JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Hydrolyzed Caseinomacropeptide Conjugated Galactooligosaccharides Support the Growth and Enhance the Bile Tolerance in *Lactobacillus* Strains

Arunachalam Muthaiyan,[†] Oswaldo Hernandez-Hernandez,[‡] F. Javier Moreno,[§] Maria Luz Sanz,[‡] and Steven C. Ricke*,[†]

[†]Center for Food Safety—Department of Food Science, University of Arkansas, Fayetteville, Arkansas 72704, United States

[‡]Instituto de Química Orgánica General (CSIC), 28006 Madrid, Spain

[§]Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), 28049 Madrid, Spain

**ABSTRACT:** In this study bioactive caseinomacropeptide was conjugated with prebiotic galactooligosaccharides (hCMP:GOS) by Maillard reaction to synthesize value added prebiotic compounds to *Lactobacillus* strains. Growth study showed the ability of hCMP:GOS to serve as a sole carbon source for *Lactobacillus* strains. A significant amount of acetate and lactate was detected in cell free culture supernatant by HPLC. It demonstrated the ability of *Lactobacillus* strains to ferment the hCMP:GOS as a carbon source. In addition, hCMP:GOS grown *Lactobacillus* cells exhibited enhanced bile tolerance and retained 90% viability. Overall results of this study indicate that the hCMP conjugated GOS can be potential multipurpose prebiotic substrates to enhance the growth and bile tolerance in *Lactobacillus* strains and serve as a fermentable substrate to produce beneficial metabolites in the host.

**KEYWORDS:** *prebiotic, probiotic, Lactobacillus, galactooligosaccharides*

## INTRODUCTION

In recent years products claiming to be healthier and to have functional and health properties have gained importance in industrial and academic research and have become specialty foods in public supermarkets.[1,2] Indeed, global sales of probiotic ingredients, supplements, and foods amounted to $21.6 billion in 2010 and have been estimated to reach $31.1 billion by 2015.[3]

Despite the availability of a range of commercial functional food ingredients, additional research and development efforts are still needed to enhance the efficacy of existing functional food ingredients. One of the promising research areas in functional food and nutraceutical products is the development of synbiotics (combination of probiotics and prebiotics).[4−6] The metabolic activity of probiotic strains and their effect on human health depends upon the strain specific properties and the dose administered. However, the availability and type of prebiotic compounds in the gastrointestinal tract play a significant role in determining the viability and beneficial metabolic activity of the probiotic strains.[7,8] Prebiotics are substances that selectively improve the activity, survival, and colonization of probiotics by providing a fermentable carbon source particularly in the large intestine. A variety of prebiotics including $\beta$-glucans, inulin, fructo-oligosaccharides, galacto-oligosaccharides, and xylo-oligosaccharides have been used to improve the growth and metabolic activity of probiotic *Lactobacillus* and *Bifidobacterium* spp.[1]

Similarly, biologically active peptides have interesting applications as supplements in food and pharmaceutical preparations.[9−11] The field of bioactive peptides has intensified during the past two decades, and numerous peptides of plant and animal origin with relevant bioactive potential have been discovered.[11] Caseinomacropeptides (CMP) obtained by enzymatic hydrolysis of $\kappa$-casein during the manufacture of cheese have been receiving more interest for the supplementation of functional foods. CMP and its hydrolyzed fragments have been shown to have various biological activities as physiological regulators, and modulating secretion of intestinal hormones.[11−15] In addition, CMP have been correlated with a wide variety of biological activities including immunosuppression capacity, inhibition of pathogen invasion,[16−18] and nutritional management of metabolic diseases.[19,20] Exploiting the potential of bioactive peptides is an important task in the discovery of novel functional foods with enhanced bioavailability through the addition and fortification of enriched bioactive peptides with other health promoting compounds. Conjugation of hydrolysates of CMP with prebiotic galactooligosaccharides by glycation could be one of the product development methods to make value added prebiotics for human nutrition and could be a potential novel ingredient for functional foods.

In the present study we used the Maillard reaction to prepare glycoconjugates of hydrolyzed caseinomacropeptide (hCMP) with lactose- and lactulose-derived galactooligosaccharides (GOS-La and GOS-Lu) and demonstrated their potential use to support the growth of six *Lactobacillus* strains. Acetate and lactate produced by the *Lactobacillus* strains were quantified to confirm the fermentability of hCMP-conjugated GOS (hCMP:GOS). In addition, the protective role of

