Master's Thesis of Science in Agriculture

Encapsulation of *Lactobacillus rhamnosus* GG by Direct Spray Drying of Fermented Reconstituted Skim Milk

발효 재수화 탈지유의 직접 분무 건조 공정을 통한

***Lactobacillus rhamnosus* GG**의 캡슐화

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Abstract

As the aging population increases, there is a strong interest in probiotics with health functional properties such as effect of killing pathogen, preventing toxic compounds secreted from pathogen, relieving irritable bowel syndrome, alleviation of adult autism, decrease the degree of allergy, anti-inflammation and cholesterol emission. Encapsulation by spray drying is an economical way to deliver probiotics to human. A common probiotic encapsulation process consists of three consecutive steps: fermentation, cell recovery, and spray drying. Fermentation has been often conducted with de Man Rogosa Sharpe (MRS) medium but also frequently performed with reconstituted skim milk (RSM) due to the lactose forming a physical barrier, and milk proteins protect probiotics from the lactic acid. Direct spray drying of fermented RSM (fRSM) could provide advantages such as ingestion of the fermented product, eco-friendly and simplified process; however, it has been rarely adopted probably due to the stickiness that occurs adversely affects productivity and product quality. In this study, *Lactobacillus rhamnosus* GG (LGG), a well-known probiotic, was fermented with 10% RSM containing 2% glucose and 1% yeast extract at 42 °C, 100 rpm for 9 h. The fRSM-SMP was prepared by adding 20% SMP to fRSM. For comparison, the rRSM′ was also prepared by recovering cells from fRSM, followed by suspending the recovered cells in 30% RSM. The three RSM samples were spray dried at a feed flow rate of 800 mL/h, feed atomization pressure of 100 kPa, and hot air flow rate of 0.65 m^3/min, and inlet and outlet temperatures of 150–160 and 80°C, respectively. The spray drying yield, the survival ratio of LGG, the shear stress-shear rate relationship of the suspension, and glass transition temperature (T_g), moisture sorption isotherm, and microstructure of spray-dried encapsulated

powder were analyzed to confirm the mechanism regarding the way that solves stickiness problem by adding SMP to fRSM, and the pH of fRSM increased from 3.9 to 5.2, and the shear stress-shear rate relationship was revealed as having shear-thinning behavior. The fRSM was not properly spray dried but the fRSM-SMP and rRSM′ were well dried with similarly high drying yields (36.1 and 35.8%, respectively) and LGG survival ratios (24.7 and 26.3%, respectively) as the added SMP limits the molecular mobility of fRSM resulting LGG cells reaching the surface of the droplets was lowered and the degree of heat transfer to the inside of the droplets seemed to be reduced, too. The fRSM powder showed a lower T_g (55.8 ^oC) than the powders of fRSM-SMP and rRSM′ (62.1 and 61.0 ^oC, respectively). The fRSM showed a sticky point of −5.8 ^oC below its T_g while the sticky point of fRSM-SMP was 7.9 ^oC above its T_g. No sticky point was observed for the rRSM′. It seems that skim milk solids enter between the interstices of the casein aggregated structure, therefore the movement of water molecules was limited, and the time it takes for the droplets to glass transition was shortened during spray drying. The results demonstrated that simple addition of SMP significantly improved the efficiency of spray drying by solving the stickiness problem via faster glass transition shift than in fRSM, thus enabled the direct spray drying which is more ecofriendly and simple way as a promising process for encapsulating LGG. This investigation could contribute to the food industry; (1) by enabling a wide range of food materials by providing information on mechanisms for solving stickiness problems during spray drying, (2) Identification of stickiness mechanism during spray drying of fermented milk using LGG, (3) The mechanism that alleviates stickiness when SMP was added into the fRSM.

Keywords: Encapsulation, *Lactobacillus rhamnosus* GG, Fermentation, Skim milk, Spray drying, Stickiness, Glass transition

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Chapter 1
Research background

1. Probiotics

By definition, probiotics are “living microorganism which, when administered in adequate amounts, confer a health benefit on the host” (Guarner et al., 1998; FAO/WHO, 2001). In the human intestine, probiotics reinforce the gut barrier functions by several ways. Non-digestible carbohydrates are metabolized by probiotics to produce carbon dioxide (CO_2) and SCFAs (short chain fatty acids; butyrate, propionates), and SCFAs thicken the mucous layer, prevent foreign substances from reaching the lamina propria directly. Also, probiotics stimulate genes that produce proteins that are involved in the tight junctions between epithelial cells or release the mucus from Goblet cells (Seth et al., 2008; Yan et al., 2007; Yan et al., 2011). Furthermore, modulation and regulation of immune systems are affected by probiotics. TLR (toll like receptor) or MAMPs (microbe-associated molecular patterns) is the site that pathogen could be entered into the lamina propria, and probiotics competitively bind to the TLR with pathogens. Dendritic cells (DCs) inform the invasion of the pathogens to T-cells so that antigen presentation is started, DCs recognize probiotics instead of pathogenic LPS (lipopolysaccharides), and the stimulation of immune systems is started by immunization. On the contrary, the anti-immunization which is important to alleviate the diseases caused from immune overreaction; IBD (inflammatory bowel disease; Crohn′s disease, ulcerative colitis, allergy could also be affected by probiotics, too. In addition, increases secretion of serotonin to affect the brain (Nature Reviews Gastroenterology and Hepatology, 2015). Due to the health functionalities of probiotics, the market has surpassed $ 35 billion in 2016 and is

Table 1. Overall taxomomical families of well known probiotics

Family	Genus	Species
Lactobacillaceae	*Lactobacillus*	*rhamnosus* GG
		reuteri
		casei 01
		paracasei NFBC 338
		plantarum CIDCA 83114
		plantarum A17
		acidophilus La-05
Bifidobacteriaceae	*Bifidobacterium*	*longum* B6
		longum subsp. Infantis
		animalis subsp. lactis AS60
		bifidum
		breve
		adolescentis
Leuconostocaceae	*Leuconostoc*	*mesenteroides subsp.*
		mesenteroides
		citreum

Ref: Parvez et al., 2006

expected to grow at an annual average of 7.4% by 2024 (Global Market Insight, 2017). Encapsulation by spray drying is a way to deliver probiotics to customers in the food industry. To provide health benefits, probiotics should contain at least 10^6–10^8 CFU/g of food supplements (FAO/WHO, 2001) and the encapsulation technologies which retain the number of viable probiotics cell are the ways to deliver probiotics to customers. Well-known probioitcs as a health promoting effect are *Lactobacillus rhamnosus* GG, *L. casei* 01, *L. paracasei* NFBC 338, *L. plantarum* CIDCA 83114, *L. plantarum* A17, *L. acidophilus* La-05, *Bifidobacterium longum* B6, and *B. lactis* AS60, etc.

2. *Lactobacillus rhamnosus* GG

Lactobacillus rhamnosus GG (LGG, ATCC53103) was first isolated in the health human fecal in 1985 by Sherwood Gorbach and Barry Goldin (GG). LGG is an anaerobic, non-sporulating, gram-positive rod, and the colony of LGG is pale yellow having a buttery odor (Saxelin, 1997; Segers and Lebeer, 2014). LGG ferments D-arabinose, galactose, D-glucose, D-fructose, D-mannose, sorbitol, etc. (Saxelin, 1997), however, LGG cannot genentically ferment lactose and casein micelles which are main ingredients in milk (Saxelin, 1997; Kankainen et al., 2009).

2.1. Health functionality

LGG has been commercialized and researched because it has excellent health functionality. LGG reinforces the integrity between intestinal epithelial cells (Marteau et al., 2001; Segers and Lebeer, 2014), and acts as an adjuvant for live-attenuated flu vaccination to stimulate immune regulation (Davidson et al., 2011; Marijke and Sarah, 2014). It is also revealed that LGG prevents or palliates five

diseases; Nosocomial rotavirus-related diarrhea, acute diarrhea, upper respiratory tract infections, atopic eczema, dental caries (Laackso et al., 2011). The hairy pili of LGG not only allows LGG to adhere to the epithelial cells well, but also increases the encapsulation efficiency as interacting to the food ingredients during the encapsulation processes. The shape of the LGG with hairy pili on the outer wall further augments their health functionality, by easily colonizing the oral, colon, and vaginal (Doron et al., 2005). AFM can be used to explain the interaction between biopolymers and bacterial cell, which is identified by the force-distance analysis between the protein coated tip and the bacterial surface (Polyakov et al., 2011). And applied this analysis to confirm the effect of abundant hairs of LGG surface on the encapsulation efficiency (Burgain et al., 2014). They have analyzed the force-distance graph using AFM between the hairy pili of the LGG surface and tip coated with whey protein isolate. When they compared wild type LGG and mutant LGG with pili removed, there was a noticeable fluctuation of force-distance graph with hairy pili.

2.2. Stress resistance during the spray drying process

In addition to the health promotion effect of LGG, they have strong stress resistance to the stress factors related to the processing. Because LGG genetically expresses heat shock proteins, which restore the damage from stress by heat, osmosis and accumulation of toxic compounds of nucleic acids and proteins. (Corcoran et al., 2004; Ananta et al., 2005; Sunny-Robberts et al., 2009). 20% (w / w) trehalose solutions containing heat stress resistant specious *L. rhamnosus* E800 and LGG was placed in a water bath set at 65–70 ^{o}C for 30 s and the survival ratio was confirmed (Sunny-Robberts et al., 2009). The viable cell count was determined by counting the number of colonies detected at 37 ^{o}C for 48 h using

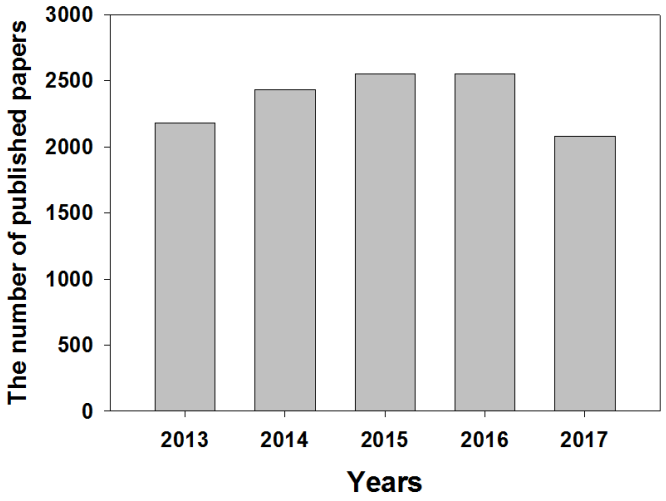


Figure 1. The number of published papers with the word *Lactobacillus rhamnosus* GG in last 5 years (Ref: Google Scholar).

de Man Rogosa Sharpe (MRS) agar plate. The survival ratio was calculated as follows:

$$\% \text{ survival} = \frac{N}{N_0} \times 100 \quad (1)$$

where N_0 represents the number of bacteria before spray drying, and N is the number of bacteria after spray drying.

As a result, L. rhamnosus E800 showed higher thermal tolerance than LGG by the viable cell count of 2.75 log CFU/mL. The survival ratio by the spray drying process was also confirmed. When spray-dried at a flow rate of 5 mL/min, outlet temperature of 65–70 °C, and various inlet temperature ranges using a co-current flow spray drier, *L. rhamnosus* E800 (55%) showed lower survival ratio than LGG (75%) and this also provides the advantage of increasing the number of live LGGs of reaching the bowel when ingesting LGG. LGG is a living microorganism and certain amounts must be maintained until it reaches the intestine in order to demonstrate its health functionality. But the number of viable LGG in processing, storage, rehydration (consumption) and digestion continues to decrease. To prevent the (Paulo et al., 2014). To protect LGGs against deteriorative factors (heat, pH, oxygen, bile salt), the encapsulation process is needed. Other than that, there is a merit that LGG capsules can be conveniently used to produce health functional foods by the simple addition of encapsulated LGG to a wide range of foods such as breads, snacks, chocolates, creams, sauces, gums and beverages. In general, the encapsulation of probiotics by spray drying, emulsion, coacervation, extrusion, gel-particle technologies, and heat induced gelation of whey proteins are commonly used in the food industry (Burgain et al., 2011; Krasaekoopt et al., 2003; Martín et al., 2015). The most widely used probiotic encapsulation process is spray drying with following advantages; (i) drying liquid directly without pretreatment, (ii) fast drying speed, (iii) drying and powderization done at once (iv) handling of

the heat-sensitive samples, and (v) massive production due to continuous operation (Ré, 1998; Gharsallaoui et al., 2007; Patel et al., 2009; Nidhi et al., 2011). Heat transfer to the droplets in spray drying with high inlet temperature (140–200 °C) does not directly damage to the probiotics due to mass transfer by moisture evaporation (Guergoletto, 2002; Jacquot and Pernette, 2004; Silva et al., 2011; Schutyser and Boom, 2012). Therefore, the outlet temperature (60–100 °C) which is the temperature at the end of spray drying process highly affects the survival ratio of probiotics. As LGG endures the exportable stresses during spray drying, the expression of heat shock proteins is increased and higher the survival ratio in simulate-gastrointestinal tract (GIT). The effect of heat treatment at 55 °C for 15 min on the survival ratio in simulate-GIT of *Lactobacillus plantarum* Lp813 and Lp998 was reported (Ferrando et al., 2016). Gastric acids which is the main reason for killing Lactobacilli treated to *L. plantarum* Lp813 and Lp998. When heat is treated to *L. plantarum* Lp813 and Lp998, the survival ratio is higher than when heat is not treated. In this case, spray drying process can be used for improving probiotic delivery to the large bowel as increasing the tolerance in human GIT.

3. Probiotics encapsulation processes by spray drying

The typical spray drying process encapsulating probiotics **(typical process)** involves three consecutive steps: fermentation in MRS broth, cell recovery (centrifugation of the culture–removing supernatant–pellet recovery to phosphate buffered saline with pH 6.6–7.0–centrifugation–removing supernatant–pellet recovery to reconstituted skim milk with 10-30% total solids), and spray drying (Lian et al., 2002; Corcoran et al., 2004; Ananta et al., 2005; Katarzyna et al., 2009; Lina et al., 2014; Rajam and Anandharamakrishnan, 2015; Sarim et al., 2016). Although the typical process shows the highest probiotics survival ratio (%) after

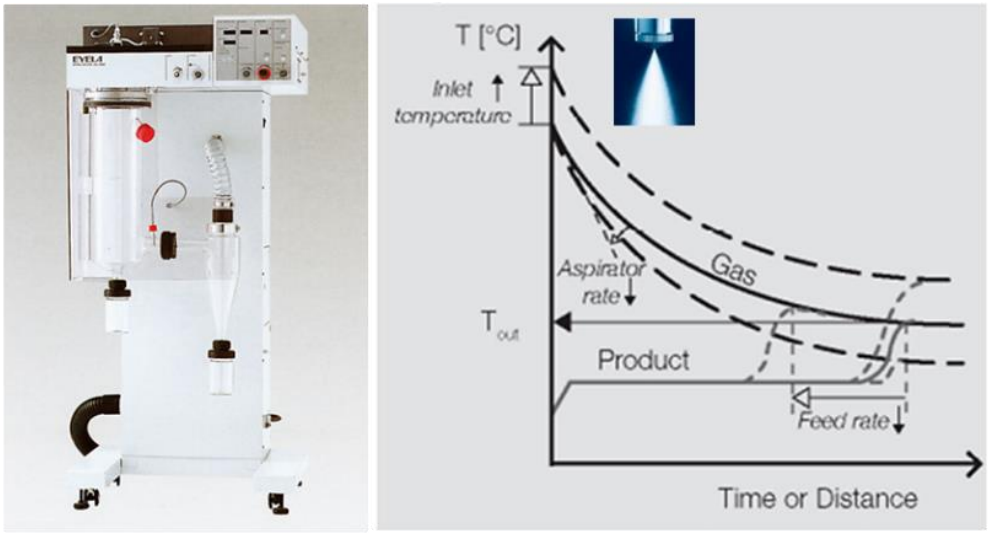


Figure 2. Spray dryer used (SD1000, Eyela, Tokyo, Japan) and its changing temperature followed by the time (distance).

