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Metabolic Profiling of Potential Probiotic or Synbiotic Cheeses by Nuclear Magnetic Resonance (NMR) Spectroscopy

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Supporting Information

ABSTRACT: To assess ripening of potential probiotic cheeses (containing either *Lactobacillus casei*-01 or *Bifidobacterium lactis* B94) or synbiotic cheeses with fructooligosaccharides (FOS) or a 50:50 mix of FOS/inulin, metabolic profiles have been obtained via classical biochemical analyses and by NMR spectroscopy. The addition of prebiotics to the cheeses resulted in lower proteolysis indices, especially in those synbiotic cheeses inoculated with *B. lactis* B94. Among synbiotic cheeses the combination of FOS and inulin resulted in an increase in lipolytic activity. The metabolic profiles of the cheeses analyzed by NMR spectroscopy, combined with multivariate statistics, allowed profiles to be distinguished by maturation time, added probiotic bacteria, or, in the case of *B. lactis* B94 cheese, added prebiotic. The NMR results are in agreement with the biochemical analyses and demonstrate the potential of NMR for the study of metabolic processes in probiotic/synbiotic food matrices.

KEYWORDS: metabolomic analysis, probiotic and synbiotic cheeses, NMR spectroscopy

INTRODUCTION

The demand for functional foods has, over recent years, increased markedly.[1] These foods, when they include probiotics and prebiotics as biologically active components, produce metabolic and physiological health benefits in addition to their nutritional properties.[2] Synbiotic products, a combination of pre- and probiotics in a given food product,[3] can provide healthy intestinal activity in a synergistic manner, with advantages over the use of either probiotics or prebiotics alone. This has driven interest and increased research into these types of novel foods.

Among different matrices studied for the delivery of probiotics to the gut, cheese has been used in several studies as a good carrier of these microorganisms, enabling their passage as viable cells through the gastrointestinal tract.[4−8] Phenomena such as glycolysis, proteolysis, and lipolysis are the main biochemical reactions that take place during cheese ripening and are responsible for the degradation of carbohydrates, proteins, and lipids present in the curdled milk matrix. These reactions occur in a controlled environment as a result of the action of different enzymes that leads to the production of a wide range of metabolic compounds that are responsible for the organoleptic characteristics of cheese during maturation.[9]

According to Wishart,[10] the metabolome is defined as the collection of molecules or small analytes that can include a wide range of chemical species from endogenous and exogenous origin, such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, and polyphenols, that can be used, ingested, or synthesized by a given cell or organism. Studying the metabolome via metabolic profiles therefore allows a snapshot of the state of the organism or substance at that particular moment to be obtained. Comparing profiles of sample groups exposed to exogenous or endogenous perturbations (disease, toxins etc.) to control groups with no perturbation can reveal differences that in many cases can be related to changes in biochemical processes resulting from that perturbation.[11]

In food science, the analysis of metabolic profiles has already been recognized as an effective tool to analyze foodstuffs and to address present and future needs in agricultural and food science.[12] Very recently the combination of NMR spectroscopy and chemometrics has been used to characterize cheeses[13,14] or fermented foods;[15] in these studies the time evolution of metabolic profiles has been monitored to assess ripening or fermentation.

In the present study, the metabolic profiles of potential probiotic or synbiotic cheeses have been analyzed by NMR spectroscopy, and it was found that they could be related to probiotic strain, type of cheese, and ripening time by the use of peak integration and multivariate statistical protocols. These results were compared with classical biochemical analyses allowing the novelty and simplicity of the NMR approach to be observed. To our knowledge, NMR spectroscopy has never been applied to study the evolution of ripening in probiotic or synbiotic cheeses.

MATERIALS AND METHODS

Cheese Manufacture. Potential probiotic cheeses, containing a single probiotic strain, *Lactobacillus casei*-01 (Chr. Hansen, Denmark) or *Bifidobacterium lactis* LAFTI B94 (Delvo_Pro DSM, Australia), and synbiotic cheeses with fructooligosaccharides (FOS) or a 50:50 mix of

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FOS/inulin (Beneo-Orafti, Belgium) were manufactured and ripened at 12 °C over a period of 60 days.

For each probiotic bacterium, 24 cheeses, each weighing between 140 and 150 g, were prepared from previously pasteurized cow's milk (at 75 °C for 10 min) and divided into 3 groups, so as to obtain probiotic, synbiotic with FOS, and synbiotic with FOS/inulin cheeses. For the probiotic cheeses, 6 mL L^{-1}_{milk} of three times diluted animal rennet (1:15000) (Naturen, Chr. Hansen), 6 mL L^{-1}_{milk} of $CaCl_2$ (Panreac), 10 g L^{-1}_{milk} of NaCl (Panreac), and 2% of *L. casei*-01 or *B. lactis* B94 were added, respectively. Each probiotic inoculum was obtained according to the method of Rodrigues et al.[16] Milk coagulation was performed at 30−32 °C for 1 h. Upon coagulation, the curd was cut, drained, and divided into three equal weight portions. One portion was distributed in perforated molds giving rise to probiotic cheeses; the other two portions were supplemented with 20 g L^{-1}_{milk} of FOS or a 50:50 mix of FOS/inulin, homogenized, and distributed in perforated molds. Upon whey draining, duplicates of probiotic cheese were withdrawn, for microbiological and chemical analyses, and labeled as 0 days. The remaining cheeses were ripened at 12 °C over a 60 day period.