**Received:** April 7, 2012
**Revised:** June 6, 2012
**Accepted:** June 11, 2012
**Published:** June 11, 2012





dx.doi.org/10.1021/jf301392y | *J. Agric. Food Chem.* 2012, 60, 6839−6845

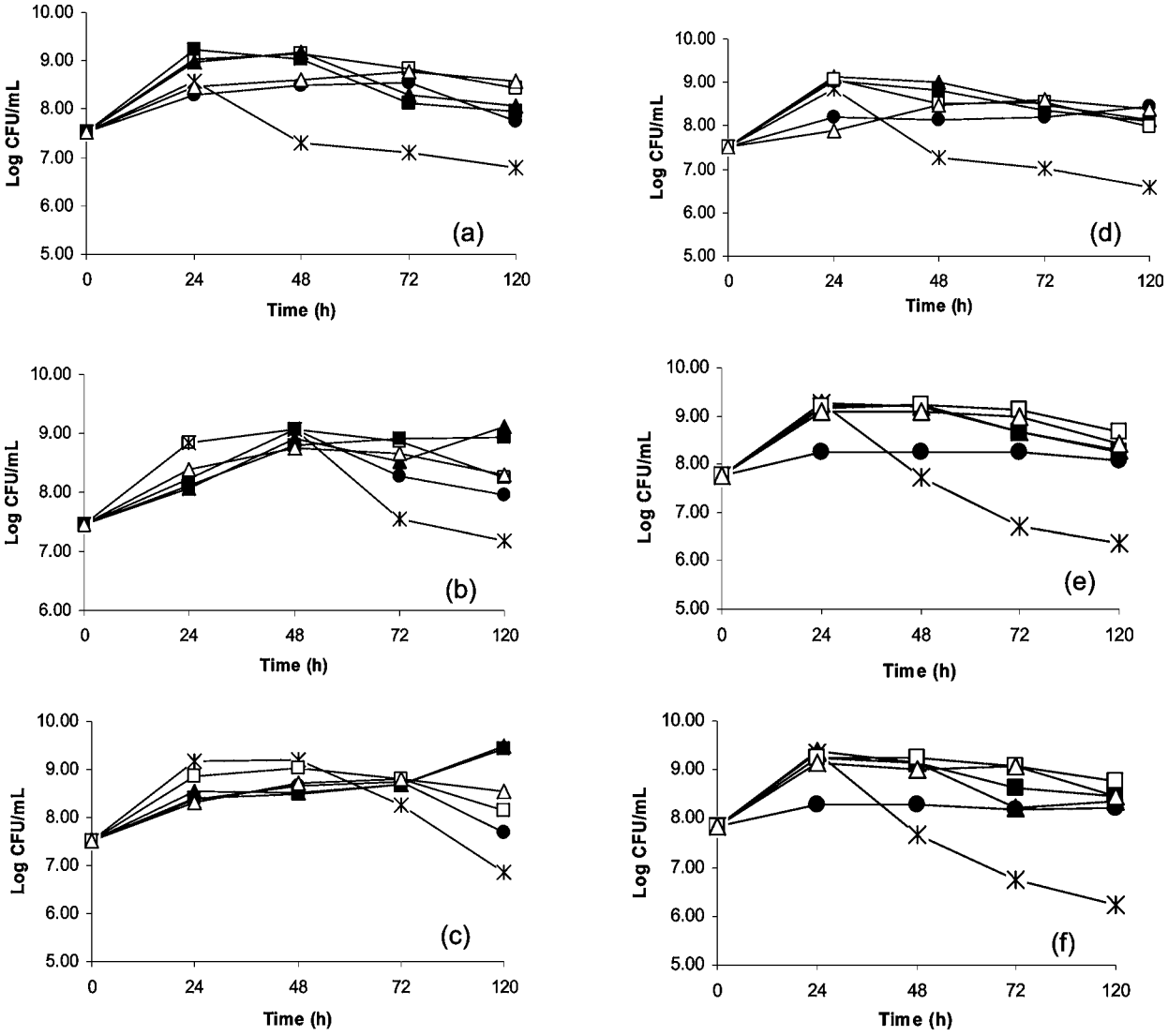


**Figure 1.** Growth of *Lactobacillus bulgaricus* ATCC 7517 (a), *L. casei* ATCC11578 (b), *L. delbrueckii* subsp. *lactis* ATCC4797 (c), *L. plantarum* ATCC8014 (d), *L. plantarum* WCFS1 (e), *L. sakei* 23K (f) in MRS broth containing glucose (×), GOS-La (▲); GOS-Lu (■); hCMP (●); hCMP:GOS-La (Δ); hCMP:GOS-Lu (□). Colony forming units per mL (CFU/mL) was calculated as an average from data obtained from three independent studies.

hCMP:GOS-La and hCMP:GOS-Lu toward the *Lactobacillus* cells in the bile extract was investigated.

## ■ MATERIALS AND METHODS

**Galactooligosaccharides and Glycoconjugates.** Lactose derived commercial galactooligosaccharide (GOS-La) Vivinal-GOS was a kind gift from Friesland Foods Domo, Zwolle, The Netherlands. Commercial prebiotic lactulose Duphalac (Solvay Pharma, Brussels, Belgium) was used to synthesize the lactulose derived galactooligosaccharides (GOS-Lu) using the method of Clemente et al.[21] GOS-La and GOS-Lu were purified by size exclusion chromatography by the method of Hernandez et al.[22] Caseinomacropeptide (CMP) was kindly provided by Davisco Foods International, Inc., Eden Prairie, MN, USA, and hydrolyzed by the method of Hernandez-Hernandez et al.[23] The glycoconjugates were prepared using the Maillard reaction by mixing aliquots of hCMP with GOS-La or GOS-Lu (2:1 w/w) in 0.1 M sodium phosphate buffer (pH 7.0). Subsequently, the mixture was lyophilized and stored in a vacuum desiccator at 40 °C for 16 days and the water activity was maintained at 0.44 with a saturated $K_2CO_3$ solution to increase the maximum formation of Amadori/Heyns compounds.[24] To confirm the formation of Amadori/Heyns compounds during the Maillard reaction, formation of 2-furoylmethyl-amino acids (2-FM-AA) were determined by the method of Moreno et al.[25] The unconjugated carbohydrate fraction was removed by ultrafiltration at 4 °C using a diafiltration unit model 8400 (Millipore) with a molecular cutoff of 1 kDa, and then the retentate was analyzed by ESI-MS to confirm the purity of glycoconjugates (unconjugated carbohydrate-free glycoconjugates).[24]

**Bacterial Strains and Media.** *Lactobacillus bulgaricus* ATCC 7517 (LB), *L. casei* ATCC11578 (LC), *L. delbrueckii* subsp. *lactis* ATCC4797 (LD), *L. plantarum* ATCC8014 (LP1), *L. plantarum* WCFS1 (LP2), and *L. sakei* 23K (LS) strains were grown in Lactobacilli MRS broth or on Lactobacilli MRS agar (EMD Chemicals, Gibbstown, NJ, USA) at 37 °C in an anaerobic chamber (10% $CO_2$:5% $H_2$:85% $N_2$) (Coy Laboratory Products, Ann Arbor, MI, USA). For the carbon source preference study, modified defined MRS medium (MRS without carbohydrate) containing 1% protease peptone, 1% beef extract, 0.5% yeast extract, 0.1% Tween-80, 0.2% ammonium citrate, 0.5% sodium acetate, 0.01% magnesium sulfate, 0.005% manganese sulfate, 0.2% dipotassium phosphate, and 0.05% cysteine-HCl was used.[26] To prepare stock solutions of GOS, hydrolyzed hCMP, hCMP:GOS-La, and hCMP:GOS-Lu (glycoconjugates), they were dissolved in sterile distilled water, filter sterilized

through a 0.2 $\mu$ filter (VWR International, Sugar Land, TX, USA), and stored at 4 °C until used.

