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Effect of different microencapsulating materials on the viability of *S. thermophilus* CCM4757 incorporated into dark and milk chocolates

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ABSTRACT

This study aimed to evaluate the viability and bioaccessibility of *Streptococcus thermophilus* CCM4757 strain supplemented in dark and milk chocolates during storage period and pass through simulated *in vitro* gastro-intestinal tract. Microencapsulated and non-microencapsulated *S. thermophilus* CCM4757 strain added into the chocolates. Emulsion technique was used to microencapsulate cells with various biopolymers; carboxymethyl-cellulose, pectin, gum arabic, and cellobiose. The microencapsulated *S. thermophilus* with these coating materials was found to be viable higher than 9 log CFU/g up to 180 days of storage at 4 °C.

Microbiological, physicochemical, and sensorial attributes of the chocolates containing microencapsulated and non-microencapsulated *S. thermophilus* CCM4757 were analyzed. The microencapsulated *S. thermophilus* showed a good survivability in milk (7.12 log CFU/g) and dark (6.90 log CFU/g) chocolate samples during 180-day storage at 4 °C. Supplementation of *S. thermophilus* did not affect significantly ($P > 0.05$) the sensory attributes of the chocolates. The results showed that *S. thermophilus* CCM4757 exhibited good cell survivability higher than 85% in chocolates under simulated gastro-intestinal fluids.

S. thermophilus supplementation into the chocolate protected the viability of cells and did not affect the sensorial characteristics and moisture content of chocolates. The present study demonstrated that the dark and milk chocolates could be used as an important matrix to carry probiotics.

1. Introduction

Nowadays, functional foods are a very popular and they are defined as foods or food ingredients which improve health and decrease the disease risks beyond nutritional values (Ozen et al., 2012). Food industries have invested resources in the development of new processed foods that may provide functional benefits to consumers' well-being (Granato et al., 2020). The functional food sector is growing rapidly with the application of probiotic bacteria as food additives. Probiotic food utilization presents many advantages related to bacterial growth, survivability, viability, stability and functionality in food processing, storage and consumption (Min et al., 2019).

Lactic acid bacteria (LAB) are most common probiotics which have therapeutic effects on human health (Parvez et al., 2006). LAB are used numerous application in food industry because they are considered as Generally Recognized as Safe (GRAS) and their probiotic potentials makes them very interesting applications as functional food (Quinto et al., 2014). Although *Lactobacillus* spp. and *Bifidobacteria* spp. are the most studied probiotic bacteria in food products; probiotic sources are not limited with these LAB. *Streptococcus thermophilus* is another conventional dairy starter bacterium which considered as important industrial bacteria (Padmanabhan et al., 2020). Recent studies showed that *S. thermophilus* have a probiotic potential (Zhang et al., 2020) and have therapeutic effects on gut related diseases (Dargahi et al., 2020; Vitetta et al., 2019).

S. thermophilus is an aerotolerant anaerobe, gram-positive and thermophilic bacterium with an optimal growth temperature of 42 °C (Facklam, 2002). The bacterium is one of the starter culture bacteria of yogurt and is the most important species of industrial LAB after *Lactococcus lactis* (Hols et al., 2005). Besides the traditional use as starter culture in preparation of yogurt, it is used in production of quark, kefir, and several cheese varieties (Chandan et al., 2017). Its main feature is the lactic acid production in a dairy fermentation, which is resulted in fast acidification and other microorganisms' inhibition (Evivie et al., 2019). *S. thermophilus* produces secondary metabolites; exopolysaccharides and acetaldehyde, which affect the texture and flavor of the food product. A large number of studies in human or animal models have shown beneficial health effects for *S. thermophilus,* such as alleviation of

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lactose intolerance (Savaiano, 2014), prevention of chronic gastritis (Rodríguez et al., 2009) and prevention of infectious diarrhea (Saavedra et al., 1994). The species act mainly *via* the production of antimicrobial compounds (Rossi et al., 2013), and also through its anti-inflammatory (Junjua et al., 2016) and antioxidant properties (Ito et al., 2003) or its capability to enhance epithelial barrier function (García-Albiach et al., 2008). According to these beneficial properties, *S. thermophilus* can be considered as a potential probiotic microorganism (Martinović et al., 2020). Probiotics are defined as "live microorganisms which confer a health benefit on the host when administered in adequate numbers" (WHO/, 2002 2002). Foods must include at least 1.0×10^6 CFU/g live probiotics to exert beneficial health effects (Prado et al., 2008). However, high amount of probiotic microorganism consumption does not guarantee the high viability rate after microorganisms arrive in the gastro-intestinal tract (Iravani et al., 2015). Maintaining viability and functionality of the probiotic in gut system, encapsulation is used to protect microorganisms from adverse environmental conditions (Burgain et al., 2011; Raddatz et al., 2020). There are different encapsulation methods such as emulsion, extrusion, spray drying and freeze-drying. The most suitable encapsulation technique is emulsion method (Heidebach et al., 2012). The encapsulation process ensures preservation of amount of the cells and long-term viability of the live microorganisms. This process is a technology that protects not only sensitive microorganisms but also components in food with edible polymer materials (Eslami et al., 2017).

Food-grade biopolymers, such as pectin, alginate, carboxymethyl cellulose, chitosan, xanthan gum, carrageenan, starch, and gelatin are largely applied using various microencapsulation techniques. Pectin is an anionic polysaccharide extracted from cell walls of higher plants and largely a linear polymer of polygalacturonic acid with varying degrees of methyl esterification (Yang et al., 2018). Cellulose is the most abundant natural raw material, which is a biodegradable, cheap and renewable polymer. It also is tough, fibrous and water insoluble and that helps in sustaining the structure of the algae and plants cell walls (Ciolacu et al., 2016). Chemically processed celluloses, such as methylcellulose, sodium carboxymethylcellulose (CMC) and hydroxypropyl methylcellulose, demonstrate a better film forming ability and chemical stability, which may offer better stabilization and protection to the microencapsulated probiotics in the harsh environmental conditions compared to the native celluloses, especially under the gastrointestinal condition (Marín-Peñalver et al., 2019; Pavli et al., 2018). Cellobiose consists of two glucose molecules that are linked by a β–1,4′glycosidic bond obtained from the partial hydrolysis of cellulose (Ouellette & Rawn, 2018). Gum arabic is an arabinogalactan polysaccharide-protein anionic complex (Randall et al., 1989). Gum arabic is commonly used due to its low viscosity, high water solubility and emulsifying features (Cano-Chauca et al., 2005).

