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NMR Relaxometry of Water in Set Yogurt During Fermentation

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Abstract The mobility of water in set yogurt during fermentation was studied using nuclear magnetic resonance (NMR) relaxometry. The spin-spin relaxation was analyzed using a 2-fraction model, resulting in 2 spin-spin relaxation time constants T_{21} and T_{22}. Both T_{21} and T_{22} exhibited rapid changes between 2 and 4 hr of fermentation, coinciding with the drop in pH and the rise in lactic acid bacteria count. The spin-lattice relaxation time T_1 increased over the fermentation period. Both T_1 and T_2 showed an increase in the mobility of water upon gel formation during fermentation. Water redistribution within the gel matrix due to casein aggregation and structure forming may be responsible for the changes in mobility.

Keywords: yogurt, fermentation, nuclear magnetic resonance, relaxometry, water mobility, protein, casein

Introduction

Set yogurt is a soft gel formed when casein micelles in milk aggregate during lactic acid fermentation. Basically, fermentation by lactic acid bacteria reduces the pH of milk towards the isoelectric point of caseins (about pH 4.6), resulting in suppression of charges on casein surfaces and, hence, reduced electrostatic repulsion between casein micelles, which eventually cause the casein micelles to form particles and then aggregate due to Van der Waals attraction (1). As the protein gel is formed, the apparent viscosity increases rapidly and then reaches a plateau as the final network forms, entrapping the fat globules and residual serum (2). The increase in the structure strength of the protein network is considered to increase the apparent viscosity.

The serum in yogurt contains mostly water. The stability of water in yogurt is important. One defect associated with water stability is syneresis, which is defined as the separation of liquid from a gel. Factors such as preheating of the milk, total solids content, pH, thickening agents, and mechanical disturbance are believed to contribute to the development of syneresis (3). These factors may affect the release of water molecules through changing the binding or association of water molecules with the gel matrix. However, no direct connection of syneresis to the states of water in yogurt has been reported in the literature.

Water is thought to exist in 3 states in yogurt; bound or structural, capillary, and free or bulk water (4). Many other terms are used in defining water molecules interacting with milk proteins (5,6), though we must recognize that these water categories are only operational terms. The structural water is the hydration water directly adhered to the casein molecules. Hydration is believed to improve the consistency of yogurt. However, hydration water in excess or insufficient would impair the consistency of yogurt by decreasing the firmness of coagulum. Capillary water is held in the spaces among the aggregated micelles. Its mobility depends on the size distribution of the capillaries. Free water refers to invisible water droplets entrapped within the protein networks. The distribution of these 3 fractions of water is likely dependent on the structure of the yogurt, which is in turn determined by the processing conditions, composition, etc (7). Yogurt shows a 3-dimensional network structure of chains and large clusters of casein micelles (8), which entraps all other constituents of the milk including the water phase within it (9). The entrapped water is very important due to its relatively large proportion in the yogurt matrix. Inappropriate mechanical treatments or degree of protein hydration could destabilize protein structure, leading to syneresis and undesirable consistency. Stabilizers or thickening agents are often used to strengthen the structure in order to improve the consistency and reduce syneresis (10). The water holding capacity of yogurt was found to be affected by polysaccharide stabilizers through their interactions with casein aggregates (11).

The objective of this study was to monitor the changes in the states of water in yogurt during fermentation using nuclear magnetic resonance (NMR) relaxometry. NMR relaxometry is commonly used to measure the mobility of water and quantify fractions of water with different distinct mobility (12,13). NMR has been used to characterize gel structure such as pore size distribution and changes as affected by temperature (14,15). Pulsed NMR has also been used to study the relaxation of water in skim milk, milk ultrafiltrate, and sodium caseinate dispersions (16). It was found that the spin-spin relaxation time T_2 showed a maximum between pH 5.0 for skim milk, decreased with decreasing pH for sodium caseinate, and changed little with pH for milk ultrafiltrate. The changes in T_2 were attributed to both the extent of casein aggregation and disassociation of micellar calcium phosphate (16).