Microbiological and Chemical Analyses. Microbiological analyses including counts of viable cells of probiotic bacteria and potential contamination, as well as total solids, pH, total nitrogen (TN), water-soluble nitrogen (WSN), nonprotein nitrogen (NPN), total free amino acids (FAA), and total free fatty acids (FFA), were monitored at 0, 7, 14, 30, 45, and 60 days in duplicate for each type of probiotic and synbiotic cheese according to protocols published in Rodrigues et al.[16] The samples have been coded according to probiotic strain (LCS for *L. casei*-01 and BLC for *B. lactis* B94), cheese type (P for probiotic, F for synbiotic cheese with FOS, and FI for synbiotic cheese with a 50:50 mix FOS/inulin), replica (1 or 2), and time (0, 7, 14, 30, 45, or 60 days). For example, LCS_P1_45 is for replica 1 for *L. casei*-01 probiotic cheese at 45 days of ripening.

Sample Preparation and NMR Analysis. All of the cheese samples studied were freeze-dried for 24 h before NMR analysis. Thereafter, for each sample, 20 mg was suspended in 700 μL of D_2O and subsequently agitated in a vortex for 10 min and finally centrifuged for 10 min (12000*g* rotation) at room temperature. After centrifugation, 650 μL of the supernatant was placed in 5 mm NMR tubes (Aldrich 528-PP, 5 mm). Due to contamination, no samples were available for the LCS probiotic cheese at the 7−14 day time points and at 0 days for synbiotic samples. The samples used in each figure are indicated in the Supporting Information.

All spectra were acquired on a Bruker Avance DRX 500 spectrometer operating at a proton frequency of 500.13 MHz. Sample temperature was continuously controlled throughout the experiments, at 298 K. Standard one-dimensional (1D) ^{1}H spectra were acquired using a 1D NOESY pulse sequence with a 3 s relaxation delay, t_m of 100 ms, and a fixed t_1 delay of 3 ms. Each spectrum co-added 256 scans containing 32K data points with a sweep width of 10000.00 Hz. The spectra were manually phased and baseline corrected, and the chemical shifts were referenced to an unassigned doublet at 1.166 ppm. In an attempt to minimize sample treatment, pH was not corrected and no internal reference was added. Previous experience has also shown us that in the presence of macromolecules a chemical shift reference compound such as TSP often binds to these species, which results in chemical shift deviations and line shape distortions. Therefore, referencing was carried out in this manner using a peak that appeared in all spectra and did not shift with pH. The chemical shift of this peak was obtained by reference to the lactose H1 peak (4.481 ppm).

Peak integration was carried out for peaks from protein/peptides/aromatic amino acids (7.50−6.75 ppm), the polysaccharide FOS (5.48−5.45 ppm), for lactose (5.30−5.20 ppm), for lactic acid (varies between 1.41 and 1.26 ppm), and for ethanol (1.23−1.18 ppm). Peak areas were calculated using the program AMIX 3.8.4 (Bruker BioSpin, Rheinstetten, Germany) with no normalization. For lactic acid, due to peak overlap with fatty acid signals, peaks were integrated using line shape fitting rather than peak area. Also, for some samples, replicate peak areas were found to differ. For these cases a visual inspection of the spectra was carried out. If the total spectral area for the sample was significantly higher (or lower) than the average for all spectra at this time point (indicating problems with sample extraction), the data were discarded.

NMR Data Processing and Multivariate Analysis. For principal component analysis (PCA),[17] initial data matrices were built using the full spectrum from 10 to 0 ppm but excluding water (4.85−4.73 ppm) and lactic acid (1.41−1.26 and 4.29−4.10 ppm). The large excluded regions used for lactic acid are a result of the pH variation that occurs during ripening (Table 1, vide infra). Each spectrum was divided into rectangular buckets of 0.005 ppm and normalized by adjusting the total area to unity using the program AMIX. An initial unsupervised PCA was carried out using SIMCA-P 11.5 (Umetrics). The first three principal components were calculated in this case.

Subsequently, a supervised orthogonal projection to latent structure (OPLS) analysis was carried out on the data using SIMCA-p 11.5. This type of approach enhances relevant information while decreasing unrelated noise in the data.[18] Using no class information, the OPLS analysis gave one predictive component (t[1]p) and three orthogonal components (t[2−4]o).

RESULTS AND DISCUSSION

Microbiological and Chemical Characterization. For all types of cheese, growth for both probiotic strains up to 15 days reached values of around 10^9−10^{10} cfu g^{-1} of cheese (Table 1). After this initial period, the number of viable cells for both strains remained almost constant until 60 days. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[19,20] The viability and metabolic activity of these microorganisms are key factors that must be controlled during processing operations, the maturation period, and storage of the food. According to Douglas and Sanders,[21] although it is not possible to accurately generalize about a minimum dose of probiotics that is needed for a beneficial effect in the gut, studies showing positive effects at levels below 10^8 cfu day^{-1} are not common. Considering these guidelines, both probiotic and synbiotic cheeses are revealed to be adequate food products to deliver probiotic concentrations capable of providing potential health benefits during a 60 day shelf life. Philips et al.[22] reported values in the range of 10^8−10^9 cfu g^{-1} for *Bifidobacterium* strains after 10 weeks of ripening in Cheddar cheese and slightly lower values for strains of *Lactobacillus*, including *L. casei* and *L. paracasei*.

