest to declare.

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