**Growth Study.** Growth studies were carried out as described by Hernandez-Hernandez et al.[27] Briefly, filter sterilized GOS, hCMP, and glycoconjugate solutions were separately added as a sole carbon source in the defined MRS broth to a final concentration of 1%. Inocula were prepared from 48 h MRS grown *Lactobacillus* cells, and approximately 1 × $10^7$ CFU/mL of each *Lactobacillus* strain (individually) was added to the medium and incubated at 37 °C in anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, USA). MRS media with glucose, unconjugated GOS-La, GOS-Lu, and hCMP were used as controls. Growth and viability of each strain in each test carbon source were monitored at regular intervals by serial dilution and plating on MRS agar plates.

**Bile Tolerance Study.** Bile tolerance of *Lactobacillus* cells grown in GOS, hCMP, and glycoconjugates as carbon sources was measured by survival studies as described by Nira et al.[28,29] Briefly, bacterial cells grown in glycoconjugates for 48 h were collected and washed twice with 1× PBS, and the cell pellets were subsequently mixed with 0.3% (w/v) porcine bile extract (Sigma Chemicals, St. Louis, MO, USA). The treatment mixtures were incubated for 3 h at 37 °C in an anaerobic chamber (10% $CO_2$:5% $H_2$:85% $N_2$) (Coy Laboratory Products, Ann Arbor, MI, USA). Samples were taken at 0, 1, and 3 h and plated onto MRS agar for viable colony count.

**Acetate and Lactate Analysis.** The concentration of acetate and lactate in the cell free culture supernatant (1.5 mL) collected at 24, 48, and 72 h was determined by the method of Sanz et al.[30] using an HPLC system with a UV detector (210 nm) (Agilent Technologies 1200 series, USA). The organic acid resin column HPX-87H HPLC (BioRad, Hercules, CA, USA) was operated with a flow rate of 0.6 mL/min of 0.005 mM $H_2SO_4$ as a mobile phase at 50 °C in isocratic mode.

**Data Analysis.** Data obtained from three independent biological experiments each performed in triplicate were used for statistical analysis. Statistical analyses were performed using Statistica for Windows version 6 (Statsoft Inc., Tulsa, OK, USA). Differences between bacterial survival, acetate, and lactate concentrations were tested using one-way ANOVA test, followed by a least significant difference (LSD) test as a post hoc comparison of means ($P < 0.05$).

## RESULTS AND DISCUSSION

**Growth of *Lactobacillus* Strains in Glycoconjugates.** In this study we investigated the potential of hCMP:GOS to support the growth of *Lactobacillus*. We used six *Lactobacillus* strains, namely, *L. bulgaricus* ATCC 7517, *L. casei* ATCC11578, *L. delbrueckii* subsp. *lactis* ATCC4797, *L. plantarum* ATCC8014, *L. plantarum* WCFS1, and *L. sakei* 23K. In the growth study variable growth patterns were observed for each bacterial strain and the carbon source used in the media (Figure 1). Overall all six *Lactobacillus* strains exhibited significant growth in GOS-La and GOS-Lu, and their glycoconjugates (hCMP:GOS-La and hCMP:GOS-Lu). Unlike GOS or glycoconjugates, cells grown in hCMP as the sole carbon source did not exhibit an increase in the number of viable cells. Strains LB, LC, and LD showed better growth in all test carbon sources and did not exhibit any differences in the growth pattern among the carbon sources (Figure 1a−c). Strains LP1, LP2, and LS exhibited better growth in GOS-La, GOS-Lu, and glycoconjugates for up to 120 h, whereas the hCMP did not support the growth after 24 h of incubation (Figure 1d−f). Strains LP2 and LS were able to utilize hCMP:GOS-La and hCMP:GOS-Lu similar to unconjugated prebiotic GOS and were able to maintain their viability up to 120 h (Figure 1e,f). Similar to our results previous studies have reported considerable differences in carbohydrate utilization abilities and kinetics by different probiotic strains.[27,31−35] In a recent study, Cardelle-Cobas et al.[36] evaluated the growth of *Streptococcus salivarius*, *Bifidobacterium breve*, *Lactobacillus reuteri*, *L. fermentum*, *L. plantarum* strains CLB7 and CLC17, *L. salivarius*, *L. animalis*, and *L. murinus* in purified fractions of trisaccharides synthesized from lactose and lactulose. In their study they found probiotic strains were able to utilize the purified GOS, however, certain trends in the GOS utilization pattern were observed for each bacterial strain. Interestingly, in our study hCMP conjugated GOS supported the growth of LB, LP2, and LS for longer time periods of up to 120 h of incubation and exhibited a significant increase in the number of viable CFUs. Since many chronic gut disorders such as colon cancer and ulcerative colitis originate in the distal colon and progress toward the proximal region, we speculate that the observed prolonged utilization of glycoconjugates by the *Lactobacillus* strains in our study could be an advantageous property to extend the prebiotic effect more effectively throughout the colon. Consequently, significant health benefits of prebiotics could be achieved if the availability of prebiotic substrates and prolonged saccharolytic fermentation are extended to the distal region of the colon.[5]

**Bile Tolerance of Glycoconjugate Grown *Lactobacillus* Cells.** The bile tolerance and relative survival patterns of all six *Lactobacillus* strains are shown in Table 1. In a bile extract treatment *L. bulgaricus* (LB) cells grown in hCMP:GOS exhibited better bile tolerance than the unconjugated GOS grown cells. After 1 h of treatment hCMP:GOS-La and hCMP:GOS-Lu grown cells remained 100 and 93% viable, respectively, whereas unconjugated GOS-La and GOS-Lu grown LB cells elicited decreased viability of 87 and 78%, respectively. After 3 h of bile treatment hCMP:GOS-La and hCMP:GOS-Lu grown LB cells retained 91 and 67% viable cells (Table 1). Glycoconjugate grown *L. casei* cells elicited better viability after 1 h of bile treatment. However, after 3 h of treatment the number of viable LC cells was decreased. When compared to glycoconjugate grown LC cells, viability of unconjugated GOS-La and GOS-Lu grown cells decreased to less than 50% at both 1 and 3 h of treatment (Table 1). *L. delbrueckii* (LD) cells grown in hCMP:GOS-Lu were 67% viable after 1 h exposure to bile. hCMP:GOS-La and GOS grown LD cells retained less than 50% of viable cells after 1 and 3 h of bile treatment. hCMP grown LD cells were only 23% viable after 1 h of bile treatment and after 3 h reached nondetectable levels (Table 1). For strain *L. plantarum* ATCC8014 (LP1), unconjugated GOS-La and GOS-Lu grown cells showed better viability in 1 and 3 h of bile treatment than the hCMP conjugated GOS. Interestingly, strain *L. plantarum* WCFS1 (LP2) and *L. sakei* 23K (LS) cells grown in all the test substrates produced significant numbers of viable cells even after 3 h of bile treatment (Table 1).