These results indicate the potential of cheese as a vector for probiotic strains such as *L. casei*-01 (LCS) or *B. lactis* B94 (BLC), supporting work reported by Kalavrouzioti et al.[5] and Kiliç et al.[6] which indicates that cheese is a food with advantages over other dairy products due in part to its solid matrix, fat content, and buffering capacity.

The metabolic activity of the different probiotic strains in the cheeses was reflected in a decrease in pH with a concomitant increase in proteolysis and lipolysis throughout ripening. The acidification profile over the 60 day period varied with the probiotic strain and as a function of the type of cheese; a lower rate of acidification was observed in cheeses inoculated with LCS compared to cheeses inoculated with BLC. In general, the pH was lower in synbiotic cheeses than in probiotic cheeses. No significant differences were observed between synbiotic cheeses

Table 1. Variation of Viable Cells, Total Solids, pH, Water-Soluble Nitrogen (WSN), Nonprotein Nitrogen (NPN), Total Free Amino Acids (FAA), and Total Free Fatty Acids (FFA), throughout Ripening, in Each Type of Cheese[a]

cheese	ripening time (days)	viable cells (cfu g^{-1})	total solids (% w/w)	pH	WSN (% TN)	NPN (% TN)	total FAA (mg gTS^{-1})	total FFA (mg g^{-1})
			Probiotic Bacterium: *B. lactis B94*					
probiotic	0	$(1.0 \pm 0.2) \times 10^8$	33.7 ± 1.2	5.92 ± 0.01	14.5 ± 0.9	5.92 ± 0.19	2.55 ± 0.05	4.57 ± 0.01
	7	$(2.4 \pm 0.3) \times 10^9$	39.3 ± 0.7	5.15 ± 0.07	45.7 ± 5.3	22.9 ± 4.0	–[b]	–
	14	$(9.67 \pm 1.2) \times 10^9$	45.8 ± 2.3	4.73 ± 0.01	65.8 ± 3.0	46.8 ± 0.3	6.4 ± 0.6	4.74 ± 0.03
	30	$(1.0 \pm 0.2) \times 10^{10}$	66.4 ± 0.4	4.9 ± 0.1	82.8 ± 3,3	71.4 ± 6.3	7.6 ± 0.6	4.78 ± 0.01
	45	$(9.1 \pm 1.3) \times 10^9$	72.7 ± 0.9	4.8 ± 0.2	79.7 ± 2.1	47.4 ± 0.7	17.9 ± 1.3	4.81 ± 0.01
	60	$(1.1 \pm 0.3) \times 10^{10}$	79.7 ± 0.8	4.76 ± 0.01	87.5 ± 1.0	75.6 ± 2.6	17.1 ± 0.4	4.81 ± 0.01
synbiotic with FOS	0	$(1.0 \pm 0.2) \times 10^8$	33.7 ± 0.2	5.92 ± 0.01	14.5 ± 0.8	5.9 ± 0.2	2.55 ± 0.05	4.57 ± 0.01
	7	$(3.5 \pm 0.4) \times 10^9$	42.5 ± 0.2	4.86 ± 0.01	37.7 ± 2.1	10.8 ± 1.6	–	–
	14	$(6.5 \pm 0.1) \times 10^9$	50.4 ± 1.6	4.52 ± 0.02	47.9 ± 0.8	34.6 ± 0.8	8.1 ± 0.5	5.01 ± 0.04
	30	$(9.94 \pm 0.09) \times 10^9$	69.9 ± 1.7	4.30 ± 0.06	52.5 ± 0.9	43.2 ± 0.7	7.6 ± 0.4	5.20 ± 0.01
	45	$(1.1 \pm 0.3) \times 10^{10}$	73.9 ± 1.6	4.1 ± 0.1	54.1 ± 4.3	47.3 ± 4.8	13.3 ± 1.5	5.29 ± 0.01
	60	$(7.3 \pm 1.7) \times 10^9$	81.8 ± 0.6	4.15 ± 0.05	51.5 ± 4.0	44.5 ± 3.6	13.1 ± 1.5	5.32 ± 0.01
synbiotic with 50:50 FOS/inulin	0	$(1.0 \pm 0.2) \times 10^8$	33.7 ± 1.2	5.92 ± 0.01	14.5 ± 0.8	5.9 ± 0.2	2.55 ± 0.05	4.57 ± 0.01
	7	$(3.27 \pm 0.01) \times 10^9$	42.7 ± 0.3	4.76 ± 0.02	44.6 ± 2.7	17.0 ± 2.2		–
	14	$(7.05 \pm 0.07) \times 10^9$	52.3 ± 0.5	4.39 ± 0.07	44.4 ± 0.5	33.7 ± 1.5	5.2 ± 0.1	5.68 ± 0.04
	30	$(1.2 \pm 0.2) \times 10^{10}$	70.5 ± 2.6	4.28 ± 0.09	47.1 ± 4.2	40.0 ± 5.2	8.3 ± 0.8	5.87 ± 0.01
	45	$(7.9 \pm 2.4) \times 10^9$	73.4 ± 2.0	4.10 ± 0.01	58.7 ± 0.4	50.5 ± 0.61	17.8 ± 0.5	5.96 ± 0.01
	60	$(7.4 \pm 1.8) \times 10^9$	81.7 ± 0.3	4.15 ± 0.02	49.7 ± 3.7	43.2 ± 3.5	15.3 ± 0.4	5.99 ± 0.01
