

**Chitooligosaccharides as novel ingredients of fermented foods**

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Chitooligosaccharides as novel ingredients of fermented foods

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Chitooligosaccharides (COSs) have been clinically evaluated for their immunostimulating effects after oral intake. As dietary supplements, prebiotics and biopreservatives, these water-soluble bioactives are easily incorporated into dairy products and beverages. Notwithstanding, the use of COS in fermented foods would be limited by its antimicrobial properties. In order to study the interaction with yoghurts as a model of fermented food, the effects of COS on chemical composition, viability, morphology and metabolism of lactic acid bacteria, fatty acid profiles and conjugated linoleic acid (CLA) were assessed over 28 days and after chemical digestion. There were no significant differences between the nutritional composition of controls and yoghurts supplemented with concentrations up to 0.1 % w/w of COS. However, the acidification of milk decreased at 0.5 % ($p < 0.05$) and the formation of yoghurt failed at 3.0 %, without affecting viable counts. Lipid hydrolysis of yoghurts supplemented with 0.1 % COS was not affected by chemical digestion. No significant differences were found between CLA percentages of controls and supplemented yoghurts after digestion. Although nutritional composition, fatty acids and viable counts were not significantly modified after COS supplementation, the present study shows that COS diminishes bacterial acidification at concentrations higher than 0.1 %, thus limiting the amounts that could be added to yoghurt.

Introduction

Chitooligosaccharides (COSs) result from the hydrolysis of chitosan, a cationic polysaccharide obtained by partial deacetylation of chitin, the natural polymer found in crustacean shells. COSs have smaller molecular size than chitosan and thus lower viscosity and greater solubility in aqueous solutions. As bioactive molecules, COSs display *in vivo* and *in vitro* antitumor effects, enhancement of calcium absorption and increase of bone strength,¹ benefits in asthma treatment,² and modulation of obesity and associated inflammation.³ The biological activities described for COS such as lowering of blood cholesterol and blood pressure depend on the molecular weight (MW) or degree of polymerization (DP), and deacetylation degree (DD).¹ It has been claimed that COS reach the systemic circulation after oral administration.⁴ The *in vivo* absorbability of the oligomers increased, while the cytotoxicity decreased when decreasing molecular weight (MW). COSs with molecular weights of 3.8 kDa or lower (DD 88 %) would display the best biological profiles.

Although commercial products based on COS have not been revised by the international regulatory organizations, some products containing chitosan polymer have been recognized as

safe dietary supplements or novel food ingredients by the FDA and EFSA.^{5,6,7} The chitosan GRAS (Generally Recognized As Safe) notice related to KiOfine[®]B, a chitosan secondary food ingredient for food processing,⁸ claims that chitosan oligomers (< 1 kDa, DD 70-95 %) are non-toxic for humans and animals, even when consumed at high dietary concentrations. A clinical trial on the efficacy of orally administered chitooligosaccharides (FACOS[™], 3.5 kDa) on immune function of healthy adults has been completed in 2007.⁹ Although no results have been provided by the sponsor, this product has been subjected to clinical trials to test the effects on the immune function of healthy adults¹⁰ and elderly.¹¹ A daily dose of 2.6 g and 5.1 g for each group respectively, led to a favorable behavior of cytokine levels in the treated group, without adverse effects. Orally administered as a dietary supplement, COS with a DD of 90 % and MW below 1.0 kDa reduced cholesterol levels in healthy men at a dose of 1 g/day.¹² Again, no adverse effects were reported.

Chitosan oligosaccharides have been proposed as potential prebiotics, since they could stimulate beneficial bacteria while inhibiting intestinal pathogens.¹³ Lee *et al.*¹⁴ showed that COS (DP 2-8, MW < 1.5 kDa, DD 99.9%) stimulated the growth of *Bifidobacterium* and *Lactobacillus* probiotic strains at concentrations ranging from 0.1 to 0.5 %. Accordingly, Liang *et al.*¹⁵ informed that COS (DP4-9, MW < 1.7 kDa, DD 60 %) stimulated the growth of *L. paracasei* and *L. kefir* at 0.1 % and concluded that oligomers with higher MWs inhibited the growth. In contrast, Fernandes *et al.*¹⁶ could not demonstrate prebiotic effects on the probiotics *Bifidobacterium animalis* and *L. acidophilus* using oligosaccharides with similar