The ability to withstand in bile is one of the essential and preferred characteristics for probiotics to use in the food applications. Once bacteria reach the duodenal section of the intestinal tract, they are exposed to deleterious bile compounds and subsequently their viability is reduced.[37,38] Results of our bile tolerance study revealed that the tolerance and survival of the *Lactobacillus* strains in the presence of bile extract was significantly changed based on the carbon source used for their growth. Interestingly, we observed enhanced bile tolerance in glycoconjugate grown LB, LP2, and LS cells than their unconjugated GOS grown cells. The toxic effects of bile toward probiotic bacteria could be alleviated partly by adding specific prebiotic substrates that can be metabolized by probiotic strains. In an earlier study, altered bile tolerance in

**Table 1. Survival of Glucose, Oligosaccharides (GOS-Lu and GOS-La), Hydrolyzed Caseinomacropeptide (hCMP), and Hydrolyzed CMP:Oligosaccharide Glycoconjugate (hCMP:GOS-Lu and hCMP:GOS-La) Grown *Lactobacillus* Strains in Bile Treatment**

| | survival (%)[a] | |
|---|---|---|
| carbon source | 1 h | 3 h |
| *Lactobacillus bulgaricus* ATCC 7517 (LB) | | |
| glucose | 100.50 (1.41) g[b] | 99.56 (3.11) g[b] |
| GOS-Lu | 78.41 (4.96) c | 43.19 (6.04) a |
| GOS-La | 87.95 (1.36) d | 62.01 (0.82) b |
| h-CMP | 96.98 (2.66) efg[b] | 68.05 (0.11) b |
| h-CMP:GOS-Lu | 93.17 (4.70) def | 67.44 (0.76) b |
| h-CMP:GOS-La | 100.88 (1.44) g[b] | 91.65 (4.26) de |
| *L. casei* ATCC11578 (LC) | | |
| glucose | 29.84 (5.77) a | ND |
| GOS-Lu | 45.08 (6.69) cd | 38.15 (4.27) abc |
| GOS-La | 44.60 (4.57) cd | 33.31 (0.85) ab |
| h-CMP | 74.05 (12.80) e | 65.87 (15.00) e |
| h-CMP:GOS-Lu | 48.40 (1.55) d | 32.57 (3.02) a |
| h-CMP:GOS-La | 72.87 (1.78) e | 42.16 (2.05) e |
| *L. delbrueckii* subsp. *lactis* ATCC4797 (LD) | | |
| glucose | 55.84 (0.38) d | 38.78 (11.65) bc |
| GOS-Lu | 37.63 (1.26) bc | 35.88 (1.40) bc |
| GOS-La | 42.36 (11.24) c | 33.22 (1.41) b |
| h-CMP | 23.37 (0.15) a | ND |
| h-CMP:GOS-Lu | 67.87 (12.01) e | 23.29 (3.34) a |
| h-CMP:GOS-La | 41.63 (12.97) c | 34.42 (3.50) bc |
| *L. plantarum* ATCC8014 (LP1) | | |
| glucose | 95.51 (3.69) ef[b] | 90.48 (2.30) e |
| GOS-Lu | 92.68 (6.73) e | 90.07 (4.30) e |
| GOS-La | 89.93 (4.46) e | 79.36 (14.44) d |
| h-CMP | ND | ND |
| h-CMP:GOS-Lu | 65.85 (0.70) c | 25.79 (0.27) a |
| h-CMP:GOS-La | 47.81 (0.44) b | ND |
| *L. plantarum* WCFS1 (LP2) | | |
| glucose | 84.45 (2.04) cd | 77.81 (0.99) b |
| GOS-Lu | 85.96 (2.51) d | 79.14 (5.15) b |
| GOS-La | 82.45 (1.18) c | 73.51 (4.04) a |
| h-CMP | 93.01 (0.11) e | 92.47 (0.31) e |
| h-CMP:GOS-Lu | 98.99 (0.31) f[b] | 94.50 (0.44) e |
| h-CMP:GOS-La | 99.35 (1.57) f[b] | 99.39 (1.20) f[b] |
| *L. sakei* 23K (LS) | | |
| glucose | 80.36 (3.32) ab | 81.58 (7.00) a |
| GOS-Lu | 87.09 (3.56) c | 87.54 (1.38) c |
| GOS-La | 83.66 (0.23) b | 83.31 (1.69) b |
| h-CMP | 95.65 (2.27) de | 90.20 (2.86) c |
| h-CMP:GOS-Lu | 99.77 (1.02) f[b] | 93.81 (0.54) d |
| h-CMP:GOS-La | 100.73 (0.96) f[b] | 97.60 (0.35) ef[b] |

[a]Survival percentage was calculated as an average with standard deviation (shown in parentheses) from data obtained from three independent studies. Different letters indicate significant differences ($P \leq 0.05$) for each strain and carbon source. [b]Significant differences with 0 h for each strain and carbon source.

*Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains grown in different types of carbohydrates was reported.[28,29] Similarly, Perrin et al.[39] observed increased bile salts resistance in bifidobacterial cells grown in fructooligosaccharides versus glucose and fructose, and this resistance pattern reportedly depended on the carbon sources used for the bacterial growth. *Lactobacillus* is the genus most commonly used as probiotics in food production.[40] After ingestion, these bacterial cells must be able to survive in the deleterious conditions of bile acids in the small intestine.[41] Our study results showed that, after 48 h of growth in glycoconjugates, *Lactobacillus* strains achieved a certain level of bile tolerance as indicated by the survival of significant percentage of viable cells even after 3 h of bile treatment. This interesting result suggests that hCMP:GOS conjugates could be potentially used as a carbon source to culture the *Lactobacillus* strains used to prepare probiotic supplemented food products. Therefore, we speculate that this approach could be used to protect the probiotic bacterial cells from the toxic effects of bile during the consumption.