(Ref: http://www.sunileyela.co.kr/, http://www.buchi.com/kr-ko/)

spray drying, it is a non-economical (due to the long process) and non-ecofriendly process by the production of the fermented waste containing chemical materials such as MRS media. If the fermented RSM is spray dried directly without LGG cell recovery step in the typical process (Tab. 1), It could be not only the simpler and environmentally friendly process (Kim and Bhowmik, 1990; Gardiner et al., 2000; Bielecka and Majkowska, 2000; Desmond et al.,2001; Desmond et al.,2002; Kumar and Mishira, 2004; Kearney et al., 2009; Izadi et al., 2014), but also has the advantages of being capable of consuming fermentation by-product which is nutritionally valuable and the acidic flavor can provide masking effect on the odor of the food ingredients. But the alternative process has the limitations caused from the stickiness phenomenon. In order to handle the stickiness problem, it has been proposed to add the drying aids (Tab. 2) such as trehalose (Sunny-Roberts et al., 2009), polydextrose, fructo-oligosaccharide (Corcoran et al., 2004; Ananta et al., 2005), dextran (Leja et al., 2009), gelatin, soluble starch, gum Arabic (Lian et al., 2002; Desmond et al., 2002) or to adjust pH of the feeding suspensions (Gardiner et al., 2000; Leja et al., 2009; Mariela et al., 2015)

4. Stickiness phenomenon during spray drying

Stickiness phenomenon is a major problem in the spray drying process as sticky powders lower the process efficiency by the nozzle clogging and wall deposition. It also negatively affects the storage stability of the powder associated with the lowered viable probiotic cells, caking phenomenon, fast contamination, and powder color change (Maillard reaction). The mechanism of stickiness phenomenon is highly affected by the properties related to the molecular mobility in the system of the feeding suspensions (Burnett et al., 2004). Molecular mobility can be controlled by temperature, moisture content and viscosity in the system of

feeding suspensions. The stickiness phenomenon during spray drying is mainly caused by the state transition from rubbery state to glassy state. When the glass transition temperature (T_g) of feeding suspensions is less than the temperature applied from spray dryer, or when water (plasticizer) content of feeding suspensions is high, the water molecules prevents to reach specific viscosity ($10^{12–14}$ Pa·s) corresponding to the glass transition. Thus, the rubbery state which fails to sustain its own weight under gravity, forming a new structure with other particles and wall (the result of stickiness phenomenon) appears over the relatively longer range in the spray dryer than the feeding suspensions having higher T_g, or lower moisture content. (Downton et al., 1982; Bhandari et al., 1997; Roos et al., 2002; Ozmen and Langrish, 2003; Palzer. 2009; Turchiuli et al., 2011; O'Callaghan and Hogan, 2013). Also the high fraction of low molar mass compounds such as sugars, acids, which are easily converted to rubbery components in the feeding suspensions prevents to transfer to glassy state (Bhandari et al., 1997; Goula et al., 2010). Describing this in spray drying, as the droplets are sprayed and the water evaporates during spray drying, the viscosity increases to become its rubbery state as the viscosity reaches at 10^7 Pa·s. When it dries further until reaches at the viscosity of $10^{12–14}$ Pa·s becoming in the glassy state, and loses its sticky property. (Downton et al., 1982; Bhandari et al., 1997; Roos et al., 2002; Palzer. 2009; Turchiuli et al., 2011).

5. Control of stickiness during spray drying

5.1. Process based approach

If the viscosity of the feeding suspensions is sufficiently high, it takes less time to reach the specific viscosity (10^{12} Pa·s) of glass transition during spray drying.

By mechanical adjustment of spray drying, the stickiness problem also can be addressed to lower the temperature, to use dehumidified air (Goula et al., 2010), nylon coating prevents the adhesive force from the particles to the spray drier wall (Ozmen and Langrish, 2017).

5.2. Material science based approach

Among the drying aids, the highest microbial viability was observed with using SMP which is a mixture composed of proteins (casein, whey protein) and carbohydrates (lactose) having protective effect for probiotics from their mechanical properties (heat conductivity and heat diffusivity) during spray drying (Gardiner et al., 2000; Desmond et al., 2001). Moreover, lactose has a cell membrane protective effect during fermentation, spray drying, and storage (Ananta et al., 2005) and casein and whey protein have pH buffering effect during fermentation, spray drying, storage, human GIT (Charteris et al., 1998).

Table 2. The quality scale of spray dried powder identified by the survival ratio (SR) of the encapsulated bacteria in the microstructure of the powder and the moisture content (X) of the powder when spray drying fermented reconstituted skim milk with different bacteria, composition, pH.

No.	Fermented RSM			Spray dried powder		Reference
	Bacteria	Composition (w/v)	pH	SR (%)	X (%, d.b.)	
1	*Lactobacillus paracasei* NFBC338	RSM 20% Yeast extract 0.5% Sucrose 1%	Adjusted to 6.8 by 4 N NaOH	50.0	3.5	Gardiner et al., 2000
2	*L. paracasei* NFBC338	RSM 16% Yeast extract 0.5% Sucrose 1%	4.7	0.2	4.0	Kearney et al., 2009
3	*L. bulgaricus* 151	RSM 18%	4.4	22.1	10.2	Bielecka and Majkowska, 2000
4	*L. bulgaricus*	RSM 14%	4.2	0.8	-	Kim and Bhowmik,1990
5	*L. acidophilus*	RSM 12%	-	5.9	4.7	Izadiet al., 2014

Table 3. The quality scale of spray dried powder identified by the survival ratio (SR) of the encapsulated bacteria in the microstructure of the powder and the moisture content (*X*) of the powder when spray drying fermented reconstituted skim milk with different bacteria, composition, pH.

No.	Fermented RSM + drying aid			Spray dried powder		Reference
	Bacteria	Drying aid (w/v)	pH	SR (%)	*X* (%, d.b.)	
1	*Lactobacillus bulgaricus*	Pectin 0.1% or K-carrageenan 0.1% or guar-gum 0.1% or locust bean gum 0.1%	4.7	3.6	-	Martha et al., 2012
2	LGG, *L.*E800	Trehalose 20%	-	68.8	4.4	Sunny-Roberts et al., 2009
3	LGG, *L.*E800	Polydextrose 10% or fructo--oligosaccharide 10% or inulin 10%	-	43.0	~4.0	Corcoran et al., 2004
4	LGG	PVP 3.6%,Dextran 2.4%	6.5	57.0	11.0	Leja, K. et al., 2009
5	*B. longum* B6	Gelatin 10% (w/w) or soluble starch 10% (w/w) or Gum arabic 10% (w/w)	-	82.7	10.3	Lian et al., 2002,
6	*L. paracasei* NFBC338	Gum arabic 10%	-	1.7	2.8	Desmond et al., 2002
7	LGG	Oligofructose 10% or polydextrose 10%	-	60.0	~4.0	Ananta et al., 2005

6. Overall objectives

The objectives of this study was to improve a consecutive LGG encapsulation process of fermentation in RSM and direct spray drying without cell recovery by controlling its glass transition and stickiness problem through simply adding more SMP to fRSM. Firstly, the efficacy of the consecutive LGG encapsulation process as compared to the traditional process including cell recovery step was evaluated by examining spray drying yield, microbial survival ratio, powder particle size, microstructure, moisture content. Secondly, the effect of additional reconstitution of SMP in the fRSM and its pH effect on the spray drying were investigated to improve the efficacy of the encapsulation process. Finally, the mechanism governing the performance of encapsulation process was investigated by examining ζ-potential, particle size of dispersed particles in feeding suspensions, shear stress-shear rate relationship, moisture sorption isotherm, glass transition zone, sticky point temperature, particle size, and microstructure of feeding suspensions and powders.

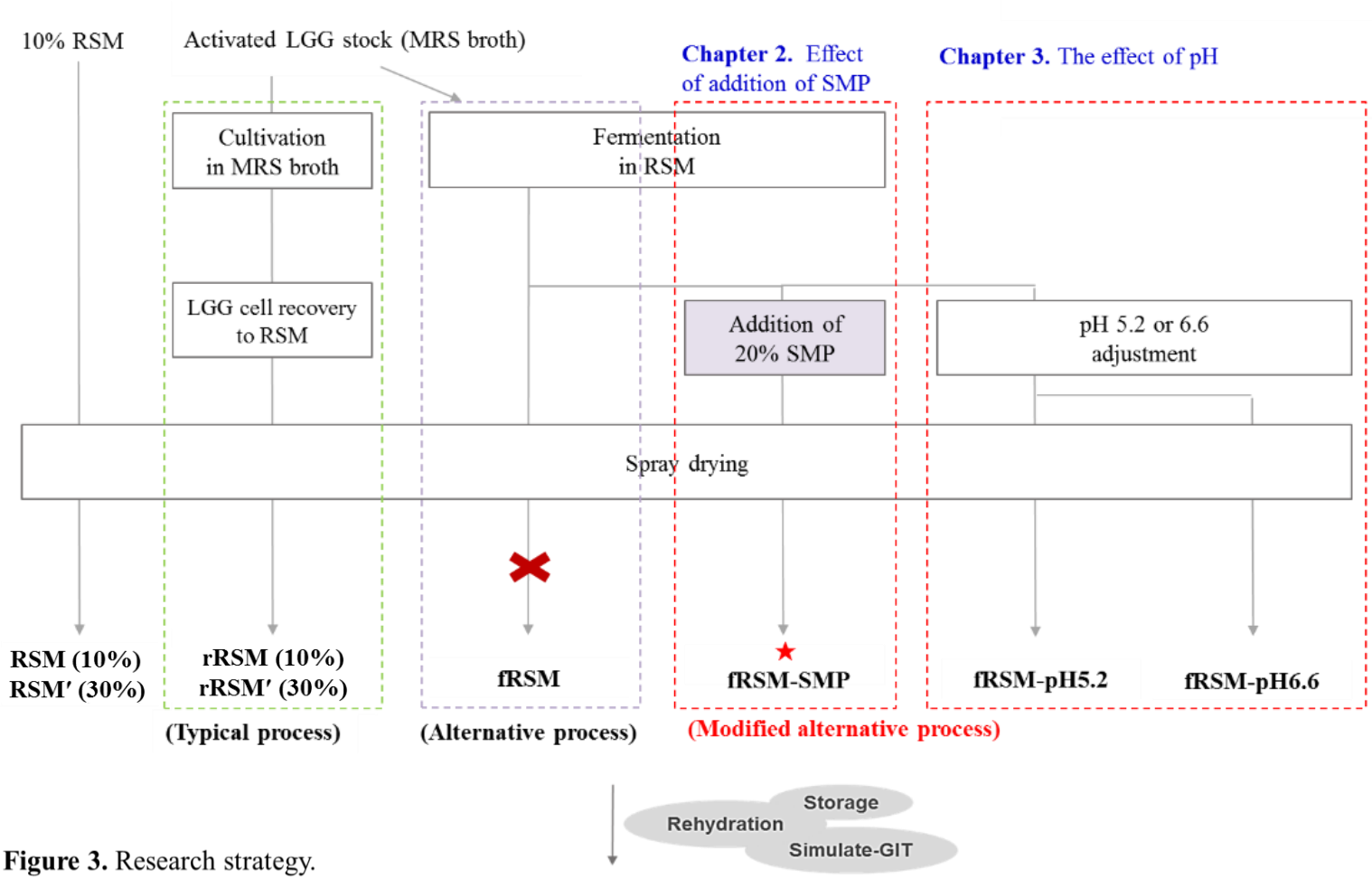


Figure 3. Research strategy.

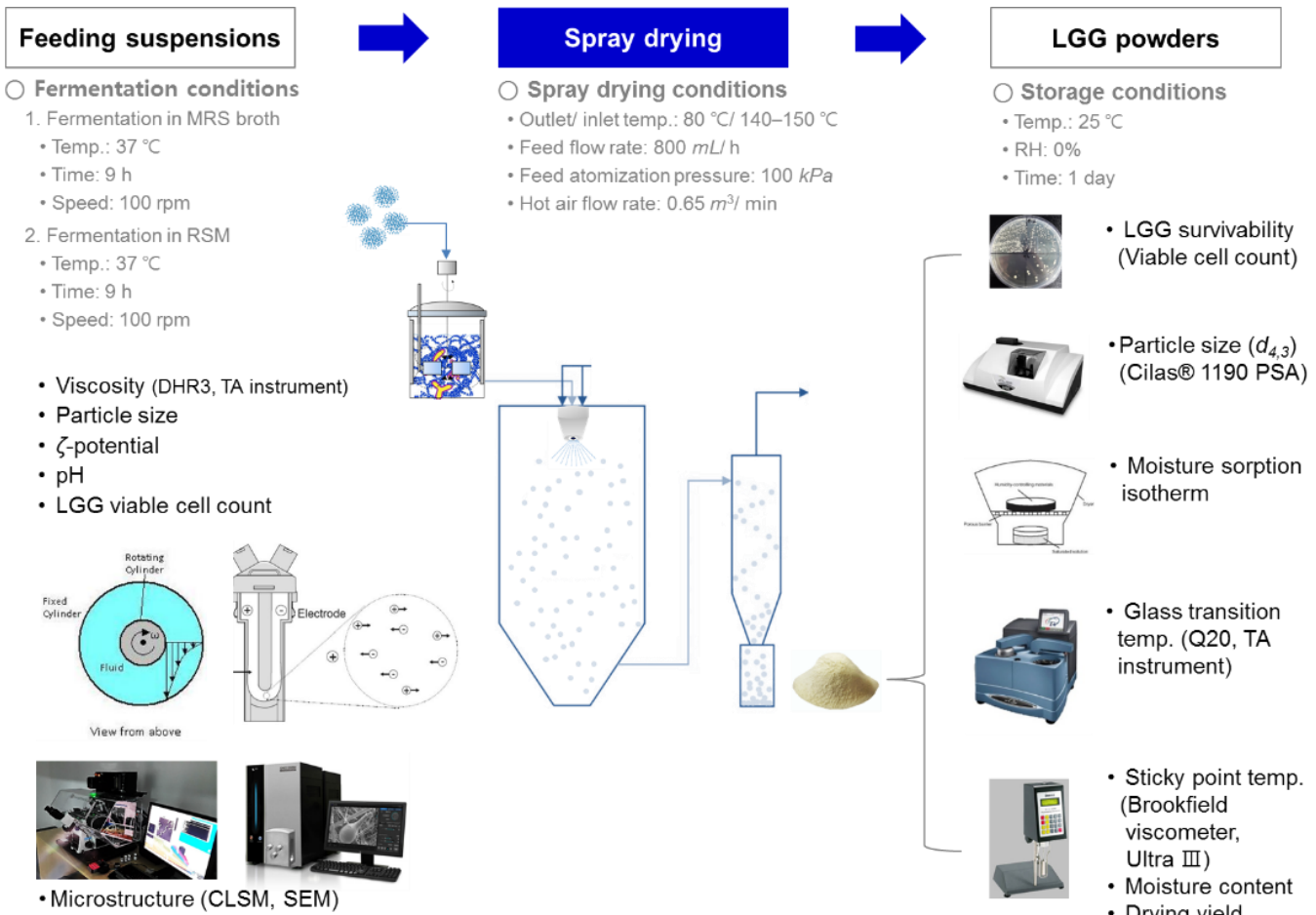


Figure 4. Schematic diagram of experimental strategy.

Chapter 2
Effect of the adding SMP on LGG encapsulation during spray drying

Introduction

In **Chapter 1**, the three encapsulation by spray drying processes were described: (i) typical process (rRSM′); (ii) alternative process (fRSM); (iii) modified alternative process (fRSM-SMP). The physicochemical properties of the feeding suspensions used in these three processes, including pH, particle size, electrophoretic mobility, and shear stress-shear rate relationship, were determined, and the drying yield, moisture content, particle size, moisture sorption isotherm, microstructure observed by CLSM and SEM, glass transition temperature, and sticky point temperature of LGG powders were measured and used to investigate the effect of fermentation step and SMP addition on the stickiness occurred in (ii) alternative process (fRSM). The objective of this chapter is to reduce the stickiness by adding additional SMP to the fRSM suspension before the direct spray drying. The productivity (dry yield) and product quality (moisture content, LGG survival ratio, and powder storage stability) of (iii) the modified alternative process (fRSM-SMP) with (i) typical process (rRSM′) to determine whether direct spray drying of fermented RSM is a substitute for the typical process were also compared.

Materials and Methods

1. Fermentation of LGG

Lactobacillus rhamnosus GG (LGG, ATCC53103) obtained from the department of food science and technology of Chungbuk National University (Cheongju, Korea) was sub-cultured for 2 times at 37 °C for 24 h in de Man, Rogosa and Sharpe (MRS) broth (Difco, Detroit, MI, USA) and inoculated 5% (w/w) of LGG culture in the enriched RSM. The enrichment of reconstituted skim milk (RSM, Seoul Milk Co., Ltd., Seoul, Korea) was achieved by adding 2% (w/w) of glucose (Ducksan, Ansan, Korea) and 1% (w/w) of yeast extract (Thermo Fisher Scientific, Erembodegem, Belgium) to the 10% (w/w) total solid (TS) RSM. Because LGG cannot metabolize lactose due to their gene frame shift (Saxelin et al., 1997, Matti et al., 2009). The enriched RSM was heat-treated at 90 °C for 30 min in water bath (BS-31, Jeiotech, Seoul, Korea) and cooled until room temperature for inoculation of LGG. The growth of LGG in the enriched RSM was compared with MRS broth, 10% (w/w) RSM, and the D.W. containing 2% (w/w) glucose and 1% (w/w) yeast extract (Fig. 1). Fermentation of enriched RSM containing LGG was performed at 42 °C for 9 h in the water bath until the pH reaches to 3.9 which was shown as the early stationary phase of growth of LGG (Fig. 1A).

1.1. Selection of LGG growth media

The growth curve of LGG during fermentation of RSM was drawn to ensure that the number of viable cells (CFU/g) in the feeding suspensions was constant. To investigate the effect of glucose, yeast extract, SMP on the growth of LGG, three different solutions were prepared; (1) medium containing 2% glucose and 1%

yeast extract dissolved in D.W. (2% G + 1% Y), (2) medium containing SMP (10% RSM), (3) medium containing 2% glucose, 1% yeast extract, and 10% RSM (10% RSM + 2% G + 1% Y). As growing LGG, the pH of enriched RSM decreased from 6.6 to 3.9 and the growth curve reached early stationary phase with 9.18 log CFU/g of viable cell count which is not significantly different from the LGG growth in MRS broth (9.43 log CFU/g) within 9 h (Fig. 1).

2. Optical density of the LGG culture

The optical density (OD) was measured at 600 nm using a spectrophotometer (DU 730, Beckman Coulter, Brea, CA, USA) for monitoring LGG growth. The 10-fold dilution was conducted when the OD value was above 1.0. MRS broth which sterilized under the same conditions as the MRS broth used for culture was used for the blank test and dilution of the culture.

3. Survivability of LGG

3.1. Viable cell count of LGG in the feeding suspensions

The feeding suspensions were diluted serially by the decimal dilution until 10^{-7} by using sterile saline (0.85% (w/v) NaCl solution). The 80 μL of the diluted feeding suspensions was spread on the sterile MRS agar plates, and placed in a rectangular jar (Mitsubishi gas chemical, Japan), and filled with nitrogen gas (N_2) and incubated at 37 °C for 24–48 h in the incubator. The yellowish white colonies with the size of above 2 mm formed were counted, and calculated as CFU/g dry basis.