			Probiotic Bacterium: *L. casei*-01					
probiotic	0	$(1.01 \pm 0.02) \times 10^8$	33.5 ± 0.7	6.00 ± 0.01	10.5 ± 0.1	4.5 ± 0.1	4.14 ± 0.1	4.56 ± 0.01
	7	$(1.00 \pm 0.03) \times 10^8$	38.4 ± 1.3	5.87 ± 0.05	34.4 ± 2.5	27.6 ± 1.8	–	–
	14	$(5.94 \pm 0.09) \times 10^9$	41.3 ± 1.0	5.35 ± 0.02	60.8 ± 0.5	49.29 ± 0.05	4.0 ± 0.4	4.72 ± 0.03
	30	$(6.9 \pm 0.5) \times 10^9$	50.6 ± 0.7	4.93 ± 0.06	85.3 ± 0.6	68.8 ± 1.2	8.2 ± 0.7	4.76 ± 0.01
	45	$(8.2 \pm 1.5) \times 10^9$	62.1 ± 7.4	5.55 ± 0.08	82.0 ± 3.5	39.0 ± 5.5	23.2 ± 2.2	4.79 ± 0.01
	60	$(7.4 \pm 1.3) \times 10^9$	75.3 ± 1.6	5.10 ± 0.07	80.2 ± 1.4	63.1 ± 1.2	37.0 ± 2.2	4.79 ± 0.01
synbiotic with FOS	0	$(1.01 \pm 0.02) \times 10^8$	33.5 ± 0.7	6.00 ± 0.01	10.5 ± 0.1	4.5 ± 0.1	4.14 ± 0.1	4.56 ± 0.01
	7	$(1.2 \pm 0.1) \times 10^9$	42.1 ± 0.2	5.80 ± 0.02	19.5 ± 0.1	14.1 ± 0.7		–
	14	$(4.6 \pm 1.2) \times 10^9$	43.0 ± 1.3	5.36 ± 0.03	49.8 ± 6.9	38.0 ± 5.4	3.9 ± 0.2	4.91 ± 0.01
	30	$(6.6 \pm 0.4) \times 10^9$	50.5 ± 0.7	4.40 ± 0.01	38.5 ± 3.5	50.4 ± 3.6	7.8 ± 0.2	5.14 ± 0.01
	45	$(7.4 \pm 0.4) \times 10^9$	68.4 ± 1.3	4.31 ± 0.05	49.0 ± 5.1	40.4 ± 7.8	10.3 ± 0.7	5.26 ± 0.02
	60	$(6.9 \pm 1.4) \times 10^9$	73.2 ± 1.1	4.46 ± 0.02	58.8 ± 7.1	54.3 ± 6.8	21.4 ± 0.2	5.33 ± 0.01
synbiotic with 50:50 FOS/inulin	0	$(1.01 \pm 0.02) \times 10^8$	33.5 ± 0.7	6.00 ± 0.01	10.5 ± 0.1	4.5 ± 0.1	4.1 ± 0.1	4.56 ± 0.02
	7	$(1.19 \pm 0.08) \times 10^9$	41.1 ± 1.2	6.17 ± 0.01	26.9 ± 1.8	20.8 ± 1.2	–	–
	14	$(4.69 \pm 0.08) \times 10^9$	44.8 ± 0.1	5.43 ± 0.02	47.2 ± 2.3	33.6 ± 2.3	3.82 ± 0.02	5.00 ± 0.01
	30	$(6.1 \pm 0.2) \times 10^9$	63.6 ± 0.1	4.54 ± 0.06	55.8 ± 4.3	47.4 ± 2.6	10.8 ± 0.7	5.23 ± 0.02
	45	$(8.6 \pm 0.5) \times 10^9$	60.8 ± 0.9	4.6 ± 0.2	64.6 ± 2.0	63.5 ± 1.2	13.8 ± 0.1	5.35 ± 0.01
	60	$(5.5 \pm 0.6) \times 10^{10}$	71.4 ± 0.4	4.42 ± 0.05	66.8 ± 5.8	61.7 ± 6.0	27.4 ± 0.5	5.43 ± 0.01

[a] Values are presented as the mean ± standard deviation of two replicas. [b] –, not determined.

with the same bacteria. A substantial increase in total solids over the 60 day period was observed for all cheeses.

Proteolytic enzyme activity (via animal rennet, plasmin in milk, probiotic proteinases and peptidases, etc.) resulted in the degradation of casein into peptides that can in turn be degraded into smaller peptides and amino acids. The WSN value indicates the fraction of large water-soluble peptides, whereas the NPN value allows us to evaluate the amount of smaller peptides and free amino acids.[23,24] In probiotic cheeses, regardless of the strain inoculated, there was a large increase in WSN (68.3 or 74.8% in those cheeses inoculated with BLC or LCS, respectively) over the first 30 days of ripening (Table 1). After this period, there were no significant changes up to 60 days. According to Ong and Shah,[25] *Bifidobacterium* sp. are bacteria that are characterized by less proteolytic potential compared to *Lactobacillus* strains. For synbiotic cheeses, in general, WSN values were lower and independent of the probiotic bacteria used or the added prebiotic. After 60 days of ripening, mean increases in WSN of 36.1 and 52.3% were observed in synbiotic cheeses inoculated with BLC or LCS, respectively.