Milk proteins have good properties such as binding small molecules, excellent gelation, self-assembly, gel swelling and capability to interact with other polymers (Livney, 2010). Therefore, whey proteins as milk proteins, exhibit a potential in the microencapsulation of LAB.

Food matrices are very important for the LAB delivery and viability during manufacturing and shelf life. The food range featuring with probiotic LAB have expanded from dairy products to meat, cereals, fruits, and chocolate (Gomand et al., 2019). Chocolate is an ideal matrix to protect encapsulated probiotics from environments because of the cocoa butter. Long shelf life and high fat content of the chocolate are the major advantages to carry probiotics. Traditional probiotic products shelf life is shorter than the chocolate and high fat content gives better protection in stomach and give more effective colonization in intestine (Toker et al., 2017).

Chocolate is prepared with cocoa, cocoa butter and sugar and its production process consists of mixing, refining, conching, tempering, molding, and packaging stages. Tempering is the most important stage to control crystallization of cocoa butter, to stabilize the polymorphic transitions of cocoa butter crystals and also to provide the shiny and smooth appearance of chocolate during storage period (Beckett, 2009).

S. thermophilus was selected for use in this study, considering its beneficial properties and potential probiotic attributes. This study involves researching microencapsulation of *S. thermophilus* by using water-in-oil emulsion technique with different coating materials and an investigation of viability levels of microencapsulated and non-microencapsulated *S. thermophilus* in dark and milk chocolate with and the main quality parameters such as texture, color, and sensorial properties during the storage period. Additionally, the survivability of *S. thermophilus* was evaluated under simulated *in vitro* gastro-intestinal tract.

2. Materials and methods

This study was carried out using 54.5% dark chocolate and milk chocolate, which were supplied by CALLEBAUT and ETİ GIDA SANAYI VE TICARET A.Ş. The dark chocolate contained 46 g carbohydrate, 5.1 g of protein and 37 g of fat per 100 g; milk chocolate contained 8.3 g protein, 50.7 g carbohydrate, 34.5 fat and 3.4 g dietary fiber per 100 g. *Streptococcus thermophilus* CCM4757 was provided from Czech Culture Collection of Microorganisms (CCM).

2.1. Microencapsulation of S. thermophilus

Water-in-oil emulsion method was carried out for the microencapsulation of *S. thermophilus* CCM4757 by making some necessary arrangements (Çabuk & Harsa, 2015). *S. thermophilus* CCM4757 was inoculated into M17 broth (Merck, Germany) at a rate of 1% and incubated for 24 h at 42 °C. The second incubation was performed with the refresh *S. thermophilus* CCM4757 inoculated into M17 broth and incubated at 42 °C for 16–18 h. After incubation, *S. thermophilus* CCM4757 cell solution (100 mL) was centrifuged (5000 rpm, 4 °C, 15 min). After the centrifugation, supernatant was removed, and pellets were kept at 4 °C.

2.1.1. Preparation of water-in-oil emulsions solutions

Whey protein concentrate (WPC) (Alfasol, Turkey) was dissolved in sterile distilled water using a magnetic stirrer. Stirring was performed for about 3 h to ensure proper dissolution, and the protein solution was denatured at 80 °C for 30 min. Denatured solution was cooled to room temperature. Crystalline methylcellulose (CMC), pectin, gum arabic, and CMC-cellobiose were dissolved separately in distilled water and the polysaccharide solutions were mixed for 3 h using a magnetic stirrer to ensure proper dissolution. Emulsions were prepared to be 9% whey protein concentrate and 9% polysaccharide (w/v) except pectin.

In the first step, primary water-in-oil emulsions were formed by emulsifying an inner aqueous phase made up by WPC/polysaccharide complex containing *S. thermophilus* into an oil phase containing 1% soy lecithin (Alfasol, Turkey) as an emulsifier. The primary emulsions were homogenized with an Ultra Turrax homogenizer for 5 min (Ultra Turrax, model T25, Janke and Kunkel, IKA Labortechnik, Staufen, Germany). The emulsions were then homogenized again in 0.1 M $CaCl_2$ solution (Applichem, Germany) for 2 min with a homogenizer. After that, these slurries were shaken with an orbital shaker for 30 min at 160 rpm to harden the microcapsules. The hardened microcapsules were separated from the solutions were performed by centrifugation at 1000 rpm for 1 h. The microcapsules were freezing at −20 °C, then lyophilized by a freeze-dryer (Lablanco Freezone 18, Kansas, USA) at −55 °C under 0.050 mBar vacuum for 48 h. The microcapsules obtained were preserved at 4 °C for further analyses.

2.1.2. Enumeration of microencapsulated S. thermophilus

In order to enumerate *S. thermophilus* CCM4757 in the microcapsules, which must be broken down. For this, microcapsules diluted 1/10 in peptone water were homogenized by homogenizer. The pour plate method was applied, after taking the samples from the appropriate

dilutions; petri dishes were incubated at 42 °C for 48 h, anaerobically with anaerobic kit (Thermo Scientific, Oxoid AnaeroGen, England).

2.2. Chocolate preparation and incorporation of S. thermophilus

S. thermophilus CCM4757 was added into chocolate after tempering process. Moreover, chocolate samples were molded in different temperature because of having different process. Therefore, milk chocolate was molded at approximately 28 °C, whereas dark chocolate was molded at 30 °C to be well-shape.

Two types of chocolate samples were prepared with *S. thermophilus* CCM4757. There are;

- DC, control dark chocolate samples without *S. thermophilus* CCM4757
- DM, samples of dark chocolate including microencapsulated *S. thermophilus* CCM4757
- DF, samples of dark chocolate including free cell (non-microencapsulated) of *S. thermophilus* CCM4757
- MC, control milk chocolate samples without *S. thermophilus* CCM4757
- MM, samples of milk chocolate including microencapsulated *S. thermophilus* CCM4757
- MF, samples of milk chocolate including free cell of *S. thermophilus* CCM4757

In order to prepare DM and MM samples, microencapsulated *S. thermophilus* cell lyophilized powder and to prepare DF and MF, *S. thermophilus* cell mass after centrifugation was used and 1:100 diluted in chocolate.