3.2. Viable cell count of LGG in the LGG powders

The powder was diluted 10-fold with sterile saline to prepare a stock solution

(10^0), and diluted serially by the decimal dilution until 10^{-7} by using sterile saline (0.85% (w/v) NaCl solution). The 80 μL of the diluted feeding suspensions was spread on the sterile MRS agar plates, and placed in a rectangular jar (Mitsubishi gas chemical, Tokyo, Japan), and filled with nitrogen gas (N_2) and incubated at 37 °C for 24–48 h in the incubator. The yellowish white colonies with the size of above 2 mm formed were counted, and calculated as CFU/g, dry basis.

3.3. Survival ratio of LGG

The LGG survival ratio before and after spray drying was calculated using Eq. (1).

4. Preparation of feeding suspensions

In the preparation of 30% (w/w) total solid (TS) RSM, 58 g of SMP was added to 200 g of heat-treated 10% (w/w) TS RSM and mixed by magnetic stirrer for 30 min. For the preparation of rRSM′ (RSM containing LGG by cell recovery step), cell recovery step is needed; 200 g of LGG sub-cultured MRS broth was centrifuged at × 4000 *g* for 10 min and the supernatant was removed. Remaining MRS broth in the pellet was washed by mixing with 200 g of 1 M phosphate buffer solution and centrifuged at × 4000 *g* for 10 min and the supernatant was removed. The pellet thus obtained was dispersed by using magnetic stirrer. 200 g of 1 M phosphate buffer solution and centrifuged at × 4000 *g* for 10 min and the supernatant was removed, and the pellet was dispersed by using magnetic stirrer for 30 min in 200 g of 30% (w/w) TS RSM. Further reconstitution of 20% SMP to the 10% (w/w) fRSM was conducted by using magnetic stirrer for 30 min to make 30% (w/w) TS fRSM-SMP.

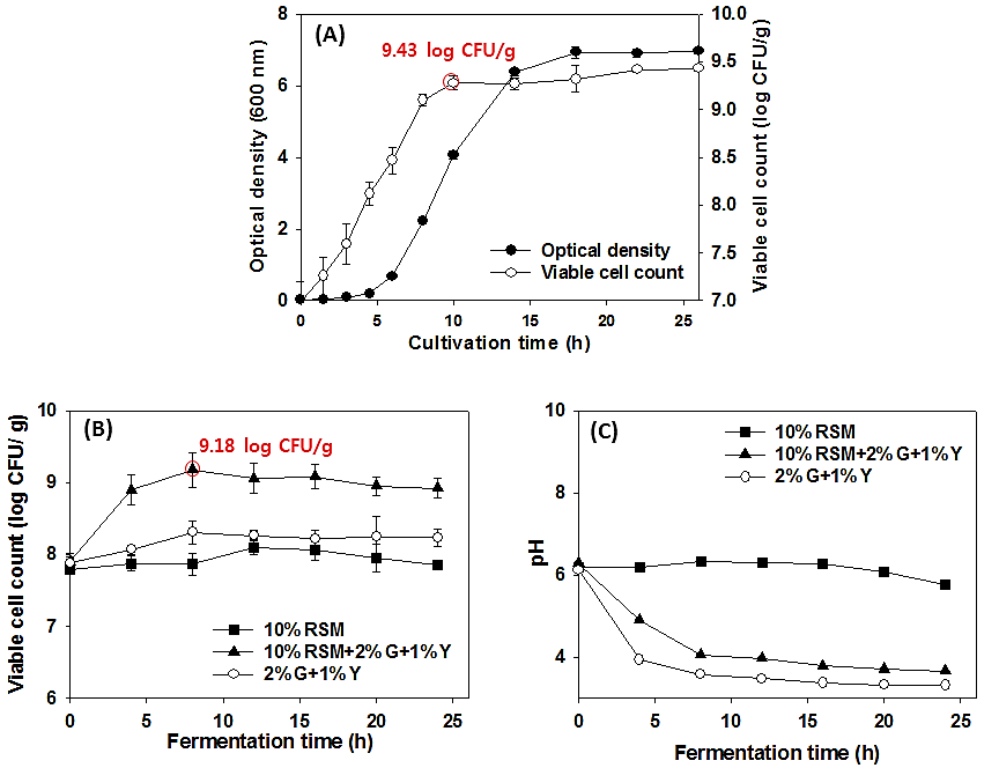


Figure 1. Growth of LGG in (A) MRS broth at 37 ºC according to optical density at 600 nm and viable cell count (log CFU/g), (B) viable cell count (log CFU/g) of LGG during fermentation, and (C) decreasing pH verses fermentation time in 10% (w/w) reconstituted skim milk (RSM), 10% (w/w) RSM+2% (w/w) G+1% (w/w) Y, and 2% (w/w) G+1% (w/w) Y. LGG=*Lactobacillus rhamnosus* GG; MRS=de, Man, Rogosa, Sharpe; G=glucose; Y=yeast extract.

5. Physicochemical properties of feeding suspensions

5.1. pH

pH meter (S220-K, Mettler Toledo International Inc., Shanghai, China) calibrated to pH of 4.01, 7.00, and 9.21 was used to measure the pH of feeding suspensions. The pH of RSM, RSM′, rRSM, and rRSM′ was almost the same, but it has adjusted to 6.6 using 10% (w/v) lactic acid solution.

5.2. Electrophoretic mobility

Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used to measure the electrophoretic mobility (μ, $10^{-5}\cdot m^2/V\cdot s$) from the Henry equation:

$$\mu=\frac{2\varepsilon z f(ka)}{3\eta} \quad (2)$$

where μ is electrophoretic mobility, ε is dielectric constant, z is zeta potential, $f(ka)$ is Henrys fuction, η is viscosity.

All feeding suspensions were diluted 10-fold and then measured at 25 °C.

5.3. Particle size

Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used to measure the volume average mean particle size ($d_{4,3}$, μm) using dynamic light scattering. The $d_{4,3}$ was calculated as follows:

$$d_{4,3}=\frac{\sum d_i^4 n_i}{\sum d_i^3 n_i}\times 100 \quad (3)$$

where n_i is the number of particles, d_i is the diameter of particles.

All feeding suspensions were diluted 10-fold and then measured at 25 °C.

5.4. Shear stress-shear rate relationship

Shear stress-shear rate relationship tests were performed using a rotational shear

rheometer (DHR-3, TA Instruments, New Castle, DE, USA) with Peltier concentric cylinder geometry (recessed/standard) having a standard cup diameter of 30.39 mm and a bob diameter of 27.99 mm at 25 °C. A shear rate ranging from 0.01 to 250·1/s was applied to investigate the shear stress of each feeding suspension. The viscosity of the feeding suspensions were compared using the *k* from the equation of Herschel-Bulkley model (Eq. (3)).

$$\tau = \tau_0 + k\,(\dot{\gamma})^n \quad \text{..... (4)}$$

where τ is shear stress, τ_0 is yield stress, k is consistency index, $\dot{\gamma}$ is shear stress, and n is flow behavior index.

The *k* was measured to compare the viscosity values of feeding suspensions which has confirmed to have non-Newtonian behavior (Fig. 3). The higher the *k*, the shorter the time it takes to reach a viscosity of $10^{12\text{-}14}$ Pa·s which is corresponding to the glassy state of materials (Downton et al., 1982; Bhandari et al., 1997), particles that are in the glassy state are no longer sticky, resulting in less stickiness problems.

6. Spray drying

The feeding suspensions were spray dried using mini spray dryer (Eyela SD-1000, Tokyo Rikakikai Co., Tokyo, Japan) equipped with a 0.7 mm diameter nozzle at a feed flow rate of 800 mL/h, feed atomization pressure of 100 kPa, and hot air flow rate of 0.65 m^3/min with inlet and outlet temperatures of 150–160 °C and 80 °C, respectively. The obtained LGG powders were stored at 25 °C in the desiccator containing saturate phosphorus pentoxide solution (0% relative humidity).

7. Drying yield

The drying yield was calculated as follows:

Drying yield (%) = $\frac{W_p}{W_i} \times 100$ ..... (5)

where W_p is weight of powder after spray drying, W_i is initial weight of total solid of feeding suspensions.

8. Moisture content

The powders were dried at 105 °C in the drying oven. When the weight of the LGG powders reached a constant weight was measured, and moisture content was calculated as follows (AOAC, 2005):

X (%, dry basis)= $\frac{W\text{-}W_s}{W} \times$ 100 ……(6)

where W total mass, W_s is mass of solid.

9. Water activity

The water activity of the LGG powders which are in the equilibrium moisture content with various relative humidities (0, 11, 23, 33, 43, 53, 69, 81, and 93%) was measured using (Aqualab water activity meter, Decagon, WA, USA) at 25 °C.

10. Particle size distribution using backscattering

The particle size and the % volume distribution was analyzed using particle size analyzer (1190LD, CILAS, Orleans, France) based on Fraunhofer theory. The 2–3 g of the LGG powders was placed on the sample distributor with the frequency of 50 Hz for matching the obscuration from 8 to 15%. All analysis were repeated for 3 times.

11. Microstructure

The microstructure of the feeding suspensions were observed using confocal

laser scanning microscope (CLSM, Applied Precision, Issaquah, WA, USA) to obtain the confocal image that makes multiple thick casein aggregated structure in one scene. An appropriate amount of the LGG powder was placed on the observation plate, and gold coated (K550; Emi-tech Ltd., Kent, UK), and the surface of the LGG powders examined in a scanning electron microscope (SEM, S-4700 Field Emission Scanning; Hitachi High Technologies, Tokyo, Japan) at an accelerating voltage of 15.0 kV.

12. Moisture sorption isotherm

The moisture sorption isotherm was analyzed with the LGG powders in the equilibrium moisture content (X_E), and it was measured**.** 2 g of LGG powders were evenly located in the aluminum plate, these were stored in the desiccators with various relative humidities (0, 11, 23, 33, 43, 53, 69, 81, and 93%) until the moisture of the powder is equilibrated (6 days). For the curve fitting, The GAB (Guggenheim-Anderson-de Boor) model was used:

$$\frac{X_e}{X_M}=\frac{Cka_w}{(1-ka_w)(1-ka_w+Cka_w)} \quad \text{.....(7)}$$

where X_e is equilibrium moisture content (kg water/kg solid), X_M is moisture content in the monolayer (kg water/kg solid), a_w is water activity, C is Guggenheim constant, k is correction factor.

13. Equilibrium moisture content of LGG powders

The moisture content (%, dry basis) LGG powders were equilibrated for 6 days at the desiccators with various relative humidities controlled by the saturated salt solutions (Tab. 1). To confirm the time when moisture equilibrium of LGG powders reached, the moisture content of LGG powders were measured, and all

LGG powders were equilibrated after storage for 6 days at 25 °C.

14. Glass transition temperature

The glass transition temperature of LGG powders were analyzed using differential scanning calorimeter (DSC; Q2000, TA Instruments, New Castle, DE, USA) equipped with a refrigerated cooling system. The LGG powders in the equilibrium moisture content were accurately weighted into aluminum pans (TA Instruments, New Castle, DE, USA), and hermetically sealed. The pans containing LGG powders were scanned for 3 cycles at the heating and cooling rates of 10 °C/min with temperature range from 2 to 100 °C, and an empty pan was used as the reference material. The temperature derivative value of heat flow (W/g·°C) was analyzed by TA Universal Analysis 2000 software version 4.5A (1998−2007 TA Instruments-Waters LLC).

15. Sticky point temperature (SPT)

The sticky point temperatures of the LGG powders were measured by the torque value (mN/m) using viscometer (LVDV III, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA), the method was based on the paper (Silalai and Roos, 2010). This method is to measure the torque value (mN/m) as a value against the force applied to the powder by the spindle rotating at 0.3 rpm, and the point at which the torque value increases sharply appears is regarded as a sticky point. The spindle used were customized to fit the design shown in the paper (Fig. 2), and the torque value were measured every 40 s per point at 10 °C intervals from 20 °C to 80 °C. Of the 40 torque values obtained for each temperature condition, the latter 20 values were averaged and plotted according to $T–T_g$ to confirm the sticky point temperature.

Table 1. Equilibrium relative humidity (RH) conditions for moisture sorption isotherm measurements.

Saturated salts in the solution	RH (%)
Phosphorus pentoxide	0
Lithium chloride	11
Potassium acetate	23
Magnesium chloride	33
Potassium chloride	43
Magnesium nitrate	53
Potassium iodide	69
Ammonium sulfate	81
Potassium nitrate	93

(Ref: Kumar et al., 2011)

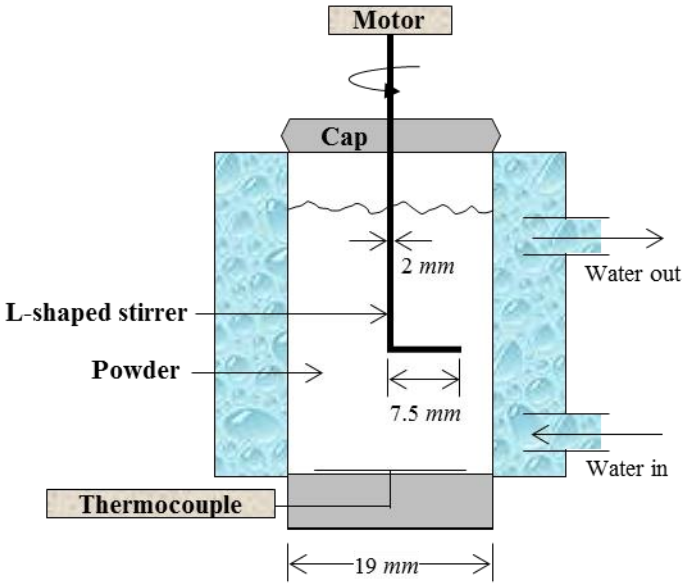


Figure 2. The holder and stirrer used for the brookfield rotational viscometer in the torque measurement for sticky point temperature.

Results and Discussion

1. Physicochemical properties of feeding suspensions

1.1. pH

After fermentation, the pH of RSM was decreased from 6.6 to 3.9, and when SMP was added to the fermented RSM, the pH increased from 3.9 to 5.2 due to the pH buffering effect of the milk proteins in the added SMP (Tab. 1).

1.2. Shear stress-shear rate relationship

In the flow behavior index (n) (Tab. 1), fRSM and fRSM-SMP shown to have the shear thinning behavior. The factors affecting the k were seemed to be the (i) fermentation step: the acidified casein micelles by the fermentation step form the aggregated structure in the RSM increases the k from 0.0015 to 0.0066 Pa·s^n. (ii) Total solid: The k of feeding suspensions having 30% TS; RSM′=0.0155 Pa·s^n, rRSM′=0.0090 Pa·s^n, fRSM-SMP=0.0583 Pa·s^n are higher than in the feeding suspensions having 10% TS; RSM=0.0015 Pa·s^n, rRSM=0.0019 Pa·s^n, fRSM=0.0066 Pa·s^n. (iii) when SMP was added after the fermentation step, and iv) LGG: as the hairy pili and flagellum of LGG physically gives external force to the system of feeding suspensions system, and lowering viscosity (Hatwalne et al., 2004; Lopez et al., 2015). In the comparison between RSM′ (30% TS) and rRSM′ (30% TS), the k value was decreased from 0.0155 to 0.0090 Pa·s^n. In the Fig. 3, fRSM-SMP showed the highest shear stress (τ). This is because the SMP particles added to the casein aggregated structure through the fermentation step interacted with more water molecules between the microstructures of the fRSM. As a result, the molecular mobility is significantly reduced and the effect of decreasing the viscosity due to the presence of LGG seems to be overcome sufficiently.

2. Microstructure of the feeding suspensions

As the net charge of the casein micelle increases in RSM during fermentation, the electrostatic repulsion between casein micelles weakens, resulting in a casein aggregated structure. As the pH decreased from 6.6 to 5.2, particle size slightly increased from 0.24 to 0.30 μm, and it was shown that particle size was increased to 7.13 μm through the fermentation step (pH 3.9). This appears to be the casein aggregated structure formed at pH of ~4.5 (pI of milk protein). In addition, electrophoretic mobility increased slightly from -1.42 to -0.20 $10^{-5}\cdot m^2/V\cdot s$ when the pH decreased from 6.6 to 5.2, and significantly increased to 0.52 $10^{-5}\cdot m^2/V\cdot s$ after the fermentation step (pH 3.9). This may be the result of increased net charge due to acidification (protonation) of κ-casein molecules located outside the casein micelle structure (Tab. 1). With these results, the microstructure of feeding suspensions identified by CLSM also showed that casein aggregated structure was formed in the fRSM, and casein aggregation was visible in fRSM-SMP but was faint (Fig. 4).

3. The efficacy of the direct spray drying of fRSM as compared to the traditional process

3.1. Drying yield

Drying yield can be used to measure the degree of stickiness problem. Because the wall deposition and nozzle clogging occur as a result of stickiness. As the RSM undergoes a fermentation step, the stickiness problem becomes serious and the process is stopped due to the gradual adherence of the sticky particles obtained during spray drying fRSM to the path where the spray drying powder moves, and the drying yield of the powder obtained from the typical process was decreased from 35.77 to 0% when the fRSM was spray dried.

Table 2. Total solid, pH, particle size by dynamic light scattering, and electrophoretic mobility (μ), shear stress-shear rate relationship of feeding suspensions.