The evolution of NPN in the different cheeses did not differ substantially from the trends seen for WSN. In the first half of the maturation period there was a significant increase in NPN that was more pronounced for the probiotic cheeses (64.2%) compared to the synbiotic cheeses (45.7%). The second part of the maturation period was characterized by very small changes. These NPN and WSN values indicate that extensive proteolysis has occurred and is comparable to values seen in curdled milk matrices.[16] The addition of prebiotics FOS or FOS/inulin to the cheeses resulted in lower WSN and NPN values. This could be related to the metabolic pathways used by probiotic bacteria. Probiotic bacteria are able to metabolize prebiotics such as FOS and inulin,[26] preferring these compounds to proteic compounds to obtain energy. This results in a lower rate of proteolysis for synbiotic cheeses.

The evolution of total free amino acids, in all types of cheese, was more pronounced for the second period of maturation

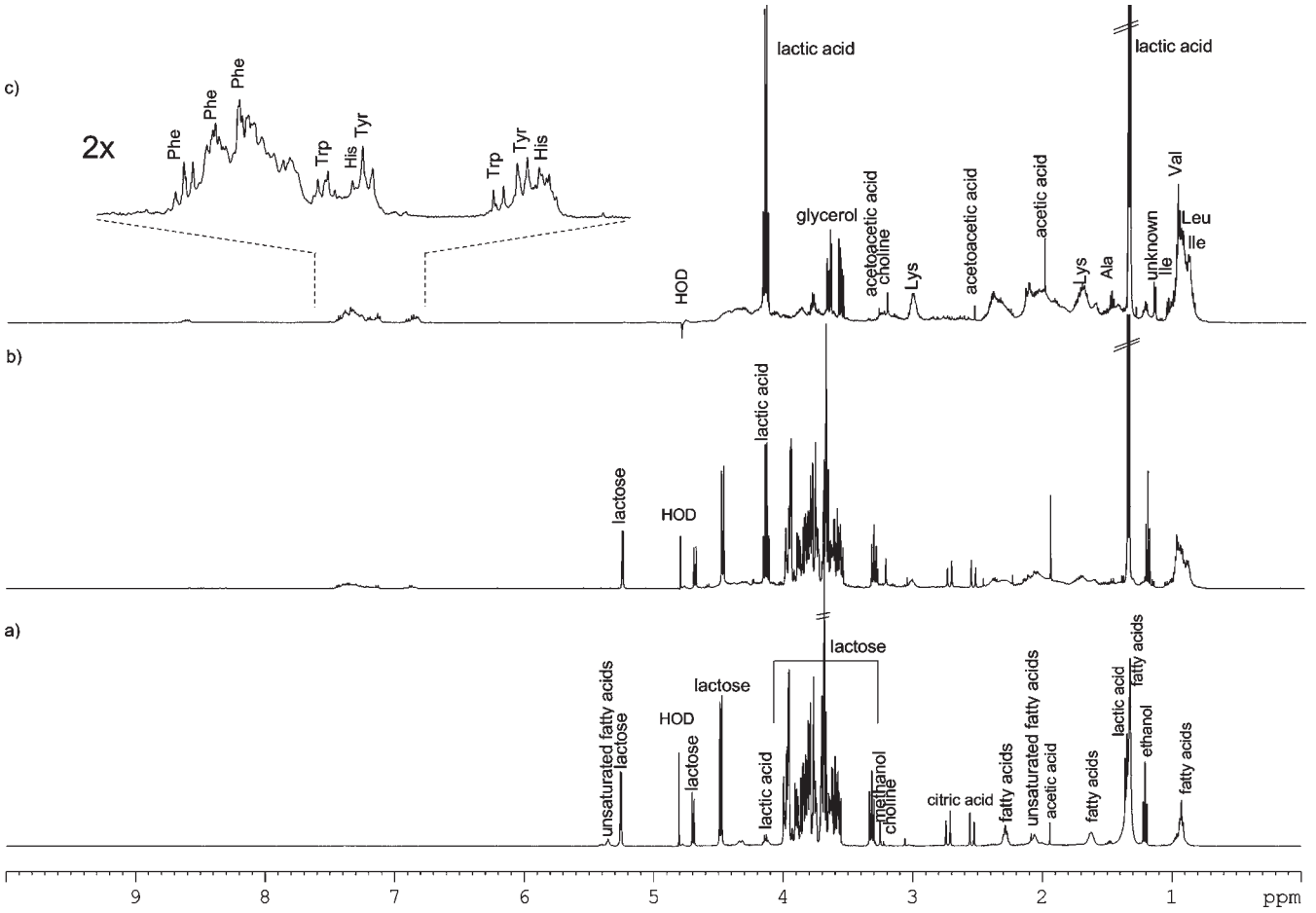

Figure 1. Complete ^{1}H NMR spectrum for the BLC probiotic cheese extract at (a) 0, (b) 7, and (c) 60 days. Assignments for the major peaks are indicated. For the spectrum at 60 days, the broad resonances that appear between 7.50 and 6.75 ppm and around 4.1, 2.4, 2.0, and 0.9 ppm are from soluble protein/polypeptide. Chemical shift values are indicated in the Supporting Information.