**Fermentability of Glycoconjugates by *Lactobacillus* Strains.** Results of HPLC analysis confirmed the presence of lactate and acetate as fermentation products in the cell free culture supernatants obtained from the *Lactobacillus* cells grown in glycoconjugates. However, significant variations in the quantity of acetate and lactate were observed in each *Lactobacillus* strain and the carbon sources used for their growth (Table 2). In general terms, higher concentrations of acetate than lactate were detected in hCMP and glycoconjugate hCMP:GOS-La and hCMP:GOS-Lu fermentations. However, glucose, GOS-Lu, and GOS-La grown cells produced higher levels of lactate than acetate (Table 2). Strain and growth substrate dependent variations in organic acid production observed in our study were comparable to results obtained from previous studies using *Lactobacillus* spp. and *Bifidobacterium* spp.[30,42−44] Similar to our results, Sanz et al.[30] also observed high levels of lactate and acetate production in lactulose and sucrose containing culture media fermented by the fecal slurry. Recently, Cardelle-Cobas et al.[44] observed increased acetic acid concentration when the fecal slurry culture was grown in lactose- and lactulose-derived oligosaccharides containing media.

The products of carbohydrate fermentation by gut bacteria, primarily organic acids such as acetate and lactate, are beneficial to host health. Acetate and lactate formation are reportedly consistent in the metabolic activities in the species of *Bifidobacterium* and *Lactobacillus*. Previous reports show that the acetate and lactate produced by the *Bifidobacterium* sp. and *Lactobacillus* sp. from the fermentation of prebiotic carbohydrates are directly utilized by other intestinal bacterial species to produce different types of short-chain fatty acids (SCFAs).[45−47] In addition, the end products of gut bacterial fermentation reactions are important because they are absorbed from the gut and influence various aspects of gut physiology beyond functioning solely as a crude caloric source.[48−50] Interestingly, in our study we observed that all the strains were able to metabolize the glycoconjugates hCMP:GOS-La and hCMP:GOS-Lu similar to unconjugated prebiotic GOS-La and GOS-Lu and produced significant amounts of both lactate and acetate.

In conclusion we have demonstrated the ability of glycoconjugates of hydrolyzed caseinomacropeptide conjugated prebiotic galactooligosaccharides to serve as a value added prebiotic source for *Lactobacillus* strains. These glycoconjugates could be used as potential functional food ingredients to support the growth and enhance the bile tolerance of *Lactobacillus* strains. In this context, further followup *in vivo* studies are required to confirm the suitability of these peptide conjugated prebiotic GOS as a multipurpose beneficial functional food component.

**Table 2. HPLC Analysis of Cell Free Culture Supernatant Obtained from Glucose, Oligosaccharides (GOS-Lu and GOS-La), Hydrolyzed Caseinomacropeptide (hCMP), and Hydrolyzed CMP:Oligosaccharide Glycoconjugate (hCMP:GOS-Lu and hCMP:GOS-La) Grown *Lactobacillus* Strains in Fermentability Studies**