Feeding suspensions	SMP* (%)	pH	Particle size(μm)	μ ($10^{-5}\cdot m^2/V\cdot s$)	Shear stress-shear rate relationship		
					k ($Pa\cdot s^n$)**	n***	R^2
RSM′[a]	30	6.6	0.24 ± 0.00	-1.42	0.0155	0.9768	0.9999
fRSM[b]	10	3.9	7.13 ± 0.51	0.52	0.0066	0.8616	0.9920
fRSM-SMP[c]	30	5.2	0.30 ± 0.11	-0.20	0.0583	0.7746	0.9995
rRSM′[d]	30	6.6	0.24 ± 0.00	-1.37	0.0090	0.9750	0.9999

* Skim milk powder; ** Consistency index; *** Flow behavior index
[a] 30% (w/w) reconstituted skim milk
[b] Fermented 10% (w/w) RSM by *Lactobacillus rhamnosus* GG (LGG) at 42 oC for 9 h.
[c] fRSM-SMP: fRSM with additional reconstitution of 20% (w/w) SMP.
[d] 30% (w/w) RSM with LGG pellet from the MRS culture.

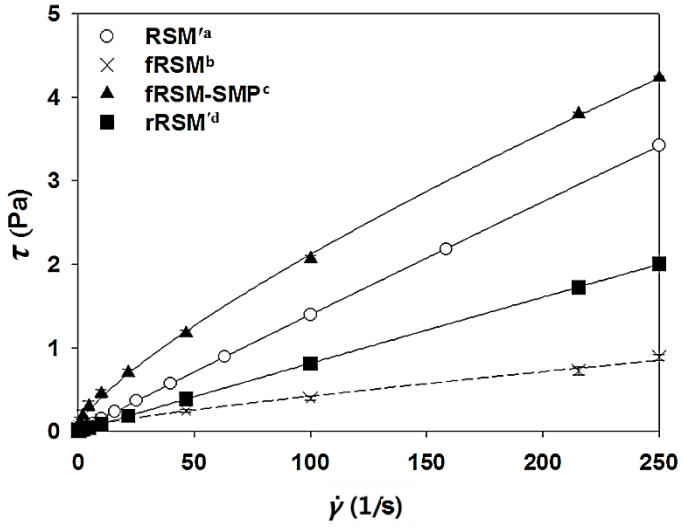


Figure 3. Shear stress (τ) for feeding suspensions as a function of shear rate ($\dot{\gamma}$) at 25 °C.

[a] 30% (w/w) reconstituted skim milk
[b] Fermented 10% (w/w) RSM by *Lactobacillus rhamnosus* GG (LGG) at 42 oC for 9 h.
[c] fRSM-SMP: fRSM with additional reconstitution of 20% (w/w) SMP.
[d] 30% (w/w) RSM with LGG pellet from the MRS culture.

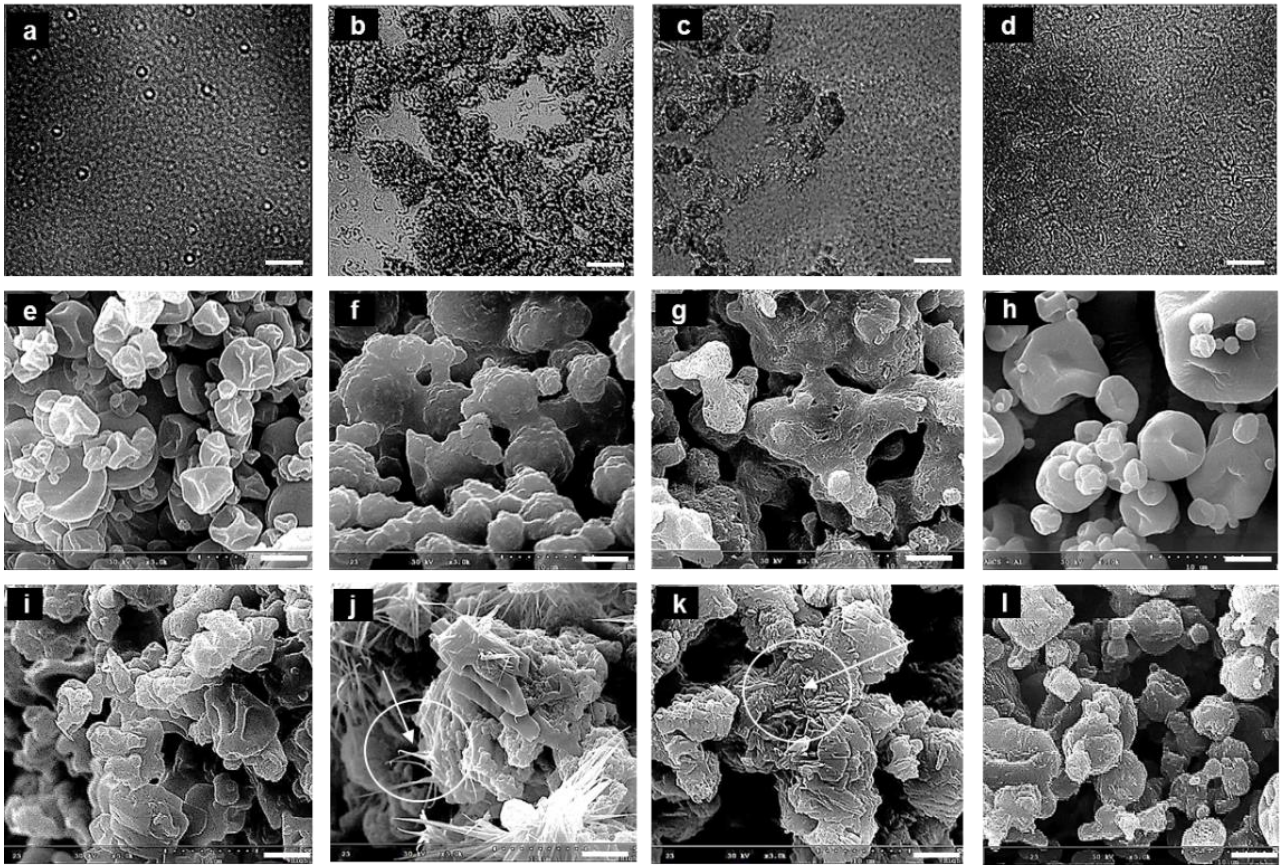


Figure 4. The microstructure of feeding suspensions and LGG powders (Bar presents 20 μm for a, b, c, d and 5 μm for the others).

a=RSM, 10% (w/w) reconstituted skim milk; b=fRSM, fermented RSM by *Lactobacillus rhamnosus* GG (LGG); c=fRSM-SMP, fRSM with additional reconstitution of 20% (w/w) skim milk powder (SMP); d=rRSM′, 30% (w/w) RSM with LGG pellet; e=LGG powder obtained from spray dried RSM stored at 0% relative humidity (RH) for 6 days; f=LGG powder obtained from spray dried fRSM stored at 0% RH for 6 days; g=LGG powder obtained from spray dried fRSM-SMP stored at 0% RH for 6 days; h=LGG powder obtained from spray dried rRSM′ stored at 0% RH for 6 days; i=LGG powder obtained from spray dried RSM stored at 69% RH for 6 days; j=LGG powder obtained from spray dried fRSM stored at 69% RH for 6 days; k=LGG powder obtained from spray dried fRSM-SMP stored at 69% RH for 6 days; l=LGG powder obtained from spray dried rRSM′ stored at 69% RH for 6 days.

3.2. Microbial survival ratio

The microbial survival ratio of the fRSM after spray drying was very low as 0.59% when comparing the survival ratio in rRSM′ (26.30%). The stickiness problem makes that the powder cannot be transferred quickly to the collection bottle, and the more heat stress was exposed to LGG resulting very low survival ratio.

3.3. LGG powder quality

The particle size of the LGG powder obtained from fRSM was non-measurable since its severe cohesiveness between particles, and the particle size of rRSM′ powder was 12.66 μm. The moisture content of fRSM powder was 13.18% which shows poor storage stability, and the moisture content of rRSM′ powder (4.69%) known as good-quality parameter for the dairy powder products (Masters, 1985).

4. The effect of adding SMP to fRSM on the spray drying

4.1. Drying yield

The drying yield of fRSM was increased from 0 to 36.10% after the additional reconstitution of SMP to fRSM, and this is almost similar to the drying yield of rRSM′ (35.77%). This is because the reduced drying yield by the fermentation step is complemented by the effect of added SMP solid particles on the stickiness problem. Moreover, the glass transition of the spray drying droplet was faster as the fRSM-SMP has the highest k (the lowest molecular mobility) among the feeding suspensions (Tab. 2).

4.2. Microbial survival ratio

The microbial survival ratio of the fRSM was increased after the addition of SMP from 0.59 to 24.71% which is almost similar value to the rRSM′ powder (26.30%). It seems that the lactose and casein proteins in SMP protect LGG cell membrane (Ananta et al., 2004), and the increased total solid from 10 to 30% may prevent heat transfer during spray drying.

4.3. LGG powder quality

The particle size of the fRSM-SMP (13.05 μm) was almost similar to the rRSM′ powder (12.66 μm). The moisture content of fRSM powder was decreased from 13.18% which shows poor storage stability to 7.87%, but it is still vulnerable when comparing the moisture content of rRSM′ powder (4.69%).

5. The mechanism governing the performance of the consecutive LGG encapsulation process with the adding SMP to fRSM

5.1. Glass transition

The moisture content of fRSM powder was as high as 13.18%, and the moisture content after storing in the desiccator having 0% RH for 6 days still much higher than that of other powders. This seems to be due to the casein aggregated structure of fRSM formed by dehydration during spray drying, this structure might allow moisture to be trapped inside the particle and it greatly affects to the glass transition temperature (T_g) of the powder resulting longer sticky zone during spray drying. In Tab. 4, the glass transition temperature of the powder which reached equilibrium moisture content after storage for 6 days in a desiccator maintained at 0.0% RH was significantly decreased from 78.5 to 55.8 °C through the fermentation step. As a result of addition of SMP into the fRSM, the temperature

increased to 62.1 °C with 6.3 °C difference. In addition, it was found that the glass transition temperature of fRSM-SMP was almost the same as that of rRSM′ (61.0 °C), which is the result of typical process. Also, the glass transition temperature and drying yield were found to be proportional, and this seems to be the glass transition temperature of fRSM increased with the addition of SMP and the time taken to switch to glassy state during spray drying decreased.

5.2. The changes in the lactose hydration property

5.2.1. Microstructure

In the feeding suspensions, the lactic acid produced after fermentation step made the charge of the κ-casein located at the surface of casein micelle lowered, shrinkage of the κ-casein, and forming a casein aggregated structure due to the weakening of the electrostatic repulsive force. When the SMP was added to fRSM, the pH increased from 3.9 to 5.2 and the structure remained unreleased even after the pI value (~ pH 4.5) was exceeded (Tab. 1, Fig. 4). In the LGG powders, the reason why a plate or a rod or a grid shaped crystals appears on the surface of the LGG powder with a certain rule is due to the formation of crystals by the crystallization phenomenon. Lactose has the simplest molecular structure among the components of LGG powder and is easy to crystallize and therefore has the highest correlation with crystal formation. The powder reaching the crystal state earlier during the storage has low glass transition temperature (Downton et al., 1982; Bhandari et al., 1997; Roos et al., 2002) such as fRSM, and no crystal growth was observed in fRSM-SMP, but partial crystallization was confirmed (Fig. 4). Lactose crystal growth was found when stored at 69% RH for 6 days after spray drying the fRSM. As the casein micelles, which were in a competitive position in the interaction with water molecules during spray drying, storage, and rehydration,

Table 3. Drying yield, moisture content (X), and volume mean diameter ($d_{4,3}$) of LGG powders.

LGG powders	Drying yield (%)	X (% d.b.)	$d_{4,3}$ (μm)
RSM′[a]	67.95 ± 1.94	9.19 ± 0.05	8.80 ± 0.41
fRSM[b]	Not measurable	13.18 ± 0.47	Not measurable
fRSM-SMP[c]	36.1 ± 1.05	7.87 ± 0.12	13.05 ± 0.36
rRSM′[d]	35.77 ± 1.09	4.69 ± 0.20	12.66 ± 0.49

[a] LGG powder obtained by spray drying of 30% (w/w) reconstituted skim milk
[b] LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 oC for 9 h.
[c] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[d] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

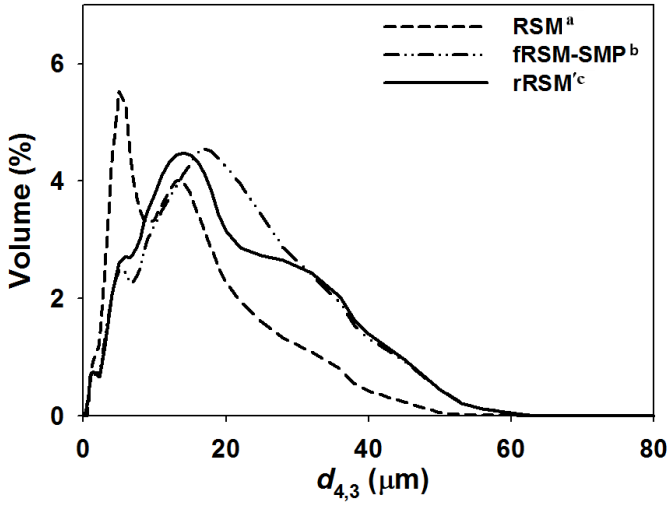


Figure 5. Particle size distributions of LGG powders as a function of volume mean diameter ($d_{4,3}$).

[a] LGG powder obtained by spray drying of 10% (w/w) reconstituted skim milk
[b] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[c] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

were denatured, the hydration property of lactose becomes better than casein micelle. For this reason, the crystal growth appeared at a high lactose content on the powder surface.

5.2.2. Particle size and moisture sorption isotherm

The curves are divided into two major groups, which may be due to the difference in the total solid of the feeding suspensions. This assumes particle size to be influenced, assuming that it is sprayed with the same size droplet, because the dried size at 30% total solid is bigger than the dried size at 10%. The RSM with 10% total solid showed the lowest particle size at 8.80 μm, and other values were similar (Tab. 3, Fig. 5). Due to the smaller powder density, the powder has more pores to interact with more water molecules. (Fig. 7) Although the powder obtained from fRSM is a big lump (due to the cohesiveness of the particles) that cannot be measured the particle size, it is presumed that the particle size forming a single mass due to the low total solid (10%) at the time of atomizing was small. It was found that crystallization occurred from the a_w of 0.04 to 0.60 of while fRSM-SMP interacted with more moisture than the other with 30% total solids and decreased a_w of 0.43–0.69. Although the glass transition temperature results show that fRSM-SMP has a pattern similar to that of rRSM′, it is expected that the internal molecular bonding force is small enough that the phase transition occurs more rapidly.

5.3. Degrees of stickiness during spray drying and storage stability

In comparison of the drying yield and the glass transition temperature, the degree of stickiness phenomenon of fRSM-SMP seems to have almost the same value with rRSM′. However, fRSM-SMP in the Fig. 7, the crystallization

phenomenon which is a transition of physical state occurred in the range of water activity from 0.43 to 0.69, and it means that the time taking until caking phenomenon when the powder is stored is faster than that in the rRSM′. In the measurement of the sticky point temperature (SPT, T-T_g) value, the meaning of SPT showing negative value is empirically sticky even after the physical glass transition occurs, and when the SPT is positive, the stickiness disappears before the glass transition occurs. Thus, it was confirmed that the fRSM has more time for occurring stickiness as -5.8 °C, and was found to be less sticky at +7.9 °C in the fRSM-SMP, and the SPT of rRSM′ was at least 20 °C (Fig. 9) The method in the Fig. 10 follows (Kaderides and Goula, 2017) and this was used for determining whether the moisture content of LGG powder is in a glass state or a rubber state when spray drying is finished in this paper. Moisture content after the spray drying of fRSM was 13.18% and the moisture content corresponding to the glass transition temperature of the fRSM in the graph was 9.9%, and fRSM was still in the rubbery state. In the proposed mechanism of stickiness phenomenon occurred in the droplets with different feeding suspensions (Fig. 12, Fig. 13), the microstructure formed by the casein acidification after fermentation prevents water evaporation and entraps water molecules inside the droplet, and this high moisture content affects greatly to decrease the glass transition temperature of the droplet, resulting the formation of liquid bridges between the wall of evaporation tube and LGG powders and it is called stickiness. When SMP is added to fRSM, the casein aggregated structure seems like to be released partially as the pH of the feeding suspension is increased from 3.9 to 5.2. In addition of this, the added SMP effectively decreases the molecular mobility of the droplet, and the glass transition of the droplet is faster, resulting the decrease in the formation of liquid bridges between the wall and the LGG powders even

though the added lactose (T_g=101 ^oC at 0% RH, Kalichevsky et al., 1993) content might lower the T_g of the feeding suspension when comparing the T_g of the rennet casein (T_g=144 ^oC at 0% RH, Lee and Lucey. 2010).