(30−60 days of ripening). This trend may be correlated with the fact that as there is a higher rate of primary proteolysis in the first period of maturation, resulting in an increase in the amount of peptides; therefore, the action of aminopeptidases or exopeptidases of microbial origin is favored in the second period of maturation. Higher values for total amino acids were recorded in probiotic and synbiotic cheeses inoculated with LCS compared to BLC. According to Requena et al.,[27] the high activity of aminopeptidases is a characteristic of *L. casei* and is probably responsible for the higher levels of free amino acids in cheeses inoculated with this bacterium.

The levels of total FFA slightly increased over the 60 day ripening period in all types of cheese; however, the total increase was higher for synbiotic cheeses than for probiotic cheeses. This was independent of the probiotic bacteria used. Among synbiotic cheeses the combination of FOS and inulin resulted in an increase in lipolytic activity and is responsible for higher triglyceride degradation seen here compared to synbiotic cheeses with FOS. With regard to the differences in the total FFA content resulting from the action of lipolytic enzymes from BLC or LCS, there were no significant differences. However, it is noteworthy that FFA values of 5.4 and 6.0 mg g^{-1} of cheese were obtained after 60 days in synbiotic cheeses with FOS and inulin inoculated with LCS or BLC, respectively.

NMR Assignment. The complete ^{1}H NMR spectra of BLC probiotic cheese at 0, 7, and 60 days of ripening are shown in Figure 1. By comparison of the three spectra, it can be seen that significant changes occur during maturation, resulting in markedly different metabolic profiles. These variations are not unexpected and have been discussed previously and, for instance, result, at later time points, in the loss of resonances for lactose, fatty acids, and ethanol and the appearance of resonances for free amino acids (sharper peaks superimposed on broader peaks), proteins/polypeptides (broader signals), lactic acid, and glycerol. These variations were confirmed for LCS probiotic cheeses as well for synbiotic LCS and BLC cheeses (vide infra).

It should be noted that in this study we attempted to minimize sample treatment; therefore, the pH of each sample was not corrected. By observing Table 1, however, we can see that the pH varies during ripening. The effect on the NMR spectra is apparent, and large shifts are seen for metabolites such as lactic acid, citric acid, and acetic acid. These shifts do not impede chemical shift assignment; however, they do affect the way the spectra are pretreated for subsequent multivariate statistical analysis (vide infra).

To obtain a more complete assignment of the metabolic profiles for these probotic and synbiotic cheeses, a database of standard compounds, previous work, and biochemical analyses were combined to assign a number of peaks.[28−30] As all spectra obtained from each cheese type (LCS, BLC, probiotic, or synbiotic cheeses) showed similar variations, these assignments were transferrable and are shown in Figure 1 and in the Supporting Information.