| carbon source | metabolite | time (h) | amt of metabolites (mM) produced by the *Lactobacillus* strains[a,b] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | LB | LC | LD | LP1 | LP2 | LS |
| glucose | lactate | 24 | 209.70 (0.20) cd | 178.54 (14.71) b | 203.52 (1.92) f | 184.33 (23.85) f | 203.18 (5.57) d | 189.12 (6.32) f |
| | | 48 | 228.67 (27.53) d | 197.93 (29.10) b | 203.09 (0.44) f | 184.55 (29.74) f | 208.12 (0.40) d | 192.89 (16.52) f |
| | | 72 | 214.89 (5.69) cd | 189.86 (23.11) b | 199.74 (0.40) f | 230.39 (41.87) g | 199.79 (3.62) d | 198.20 (9.37) f |
| | acetate | 24 | 36.76 (2.89) abcd | 34.34 (2.17) | 33.61 (1.15) ab | 32.68 (4.37) a | 32.82 (0.49) ab | 37.37 (4.84) abc |
| | | 48 | 42.65 (0.91) bcdef | 31.71 (1.34) abcd | 31.97 (1.13) ab | 33.67 (3.10) ab | 34.87 (0.22) ab | 35.42 (0.44) abc |
| | | 72 | 38.52 (3.94) abcd | 31.04 (0.14) ab | 39.16 (14.93) abc | 40.65 (8.53) abcd | 31.35 (0.89) ab | 37.11 (2.68) abc |
| GOS-Lu | lactate | 24 | 69.20 (1.52) b | 183.87 (29.23) b | 42.90 (6.90) cde | 63.61 (0.94) bcd | 66.72 (6.73) b | 67.42 (4.53) c |
| | | 48 | 80.73 (4.40) b | 40.42 (2.05) a | 35.56 (9.37) bcd | 75.06 (5.23) cde | 66.87 (6.21) b | 65.58 (6.10) c |
| | | 72 | 77.09 (2.50) b | 43.80 (0.77) a | 44.51 (0.99) de | 87.71 (12.00) de | 67.30 (6.55) b | 71.26 (8.22) cd |
| | acetate | 24 | 43.02 (5.75) cdefg | 42.49 (6.04) bcd | 42.04 (0.94) abc | 63.07 (2.84) f | 31.61 (2.12) ab | 58.52 (2.72) fg |
| | | 48 | 54.13 (2.07) hi | 44.37 (2.05) bcd | 43.64 (3.40) bc | 51.95 (0.09) bcdef | 44.42 (13.69) bcd | 53.11 (0.12) efg |
| | | 72 | 48.56 (10.26) defgh | 42.32 (5.80) bcd | 52.51 (13.54) c | 59.21 (11.69) def | 42.42 (14.22) abcd | 54.94 (2.83) fg |
| GOS-La | lactate | 24 | 65.66 (2.90) b | 33.06 (11.05) a | 54.12 (1.89) e | 46.91 (1.55) abc | 76.81 (2.04) bc | 77.96 (3.09) cde |
| | | 48 | 83.71 (9.03) b | 33.20 (10.09) a | 36.14 (12.14) bcd | 102.97 (22.16) e | 82.53 (0.36) c | 80.25 (0.28) de |
| | | 72 | 76.73 (7.55) b | 32.87 (9.92) a | 33.52 (10.84) abcd | 91.42 (16.87) de | 85.21 (2.95) c | 84.91 (1.25) e |
| | acetate | 24 | 45.22 (0.25) defgh | 40.21 (12.37) bcd | 46.47 (2.11) bc | 57.36 (1.97) def | 61.59 (10.40) e | 64.06 (3.85) g |
| | | 48 | 52.99 (2.45) ghi | 47.05 (1.43) d | 42.46 (1.66) abc | 59.38 (9.52) ef | 52.35 (0.34) de | 52.84 (1.89) defg |
| | | 72 | 51.69 (0.32) fgh | 47.69 (1.31) d | 46.59 (2.88) bc | 57.10 (10.38) def | 50.01 (1.82) cde | 53.46 (1.79) efg |
| h-CMP | lactate | 24 | 26.22 (4.07) a | 24.56 (2.82) a | 26.58 (3.15) bc | 23.84 (1.91) a | 23.37 (0.20) a | 24.86 (0.79) ab |
| | | 48 | 19.85 (0.14) a | 23.22 (1.73) a | 23.92 (0.07) a | 24.39 (0.42) a | 23.63 (1.50) a | 24.52 (0.48) ab |
| | | 72 | 28.42 (2.44) a | 23.06 (4.42) a | 26.07 (4.50) bc | 23.59 (0.68) a | 20.91 (2.59) a | 21.99 (2.63) ab |
| | acetate | 24 | 30.74 (1.41) a | 41.02 (5.85) bcd | 38.73 (4.00) abc | 28.32 (3.49) | 31.90 (5.57) ab | 31.72 (2.84) ab |
| | | 48 | 32.76 (1.11) ab | 39.72 (2.71) bcd | 33.88 (1.21) ab | 36.52 (2.67) abc | 30.67 (3.80) ab | 30.93 (1.49) a |
| | | 72 | 40.67 (1.18) abcde | 41.31 (8.04) bcd | 35.58 (9.66) ab | 37.49 (3.99) abc | 29.12 (3.16) a | 45.19 (17.86) bcdef |
| h-CMP-GOS-Lu | lactate | 24 | 38.44 (2.73) a | 27.32 (6.82) a | 33.83 (2.42) abcd | 30.26 (2.29) a | 28.97 (7.51) a | 31.95 (1.06) b |
| | | 48 | 36.81 (6.41) a | 25.25 (5.15) a | 23.43 (0.84) a | 28.30 (2.65) a | 25.45 (0.11) a | 28.22 (0.42) b |
| | | 72 | 41.85 (0.37) a | 32.91 (4.07) a | 32.60 (0.83) abc | 37.66 (10.01) ab | 28.32 (6.38) a | 31.25 (1.18) b |
| | acetate | 24 | 39.45 (6.41) abcd | 40.49 (10.58) bcd | 38.93 (6.49) abc | 45.55 (3.28) bcdef | 42.10 (9.24) abcd | 40.16 (7.22) abcde |
| | | 48 | 50.58 (2.58) efgh | 24.98 (11.98) a | 27.83 (1.94) a | 42.00 (12.61) abcde | 35.31 (2.29) abc | 47.94 (8.83) cdef |
| | | 72 | 62.01 (12.37) i | 45.84 (5.21) cd | 41.00 (6.17) abc | 54.95 (8.94) cdef | 41.77 (10.27) abcd | 52.21 (4.05) defg |
| h-CMP-GOS-La | lactate | 24 | 27.50 (7.74) a | 26.29 (9.45) a | 28.54 (3.23) bc | 20.01 (0.12) a | 23.60 (1.05) a | 23.77 (1.33) a |
| | | 48 | 26.35 (7.42) a | 23.82 (3.84) a | 25.10 (8.57) bc | 18.53 (1.97) a | 20.39 (9.22) a | 15.32 (4.19) ab |
| | | 72 | 30.52 (0.94) a | 24.96 (3.82) a | 26.24 (4.74) bc | 19.74 (5.80) a | 20.21 (7.67) a | 20.21 (7.67) ab |
| | acetate | 24 | 31.90 (9.00) a | 36.63 (2.30) abcd | 39.58 (7.27) abc | 30.30 (2.79) | 37.95 (2.19) abcd | 36.81 (6.06) abc |
| | | 48 | 33.94 (6.36) abc | 37.97 (12.05) abcd | 37.71 (10.61) abc | 43.60 (1.59) abcde | 38.43 (6.24) abcd | 35.35 (7.24) abc |
| | | 72 | 45.34 (3.42) defgh | 42.14 (7.54) bcd | 39.00 (4.50) abc | 43.59 (14.76) abcde | 39.34 (10.63) abcd | 39.34 (10.63) abcd |

[a]Standard deviations are shown in parentheses. Different letters indicate significant differences ($P \leq 0.05$) for each strain and metabolite. [b]*Lactobacillus bulgaricus* ATCC 7517 (LB), *L. casei* ATCC11578 (LC), *L. delbrueckii* subsp. *lactis* ATCC4797 (LD), *L. plantarum* ATCC8014 (LP1), *L. plantarum* WCFS1 (LP2), *L. sakei* 23K (LS).



dx.doi.org/10.1021/jf301392y | J. Agric. Food Chem. 2012, 60, 6839−6845

## AUTHOR INFORMATION


### Corresponding Author

*Center for Food Safety—Department of Food Science, University of Arkansas, Fayetteville, AR 72704, USA. Tel: 479-575-4678. Fax: 479-575-6936. E-mail: sricke@uark.edu.



### Funding

We gratefully acknowledge financial support from Junta de Castilla-La Mancha and European regional development fund (ERDF) (POII10-0178-4685), from Comunidad Autónoma de Madrid (ALIBIRD S2009/AGR-1469), and from Ministerio de Ciencia e Innovación (AGL2011-27884). S.C.R. received support from a USDA Food Safety Consortium and a USDA-NIFSI (2008-51110-04339) grant. O.H.-H. thanks the CSIC for a JAE-PreDoc PhD grant.


### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS


We thank Dr. G. Klarenbeek and Dr. J. de Slegte from Friesland Foods Domo for providing us with Vivinal-GOS, and we thank Laurie Davis from Davisco Foods International, Inc., for kindly providing us with CMP.


## REFERENCES


(1) de Sousa, V. M. C.; dos Santos, E. F.; Sgarbieri, V. C. The importance of prebiotics in functional foods and clinical practice. *Food Nutr.* **2011**, *2*, 133−144.

(2) Holzman, D. C. Major host health effects ascribed to gut microbiome. *Microbe* **2011**, *6*, 259−260.

(3) Agheyisi, R. The Probiotics Market: Ingredients, Supplements. *Foods*; Report Code: FOD035C, 2011; URL (http://www.bccresearch.com/report/probiotics-market-ingredients-foods-fod035c.html), accessed 31 March 2012.

(4) Rastall, R. A.; Maitin, V. Prebiotics and synbiotics: towards the next generation. *Curr. Opin. Biotechnol.* **2002**, *13*, 490−496.

(5) Rastall, R. A.; Gibson, G. R.; Gill, H. S.; Guarner, F.; Klaenhammer, T. R.; Pot, B.; Reid, G.; Rowland, I. R.; Sanders, M. E. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. *FEMS Microbiol. Ecol.* **2005**, *52*, 145−152.

(6) Thammarutwasik, P.; Hongpattarakere, T.; Chantachum, S.; Kijroongrojana, K.; Itharat, A.; Reanmongkol, W.; Tewtrakul, S.; Ooraikul, B. Prebiotics − a review. *Songklanakarin J. Sci. Technol.* **2009**, *31*, 401−408.

(7) Cummings, J. H.; Macfarlane, G. T.; Englyst, H. N. Prebiotic digestion and fermentation. *Am. J. Clin. Nutr.* **2001**, *73*, 415S−419S.

(8) Langlands, S. J.; Hopkins, M. J.; Coleman, N.; Cummings, J. H. Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel. *Gut* **2004**, *53*, 1610−1616.

(9) FitzGerald, R. J.; Meisel, H. Milk protein hydrolysates and bioactive peptides. In *Advanced Dairy Chemistry: Proteins*, 3rd ed.; Fox, P. F. McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum Publisher: New York, NY, 2003; Vol. *1*, Part A, pp 675−698.

(10) FitzGerald, R. J.; Murray, B. A. Bioactive peptides and lactic fermentations. *Int. J. Dairy Technol.* **2006**, *59*, 118−125.

(11) Hartmann, R.; Meisel, H. Food-derived peptides with biological activity: from research to food applications. *Curr. Opin. Biotechnol.* **2007**, *18*, 163−169.

(12) Meisel, H.; Schlimme, E. Milk proteins: precursors of bioactive peptides. *Trends Food Sci. Technol.* **1990**, *1*, 41−43.

(13) Meisel, H. Biochemical properties of bioactive peptides derived from milk proteins: Potential nutraceuticals for food and pharmaceutical applications. *Livest. Prod. Sci.* **1997**, *50*, 125−138.

(14) Meisel, H. Overview on milk protein-derived peptides. *Int. Dairy J.* **1998**, *8*, 363−373.

(15) Thomä-Worringer, C.; Sørensen, J.; López-Fandiño, R. Health effects and technological features of caseinomacropeptide. *Int. Dairy J.* **2006**, *16*, 1324−1333.

(16) LaClair, C. E.; Ney, D. M.; MacLeod, E. L.; Etzel, M. R. Purification and use of glycomacropeptide for nutritional management of phenylketonuria. *J. Food Sci.* **2009**, *74*, E199−E206.

(17) Mikkelsen, T. L.; Rasmussen, E.; Olsen, A.; Barkholt, V.; Frøkiær, H. Immunogenicity of κ-casein and glycomacropeptide. *J. Dairy Sci.* **2006**, *89*, 824−830.

(18) Requena, P.; Daddaoua, A.; Guadix, E.; Zarzuelo, A.; Suárez, M. D.; De Medina, F. S.; Martínez-Augustin, O. Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-?B signal transduction pathways. *Br. J. Pharmacol.* **2009**, *157*, 1232−1240.

(19) Abdel-Salam, A. M.; Effat, L. K. Preparation and evaluation of a novel therapeutic dairy-based drink for phenylketonuria. *North Am. J. Med. Sci.* **2010**, *2*, 66−70.

(20) Ney, D. M.; Hull, A. K.; van Calcar, S. C.; Liu, X.; Etzel, M. R. Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenylketonuria. *J. Nutr.* **2008**, *138*, 316−322.

(21) Clemente, A.; Rubio, L. A.; Sanz, Y.; Laparra, J. M.; Sanz, M. L.; Hernández, O. J.; Montilla, A.; Olano, A.; Moreno, F. J. Multifunctional galacto-oligosaccharides derived from lactulose with immunomodulatory and prebiotic activities. 2011, Spanish Patent P201130784.

(22) Hernández, O.; Ruiz-Matute, A. I.; Olano, A.; Moreno, F. J.; Sanz, M. L. Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. *Int. Dairy J.* **2009**, *19*, 531−536.

(23) Hernandez-Hernandez, O.; Lebron-Aguilar, R.; Quintanilla-Lopez, J. E.; Sanz, M. L.; Moreno, F. J. Development of a new method using HILIC-tandem mass spectrometry for the characterization of O-sialoglycopeptides from proteolytically digested caseinomacropeptide. *Proteomics* **2010**, *10*, 3699−3711.

(24) Hernandez-Hernandez, O.; Sanz, M. L.; Kolida, S.; Rastall, R. A.; Moreno, F. J. In vitro fermentation by human gut bacteria of proteolytically digested caseinomacropeptide non-enzymatically glycosylated with prebiotic carbohydrates. *J. Agric. Food Chem.* **2011**, *59*, 11949−11955.