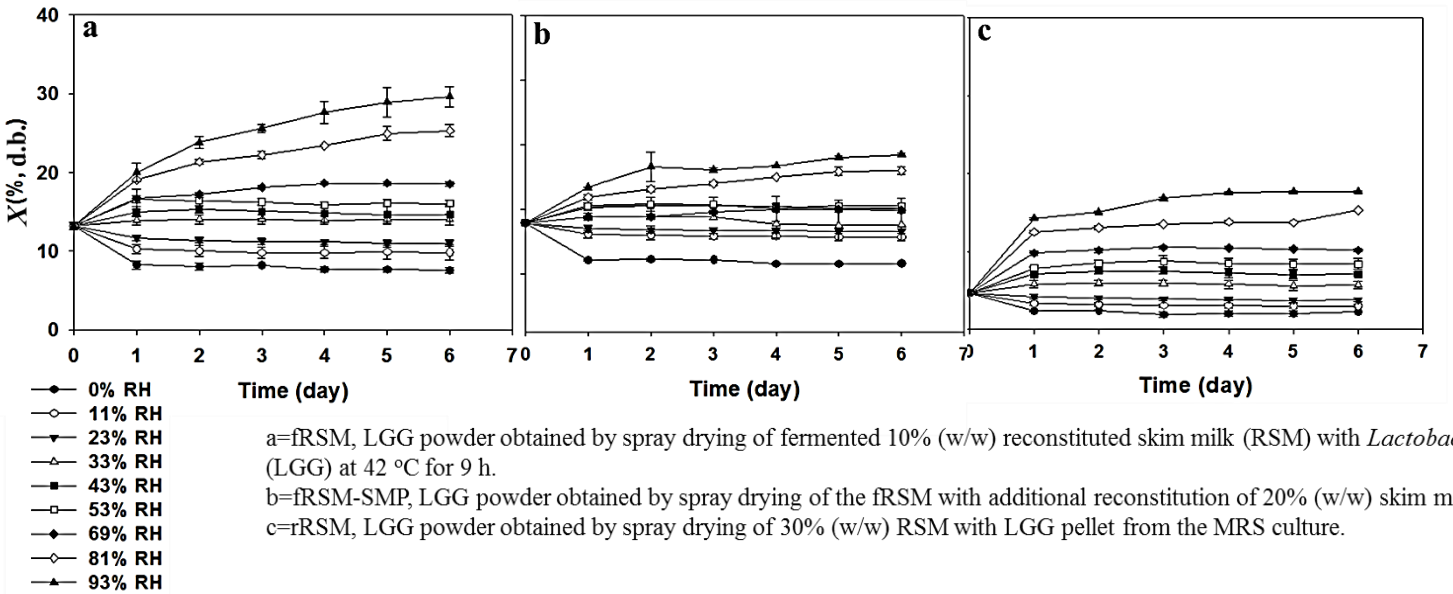


Figure 6. Moisture sorption isotherm, the moisture content (X) of LGG powders after storing at 25 °C in various relative humidity from 0 to 93% for 6 days.

a=fRSM, LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 °C for 9 h.

b=fRSM-SMP, LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.

c=rRSM', LGG powder obtained by spray drying of RSM' with LGG pellet from the MRS culture.

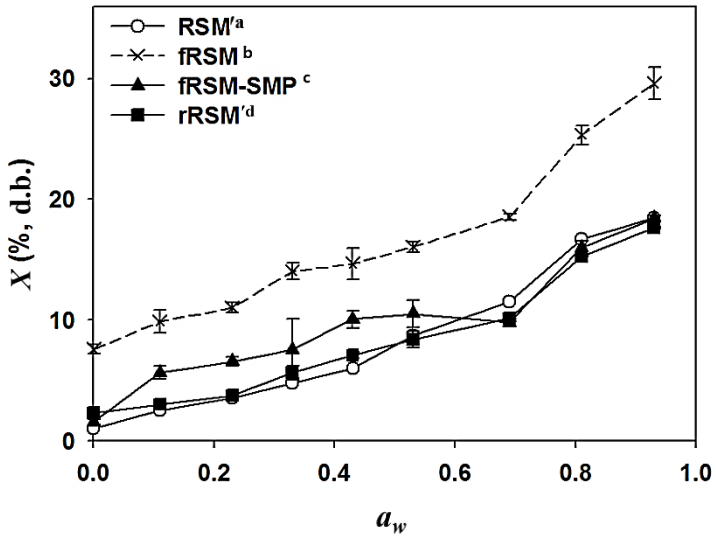


Figure 7. Moisture sorption isotherm, the moisture content (X) of the LGG powders after storing at 25 °C for 6 days in the desiccators having various relative humidity (0, 11, 23, 33, 43, 53, 69, 81, and 93%) as a function of water activity (a_w).

[a] LGG powder obtained by spray drying of 30% (w/w) reconstituted skim milk
[b] LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 °C for 9 h.
[c] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[d] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

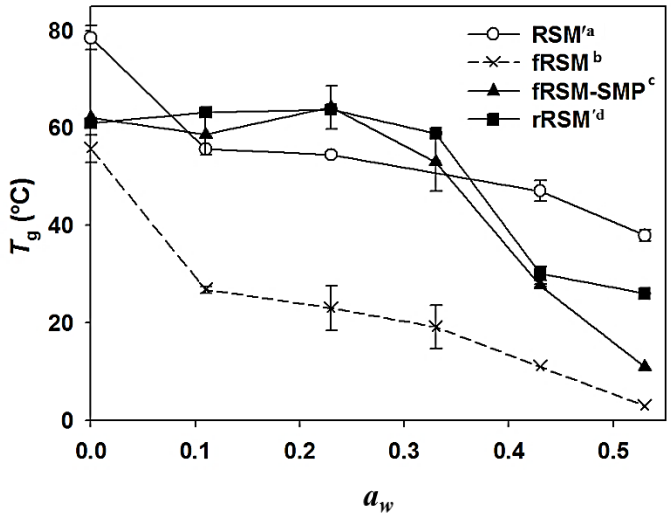


Figure 8. Glass transition temperature (T_g) of the LGG powders stored at 25 °C for 6 days in the desiccators with various relative humidities (0, 11, 23, 33, 43, and 53%) as a function of water activity (a_w).

[a] LGG powder obtained by spray drying of 30% (w/w) reconstituted skim milk
[b] LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 °C for 9 h.
[c] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[d] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

Table 4. Glass transition temperature (T_g) of the LGG powders stored at 25 °C for 6 days in the desiccator with relative humidity (RH) of 0%.

LGG powders	T_g (°C), (RH=0%)
RSM′	78.5 ± 2.5
fRSM	55.8 ± 2.7
fRSM-SMP	62.1 ± 0.2
rRSM′	61.0 ± 0.3

[a] LGG powder obtained by spray drying of 30% (w/w) reconstituted skim milk
[b] LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 °C for 9 h.
[c] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[d] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

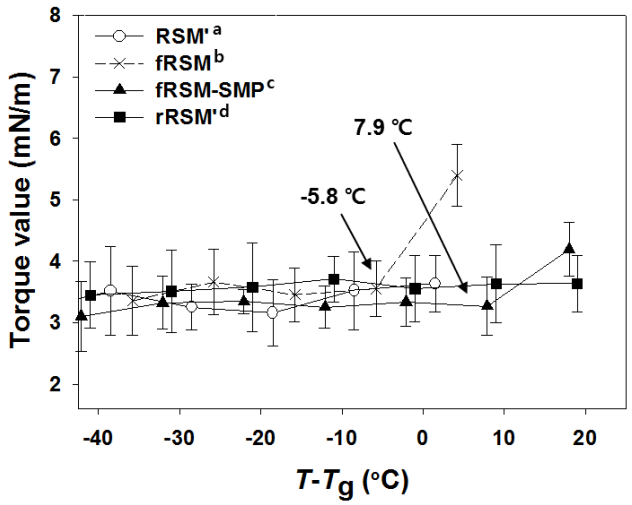


Figure 9. Torque values at various water activities for the LGG powders as a function of temperature difference to the glass transition temperature ($T–T_g$).

[a] LGG powder obtained by spray drying of 30% (w/w) reconstituted skim milk
[b] LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 ºC for 9 h.
[c] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[d] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

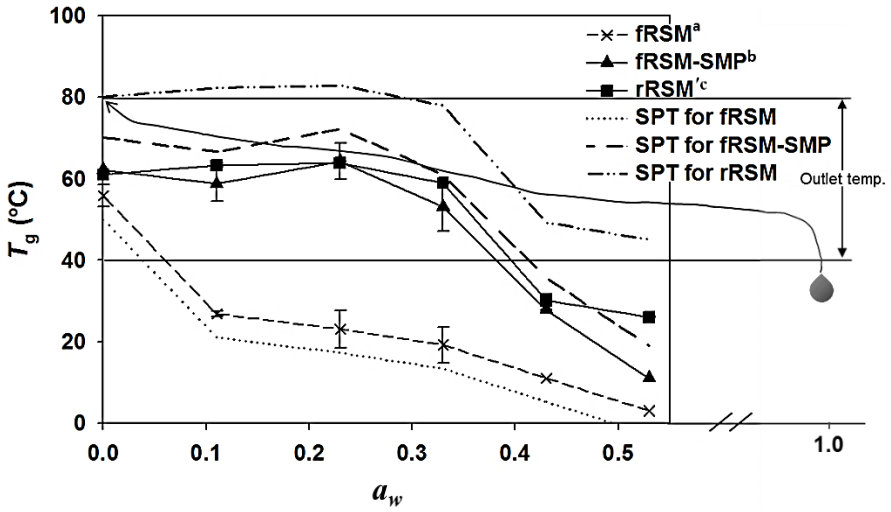


Figure 10. Glass transition temperature (T_g) and sticky point temperature (SPT) for LGG powders as a function of water activity (a_w).

[a] LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 °C for 9 h.
[b] LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
[c] LGG powder obtained by spray drying of RSM′ with LGG pellet from the MRS culture.

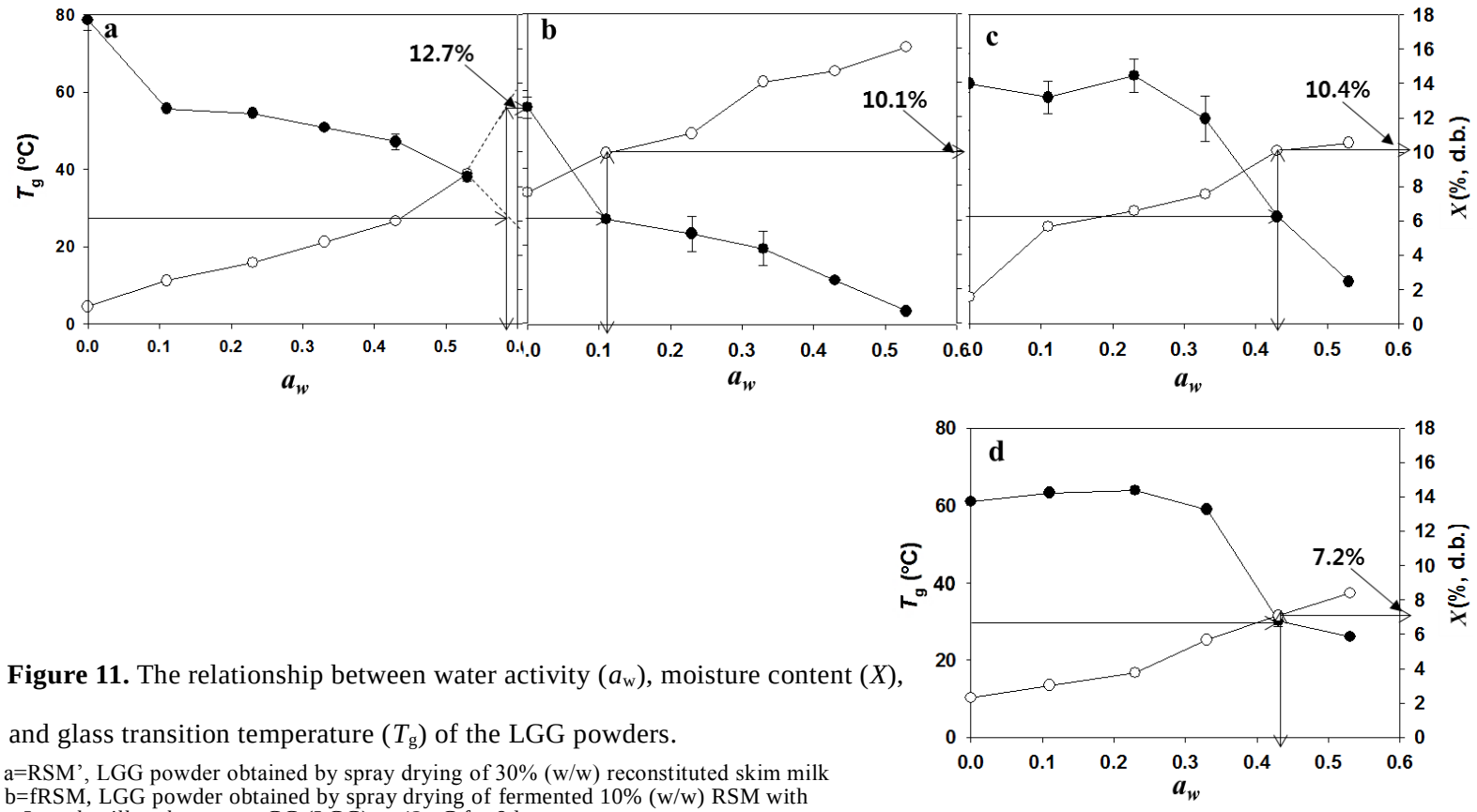


Figure 11. The relationship between water activity (a_w), moisture content (X), and glass transition temperature (T_g) of the LGG powders.

a=RSM', LGG powder obtained by spray drying of 30% (w/w) reconstituted skim milk
b=fRSM, LGG powder obtained by spray drying of fermented 10% (w/w) RSM with *Lactobacillus rhamnosus* GG (LGG) at 42 oC for 9 h.
c=fRSM-SMP, LGG powder obtained by spray drying of the fRSM with additional reconstitution of 20% (w/w) SMP.
d=rRSM', LGG powder obtained by spray drying of RSM' with LGG pellet from the MRS culture.

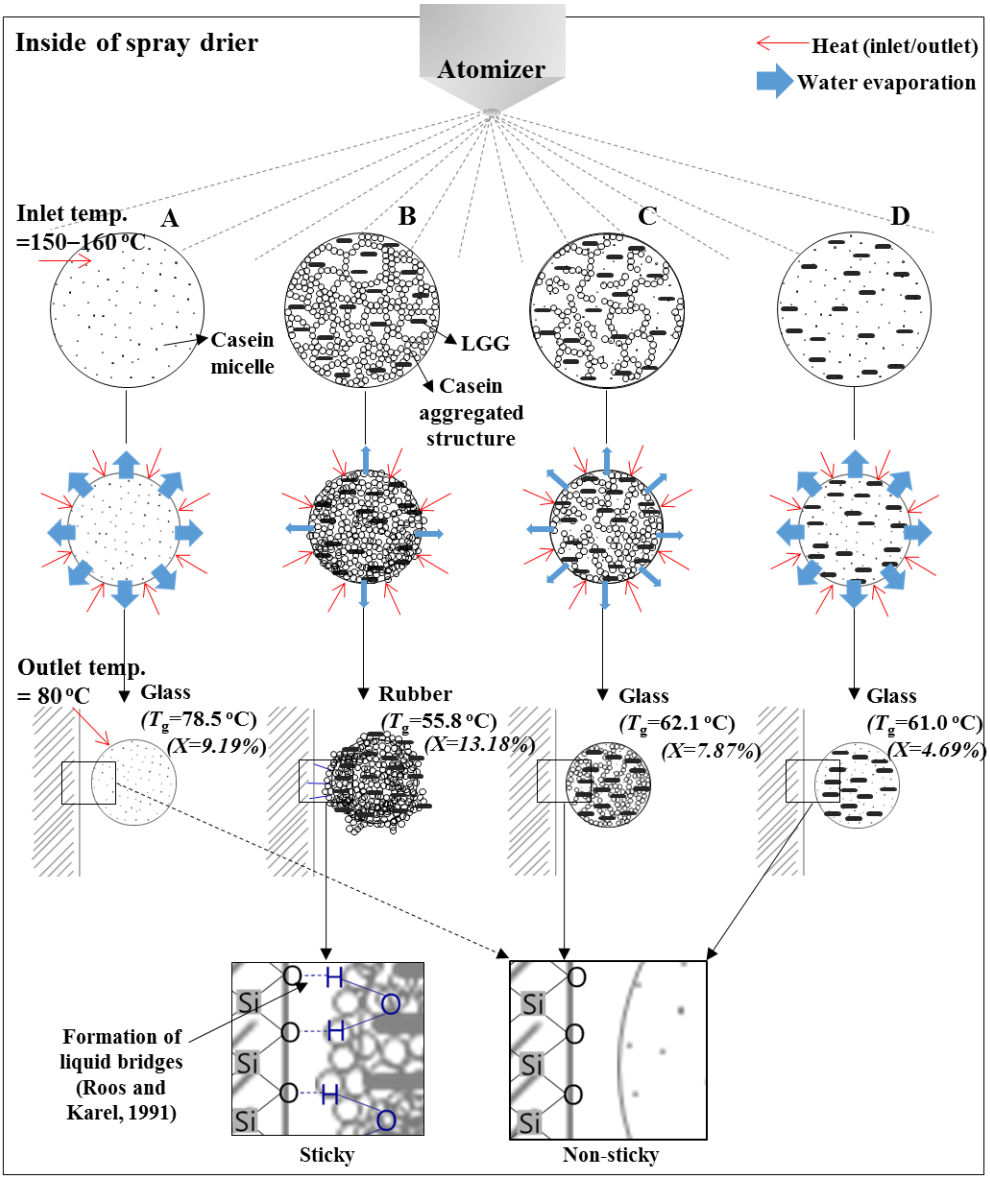


Figure 12. Proposed mechanism of stickiness phenomenon (adhesion) occurring between the droplet and wall in the spray dryer with different feeding suspensions: A=RSM′, 30% (w/w) reconstituted skim milk; B=fRSM, fermented 10% (w/w) RSM; C=fRSM-SMP, fRSM added with 20% (w/w) skim milk powder; D=rRSM′, 30% (w/w) RSM with LGG pellet from the MRS culture.