These chocolate samples were stored at 4 °C for 180 days. All types of chocolate samples were analyzed after 7 days of storage time so that these can get the chocolate stabilization. All chocolate types were prepared in at least two replicates.

2.3. Chocolate characterization

2.3.1. Microbiological evaluation

Chocolate samples of each formulation were suspended in peptone water; serial dilutions were prepared and used for microbiological viability analysis. Enumeration of *S. thermophilus* CCM4757 in samples was determined by cultivating on M17 agar, after that incubated at 42 °C for 48 h, anaerobically with anaerobic kit. The counts were done on the 0^{th}, 7^{th}, 30^{th}, 60^{th}, 90^{th}, 120^{th} and 180^{th} days, were expressed as log CFU/g.

Microbiological quality of chocolate samples was determined by counting yeasts, molds, and *Salmonella*. Brilliant Green Agar (Merck, Germany) was used *Salmonella* counting and incubated at 35 °C, for 24 h. The mold and yeast counting were performed in Potato Dextrose Agar (Oxoid, England) and incubated at 30 °C, for 120 h.

2.3.2. Physicochemical assessments

Color measurements of chocolate samples were determined using Konica Minolta colorimeter (CR 410, Konica Minolta, Tokyo, Japan).

To determine the moisture content of chocolate samples, which were dried at 105 °C for 24 h (Rajam et al., 2012). The average moisture content (%) was calculated as

$$\text{Moisture content } (\%) = [(W_{wet} - W_{dry})/(W_{wet})] \text{ x } 100$$

Where wet chocolate sample weight is W_{wet} and dry chocolate sample weight is W_{dry}.

The hardness of milk and dark chocolates were analyzed by using the Texture Analyzer (TA-XT PLUS, Stable Micro Systems, Godalming, UK) with a load cell of 50 N. Hardness is the highest penetrating force necessary for penetration of needle into the chocolate (26 × 20 mm, depth 20 mm). The hardness measurements of chocolates were performed by a needle probe at 25 °C. The chocolates had the same dimensions and smooth surfaces. The analysis was performed with three replications and five repeat measurements, mean values and standard errors were calculated (Cikrikci et al., 2016).

2.4. Simulated in vitro gastro-intestinal digestion

The experiment was performed on milk and dark chocolates supplemented microencapsulated and non-microencapsulated *S. thermophilus* CCM4757, following the protocols described by Paz-Yepez et al. (2019) with some modifications. Three digestion fluids were prepared: salivary, gastric, and intestinal.

Simulated salivary fluid (SSF) were prepared with 15.1 mmol/L KCl, 13.6 mmol/L $NaHCO_3$, 3.7 mmol/L KH_2PO_4, 0.15 mmol/L $MgCl_2(H_2O)_6$, 1.5 mmol/L $CaCl_2$, 0.06 mmol/L $(NH_4)_2CO_3$ and α-Amylase (75 Unit/mL) (Sigma-Aldrich). The SSF was adjusted to pH 8.0 at 37 °C. Milk and dark chocolates (containing microencapsulated/non-microencapsulated cells) were mechanically broken by a Stomacher. 5 mL SSF and 5 mg chocolate sample were homogenized and incubated at 37 °C for 3 min. Non-microencapsulated cells within the SSF were also used as control.

Simulated gastric fluid (SGF) were prepared with 6.91 mmol/L KCl, 25 mmol/L $NaHCO_3$, 0.9 mmol/L KH_2PO_4, 47.2 mmol/L NaCl, 0.1 mmol/L $MgCl_2(H_2O)_6$, 0.15 mmol/L $CaCl_2$ and 0.5 mmol/L $(NH_4)_2CO_3$. Pepsin (Sigma-Aldrich) was added into the SGF to achieve a concentration in gastric mixture of 2000 Unit/mL and adjusted to pH 3.0 with 1 N HCl. After SSF, oral bolus in a ratio 1:1 (v/w) was added to the SGF. Samples were mixed orbital shaker at 55 rpm and incubated at 37 °C for 120 min.

Simulated intestinal fluid (SIF) were prepared with 6.8 mmol/L KCl, 85 mmol/L $NaHCO_3$, 0.8 KH_2PO_4, 38.4 mmol/L NaCl, 0.6 mmol/L $CaCl_2$, 0.33 mmol/L $MgCl_2(H_2O)_6$, 1 mM bile salts (Sigma-Aldrich) and pancreatin (2000 LU/g of fat) (Sigma-Aldrich). The SIF and gastric chime were mixed in a ratio 1:1 (v/w) and adjusted pH 7.0 with 1 N NaOH. Samples were mixed orbital shaker at 55 rpm and incubated at 37 °C for 120 min. The pH value was monitored during the digestion process.

The survivability of *S. thermophilus* CCM4757 (microencapsulated/non-microencapsulated) in milk and dark chocolate samples were evaluated at the end of the incubations within SSF, SGF and SIF. Mean values and standard errors were calculated. Survival rate (%) was calculated by following equation:

$$\text{Survival rate } (\%) = (\log \text{CFU } N_1 / \log \text{CFU } N_0) * 100$$

where N_1 is the viable cell counts after treatment by SSF, SGF, or SIF and N_0 is the viable cell counts before treatment.

2.5. Sensory analysis

The sensory analysis was performed after a week of the chocolate production and the panel consisted of 20 untrained individuals. Acceptance test of qualifications (color, smell, texture, taste, and overall acceptance) using a 7-point hedonic scale (1 = very bad and 7 = excellent) was carried out (Granato et al., 2010). Panelists evaluated 6 different chocolate samples at one time. Each of chocolate samples were encoded with a 3-digit arbitrary number and presented properly to the panelists.

2.6. Statistical analysis

All experiments were carried out in parallel. Data analysis was performed using Minitab 19.0 software (Minitab Inc., State College, PA, USA). The results were expressed with standard deviations. Variance analysis (ANOVA) test and Tukey's test were used for the differences

between the chocolate samples.