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characteristics (MW 1.7 KDa, DD 65 %). These results would suggest that COS effects on bacterial growth are strain specific. Indeed, previous studies showed that COSs (MW<5 KDa, MW<3KDa, DD 80-85%) inhibit Gram negative bacteria more than Gram positive bacteria.^{17,18} This could be attributed to differences in cell wall, since COS but not chitosan could penetrate the cell wall of Gram negatives and exert intracellular damage. Regarding inhibition of undesirable bacteria, Wang *et al.*¹⁹ recently reported that COS had the potential to reduce intestinal pathogen adhesion. Šimůnek *et al.* showed that oligomers (MW<2KDa, MW<3KDa, MW<6KDa, DD 88%) inhibit non-pathogenic human colonic bacteria weakly, while the growth rate of Bifidobacteria of human origin is not affected.^{20,21}

Due to their antimicrobial properties against foodborne pathogens, COSs have been proposed as food biopreservatives. Tsai *et al.*²² reported that COSs (DP 1-8) prevented milk spoilage by inhibiting the growth of undesirable microbial species at 0.24 to 0.48 %. In another study, COS (DP < 5, DD 80-85 %) displayed MICs and MLCs between 0.1 and 0.5 % w/v against *Escherichia coli* and *Staphylococcus aureus* in milk and apple juice.²³ According to Barreateau *et al.*,²⁴ COSs showed better properties than chitosan polymer against food borne pathogens. These authors also suggested that the antimicrobial properties of COS may not permit its use in fermented foods. A profitable product should be active against pathogens and innocuous to beneficial microorganisms such as starters, probiotics and intestinal bacteria associated with health. Regarding microbial strains used in the manufacture of yoghurts, it has been shown that COSs (MW < 1.5 KDa, 85%) activity against yoghurt starters *L. delbrueckii* and *S. thermophilus* depends on COS concentration and initial bacterial load. It is worth to mention that these oligomers were more tolerable than those of higher molecular weights.²⁵

In the present study, we evaluate for the first time the effects of COS on yoghurt as the selected model of fermented food. Traditionally, yoghurt is produced by incubating concentrated milk with *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*.²⁶ According to the standards for fermented milk,²⁷ yoghurt must possess a high number of viable bacteria.^{28,29,30} Among yoghurt lipids, conjugated linoleic acid (CLA) has many biological properties that contribute to its nutritional value.²⁹ It accumulates preferably in cheeses and yoghurt in quantities ranging from 1 to 9 mg/g of lipid.³¹ CLA comprises those isomers of linoleic acid possessing conjugated double bonds, a structural feature that confers chemical instability to the molecule and the ability to react against free radicals.³²

Bearing in mind the increasing interest in the use of COS as novel food ingredients, the present study aims to evaluate whether COS modifies the nutritional properties of yoghurt, such as nutritional composition, viable counts of lactic acid bacteria (LAB) and fatty acid profiles. In order to determine the effects of COS thoroughly, fatty acid profiles and viable counts were followed over a storage period of 28 days. Additionally, a

sensory acceptance study on yoghurts supplemented with COS was performed.

Taking into account that chitosan, a polycationic polymer, interferes with lipid absorption by binding lipids and fatty acids,^{33,34} the lipolysis, fatty acid profiles and CLA content were studied after chemical digestion of fresh yoghurts supplemented with COS.

Materials and methods

Preparation of chitooligosaccharides

Chitosan was obtained from shrimp (*Pleoticus mülleri*) waste as previously reported.³⁵ The chitosan average molecular weight was 468,186 KDa, assessed by viscometry and calculated from experimental intrinsic viscosity by utilizing the Mark-Houwink-Kuhn-Sakurada.³⁶ The moisture content was 6.58 % and ash content 0.53 %. The degree of deacetylation (DD) was assessed by procedures previously described.³⁷ Hydrolysis of chitosan (2 g) was performed with 12.5 % v/v H₂O₂ for 20 min at room temperature. After being irradiated in a microwave oven at 700W for 4 min, the sample was cooled at room temperature and filtrated under reduced pressure. The chitooligosaccharides were precipitated with 150 mL of ethanol 98% v/v from the filtrate. After 15 min, the residue was filtrated under reduced pressure and washed several times with ethanol until a negative reaction with KMnO₄ (0.02 M) confirmed the absence of H₂O₂. The resulting product was dissolved in distilled water and freeze dried. All reagents were of analytical grade. The COSs average molecular weight was 2.764 KDa, moisture content was 13.3 %, ash content 2.60 % and DD 68%.