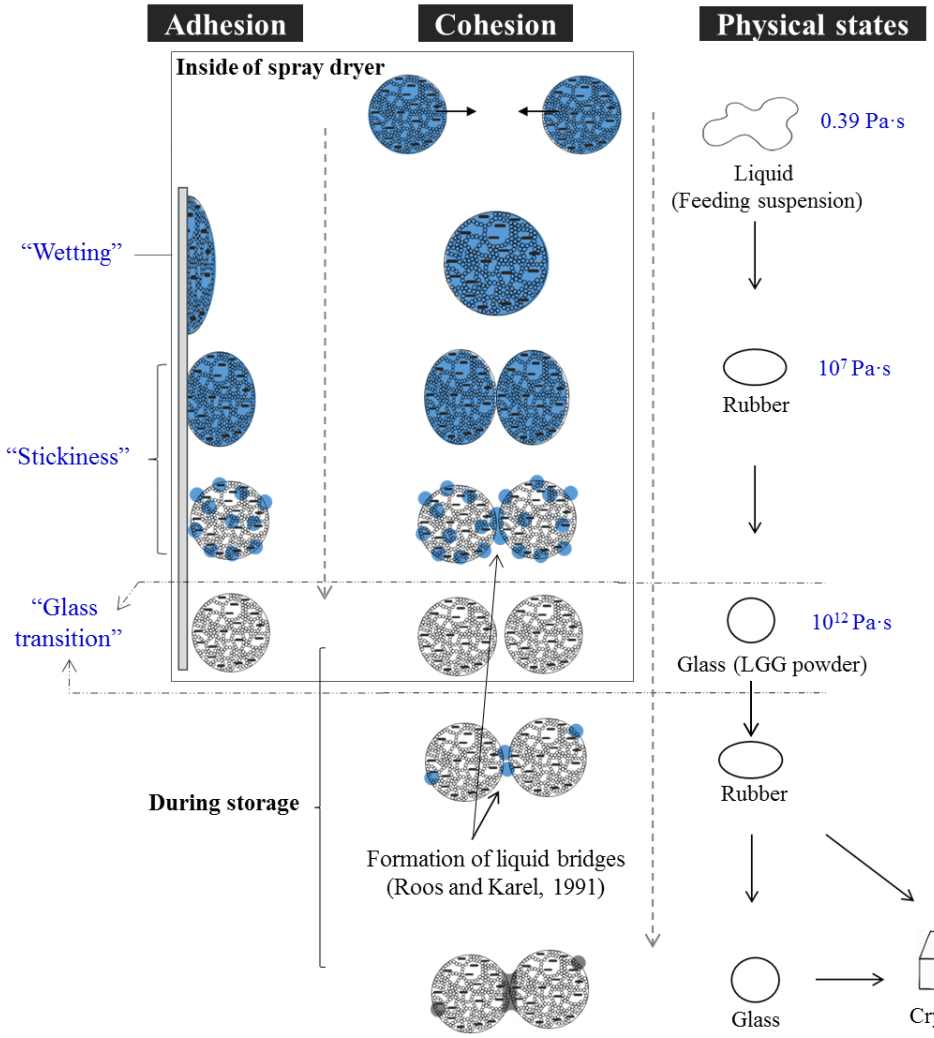


Figure 13. Proposed mechanism of stickiness phenomenon (cohesion) occurring between the spray drying droplets or LGG powders and their physical states.

Conclusions

We have developed LGG encapsulation process as a more economical, simpler, and environmentally friendly (fRSM-SMP) process. The effect of adding SMP to fRSM was achieved by the enhancement of k (i) by casein aggregated structure through fermentation, (ii) as the increase in the TS of fRSM, and (iii) as casein micelles released partially when the pH was increased from 3.9 to 5.2 identified by reduced particle size and μ. The increased k affects to the increases in the T_g and SPT, and the time to reach the glassy state of the spray drying droplets has been faster. In the theoretical level of stickiness, the k of fRSM (0.0066 Pa·s^n) was significantly increased by adding SMP (0.0583 Pa·s^n), and it was shown to highly surpass the rRSM (0.090 Pa·s^n). The T_g of fRSM (55.8 ^{o}C) was also increased by adding SMP (62.1 ^{o}C), and it was shown to replace rRSM (61 ^{o}C). In the empirical degree of stickiness, SPT of fRSM (-5.8 ^{o}C) was increased by adding SMP (7.9 ^{o}C), but it was not appear to be comparable to the rRSM (≥20 ^{o}C). The drying yield of fRSM (0%) was also dramatically increased by adding SMP (36.1%), and it was shown to replace rRSM (35.77%). In the LGG powder quality, the microbial survival ratio of fRSM (0.59%) was significantly improved in fRSM-SMP (24.71%), and it was appeared to be comparable to rRSM (26.30%), and the X of fRSM (13.18%) was decreased in fRSM-SMP (7.87%) which means still the vulnerable quality as a dairy product. Although the high X was shown, all the LGG powders were in the glassy state after spray drying except for fRSM. Simple addition of SMP significantly improved the efficiency of spray drying by lowering the stickiness via glass transition shift, and thus enabled the direct spray drying of fRSM as a promising encapsulation method for LGG.

Chapter 3
Effect of pH on LGG encapsulation during spray drying

Introduction

In Chapter 2, the additional SMP reconstitution to the fRSM solved the stickiness problem occurred from the spray drying of fRSM. The effects of addition of SMP to fRSM are both increase in total solids and increase in pH. Thus, the solution of stickiness problem may have been due to the increase in pH simply. It is also found that spray drying of feeding suspensions containing high lactic acid content and the low pH are not an easy way because powder sticks to the spray dryer and cyclone walls of the high lactic acid content and low pH (Modler and Emmons, 1978; Salameh and Taylor, 2006; Bhandari, 2008). The objective of this chapter is to reduce the stickiness by adjusting pH of the feeding suspensions to the pH of 5.2 or 6.6 before the direct spray drying. The physicochemical properties of the feeding suspensions including pH, particle size, electrophoretic mobility, and shear stress-shear rate relationship were determined, and the drying yield, moisture content, particle size, moisture sorption isotherm, microstructure observed by CLSM and SEM, glass transition temperature, and sticky point temperature of LGG powders were measured for the investigation of pH effect on the solving stickiness problem.

Materials and Methods

1. Fermentation of LGG

Lactobacillus rhamnosus GG (LGG, ATCC53103) obtained from the department of food science and technology of Chungbuk National University (Cheongju, Korea) was sub-cultured for 2 times at 37 °C for 24 h in de Man, Rogosa and Sharpe (MRS) broth (Difco, Detroit, MI, USA) and inoculated 5% (w/w) of LGG culture in the enriched RSM. The enrichment of reconstituted skim milk (RSM, Seoul Milk Co., Ltd., Seoul, Korea) was achieved by adding 2% (w/w) of glucose (Ducksan, Ansan, Korea) and 1% (w/w) of yeast extract (Thermo Fisher Scientific, Erembodegem, Belgium) to the 10% (w/w) total solid (TS) RSM. Because LGG cannot metabolize lactose due to their gene frame shift (Saxelin et al., 1997; Matti et al., 2009). The enriched RSM was heat-treated at 90 °C for 30 min in water bath (BS-31, Jeiotech, Seoul, Korea) and cooled until room temperature. The growth of LGG in the enriched RSM was compared with MRS broth, 10% (w/w) RSM, and the D.W. containing 2% (w/w) glucose and 1% (w/w) yeast extract. Fermentation of enriched RSM containing LGG was performed at 42 °C for 9 h in the water bath until the pH reaches to 3.9.

2. Survivability of LGG

2.1. Viable cell count of LGG in the feeding suspensions

The feeding suspensions were diluted serially by the decimal dilution until 10^{-7} by using sterile saline (0.85% (w/v) NaCl solution). The 80 μL of the diluted feeding suspensions was spread on the sterile MRS agar plates, and placed in a rectangular jar (Mitsubishi gas chemical, Tokyo, Japan), and filled with nitrogen gas (N_2) and incubated at 37 °C for 24–48 h in the incubator. The yellowish white

colonies with the size of above 2 mm formed were counted, and calculated as CFU/g, dry basis.

2.2. Viable cell count of LGG in the LGG powders

The powder was diluted 10-fold with sterile saline to prepare a stock solution (10^0), and diluted serially by the decimal dilution until 10^{-7} by using sterile saline (0.85% (w/v) NaCl solution). The 80 μL of the diluted feeding suspensions was spread on the sterile MRS agar plates, and placed in a rectangular jar (Mitsubishi gas chemical, Tokyo, Japan), and filled with nitrogen gas (N_2) and incubated at 37 °C for 24–48 h in the incubator. The yellowish white colonies with the size of above 2 mm formed were counted, and calculated as CFU/g, dry basis.

2.3. Survival ratio of LGG

The LGG survival ratio before and after spray drying was calculated using Eq. (1).

3. Preparation of feeding suspensions

In the preparation of 10% (w/w) total solid (TS) RSM, 20 *g* of SMP was added to 180 *g* of D.W. and reconstituted for 2 h at 25 °C, and heat-treated at 90 °C for 30 min. For the preparation of 10% TS rRSM (RSM containing LGG by cell recovery step), cell recovery step is needed; 200 g of LGG sub-cultured MRS broth was centrifuged at × 4000 *g* for 10 min and the supernatant was removed. Remaining MRS broth in the pellet was washed by mixing with 200 g of 1 M phosphate buffer solution and centrifuged at × 4000 *g* for 10 min and the supernatant was removed. The pellet thus obtained was dispersed by using magnetic stirrer in 200 g of 1 M phosphate buffer solution and centrifuged at ×

4000 *g* for 10 min and the supernatant was removed. The pellet thus obtained was dispersed by using magnetic stirrer for 30 min in 200 g of 10% (w/w) TS RSM. The pH of the fRSM was adjusted by adding 10 M NaOH solution (Daejung Chemicals and Metals co., Ltd, Gyeonggi-do, Korea) from 3.9 (fRSM) to 5.2 or 6.6 (fRSM-pH 5.2 or fRSM-pH 6.6).

4. Physicochemical properties of feeding suspensions

4.1. pH

pH meter (S220-K, Mettler Toledo International Inc., Shanghai, China) calibrated to pH of 4.01, 7.00, and 9.21 was used to measure the pH of feeding suspensions. The pH of RSM, RSM′, rRSM, and rRSM′ was almost the same, but it has adjusted to 6.6 using 10% (w/v) lactic acid solution.

4.2. Electrophoretic mobility

Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used to measure the electrophoretic mobility (μ, $10^{-5}\cdot m^2/V\cdot s$) from the Henry equation (Eq. (2)). All feeding suspensions were diluted 10-fold and then measured at 25 °C.

4.3. Particle size

Zetasizer nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used to measure the volume average mean particle size ($d_{4,3}$, μm) using dynamic light scattering. The $d_{4,3}$ was calculated by Eq. (3)**.** All feeding suspensions were diluted 10-fold and then measured at 25 °C.

4.4. Shear stress-shear rate relationship

Shear stress-shear rate relationship tests were performed using a rotational shear

rheometer (DHR-3, TA Instruments, New Castle, DE, USA) with Peltier concentric cylinder geometry (recessed/standard) having a standard cup diameter of 30.39 mm and a bob diameter of 27.99 mm at 25 °C. A shear rate ranging from 0.01 to 250 1/s was applied to investigate the shear stress of each feeding suspension. The viscosity of the feeding suspensions were compared using the *k* from the equation of Herschel-Bulkley model (Eq. (4)). The *k* was measured to compare the viscosity values of feeding suspensions which has confirmed to have non-Newtonian behavior (Fig. 1). The higher the *k*, the shorter the time it takes to reach a viscosity of $10^{12\text{-}14}$ Pa·s which is corresponding to the glassy state of materials (Downton et al., 1982; Bhandari et al., 1997), particles that are in the glassy state are no longer sticky, resulting in less stickiness problems.

5. Spray drying

The feeding suspensions were spray dried using mini spray dryer (Eyela SD-1000, Tokyo Rikakikai Co., Tokyo, Japan) equipped with a 0.7 mm diameter nozzle at a feed flow rate of 800 mL/h, feed atomization pressure of 100 kPa, and hot air flow rate of 0.65 m^3/min with inlet and outlet temperatures of 150–160 °C and 80 °C, respectively. The obtained LGG powders were stored at 25 °C in the desiccator containing saturate phosphorus pentoxide solution (0% relative humidity).

6. Drying yield

The drying yield was calculated as Eq. (5)

7. Moisture content

The powders were dried at 105 °C in the drying oven. When the weight of the

LGG powders reached a constant weight was measured, and moisture content was calculated as Eq. (6) (AOAC, 2005).

8. Water activity

The water activity of the LGG powders which are in the equilibrium moisture content with different relative humidities (0, 11, 23, 33, 43, 53, 69, 81, and 93%) was measured using (Aqualab water activity meter, Decagon, WA, USA) at 25 °C.

9. Particle size distribution using backscattering

The particle size and the % volume distribution was analyzed using particle size analyzer (1190LD, CILAS, Orleans, France) based on Fraunhofer theory. The 2–3 g of the LGG powders was placed on the sample distributor with the frequency of 50 Hz for matching the obscuration from 8 to 15%. All analysis were repeated for 3 times.

10. Microstructure

The microstructure of the feeding suspensions were observed using confocal laser scanning microscope (CLSM, Applied Precision, Issaquah, WA, USA) to obtain the confocal image that makes multiple thick casein aggregated structure in one scene. An appropriate amount of the LGG powder was placed on the observation plate, and gold coated (K550; Emi-tech Ltd., Kent, UK), and the surface of the LGG powders examined in a scanning electron microscope (SEM, S-4700 Field Emission Scanning; Hitachi High-Technologies, Tokyo, Japan) at an accelerating voltage of 15.0 kV.

11. Moisture sorption isotherm

The moisture sorption isotherm was analyzed with LGG powders in the equilibrium moisture content (X_E), and it was measured**.** 2 g of LGG powders were evenly located in the aluminum plate, these were stored in the desiccators with various relative humidities (0, 11, 23, 33, 43, 53, 69, 81, and 93%) until the moisture of the powder is equilibrated (6 days). For the curve fitting, the GAB (Guggenheim-Anderson-de Boor) model was used (Eq. (7))

12. Glass transition temperature

The glass transition temperature of LGG powders were analyzed using differential scanning calorimeter (DSC; Q2000, TA Instruments, New Castle, DE, USA) equipped with a refrigerated cooling system. The LGG powders in the equilibrium moisture content were accurately weighted into aluminum pans (TA Instruments, New Castle, DE, USA), and hermetically sealed. The pans containing LGG powders were scanned for 3 cycles at the heating and cooling rates of 10 °C/min with temperature range from 2 to 100 °C, and an empty pan was used as the reference material. The temperature derivative value of heat flow (W/g·°C) was analyzed by TA Universal Analysis 2000 software version 4.5A (1998−2007 TA Instruments-Waters LLC).

13. Sticky point temperature (SPT)

The sticky point temperatures of the LGG powders were measured by the torque value (mN/m) using viscometer (LVDV III, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA), the method was based on the paper (Silalai and Roos, 2010). This method is to measure the torque value (mN/m) as a value against the force applied to the powder by the spindle rotating at 0.3 rpm,

and the point at which the torque value increases sharply appears is regarded as a sticky point. The spindle used were customized to fit the design shown in the Chapter 2-Fig. 2, and the torque value were measured every 40 s per point at 10 °C intervals from 20 °C to 80 °C. Of the 40 torque values obtained for each temperature condition, the latter 20 values were averaged and plotted according to $T–T_g$ to confirm the sticky point temperature.

Results and Discussion

1. Physicochemical properties of feeding suspensions

As the pH increased from 3.9 to 5.2 and 6.6, unexpectedly, the *k* were decreased from 0.0066 to 0.0087 $Pa \cdot s^n$ and 0.237 $Pa \cdot s^n$ from the confirmation of microstructure, particle size and electrophoretic mobility (Tab. 1). The reason for this is presumably because the casein aggregated structure is not completely restored to the casein micelles by increasing pH, and some casein micelles released performed a role that increases molecular mobility of the feeding suspensions.

2. Microstructure of feeding suspensions

In Fig. 2, the casein aggregated structure formed after the fermentation step was formed as the pH decreased from 6.6 to 3.9, which is below the pI value of milk protein (4.5), and seems to be slightly loosened but not released visibly although the pH was again increased from 3.9 to 5.2 or 6.6 (casein denaturation was confirmed to be irreversible). In accordance to this, Tab. 1 shows that the increased particle size from 0.21 to 7.13 μm by fermentation step, and it was decreased to 3.43 and 0.21 μm, respectively, when the pH was increased to 5.2 and 6.6. The electrophoretic mobility of fRSM-pH 5.2 and fRSM-pH 6.6 were decreased from 0.52 to 0.16 and 0.85 $10^{-5} \cdot m^2/V \cdot s$, respectively. The decrease in particle size and electrophoretic mobility seems to be due to an increase in the negative charge between casein micelles resulting in a loosened casein aggregated structure.

Table 1. Total solid, pH, particle size by dynamic light scattering, and electrophoretic mobility (μ), shear stress-shear rate relationship of feeding suspensions.