3. Results and discussion

3.1. Viability of S. thermophilus after microencapsulation

The effect of microencapsulation by emulsion method using different coating materials (carboxymethylcellulose, pectin, gum arabic and crystalline methylcellulose-cellobiose) on the viability of *S. thermophilus* CCM4757 after freeze-drying is shown in Fig. 1. Different coating materials using for microencapsulation differently protect cells from freeze-dryer conditions (at −55 °C under 0.050 mBar vacuum), so the 0th cell counts showed difference. During 14-day storage the viable cell counts increased, this might be explained owing to the prebiotic properties, higher moisture absorption capacity and moisture content of the coating materials. In the case of 14th and 180th days, no differences in survival were found among four coating materials samples with whey protein concentrate (WPC). According to Fig. 1, the viable cell counts ranged from 9.84 log CFU/g to 9.88 log CFU/g when using CMC-cellobiose as a coating material, ranged from 10.05 log CFU/g to 9.68 log CFU/g when using CMC without cellobiose. In the case of gum arabic was used as a coating material, the viability ranged from 10.26 log CFU/g to 9.94 log CFU/g, likewise, when using pectin, the viable cell count changed between 10.09 log CFU/g and 9.88 log CFU/g. In the light of experiments performed the microencapsulated *S. thermophilus* CCM4757 exhibited high viability.

El-Shafei et al. (2018) investigated various biopolymers (skim milk, chitosan, dextrin, and whey protein) to efficient encapsulation of *Streptococcus thermophilus* CH-1 by using extrusion method. Authors revealed that alginate-chitosan and alginate-denatured whey protein were better in protecting *S. thermophilus* under simulated gastric fluid, while alginate-denatured whey protein exhibited good cell survivability under simulated intestinal fluid, the viability loss was 0.34 log cycle.

Singh et al. (2017) demonstrated that, carboxymethyl cellulose–chitosan matrices were used for the microencapsulation of the probiotic *L. rhamnosus* GG through a nozzle-spray method. The results confirmed the matrices had potential for microencapsulation and delivery of *L. rhamnosus* GG with an acceptable survival rate.

de Almeida Paula et al. (2019) obtained a microencapsulation efficiency of 97.8% in their study that addressed the microencapsulation of *L. plantarum* in the double emulsion followed by complex coacervation, using gum arabic and gelatin and the viability of microencapsulated cells was 8.6 log CFU/g. Silva et al. (2019) reported that microencapsulation of *L. acidophilus* La-5 within gelatin and gum arabic using complex coacervation technique demonstrated a high microencapsulation efficiency ranged from 77% to 87%. The microencapsulation was an important process to achieve significant protection of probiotic cells against simulated gastro-intestinal conditions compared with non-microencapsulated cells. Microencapsulation also enhanced the survivability of probiotic cells during period of storage at −18 °C for 120 days, 7 °C for 105 days and 25 °C for 45 days. Similar results found by Holkem et al. (2016), who reported the use of emulsification to microencapsulate *B. lactis* with a microencapsulation efficiency of 89%.

Heidebach et al. (2009) examined the probiotics microencapsulated in milk protein complexes. They reported that the survivability of *L. paracasei* and *Bifidobacterium lactis* microencapsulated in milk protein significantly increased compared with non-microencapsulated cells. The viable cell counts of microencapsulated *L. paracasei* and *B. lactis* were higher by 0.8 and 2.8 log CFU/g, respectively, than non-microencapsulated cells after 90 min under low pH at 37 °C.

Microencapsulation of probiotic *L. rhamnosus* GG in microgels prepared with pectin has been exhibited to improve viability of cells within simulated gastro-intestinal tract conditions (Li et al., 2016). In a study, Gerez et al. (2012) demonstrated that *L. rhamnosus* CRL 1505 into pectin coated with whey protein enhanced the survivability of the cells after exposure to simulated gastric conditions when compared to non-microencapsulated free cells. *L. acidophilus* La5 microencapsulation by ionotropic gelation with using pectin and whey protein showed great microencapsulation efficiency (84%) and protected the viability of the probiotic when exposed to simulated gastro-intestinal conditions (Gebara et al., 2013). Odun-Ayo et al. (2016) found that the citrus pectin and alginate probiotic microbeads improved the survivability of *L. acidophilus* ATCC 4356 compared to the free cells, exposure to simulated gastric juice for 3 h, resulted in 82.7% survival of probiotic. When compared to the previous literature results these findings gave similar microencapsulation efficiency with food grade biopolymer pectin. Microencapsulated *S. thermophilus* cells using pectin, incorporated into chocolates for further experiments.

3.2. Viability of S. thermophilus CCM4757 in milk and dark chocolate

As indicated in Table 1, initial viable cell count of *S. thermophilus* (form as non-microencapsulated) in chocolate samples ranged from 6.30 to 7.0 log CFU/g and microencapsulated bacteria ranged from 6.77 to 7.55 log CFU/g.