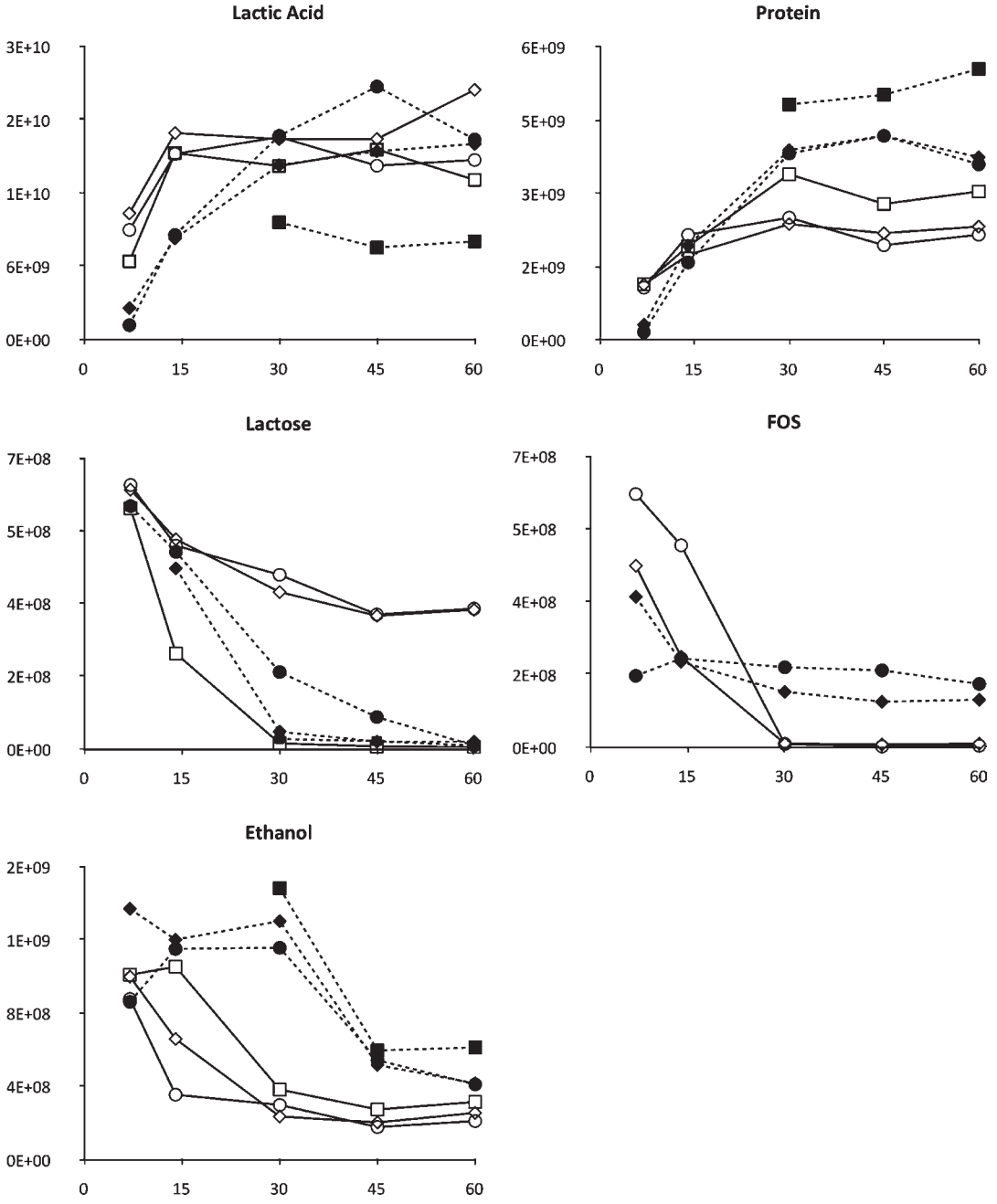

Figure 2. Peak areas (arbitrary units) for selected metabolite resonances over the 60 day ripening period for probiotic and synbiotic cheeses. Dashed lines and solid symbols indicate LCS cheeses, whereas continuous lines and open symbols indicate BLC. The squares are probiotic cheese, the diamonds are synbiotic cheese with added FOS/inulin, and the circles are synbiotic cheese with added FOS.

NMR spectra for cheeses at early ripening times were characterized by higher amounts of lactose and ethanol as well as by the presence of some acids, such as lactic, acetic, and citric acid, resulting from fermentation via probiotic bacteria cells. Other components related to milk composition such as fatty acids were also identified. The added FOS or FOS/inulin in synbiotic cheese was identified by comparison with spectra from standard compounds (Supporting Information). The presence of fatty acid peaks (at ca. 1.2, 1.5, and 2.2 ppm and a peak at 5.5 ppm from unsaturated fatty acids) at 0 days is not wholly unexpected as the concentration of fats in cheese is very high, and therefore even though longer chain FFA are fairly insoluble in water, they appear in the NMR spectrum. FFA were also seen in Reggiano cheese by aqueous extract (using no buffer) by Consonni et al.[13] From Figure 1 it can be seen that the fatty acids signals disappear before 7 days and are absent at 60 days. The biochemical analyses indicate that FFA do not vary greatly over the 60 day ripening period; therefore, the disappearance of fatty acids from the NMR spectra are most probably a result of the drop in pH that occurs during ripening, reducing the solubility of the FFA.

Cheeses with 60 days of ripening were characterized by lower amounts of lactose and organic acids except lactic acid (Figure 1c), both resulting from fermentation occurring via the viable probiotic bacteria cells. The amount of soluble protein/peptides (broad

resonances at 7.50−6.75 ppm and around 4.1, 2.4, 2.0, 0.9 ppm) and free amino acids (sharp weak signals superimposed on broader protein signals) also increased in ripened cheeses. Similar results have been reported by Choi et al.[15] in a fermented food.

These changes were quantified by integrating selected peaks (Figure 2), which allows us to confirm some results from the biochemical analyses. We found that lactose decreases during ripening, although BLC synbiotic cheeses have higher levels compared to the other cheeses after 14 days. Lactic acid was found to increase during ripening, with BLC cheeses having higher initial amounts that level off at 14 days, whereas LCS cheeses show a slower increase in lactic acid, although they have 30−60 day levels similar to those of BLC cheeses. LCS probiotic cheese was found to have a lower final amount of lactic acid when compared to the other cheeses. Ethanol was found to decrease over time, with LCS cheeses having consistently higher levels over the 15−60 day ripening period. With regard to proteins in solution, due to the fact that the resonances found in the 7.50−6.75 ppm region of the spectra result from NH and aromatic groups from amino acids, peptides, or proteins, the peak areas measured here are the sum of these species present at any one time point, although proteins can normally be distinguished from small peptides and amino acids due to their much greater line width (sharp lines arise from smaller species such as amino acids and peptides). Figure 2 shows that our measured "protein" levels show a larger variation, and higher final amounts, for synbiotic and probiotic LCS cheeses compared to BLC cheeses, with BLC cheeses having slightly higher initial levels compared to LCS cheeses. Synbiotic cheeses could also be distinguished from probiotic cheeses, with synbiotics having consistently lower protein levels than probiotic cheeses from 30 days onward. Finally, for the synbiotic cheeses, the level of FOS varies to a much greater extent in BLC cheeses compared to LCS cheeses, and the 30−60 day levels in BLC cheeses are lower than that for LCS cheeses.

In general, these results confirm our biochemical analyses in that the pH falls during ripening (with a lower rate observed for LCS compared to BLC, due to production of lactic acid), and the BLC cheeses have less proteolytic potential than LCS (lower final levels of soluble protein), and the addition of FOS or FOS/inulin results in a further reduction in proteolysis due to the probiotics using these polysaccharides as an alternative source of energy (circles and diamonds lower than squares for BLC and LCS cheeses). These results also suggest that we can use peak areas in the 7.50−6.75 ppm region of the NMR spectrum to follow proteolytic activity in these cheeses.