Feeding suspensions	SMP* (%)	pH	Particle size (μm)	μ (10^{-5}·m^2/V·s)	Shear stress-shear rate relationship		
					k (Pa·s^n)**	n***	R^2
RSM[a]	10	6.6	0.21 ± 0.00	-1.61	0.0015	1.0480	0.9979
fRSM[b]	10	3.9	7.13 ± 0.51	0.52	0.0066	0.8616	0.9920
fRSM-pH 5.2[c]	10	5.2	3.43 ± 2.93	0.16	0.0087	0.8680	0.9937
fRSM-pH 6.6[d]	10	6.6	0.35 ± 0.11	-0.85	0.0237	0.6916	0.9776
rRSM[e]	10	6.6	0.21 ± 0.00	0.01	0.0019	1.1450	0.9987

* Skim milk powder; ** Consistency index; *** Flow behavior index
[a] 10% (w/w) reconstituted skim milk
[b] Fermented 10% (w/w) RSM by *Lactobacillus rhamnosus* GG (LGG) at 42 ºC for 9 h.
[c] fRSM after pH adjustment from 3.9 to 5.2.
[d] fRSM after pH adjustment from 3.9 to 6.6.
e 10% (w/w) RSM with LGG pellet from the MRS culture.

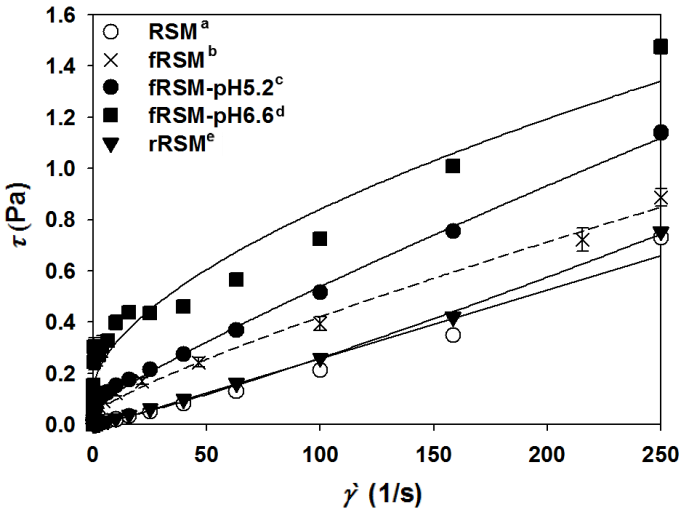


Figure 1. Shear stress (τ) for feeding suspensions as a function of shear rate ($\dot{\gamma}$) at 25 °C.

[a] 10% (w/w) reconstituted skim milk
[b] Fermented 10% (w/w) RSM by *Lactobacillus rhamnosus* GG (LGG) at 42 ºC for 9 h.
[c] fRSM after pH adjustment from 3.9 to 5.2.
[d] fRSM after pH adjustment from 3.9 to 6.6.
e 10% (w/w) RSM with LGG pellet from the MRS culture.

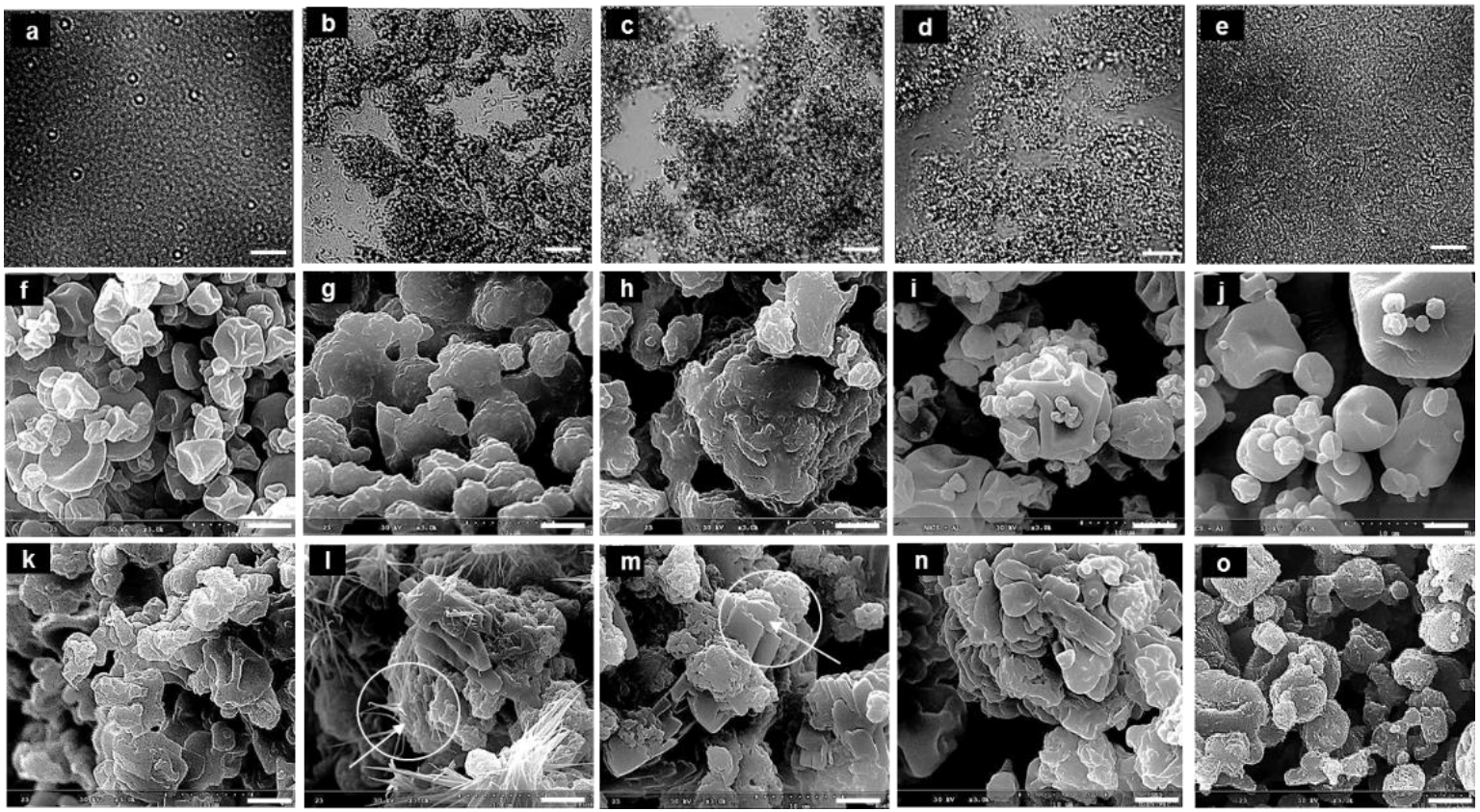


Figure 2. The microstructure of feeding suspensions and LGG powders (bar presents 20 μm for a, b, c, d and 5 μm for the others).

a=RSM, 10% (w/w) reconstituted skim milk; b=fRSM, fermented RSM by *Lactobacillus rhamnosus* GG (LGG); c=fRSM-pH5.2, fRSM with pH adjustment from 3.9 to 5.2; d=fRSM-pH6.6, fRSM with pH adjustment from 3.9 to 6.6; e=rRSM′, 30% (w/w) RSM with LGG pellet; f=LGG powder obtained from spray dried RSM stored at 0% relative humidity (RH) for 6 days; g=LGG powder obtained from spray dried fRSM stored at 0% RH for 6 days; h=LGG powder obtained from spray dried fRSM-pH5.2 stored at 0% RH for 6 days; i=LGG powder obtained from spray dried fRSM-pH6.6 stored at 0% RH for 6 days; j=LGG powder obtained from spray dried rRSM′ stored at 0% RH for 6 days; k=LGG powder obtained from spray dried RSM stored at 69% RH for 6 days; l=LGG powder obtained from spray dried fRSM stored at 69% RH for 6 days; m=LGG powder obtained from spray dried fRSM-pH5.2 stored at 69% RH for 6 days; n=LGG powder obtained from spray dried fRSM-pH6.6 stored at 69% RH for 6 days; o=LGG powder obtained from spray dried rRSM′ stored at 69% RH for 6 days.

3. The pH effect of fRSM on the spray drying

3.1. Drying yield

In Tab. 2, the calculated drying yield of fRSM-pH 5.2 and fRSM-pH 6.6 were significantly lower than those of rRSM (36.74%), which were 21.95% and 28.74%, respectively. This has matched to the results of microstructure, particle size, and electrophoretic mobility as previously described, in which the increase in pH did not completely restore the casein aggregated structure, but still the pH of the feeding suspensions affect to the glass transition of the droplets during spray drying.

3.2. Microbial survival ratio

The microbial survival ratio of the fRSM was increased after the pH adjustment from 0.59 to 10.23% (fRSM-pH 5.2) and 17.74% (fRSM-pH 6.6). It seems that the neutralized pH may affect to prevent pH stress to the LGG cell membrane during spray drying.

3.3. LGG powder quality

The LGG powder particle size was decreased significantly from the size of not-measurable to 186.45 μm (fRSM-pH 5.2) and 12.24 μm (fRSM-pH 6.6) which is almost similar to the rRSM powder (12.66 μm). But the moisture content of fRSM powder was increased from 13.18% to 14.85% (fRSM-pH 5.2) and 16.65% (fRSM-pH 6.6) which shows poor storage stability. It seems to be the result of water hydration property of added NaOH for the pH adjustment step.

4. The mechanism governing the performance of the consecutive LGG encapsulation process with the pH adjustment of fRSM

4.1. Glass transition

The glass transition temperature of LGG powder increased from 50.8 to 55.0 °C as the pH increased from 5.2 to 6.6, which was almost the same value as compared to the glass transition temperature of 56.0 °C of rRSM (Tab. 4). Therefore, it was confirmed that the effect of increasing the glass transition temperature of the LGG powder occurs only by the pH adjustment step from the pH of 3.9 to 5.2 and 6.6. In Fig. 7, all samples were found to be in the glassy state after spray drying except fRSM powder. The moisture content of fRSM powder was as high as 13.18%, and the moisture content after storing in the desiccator having 0% RH for 6 days still much higher than that of other powders. This seems to be due to the casein aggregated structure of fRSM formed by dehydration during spray drying, this structure might allow moisture to be trapped inside the particle and it greatly affects to the glass transition temperature (T_g) of the powder resulting longer sticky zone during spray drying. In this data, pH adjustment step seems rarely affects to the T_g as comparing the T_g of rRSM′ (61.0 ^oC) and fRSM-SMP (62.1 ^oC).

4.2. The changes in the lactose hydration property

4.2.1. Microstructure

In the feeding suspensions, the lactic acid produced after fermentation step made the charge of the κ-casein located at the surface of casein lowered, shrinkage of the κ-casein, and forming a casein aggregated structure due to the weakening of the electrostatic repulsive force. When the pH was adjusted to 5.2,

the structure remained unreleased even after the pI value (~ pH 4.5) was exceeded (Fig. 2). In the LGG powders, the reason why a plate or a rod or a grid shaped crystals appears on the surface of the LGG powder with a certain rule is due to the formation of crystals by the crystallization phenomenon. Lactose has the simplest molecular structure among the components of LGG powder and is easy to crystallize and therefore has the highest correlation with crystal formation. The powder reaching the crystal state earlier during the storage has low glass transition temperature (Downton et al., 1982; Bhandari et al., 1997; Roos et al., 2002) such as fRSM, fRSM-pH 5.2, fRSM-pH 6.6 (Fig. 2). And the level of formed lactose crystals was decreased as the pH of feeding suspensions increased from the pH of 3.9 to 5.2 and 6.6.

4.2.2. Moisture sorption isotherm

There was no significant difference between LGG powders in moisture sorption isotherm (Fig. 4), but it was found that the more moisture absorbing was shown as the pH of the feeding suspenstions is increased from 3.9 to 5.2 and 6.6. This seems to be due to the hydration property of the NaOH content used for the pH adjustment step (Fig. 4).

4.3. Degrees of stickiness during spray drying and storage stability

In comparison of the drying yield, the degree of stickiness phenomenon of fRSM-pH 5.2 and 6.6 was increased from 21.95 to 28.74% as the pH is increased, but it was still not comparable to the value of rRSM (36.74%). Even fRSM- pH 6.6 in the Fig. 4 showed the best water holding property among LGG powders, and it means that the time taking until caking phenomenon when the powder is stored is fast. In the measurement of the sticky point temperature (SPT), it was

confirmed that the fRSM has the longest time for occurring stickiness as its SPT is -5.8 °C, and was found to be less sticky in the fRSM-pH 5.2 and fRSM-pH 6.6 as its SPT are -0.8 °C and 5 °C, respectively, and the SPT of rRSM was at least 14 °C (Fig. 7). In this data, the level of stickiness problem was simply decreased by the increase in the pH of feeding suspensions, but, it is still not reaches to the degree of fRSM-SMP (SPT=7.9 °C), even though the pH of fRSM-SMP (pH 5.2) is lower than that of in the fRSM-pH 6.6 (pH 5.2). In this reason, the pH adjustment of feeding suspensions is an effective way to reduce the degree of stickiness for the direct spray drying of fRSM, but the pH effect is still lesser than the effect of adding SMP to fRSM. The method in the Fig. 7 follows (Kaderides and Goula, 2017) and this was used for determining whether the moisture content of LGG powder is in a glass state or a rubber state. Moisture content after the spray drying of fRSM was 13.18% and the moisture content corresponding to the glass transition temperature of the fRSM in the graph was 10.1%, and only fRSM was still in the rubbery state.

Table 2. Drying yield, moisture content (X) and volume mean diameter ($d_{4,3}$) of LGG powders.

LGG powders	Drying yield (%)	X (%, d.b.)	$d_{4,3}$ (μm)
RSM[a]	67.95 ± 1.94	9.19 ± 0.05	8.80 ± 0.41
fRSM[b]	Not measurable	13.18 ± 0.47	Not measurable
fRSM-pH 5.2[c]	21.95 ± 3.46	14.85 ± 0.52	186.45 ± 8.35
fRSM-pH 6.6[d]	28.74 ± 2.22	16.65 ± 0.79	12.24 ± 0.13
rRSM[e]	36.74 ± 1.64	12.01 ± 0.00	9.40 ± 0.51

[a] *Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM)
[b] LGG powder obtained from spray drying of fermented 10% (w/w) RSM by LGG.
[c] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2.
[d] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6.
[e] LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

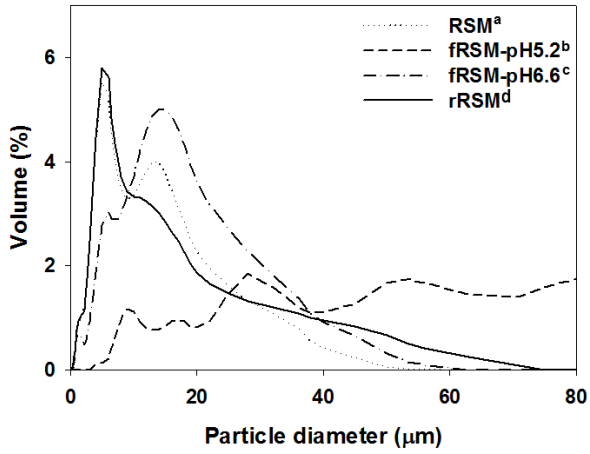


Figure 3. Particle size distributions of LGG powders as a function of volume mean diameter ($d_{4,3}$).

[a] *Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM)
[b] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2.
[c] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6.
[d] LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

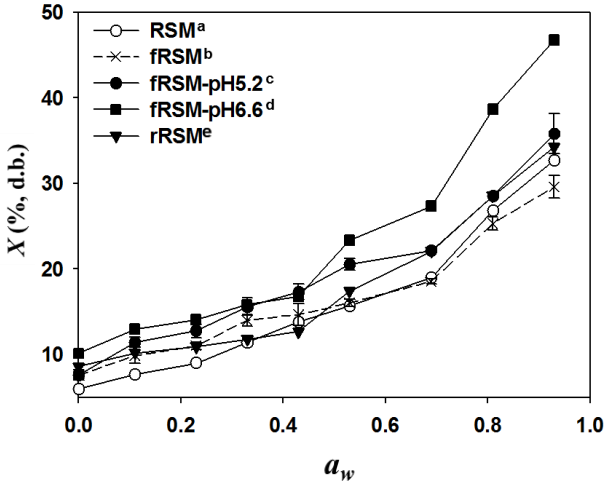


Figure 4. Moisture sorption isotherm, the moisture content (X) of the LGG powders after storing at 25 °C for 6 days in the desiccators having various relative humidity (0, 11, 23, 33, 43, 53, 69, 81, and 93%) as a function of water activity (a_w).

[a] *Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM)
[b] LGG powder obtained from spray drying of fermented 10% (w/w) RSM by LGG.
[c] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2.
[d] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6.
[e] LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

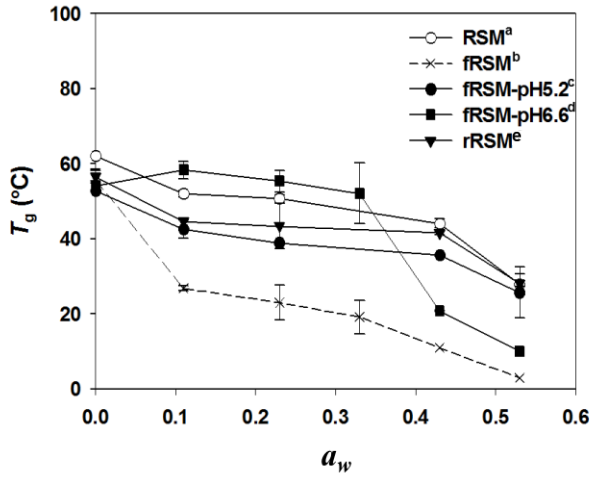


Figure 5. Glass transition temperature (T_g) of the LGG powders stored at 25 °C for 6 days in the desiccators with various relative humidities (0, 11, 23, 33, 43, and 53%) as a function of water activity (a_w).

[a] *Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM)
[b] LGG powder obtained from spray drying of fermented 10% (w/w) RSM by LGG.
[c] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2.
[d] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6.
[e] LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

Table 3. Glass transition temperature (T_g) of the LGG powders stored at 25 °C for 6 days in the desiccator with relative humidity (RH) of 0%.