The production of chocolate process involves some steps that can

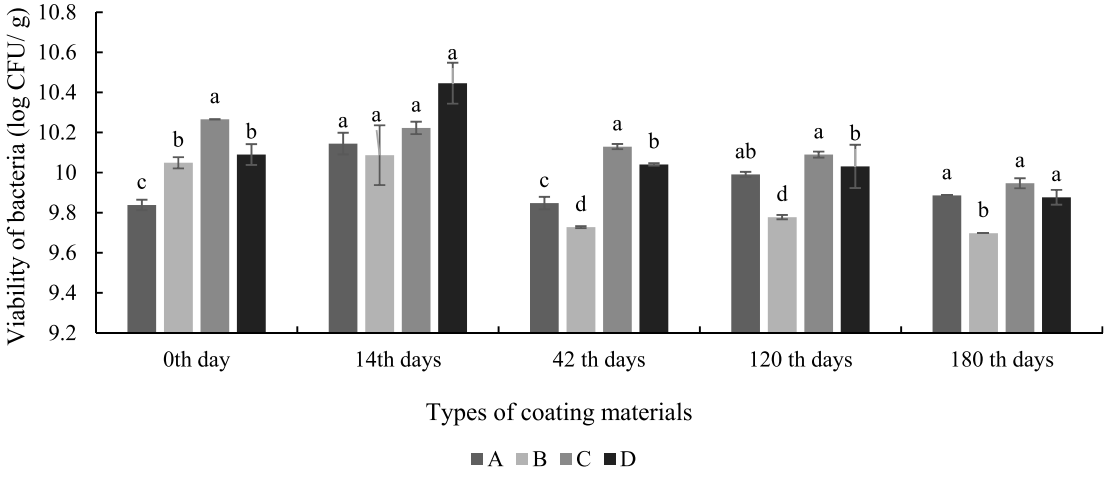

Fig. 1. Change in viability of *S. thermophilus* CCM4757 microencapsulated with different coating materials.
Viability of *S. thermophilus* CCM4757 microencapsulated with different coating materials. A) Crystalline methylcellulose-cellobiose and WPC, B) Crystalline methylcellulose and WPC, C) Gum arabic and WPC, D) Pectin and WPC. An initial inoculum of 11 log CFU/mL was used; enumeration was conducted at 0th, 14th, 42nd, 120th and 180th days.

Table 1
Viability changes of *S. thermophilus*CCM4757 in milk and dark chocolates.

	0^{th} day	7^{th} day	30^{th} day	60^{th} day	90^{th} day	120^{th} day	180^{th} day
MM	7.53 ± 0.029^{ABa}	7.34 ± 0.015^{ABab}	7.49 ± 0.11^{Aa}	7.53 ± 0.15^{Aa}	7.45 ± 0.14^{ABa}	7.32 ± 0.017^{ABab}	$7,15 \pm 0.10^{Bb}$
MF	6.845 ± 0.01^{ABc}	6.96 ± 0.23^{ABb}	7.04 ± 0.04^{Ab}	7.095 ± 0.06^{Aa}	7.05 ± 0.04^{ABa}	7.047 ± 0.03^{ABab}	6.684 ± 0.03^{Bd}
DM	6.83 ± 0.07^{ABbc}	6.98 ± 0.09^{ABb}	7.43 ± 0.12^{Aa}	7.577 ± 0.11^{Aa}	6.90 ± 0.04^{ABbc}	6.65 ± 0.22^{ABc}	6.90 ± 0.05^{Bbc}
DF	6.778 ± 0.001^{ABab}	6.54 ± 0.07^{ABcd}	6.68 ± 0.13^{Abc}	6.68 ± 0.026^{Abc}	6.65 ± 0.08^{ABbc}	6.92 ± 0.13^{ABa}	6.36 ± 0.07^{Bd}

Results are shown as mean ± standard deviation. Different capital letters (A, B, C, D) in the same column show a significant difference in the Tukey test ($P < 0.05$). Means with different superscripts (a, b, c, d) within a row were significantly different ($P < 0.05$).

damage to probiotic cells (Silva et al., 2017). Thus, the supplementation of *S. thermophilus* into milk and dark chocolates were performed after the tempering step to avoid to adverse effect of high temperature on bacterial cells. The viable cell counts of fresh milk chocolates were determined (0^{th} day), for MF count was 6.85 log CFU/g and for MM count was 7.52 log CFU/g, end of the storage period (180^{th} day) the cell counts decreased 0.17 log CFU/g and 0.40 log CFU/g, respectively. The fresh dark chocolates were also evaluated, initially (0^{th} day) the cell counts of DF and DM were 6.78 log CFU/g and 6.83 log CFU/g, respectively. After 180-day storage, when the viable cell count of DF reduced to 6.36 log CFU/g, the viable cells increased in DM to 6.90 log CFU/g. The results showed that, *S. thermophilus* CCM4757 exhibited an excellent survivability in milk and dark chocolate samples during 180-day storage period at 4 °C. Milk chocolate which contains higher protein and carbohydrate content might lead to high viable cell count during storage when compare to the dark chocolate.

Chocolate is an ideal matrix to protect encapsulated probiotics from environments because of the cocoa butter. Chocolates contain antioxidant compounds, so they might serve as a better probiotic cell carrier than dairy products for gastro-intestinal tract (Possemiers et al., 2010). In this regard, Klindt-Toldam et al. (2016) found that chocolate can be a good carrier for the microencapsulated probiotics *B. lactis* HN019 and *L. acidophilus* NCFM. In another study, Yonejima et al. (2015) demonstrated that *L. brevis* was shown a better stability against gastric acid treatment in milk chocolate than in beverages or in probiotic powder. Additionally, Kemsawasd et al. (2016) found that milk and dark chocolates were suitable carriers to protect immobilized probiotic cells from the gastro-intestinal injuries. These authors assumed that high levels of polyphenols might be able to increase the probiotic cell survivability in dark chocolate, to protect them from the toxicity of oxygen (Kemsawasd et al., 2016).

In a similar study, Erdem et al. (2014) observed lower than 1 log CFU/g reduction in *B. indicus* HU36 concentration compare to the initial count in chocolate samples, all samples contained more than 5 log CFU/g bacteria survival. Probiotic strains *L. paracasei* F19 and *L. rhamnosus* GG exhibited a good survivability when added to dark chocolate. These two strains were substantially constant during the storage period, with final loads of about 8 log CFU/g (Succi et al., 2017). Maragkoudakis et al. (2006) considered that 5 and 8 log CFU/g probiotic cell load in dairy products was acceptable.

Nebesny, Zyzelewicz, Motyl, and Libudzisz (2006) evaluated the survivability of *L. paracasei* and *L. casei* strains in dark chocolate, after 12 months storage of chocolate at different temperatures, survival rates of the strains were 89–94% at 4 °C, 80–87% at 18 °C and 60–67% at 30 °C, respectively. Based on the findings reported by Zarić et al. (2016), the viability in milk chocolate samples supplemented with *L. acidophilus* NCFM and *L. rhamnosus* HN001 strains remained at a satisfactory level, during 6 months of storage period, the survivability of these strains was more than 90%, with the viable cell count of approximately 8.1 log CFU/g.