Preparation of yoghurt

Yoghurt was prepared according to a method previously reported.³⁸ Reconstituted skim milk (15 % w/w, La Serenísima, Argentina) with sucrose 5 % was heated to 80 °C for 30 min, cooled to room temperature and supplemented with 0.05, 0.1, 0.5 and 3 % w/w COS. After homogenization, 1 mL of a fresh culture of *Streptococcus thermophilus* (CP2 CIDCA collection 321) and 1 mL of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lbp, CIDCA collection 332), were added to 100 g of milk with sucrose and supplemented with COS. Controls were obtained by the same procedures without the addition of COS. Samples were incubated at 40 ± 1 °C until pH 4.4–4.6 and stored at 4 ± 1 °C after completion of the fermentation process. The starters were activated for 24 to 48 h at 40 ± 1 °C in autoclaved skim milk (120 °C, 20 min) before inoculating the yoghurts, until a biomass of ~10⁸ CFU/mL was reached. All analyses were performed in triplicates.

Viable counts

Aliquots of 1g of yoghurt were suspended in 100 mL of sterile physiologic solution. Serial dilutions were prepared and 100 µL of each dilution was spread over De Man, Rogosa and Sharpe agar plates (Laboratorios Britania S.R.L., Buenos Aires, Argentina). The plates were incubated for 24 h at 40 ± 1 °C.

Colonies growing at these conditions were counted and informed as lactic acid bacteria. Samples were analyzed in triplicates.

Microscopy

Gram staining was performed in order to check for general morphology and contamination. Aliquots of 5 μL of a dilution 1/10 v/v in sterile physiologic solution (0.85 % NaCl, analytical grade) of yoghurt samples were fixed on glass slides and stained according to the instructions of the kit supplier (Gram Britania, Argentina). The samples were observed under optical microscope. The morphology of lactobacilli and streptococci of controls and yoghurt samples treated with COS was observed with a Scanning Electron Microscope LEO EVO 40 (Zeiss, Germany). Cells were adhered using polylysine surfaces by a method previously reported.^{39,40}

Compositional analyses

Yoghurt samples were analyzed after 24 h storage at 4° C. Protein content was measured by the Kjeldahl method using the conversion factor 6.38 (method 24.027, AOAC),⁴¹ and fat was determined by the Gerber method. Acidity was measured by titration with 0.1 N NaOH, and expressed as percentage of lactic acid (g lactic acid/100 g, method 907.124, AOAC).⁴² Ash content was quantified by dry ashing the samples in a muffle furnace at 550° C for 24 h (method 24.009, AOAC).⁴¹ Water content was determined by oven drying at 105 °C (method 24.002, AOAC).⁴¹ All analyses were performed in triplicates. All reagents were of analytical grade.

Lipid extraction and fatty acid methylation

Aliquots of 700 mg of yoghurt were extracted three times with 700 μL of a chloroform/methanol solution (2:1 v/v, analytical grade, Cicarelli, ≥ 99.0 %, Buenos Aires, Argentina). In order to improve the extraction, samples were shaken at 230 rpm for 15 min. To disrupt the emulsion, samples were centrifuged at 4200 rpm for 5 min. The organic phase was collected and poured into a clean glass tube. Samples were reduced under nitrogen stream, and 1 mL of 10 % methanolic HCl (v/v) was added. This solution was prepared with 37 % HCl (w/v) (for analysis, Merck, Buenos Aires, Argentina) and anhydrous methanol (analytical grade, Biopack, ≥ 99.8 %, Buenos Aires, Argentina). The reaction was held at 50 °C for 20 min. After cooling the samples to room temperature, fatty acid methyl esters were extracted twice with 1 mL of n-hexane (chromatographic grade, U.V.E. Dorwil, Buenos Aires, Argentina) and washed once with 1 mL of 1.2 % NaOH (w/v) (analytical grade, Anedra, ≥ 99.8 %, Buenos Aires, Argentina). Samples were immediately dried under nitrogen stream and subjected to chromatographic analysis or stored under a nitrogen atmosphere. Lipids were extracted by similar procedures after chemical digestion. Aliquots of 15 mL of the digested suspension were extracted three times with the same volume of the chloroform/methanol solution (2:1, v/v), mixed and centrifuged. The organic phase was concentrated with a rotatory evaporator at 45 °C and the resulting extracts were