In the present study, NMR relaxometry was used to determine spin-spin and spin-lattice relaxation time constants and corresponding amplitudes of set yogurt during fermentation. The changes in pH and bacteria colonies were also monitored.

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Materials and Methods

Materials Whole milk was mixed with 4% defatted dried milk and heated to 82°C for 30 min for pasteurization and then cooled to 40°C. A yogurt culture (Yogourmet; LYO-SAN Inc., Lachute, Canada) containing *Lactobacillus bulgaricus*, *Streptococcus thermophilus,* and *Lactobacillus acidophilus* was added to the pasteurized milk at 0.5%(w/v) level and mixed for 5 min on a magnetic stirrer. Aliquots of the prepared milk (10.0 mL) were transferred into sterile tubes sealed with stoppers in a clean bench. The tubes were then incubated at 37°C for lactic acid fermentation. Samples were taken at designated times for NMR, pH, and microbiological tests. The samples were used for the NMR tests first. After the NMR test, the NMR tube was vigorously mixed on a vortex mixer until the gel was broken. A part of the sample was transferred to a short test tube for the pH measurement. The remaining material in the NMR tube was quickly cooled down in an ice bath and used for the lactic acid bacteria enumeration analysis.

NMR analysis The NMR experiments were performed on a MARAN DRX bench top imager with a high quality permanent magnet of 21.4 MHz proton resonance frequency (Resonance Instruments Ltd., Witney, Oxon, UK). Spin-spin relaxation time (T_2) measurements were performed with the Carr Purcell Meiboom Gill (CPMG) using the echoes of 4,096 and an echo time of 0.4 msec. Inversion recovery (IR) pulse sequence was used to determine the spin-lattice relaxation time (T_1) using 12 pulse spacing times between 1 msec and 4 sec to describe all the recovery curve. For all NMR experiments, a sample relaxation delay of 5 sec ($\cong$ 5 T_1), a 90° pulse of 9 μsec, and 2 consecutive scans were used. The NMR relaxation data were fitted to a single exponential function for T_1 and a 2-exponential function for T_2 using the RI WinFit software provided by the vendor. T_1 and T_2 were collected in duplicate, and the error was always less than 2%.

pH assay The pH of the yogurt was determined with a pH meter (Model 340; Corning Inc., Corning, NY, USA). The results were expressed as the average values of 3 independent measurements.

Lactic acid bacteria enumeration The growth of lactic acid bacteria during fermentation was examined by the pour-plate colony count method (17) on plate count agar (Difco, Becton Dickinson Microbiology Systems, Sparks, MD, USA). Serial dilutions were made with sterile saline solution, and 1 mL of the dilute was placed in a petri dish and the warm plate count agar was poured and well mixed. The petri dishes were incubated at 37°C. The number of colonies was counted after 72 hr. All the enumerations were done in triplicate.

Results and Discussion

Changes in pH and lactic acid bacteria during fermentation The changes in pH and the number of lactic acid bacteria during fermentation are shown in Fig. 1. The pH decreased very slowly in the first 1 hr before it started to drop rapidly. The final pH was observed at 3.78 after 4.5 hr of fermentation. The number of lactic acid bacteria remained constant in the initial stage of the fermentation for 3 hr, then started to increase very rapidly until it reached a maximum of 2.38×10^8 CFU/mL after 7 hr and remained the same afterward. The decrease in pH preceded the increase in the number of the lactic acid bacteria by 1 hr, but the patterns matched very closely each other.