In another study, during the storage period of 180 days, the viable cell number of *L. plantarum* HM47 remained greater than 8 log CFU/g in milk chocolate matrix (Nambiar et al., 2018). Lalicic-Petronijevic et al. (2015) studied viability on *L. acidophilus* NCFM® and *B. lactis* HN019 in both milk and dark chocolates. They found that, these two probiotic strains showed good survivability during storage at two different temperatures 4 °C and 20 °C. In a study carried out by Coman et al. (2012) *L. rhamnosus* and *L. paracasei* combination was used to produce chocolate. The products contained 7–9 log CFU/g viable probiotic at the end of the shelf life.

In this study, the chocolates were wrapped in an aluminum foil to protect the chocolates from humidity and other damages during storage period and to minimize the contact of bacterial cells with oxygen. According to Lalicic-Petronijevic et al. (2015), the aluminum package could play an important role during the storage period, since oxygen may adversely affect the probiotic survivability, especially those on the chocolate surface.

3.3. Microbial quality of chocolate

Salmonella, mold, and yeast counts were analyzed to determine whether all samples were produced under the hygienic conditions. *Salmonella* is a crucial criterion for chocolate products. Because *Salmonella* led to outbreaks, although its concentration in the foods was as low as 0.005 CFU/g (Hockin et al., 1989). In our study, the findings demonstrated that, *Salmonella*, mold and yeast were not detected.

3.4. Color analysis

Color is one of the most important features for consumer acceptance. Color changes in chocolates are because of the difference in their composition and production process. In order to evaluate the effect of the storage period on the chocolate color, the CIELab parameters (L*, a* and b*) have been analyzed (Table 2). In terms of L* (brightness) values, all milk chocolate samples showed higher L* values than dark chocolates during all storage periods. a* (redness) and b* (yellowness) values showed similar results to L* values, the dark chocolate samples demonstrated lower values than all milk chocolate samples. The L* values for all chocolate samples decreased with the period of storage. As it was expected, L* values decreased with the increase of cocoa concentration (dark chocolate samples) because lower values for L* values indicate a darker appearance. Regarding a* and b* values showed a reduction correlated with the cocoa concentration and the period of storage.

The microencapsulated *S. thermophilus* CCM4757 addition caused a decrease in L* values for the milk chocolates and an increase in L* values for the dark chocolates. This color change might be attributed to microcapsules are lighter in color than cacao. Tolve et al. (2018) demonstrated that, microencapsulated polysterol addition caused an increase in L* values for dark chocolates. The high-temperature conditions could cause chocolate samples to be lighter in color (Nightingale et al., 2011). According to the Gul (2017) higher L* values indicated that the microcapsules showed light color and Aryana and Mcgrew (2007) suggested that higher L* values of microcapsules were desired.

3.5. Moisture content

The results related to the moisture content of the milk and dark chocolate samples are reported in Table 2. The obtained results demonstrated no significant effect of the cocoa content and

Table 2
Color analysis and Moisture content.

		MC	MF	MM	DC	DF	DM
1st week	L*	31.92 ± 0.255^{Aa}	31.80 ± 0.225^{Aa}	30.92 ± 0.032^{Ba}	22.92 ± 0.731^{Eb}	24.46 ± 0.102^{Ca}	23.43 ± 0.183^{Da}
	a*	10.30 ± 0.173^{Aa}	10.48 ± 0.046^{Aa}	10.22 ± 0.133^{Aa}	6.57 ± 0.539^{Da}	7.80 ± 0.553^{Ba}	7.18 ± 0.057^{Ca}
	b*	11.88 ± 0.403^{Ba}	12.53 ± 0.691^{Aa}	11.67 ± 0.160^{Ba}	5.36 ± 0.495^{Cab}	6.75 ± 0.446^{Ea}	6.00 ± 0.111^{Da}
2nd month	L*	30.90 ± 0.692^{Aa}	29.79 ± 1.059^{Bb}	29.99 ± 0.481^{Bb}	22.40 ± 0.539^{Db}	23.58 ± 0.323^{Cb}	23.42 ± 0.391^{Ca}
	a*	9.56 ± 0.180^{Ab}	9.64 ± 0.344^{Ab}	9.43 ± 0.292^{Ab}	6.90 ± 0.465^{Ca}	7.34 ± 0.204^{Bb}	7.14 ± 0.208^{BCa}
	b*	10.64 ± 0.463^{Ab}	10.61 ± 0.590^{Ab}	10.45 ± 0.111^{Ab}	5.80 ± 0.677^{Ba}	6.16 ± 0.274^{Bb}	6.00 ± 0.223^{Ba}
	L*	27.34 ± 1.804^{Bb}	28.83 ± 0.635^{Ac}	26.98 ± 1.314^{Bc}	24.16 ± 1.128^{Ca}	22.21 ± 1.00^{Dc}	21.14 ± 0.764^{Db}
6th month	a*	9.33 ± 0.497^{Ab}	9.07 ± 0.155^{Ac}	9.01 ± 0.236^{Ac}	6.01 ± 0.384^{Bb}	5.68 ± 0.371^{Bc}	5.93 ± 0.142^{Bb}
	b*	9.80 ± 0.541^{Ac}	9.98 ± 0.189^{Ac}	10.15 ± 0.328^{Ac}	5.00 ± 0.556^{Bb}	5.30 ± 0.341^{Bc}	5.31 ± 0.135^{Bb}
Moisture content (%)		1.165 ± 0.05^{A}	1.160 ± 0.00^{A}	1.084 ± 0.235^{A}	1.442 ± 0.036^{A}	1.381 ± 0.079^{A}	1.421 ± 0.006^{A}

Results are shown as mean ± standard deviation. Different capital letters (A, B, C, D) in the same column show a significant difference in the Tukey test ($P < 0.05$). Means with different superscripts (a, b, c, d) within a row were significantly different ($P < 0.05$).

S. thermophilus cells (non-microencapsulated and microencapsulated) on the moisture of chocolates.

3.6. Textural properties

The perception of chocolate texture during chewing is of great importance. Hardness is one of the textural properties, refers to rigidity of chocolate sample and is directly related to perception of sensory during consumption (Shah et al., 2010).