Principal Component Analysis. To analyze the NMR metabolic profiles further, and to identity any underlying variability that could be related to metabolic differences in probiotic or synbiotic cheeses, multivariate analyses were employed. The full ^{1}H NMR spectra obtained for all of the cheese samples were used to obtain a data matrix (see Materials and Methods); an initial PCA of all profiles identified probiotic samples at 0 days as outliers, and visual inspection of the spectra confirmed this. The subsequent PCA scores plot for all 7−60 day samples (Supporting Information) was difficult to interpret without performing a rotation operation on the data. We therefore ran an OPLS analysis of the 7−60 day data, which resulted in one predictive and three orthogonal components. The predictive and first orthogonal components are shown in a scores plot in Figure 3a, which has a Q^2 of 0.89. OPLS facilitates interpretation of the model produced from the data, and metabolic profiles could be distinguished by bacterial strain, by maturation time, and by probiotic/synbiotic cheese in the case of BLC. Ripening dominates the profiles as seen by the vertical trend from small to large symbols in Figure 3a. Observation of

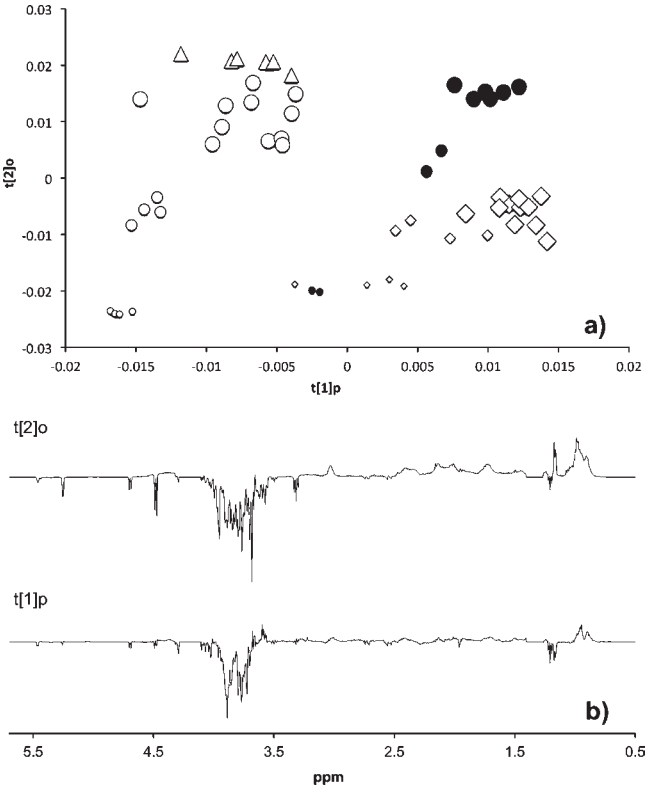

Figure 3. (a) OPLS scores plot for the predictive and first orthogonal component (Q^2 of 0.89) and (b) loadings plot (6−0 ppm) for t[1]p (17.3%) and t[2]o (64.4%) for all samples at 7−60 days. Ripening time is indicated by symbol size: small (7 days), medium (14 days), and large (30−60 days). LCS synbiotic samples are open cicles, LCS probiotic samples are open triangles, BLC synbiotic samples open diamonds, and BLC probiotic samples are solid circles.

the loadings for t[2]o (Figure 3b) confirms that protein proteolysis increases relatively during ripening (broad peaks at 7.5−6.8, 2.5−1.5, and 1.1−0.8 ppm) and also indicates that lactose, ethanol, citric acid, and FOS/inulin (for synbiotics) decrease with ripening for all cheeses. For BLC cheese it can be seen that synbiotic cheese has a lower level of proteolysis relative to probotic cheese. For LCS cheese the lack of data points precludes any definite conclusions about proteolysis, although the fact that the 30−60 day profiles are similar for probiotic and synbiotic cheeses may suggest that proteolysis rates are similar in LCS cheese. The t[1]p loadings (Figure 3b), which distinguish, to some extent, bacterial type, suggest that species with resonances at around 3.6−3.8 ppm appear to be found in higher amounts in LCS relative to BLC cheeses. To a lesser extent, ethanol and citric acid are also seen as having slightly higher levels in LCS relative to BLC cheese.

The NMR results from the multivariate analysis are in agreement with the biochemical data collected for the same samples, with the analysis of the metabolic profiles by NMR confirming that proteolytic activity is greatest up to 30 days (30−60 day samples cluster together) and that, in the case of BLC cheese, probiotic samples have higher proteolytic rates compared to synbiotic samples.

In conclusion we have shown that metabolic profiling by NMR using a simple aqueous extraction procedure and no postextraction manipulation (adjusting the pH, for example) combined with peak integration and multivariate statistical analysis allows profiles to be distinguished in terms of maturation time, added probiotic bacteria, and added prebiotic (FOS or FOS/inulin). These NMR results are

in full agreement with the biochemical analyses carried out on the same samples and demonstrate the potential of NMR for the study of metabolic processes in probiotic/synbiotic food matrices.

■ ASSOCIATED CONTENT

Supporting Information. Scores plot from an unsupervised PCA analysis of the data (all cheeses 7−60 days) and ^{1}H NMR spectra for the polysaccharides FOS, inulin, and a mixture of FOS/inulin as well as tables containing information on the samples used in our experiments and chemical shift data. This material is available free of charge via the Internet at http://pubs.acs.org.

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