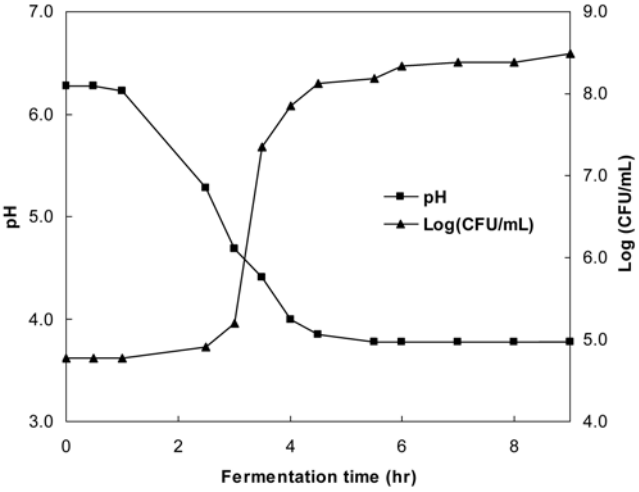

Fig. 1. Changes in pH and lactic acid bacteria counts of yogurt during fermentation.

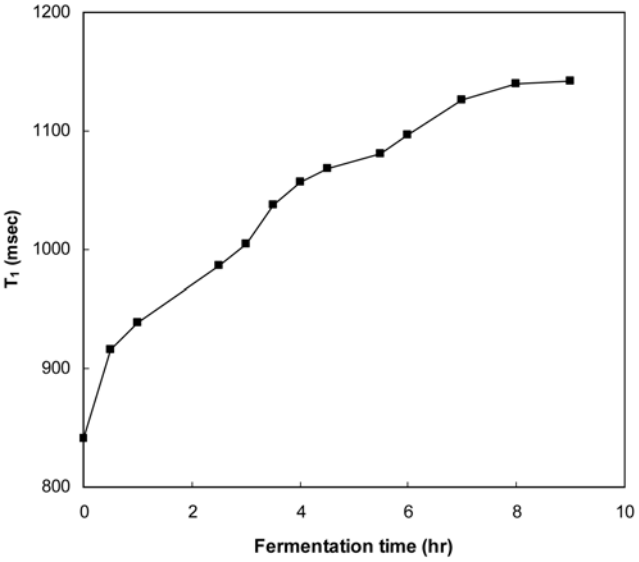

Fig. 2. Spin-lattice relaxation time as a function of fermentation time.

NMR relaxometry The T_1, which is related to the overall mobility of the yogurt matrix, showed a rapid increase in mobility, as shown in Fig. 2. The T_1 started to increase before the pH of the milk began its rapid fall. The increase in mobility as the fermentation proceeds might be related to other observations, such as increased apparent viscosity, gel strength, gel firmness, storage modulus, etc (7).

The spin-spin relaxation data obtained with the CPMG sequence were fitted into a 2-fraction model, resulting in

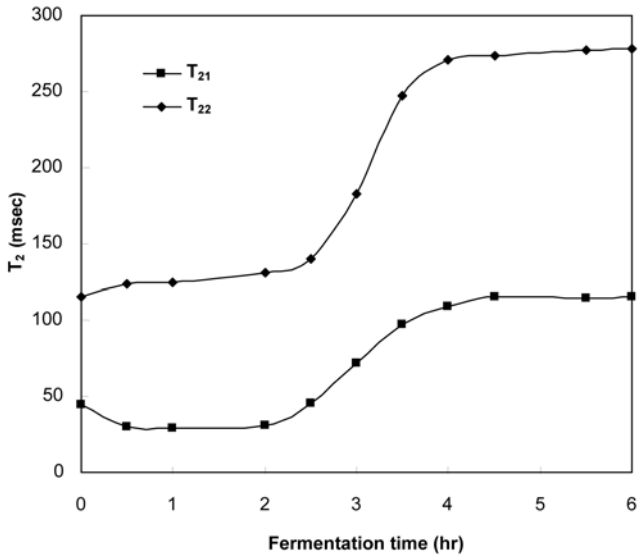

Fig. 3. Spin-spin relaxation time (T_2, CPMG, 2-fraction model) as a function of fermentation time.