(25) Moreno, F. J.; López-Fandiño, R.; Olano, A. Characterization and functional properties of lactosyl caseinomacropeptide conjugates. *J. Agric. Food Chem.* **2002**, *50*, 5179−5184.

(26) Man, J. C.; Rogosa, M.; Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **1960**, *23*, 130−135.

(27) Hernandez-Hernandez, O.; Muthaiyan, A.; Moreno, F.; Montilla, A.; Sanz, M.; Ricke, S. Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus*. *Food Microbiol.* **2012**, *30*, 355−361.

(28) Kimoto-Nira, H.; Kobayashi, M.; Nomura, M.; Sasaki, K.; Suzuki, C. Bile resistance in *Lactococcus lactis* strains varies with cellular fatty acid composition: Analysis by using different growth media. *Int. J. Food Microbiol.* **2009**, *131*, 183−188.

(29) Kimoto-Nira, H.; Suzuki, C.; Sasaki, K.; Kobayashi, M.; Mizumachi, K. Survival of a *Lactococcus lactis* strain varies with its carbohydrate preference under in vitro conditions simulated gastrointestinal tract. *Int. J. Food Microbiol.* **2010**, *143*, 226−229.

(30) Sanz, M. L.; Gibson, G. R.; Rastall, R. A. Influence of disaccharide structure on prebiotic selectivity in vitro. *J. Agric. Food Chem.* **2005**, *53*, 5192−5199.

(31) Poolman, B. Energy transduction in lactic acid bacteria. *FEMS Microbiol. Rev.* **1993**, *12*, 125−147.

(32) Holt, S. M.; Miller-Fosmore, C. M.; Côté, G. L. Growth of various intestinal bacteria on alternansucrase-derived oligosaccharides. *Lett. Appl. Microbiol.* **2005**, *40*, 385−390.

(33) Amaretti, A.; Tamburini, E.; Bernardi, T.; Pompei, A.; Zanoni, S.; Vaccari, G.; Matteuzzi, D.; Rossi, M. Substrate preference of *Bifidobacterium adolescentis* MB 239: compared growth on single and mixed carbohydrates. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 654−662.

(34) Su, P.; Henriksson, A.; Mitchell, H. Selected prebiotics support the growth of probiotic mono-cultures in vitro. *Anaerobe* **2007**, *13*, 134−139.
(35) Pastell, H.; Westermann, P.; Meyer, A. S.; Tuomainen, P.; Tenkanen, M. In vitro fermentation of arabinoxylan-derived carbohydrates by Bifidobacteria and mixed fecal microbiota. *J. Agric. Food Chem.* **2009**, *57*, 8598−8606.
(36) Cardelle-Cobas, A.; Corzo, N.; Olano, A.; Peláez, C.; Requena, T.; Ávila, M. Galactooligosaccharides derived from lactose and lactulose: Influence of structure on *Lactobacillus, Streptococcus* and *Bifidobacterium* growth. *Int. J. Food Microbiol.* **2011**, *149*, 81−87.
(37) Begley, M.; Gahan, C. G. M.; Hill, C. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* **2005**, *29*, 625−651.
(38) Begley, M.; Hill, C.; Gahan, C. G. M. Bile Salt Hydrolase Activity in Probiotics. *Appl. Environ. Microbiol.* **2006**, *72*, 1729−1738.
(39) Perrin, S.; Grill, J. P.; Schneider, F. Effects of fructooligosaccharides and their monomeric components on bile salt resistance in three species of Bifidobacteria. *J. Appl. Microbiol.* **2000**, *88*, 968−974.
(40) Turpin, W.; Humblot, C.; Thomas, M.; Guyot, J. Lactobacilli as multifaceted probiotics with poorly disclosed molecular mechanisms. *Int. J. Food Microbiol.* **2010**, *143*, 87−102.
(41) Whitehead, K.; Versalovic, J.; Roos, S.; Britton, R. A. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. *Appl. Environ. Microbiol.* **2008**, *74*, 1812−1819.
(42) Marx, S. P.; Winkler, S.; Hartmeier, W. Metabolization of $\beta$-(2, 6)-linked fructose-oligosaccharides by different bifidobacteria. *FEMS Microbiol. Lett.* **2000**, *182*, 163−169.
(43) Wichienchot, S.; Prasertsan, P.; Hongpattarakere, T.; Gibson, G. R.; Rastall, R. A. In vitro fermentation of mixed linkage gluco-oligosaccharides produced by *Gluconobacter oxydans* NCIMB 4943 by the human colonic microflora. *Curr. Issues Intest. Microbiol.* **2006**, *7*, 7−12.
(44) Cardelle-Cobas, A.; Fernández, M.; Salazar, N.; Martínez-Villaluenga, C.; Villamiel, M.; Ruas-Madiedo, P.; de los Reyes-Gavilán, C. G. Bifidogenic effect and stimulation of short chain fatty acid production in human faecal slurry cultures by oligosaccharides derived from lactose and lactulose. *J. Dairy Res.* **2009**, *76*, 317−325.
(45) Edwards, C. A.; Parrett, A. M. Intestinal flora during the first months of life: new perspectives. *Br. J. Nutr.* **2002**, *88*, s11−s18.
(46) Belenguer, A.; Duncan, S. H.; Calder, A. G.; Holtrop, G.; Louis, P.; Lobley, G. E.; Flint, H. J. Two routes of metabolic cross-feeding between *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human gut. *Appl. Environ. Microbiol.* **2006**, *72*, 3593−3599.
(47) Falony, G.; Vlachou, A.; Verbrugghe, K.; Vuyst, L. D. Cross-feeding between *Bifidobacterium longum* BB536 and acetate-converting, butyrate-producing colon bacteria during growth on oligofructose. *Appl. Environ. Microbiol.* **2006**, *72*, 7835−7841.
(48) Cummings, J. H.; Macfarlane, G. T. The control and consequences of bacterial fermentation in the human colon. *J. Appl. Microbiol.* **1991**, *70*, 443−459.
(49) Macfarlane, G. T.; Steed, H.; Macfarlane, S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J. Appl. Microbiol.* **2008**, *104*, 305−344.
(50) Neish, A. S. Microbes in gastrointestinal health and disease. *Gastroenterology* **2009**, *136*, 65−80.