LGG powders	T_g (°C), (RH=0)
RSM[a]	62.0 ± 0.4
fRSM[b]	55.8 ± 2.7
fRSM-pH 5.2[c]	50.8 ± 0.4
fRSM-pH 6.6[d]	55.0 ± 1.3
rRSM[e]	56.0 ± 1.6

[a] *Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM)
[b] LGG powder obtained from spray drying of fermented 10% (w/w) RSM by LGG.
[c] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2.
[d] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6.
[e] LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

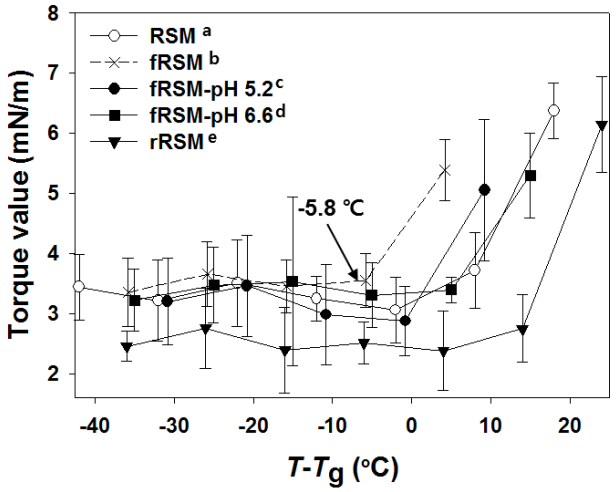


Figure 6. Torque values at various water activities for the LGG powders as a function of temperature difference to the glass transition temperature ($T–T_g$).

[a] *Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM)
[b] LGG powder obtained from spray drying of fermented 10% (w/w) RSM by LGG.
[c] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2.
[d] LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6.
[e] LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

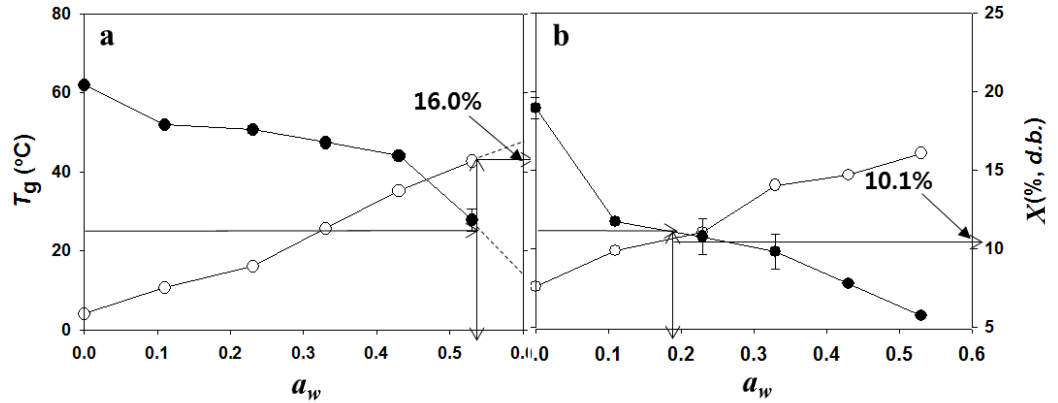
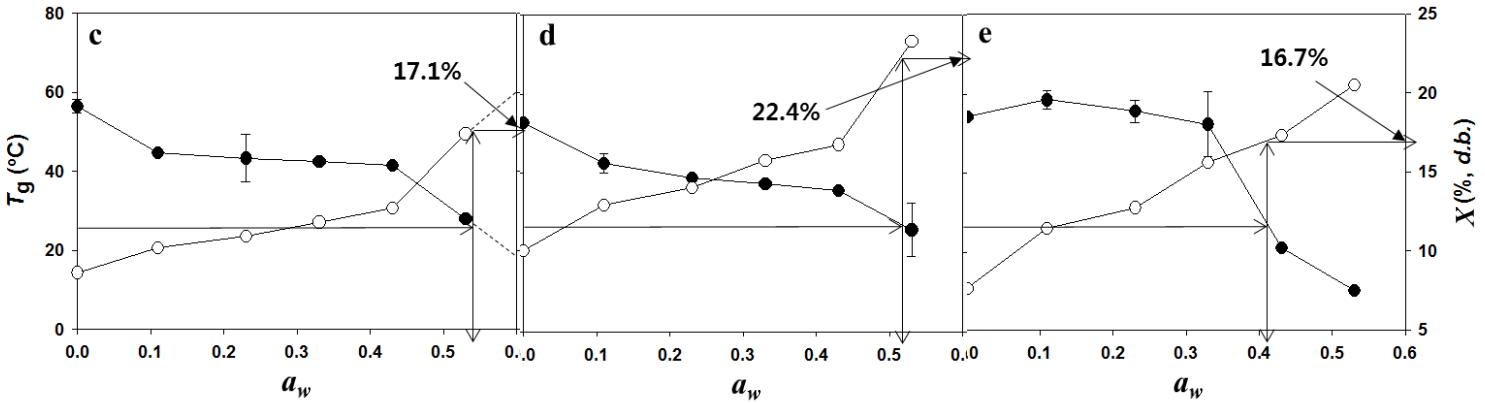


Figure 7. The relationship between water activity (a_w), moisture content (X), and glass transition temperature (T_g) of the LGG powders.



a=*Lactobacillus rhamnosus* GG (LGG) powder obtained from spray drying of 10% (w/w) reconstituted skim milk (RSM); b=LGG powder obtained from spray drying of fermented 10% (w/w) RSM by LGG; c=LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 5.2; d=LGG powder obtained from spray drying of fRSM after pH adjustment from 3.9 to 6.6; e=LGG powder obtained from spray drying of 10% (w/w) RSM with LGG pellet from the MRS culture.

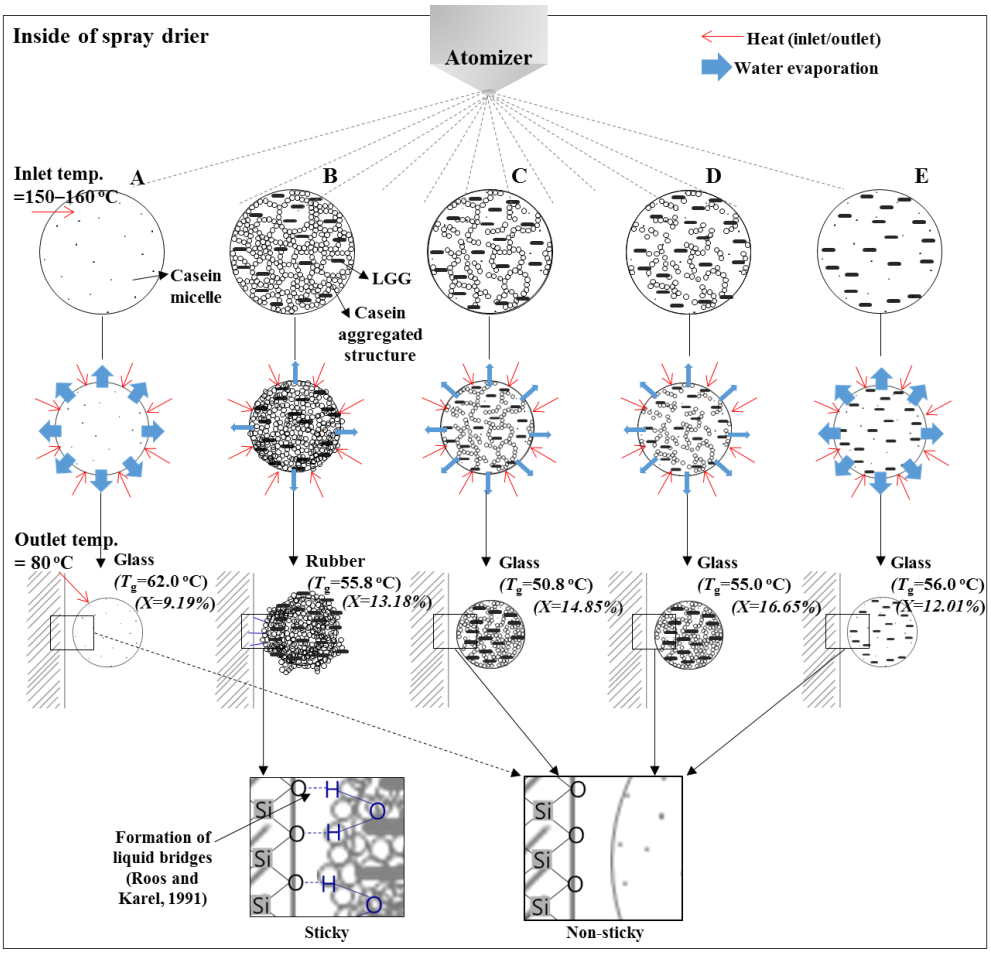


Figure 8. Proposed mechanism of stickiness phenomenon (adhesion) occurring between the droplet and wall in the spray dryer with different feeding suspensions: A=RSM, 10% (w/w) reconstituted skim milk; B=fRSM, fermented 10% (w/w) RSM; C=fRSM-pH5.2, fRSM with pH adjustment to 5.2; D=fRSM-pH6.6, fRSM with pH adjustment to 6.6; E=rRSM, 10% (w/w) RSM with LGG pellet from the MRS culture.

Conclusions

In the theoretical level of stickiness, the *k* of fRSM (0.0066 $Pa \cdot s^n$) was increased by adjusting pH, and fRSM-pH 5.2 (0.0087 $Pa \cdot s^n$) and 6.6 (0.0237 $Pa \cdot s^n$) were shown to replace the rRSM (0.019 $Pa \cdot s^n$), and the T_g of fRSM-pH 5.2 (50.8 ^oC) and 6.6 (55 ^oC) were also appear to be comparable to the rRSM (56 ^oC).

In the empirical degree of stickiness, SPT of fRSM (-5.8 ^oC) was increased by adjusting pH, but fRSM-pH 5.2 (-0.8 ^oC) and 6.6 (5 ^oC) were not appear to be comparable to the rRSM (≥14 ^oC), and the drying yield of fRSM (0%) was also increased by the pH adjustment, but fRSM-pH 5.2 (21.95%) and 6.6 (28.74%) were also not appear to be comparable to the rRSM (36.74%).

In the LGG powder quality, the microbial survival ratio of fRSM (0.59%) was significantly improved in fRSM-pH 5.2 (10.23%) and 6.6 (17.74%), and it was shown to replace the rRSM (26.30%), and the *X* of fRSM (13.18%) was increased in fRSM-pH 5.2 (14.85%) and 6.6 (16.65%) which mean the vulnerable quality as a dairy product. Although the high *X* was shown, all the LGG powders were in the glassy state after spray drying except for fRSM.

In overall, the effect of pH adjustment from pH 3.9 to 5.2 and 6.6 were not shown to replace the typical LGG encapsulation process, but, it was still found to be improved the efficiency of spray drying by lowering the stickiness via glass transition shift, and thus enabled the direct spray drying of fRSM.

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Abstract in Korean

고령화 인구 증가에 따라 정장, 정균 효과, 과민성 대장 증후군 (IBS) 완화, 성인자폐증 개선, 알러지 개선, 염증 완화, 콜레스테롤 배출 효과를 가지는 프로바이오틱의 건강기능성에 대한 관심이 높아지고 있으며, 그 시장 규모는 2016년에 350억 달러를 돌파했다. 분말 식품은 프로바이오틱을 고령화 인구에 전달하는 경제적인 방법으로, 일반적으로 de Man Rogosa Sharpe (MRS) medium에서 발효 하여 cell recovery 후 분무건조하여 생산한다. 특히 cell recovery는 4,000 rpm, 10 min에서 원심분리하여 MRS를 제거하고 LGG pellet을 회수한 후 PBS에 재분산하여 다시 같은 조건으로 얻은 LGG pellet을 새로운 30% total solid reconstituted skim milk (30% RSM)에 재분산하는 매우 복잡하고 여러 단계로 이루어져 있어서(rRSM′), 발효액을 직접 분무건조 공정을 통해 분무건조하는 것 (fRSM)보다 비효율적이고, 비경제적이고, 발효폐기물의 발생으로 인해 비환경친화적이다. 재분산액에 첨가하는 drying aid는 열풍건조로 유발되는 heat, acid, osmloar stress로부터 LGG cell protective effect를 가지는 식품소재로서, 주로 skim milk powder (SMP), whey protein isolate, maltodextrin, starch, gum arabic, gelatin을 사용하는데, 그 중 SMP는 lactose의 수산기가 LGG의 세포막 인지질 이중층과 다량의 수소결합을 이루어 물리적 방어벽 역할을 하고, casein micelle 및 whey protein이 건조농축으로 인해 낮아진 pH에 대해 pH buffer 효과를 보이며, 값이 싸고, 쉽게 구할 수 있기 때문에 많이 사용되는 drying aid이다. *Lactobacillus rhamnosus* GG (LGG, ATCC53103)는 사람의 장내에서 항염 효과 및 아토피 개선 효과가 뛰어난 종으로서, 가장 많은 연구가 된 프로바이오틱 중 하나이다. LGG 분말의 제조에 spray drying을 사용했을 때 ① LGG의 hairy pili가 분무건조를 통한 캡슐화 효율을 향상시키고, ② LGG가 유전학적으로 건조와 연관되는 stress (heat, osmolar, acid accumulation)에 대해서 다른

probiotics (*Bifidobacterium* 속, *Lactobacillus rhamnosus* E800)와 비교했을 때 heat shock protein (HSP)의 발현으로써 그 저항성이 우수하고, ③ 건조 및 분말화 속도가 빠르고, ④ 전처리 및 별도의 분말화 과정이 필요 없어서 공정이 간소하고, 속도가 빠르기 때문에 가장 많이 사용되는 캡슐화 공정으로 분무건조공정이 사용된다. 위에서 설명한 일반적인 LGG 분말 제조법에 비해서 fRSM을 직접분무건조공정을 통해 분무건조 할 때 ① 공정의 간소화, ② LGG 발효산물의 섭취, ③ 산미로 인한 이미, 이취의 masking 혹은 flavoring 효과, ④ 버리는 배양액이 거의 없어서 친환경적인 장점이 있다. 그러나, 발효액의 직접분무건조공정에는 끈적임이 발생하여 건조 수율이 낮고, 기계 막힘으로 인해 공정이 중단 될 뿐만 아니라, LGG 생존율이 낮아지므로, 공정효율성 및 분말의 품질에 악영향을 미치는 치명적인 약점이 있다. 발효액에 drying aid로 SMP를 첨가하면, 앞서 설명한 LGG cell protective effect와 더불어 유리전이온도 증가 효과로 인하여 끈적임 현상이 덜 일어난다. LGG는 10% (w/w) 탈지분유, 2% (w/w) glucose, 그리고 1% (w/w) yeast extract를 넣고 만든 재수화 탈지유에서 42 °C, 100 rpm, 9 h의 조건으로 발효되었다. 그 후, 20% (w/w) SMP가 발효액에 첨가되고 25 °C에서 30분간 완전히 분산되었다. 분무건조는 feed flow rate가 800 mL/h, feed atomization pressure가 100 kPa, 그리고 hot air flow rate가 0.65 m^3/min이며, inlet 온도가 150–160 °C, outlet 온도가 80 °C로 유지되어 수행했다. SMP를 발효액에 첨가하여 끈적임 현상이 덜 나타나는 메커니즘을 확인하기 위해 분무건조수율, LGG 생존율, shear stress-shear rate relationship을 feeding suspension에서 확인하였고, LGG powder에서는 유리전이온도, 등온흡습곡선, 그리고 미세구조를 분석하였다. SMP를 첨가하면서, fRSM의 pH가 3.9에서 5.2로 증가하면서 consistency index (k)가 0.0066 Pa·s^n에서 0.0583 Pa·s^n으로 약 9배 이상 증가하였고, 유리전이온도 (T_g)는 55.75 °C에서 62.13 °C로 증가하였다. 이는 SMP가 casein aggregated structure 사이에 끼어들어 물분자의 움직임에

제한을 둔 효과로 보이며, 그로 인해 분사된 droplet의 유리전이까지 걸리는 시간이 단축된 효과로 보인다. T_g가 증가한 효과로, 건조수율이 0% (끈적임 현상으로 인함)에서 36.1%로 증가하였고, LGG 생존율도 0.59%에서 24.71%로 증가하였다. 이는 SMP로 인해 낮아진 droplet 내의 분자이동도가 LGG까지의 heat transfer 및 LGG가 droplet의 표면에 도달하는 빈도를 낮춘 효과로 보인다. 이 효과는 rRSM′에서의 건조수율이 35.77%이고, LGG 생존율이 26.30%인 것과 비교하였을 때 기존의 공정을 대체할만한 공정효율성 및 분말의 품질을 충족시키는 것으로 판단된다. 단순히 SMP를 첨가하는 것으로, 분무건조 시 유리전이까지 걸리는 시간이 단축되어 fRSM에서의 끈적임 문제점을 해결했고, 더불어 fRSM의 직접분무건조공정이 가능하게 됨으로써, 더욱 간단하고 친환경적인 공정으로써 LGG를 캡슐화하였다. 이 연구의 내용은 (1) 분무건조에서 발생하는 끈적임 현상이 나타나는 메커니즘에 대한 정보를 제공하여 더 넓은 범위의 식품소재를 분무건조할 수 있는 점, (2) LGG를 사용한 발효액을 분무건조하는 데 발생하는 끈적임 현상의 메커니즘을 제공한 점, (3) fRSM에 SMP를 첨가하여 끈적임 현상으로 인한 문제를 해결한 메커니즘을 제공한 점에서 식품산업에 기여할 수 있다.