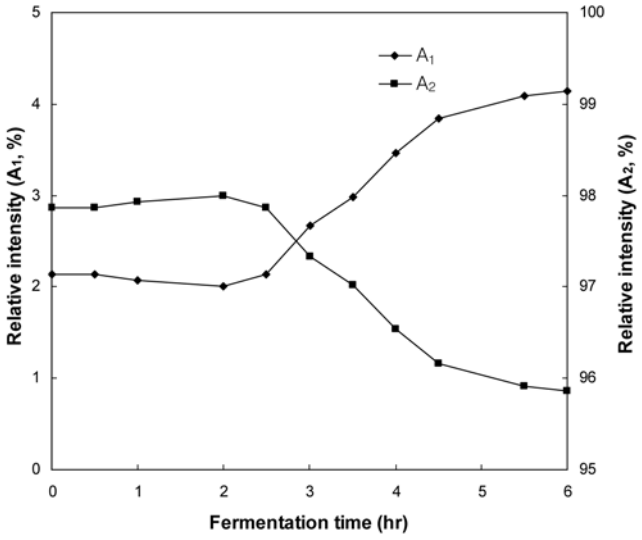

Fig. 4. Amplitudes of spin-spin relaxation (A, CPMG, 2-fraction model) as a function of fermentation time.

two T_2 values and the associated amplitudes as shown in Fig. 3 and 4, respectively. T_{21} is smaller than T_{22}, indicating that T_{21} represents protons with lower mobility than protons represented by T_{22}. This suggests that there are proton exchanges between the two fractions of water and between water molecules and other molecules such as caseins (18). Both T_{21} and T_{22} relaxation times behaved similarly, i.e., they remained relatively constant during the initial period before the pH fell appreciably. After the onset of the pH fall, the relaxation times rose rapidly up to 4 hr, followed by plateaus. This suggests that the protons became very mobile during the network forming process and stayed at a high mobility level after the network was set. This may be attributed to the characteristics of gradual casein micelle aggregation. Before the inoculation of the starting culture, water molecules are uniformly associated with casein micelles and other compounds in milk. After milk is inoculated with the culture, the colloidal calcium, which binds the casein micelles together, is leached out into the serum as the pH decreases due to the activity of the lactic acid bacteria. This may cause the casein micelles to lose some of their hydration water molecules into the serum (19), increasing the mobility of these water molecules.

A similar phenomenon was noticed in heat-induced changes in water holding capacity of protein gels (20), where a 2-stage mechanism was proposed as being responsible for the changes. In the first stage, denaturation increases the water holding capacity by exposing hydrophilic groups, while in the second stage, continued heating initiates the formation of hydrophobic groups which expel water from the protein surfaces, resulting in a reduced water holding capacity. This may be applied to acid-induced protein changes where hydrophobic interactions play an important role in casein aggregation on initial suppression of electrostatic repulsion by acidification due to fermentation (21,22).

Changes in water mobility during fermentation One interesting observation from Fig. 4 is that A_1 and A_2, which represent the quantity of the proton fractions with T_{21} and T_{22}, respectively, changed differently. In general, A_1 is much smaller than A_2. However, A_1 increased rapidly while A_2 decreased rapidly as the samples began to set. These changes suggest that there was a shift of protons from the more mobile fraction to the less mobile fraction during that rapid change period.

It has been known that casein micelles tend to disintegrate into smaller units that aggregate as a co-precipitate as the pH of milk continues to drop during fermentation (1,9). Initially, small aggregates form and eventually coagulate into a network of small chains as the casein is precipitated. Finally, the strands join up in a honeycomb-like mesh (23). The formation of the honeycomb-like mesh may have two contrasting effects, one providing more pores where free water could reside, and the other one, for example, the large casein clusters, providing a matrix structure which may hold more water whose mobility is much less than that of water in the pores but slightly greater than those in fresh milk or heated milk.

The results of this study indicated that the overall mobility of water in the honeycomb-like mesh structure is much higher than that in the heated milk. This could be an explanation for the increases in T_{21} and T_{22} as the fermentation proceeded (Fig. 3). On the other hand, the formation of the honeycomb-like network indeed increased the proportion of the strengthened structure (casein clusters) capable of trapping more water molecules, which may contribute to the slower increase in T_1 (Fig. 2) and the increase in less mobile water (A_1) (Fig. 4) during the setting process.

Acknowledgments

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