Table 3 shows the effects of cacao concentration and addition of microencapsulated/non-microencapsulated cells on the hardness of the chocolates. Results showed that *S. thermophilus* CCM4757 supplementation had significant effect on chocolate hardness when compared with control chocolate samples (without bacteria) ($P < 0.05$). According to the table, as the cocoa concentrate increased, the hardness of the chocolate increased. The table also illustrated the effects of storage period on the hardness of the chocolate samples stored at refrigerator temperature. The hardness of all chocolate samples increased during storage and after 180 days no significant difference was observed between milk and dark chocolate samples.

3.7. Simulated in vitro gastro-intestinal digestion

The survivability of *S. thermophilus* CCM4757 in the milk and dark chocolate samples has been evaluated. Significant differences in the *S. thermophilus* release from chocolate samples into simulated gastric and intestinal fluids have been observed ($P < 0.05$). As reported in Fig. 2, the survivability of *S. thermophilus* in the simulated gastric fluid approximately 95% except DM sample, similarly in the simulated intestinal fluid the survivability ranged from 85 to 95%.

The effect of simulated intestinal fluid on survivability of *S. thermophilus* CCM4757 in chocolate samples with both free and microencapsulated cells, is shown in Fig. 3. This study showed that the microencapsulated *S. thermophilus* CCM4757, in dark and milk chocolates were found to have high survivability after being exposed to the SIF for 120 min. This may suggest that either pectin-WPC complex and/or chocolates were beneficial materials to protect *S. thermophilus* from the harsh conditions.

Table 3
Changes in hardness of milk and dark chocolate samples.

	7th day	60th day	120th day	180th day
MC	32.246 ± 0.672^{Cc}	32.128 ± 2.041^{Cb}	42.66 ± 1.556^{Bab}	53.192 ± 1.165^{Aa}
MM	27.58 ± 1.52^{Cc}	24.64 ± 5.03^{Cc}	36.053 ± 2.164^{Bbc}	47.465 ± 1.445^{Aa}
MF	30.065 ± 2.201^{BCc}	21.44 ± 5.58^{Cc}	35.22 ± 6.49^{Bc}	48.99 ± 7.68^{Aa}
DC	52.41 ± 4.81^{ABa}	39.43 ± 2.28^{Ca}	46.654 ± 2.231^{Ba}	53.87 ± 4.08^{Aa}
DM	43.301 ± 0.701^{BCb}	27.43 ± 2.68^{Dbc}	39.211 ± 1.085^{Cbc}	50.99 ± 3.3^{Aa}
DF	43.15 ± 3.62^{ABb}	23.76 ± 2.92^{Cc}	37.4 ± 5.01^{Bbc}	51.05 ± 7.26^{Aa}

Results are shown as mean ± standard deviation. Different superscripts letters (A, B, C, D) in the same column show a significant difference in the Tukey test ($P < 0.05$). Means with different superscripts (a, b, c, d) within a row were significantly different ($P < 0.05$).

When *S. thermophilus* CCM4757 cells pass through the stomach and reach the intestine, they are exposed to different conditions (especially pH). Despite of these changes in conditions, the microencapsulated cells show better survivability when compared to non-microencapsulated free cells. The findings from an investigation showed that when the probiotic cells were combined with chocolates, their survivability were tended to increase under simulated *in vitro* gastro-intestinal conditions (Possemiers et al., 2010).

Figs. 2 and 3 gives us a clear indication of the advantage of microencapsulation of bacteria within the matrix of pectin-whey protein concentrate. Non-microencapsulated cells have low survivability in harsh conditions. Exposing into the simulated gastric fluid for 120 min, number of microencapsulated cells increased up to 8.65 log CFU/g from an initial count of 8.15 log CFU/g.

Possemiers et al. (2010) found that the addition of encapsulated probiotic cells into chocolate sample can be a great medium to protect the cells from environmental conditions. Earlier, researchers suggested that the probiotic addition into chocolate improves its survivability under simulated *in vitro* gastrointestinal conditions (Succi et al., 2017; Valencia et al., 2016).

3.8. Sensory analysis

Sensory quality is a crucial property for consumers. The attitudes of consumers towards functional foods depend not only on the healthiness of the food consumed, but also on sensory quality and price like a conventional product (Ares et al., 2010). *S. thermophilus* supplemented chocolates and controls were evaluated, at the beginning of storage, by 20 untrained panelists with regard to color, smell, texture, taste and overall acceptance. The results are reported in Table 4. Panelists attributed to all samples very good scores for all sensory properties. This result showed that a concentration of *S. thermophilus* of about 6–7 log CFU/g did not affect the characteristics of white and dark chocolates. The sensorial attributes of these white and dark chocolates received scores between 4.75 and 5.75 on the 7-point hedonic scale. The interaction between the chocolate cocoa concentration and free and microencapsulated *S. thermophilus* did not show any significant effect ($P > 0.05$). According to Callebaut (2008) the chocolate supplemented with probiotics has no effect on chocolate texture, taste and mouth feel.

It can be concluded that both microencapsulated *S. thermophilus* dark and milk chocolates had similar sensory quality and that encapsulated bacteria did not have an effect on sensory and texture of final products in 180-day storage period.

Mirkovic et al. (2018) studied on quality parameters of dark

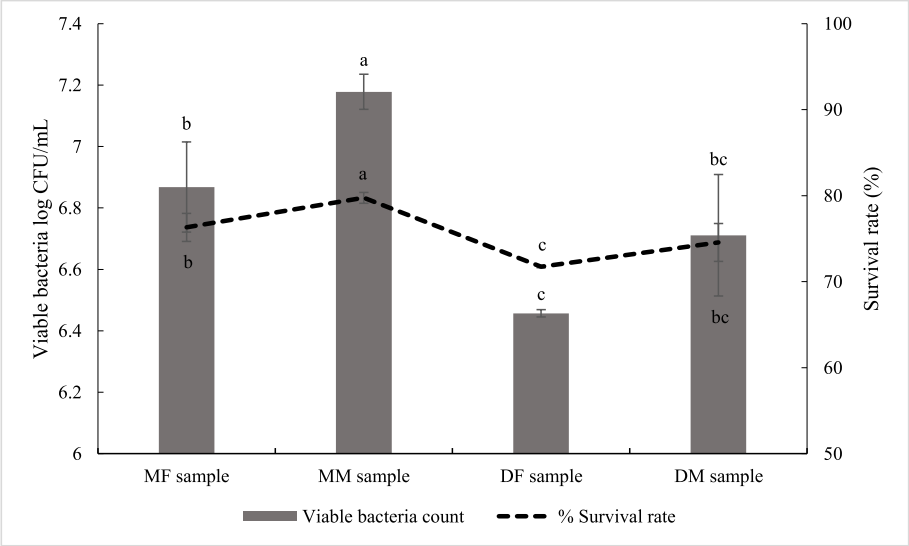

Fig. 2. Viability and survival rate of bacteria and chocolate samples in simulated gastric fluid at 37 °C.
S. thermophilus incorporated chocolate samples in simulated gastric fluid at 37 °C.Viability of *S. thermophilus* showed by columns and survival rate illustrated by dashed line. Different letters show a significant difference by the Tukey's test ($P < 0.05$).

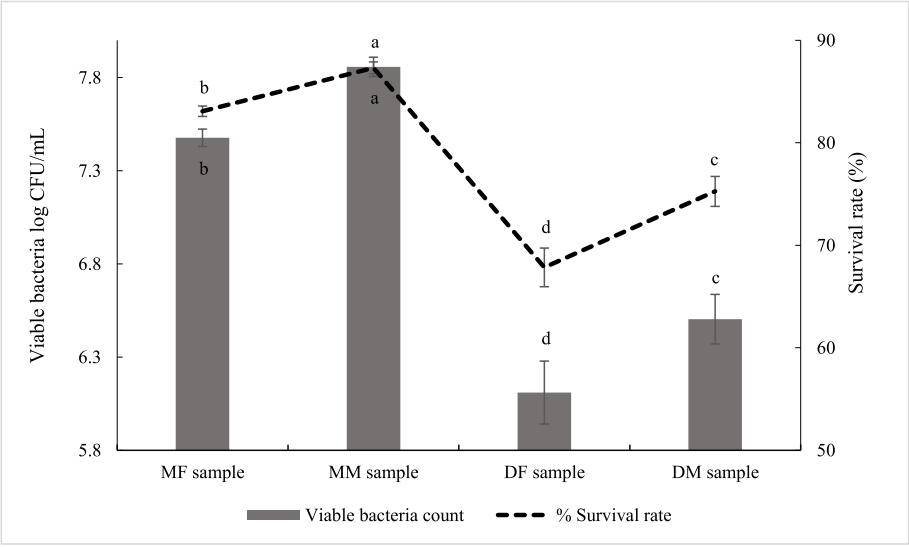

Fig. 3. Viability and survival rate of bacteria and chocolate samples in simulated intestinal fluid at 37 °C.
S. thermophilus incorporated chocolate samples in simulated intestinal fluid at 37 °C. Viability of *S. thermophilus* showed by columns and survival rate illustrated by dashed line. Different letters show a significant difference by the Tukey's test ($P < 0.05$).

Table 4
Sensory evaluation results of milk and dark chocolate formulations.

	MC	MF	MM	DC	DF	DM
Color	5.7 ± 0.571^{A}	5.4 ± 0.754^{A}	5.2 ± 0.894^{A}	5.3 ± 0.865^{A}	5.4 ± 0.995^{A}	5.7 ± 1.302^{A}
Smell	5.2 ± 0.768^{A}	5.5 ± 0.889^{A}	5.05 ± 0.826^{A}	5.0 ± 1.170^{A}	5.45 ± 0.999^{A}	5.05 ± 1.191^{A}
Texture	5.05 ± 0.945^{A}	4.85 ± 1.040^{A}	4.95 ± 1.146^{A}	5.15 ± 1.182^{A}	5.15 ± 0.988^{A}	5.1 ± 1.334^{A}
Taste	5.65 ± 1.137^{A}	5.75 ± 0.639^{A}	5.1 ± 1.210^{A}	4.75 ± 1.585^{A}	5.25 ± 1.209^{A}	5.3 ± 1.418^{A}
Overall acceptance	5.45 ± 0.999^{A}	5.55 ± 0.887^{A}	5.2 ± 1.152^{A}	5.2 ± 1.322^{A}	5.45 ± 1.146^{A}	5.35 ± 1.182^{A}

Results are shown as means ± standard deviation. Different capital letters on the same line show a significant difference by the Tukey's test ($P < 0.05$), $n = 20$.

chocolates enriched with encapsulated probiotic *L. plantarum*. It can be demonstrated that the dark chocolate enriched with microencapsulated probiotic gave a functional product with very good sensory and compositional characteristics.

4. Conclusion

In the present study, milk and dark chocolates were successfully enriched with microencapsulated and non-microencapsulated *S. thermophilus* CCM4757 strain, the viability of *S. thermophilus* was

studied during storage and under a simulated *in vitro* gastro-intestinal model. Microencapsulation process carried out with different biopolymers as coating materials, and all materials showed great cell viability (>9 log CFU/g). The microencapsulated *S. thermophilus* CCM4757 was successfully incorporated into dark and milk chocolates, which was found to be a potential carrier for the bacterial cells by keeping the cells viable for 180 days at 4 °C. The obtained results demonstrated no significant effect of the free and microencapsulated cells on the moisture content and sensorial evaluation of chocolates, regardless of chocolate types.

In the light of this study, milk and dark chocolates were found to be appropriate carriers for maintaining the viability of probiotic during storage period and passing through the simulated *in vitro* gastrointestinal conditions. The results of this work demonstrated that, chocolates enriched with microencapsulated *S. thermophilus* CCM4757 represent a new functional food formulation and it is formed as a reference work about the selection of appropriate coating materials for microencapsulation. As a future perspective, *in vivo* studies can be carried out to show beneficial health effects of *S. thermophilus incorporated* into functional chocolate.

CRediT authorship contribution statement

Burcu Ozturk: Conceptualization, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Menşure Elvan:** Investigation, Methodology, Formal analysis, Writing – original draft. **Merve Ozer:** Investigation, Methodology. **Sebnem Tellioglu Harsa:** Supervision, Writing – review & editing.

Declaration of competing interest

There are no conflicts of interest to declare.

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