dried under nitrogen stream. Samples were dissolved in 500 μL of deuterated chloroform for NMR analyses. Once the spectra were recorded, 100 μL of the total volume was methylated for GC-MS analyses. All analyses were performed in triplicates.

Gas chromatography analyses

Fatty acid methyl esters were analyzed by GC-MS with a 7890B chromatograph equipped with a mass spectrometer 5977A (Agilent Technologies). The ionization mode was electron impact and the ionization energy was 70 eV. Samples (1 μL) were injected into a HP-5MS capillary column (30 m \times 0.25 mm i.d. \times 0.25- μm film thickness). Temperature was programmed from 150 °C to 250 °C at a rate of 4 °C/min and held at the final temperature for 15 min. The injector and detector temperature was 280 °C; the carrier gas was helium at a flow rate of 1 mL/min and the split ratio of 20:1. Fatty acid compositions were expressed as relative percentages (w/w). CLA isomers were identified by comparing their retention times and mass fragmentation patterns with those of the standards: c9,t11-octadecadienoic acid, t10,c12 and other minor isomers, including t9,t11. Standards of methyl esters of conjugated linoleic acid were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA (CODE O5507).

Nuclear Magnetic Resonance experiments

¹H NMR spectra of lipid extracts were acquired with a Bruker Avance Ultrashield 300 MHz NMR spectrometer using CDCl₃ (99.8 atom % D, Sigma-Aldrich Chemical Co., St. Louis, MO, USA, CODE 151823) as solvent and residual protons as internal reference (7.26 ppm). Spectra were recorded at 300 K, 16 scans with a spectral width of 6000 Hz.

Digestive chemical experimental model

The chemical digestion has been previously described by Rodríguez *et al.*³⁵ Procedures were performed as follows: 12.5 g of yoghurt was stirred in 50 mL of 0.1 M HCl (Merck) for 1 h at pH 1.0–2.0, at 30 rpm and 37 °C to reproduce the gastric environment. The pH was adjusted to 6.8–7.2 with 15 g/L of NaHCO₃ (Sigma Chemical Co., St. Louis, MO, USA) and the stirring speed was increased from 30 to 300 rpm, while the temperature was maintained at 37 °C to reproduce the duodenal environment. All reagents were of analytical grade.

Sensory evaluation

Sensory evaluation was done in the morning in a standardized test room, by an affective test with 9-point structured scale (1 for 'dislike extremely' and 9 for 'like extremely'). Yoghurt samples (10 g) with and without 0.1 % COSs were served at 10 °C in white plastic cups coded with random three digit numbers. Mineral water was provided for mouth-rinsing. The attributes of overall acceptability, flavor, color and texture, were evaluated by a panel of 30 non trained judges to determine the acceptability of the yogurts.⁴³

Statistical analyses

Relative percentages of compositional analysis are expressed as the mean and standard error of the mean. Significant differences were tested using two-way ANOVA. Post tests were performed using the Bonferroni method for multiple comparisons.

Results and discussion

The composition of yoghurts obtained by fermentation of milk supplemented with 0.1 and 0.05 % w/w of COS satisfied the

At a concentration of 0.5 % w/w, the lactic acid percentage decreased significantly ($p < 0.05$, Table 1) to reach the minimum accepted by the Codex.²⁷ When COS were added to 3.0 % w/w, the acidification failed in such a way that protein coagulation did not occur and product consistence resembled that of milk (data not shown). This suggests that lactic acid production, and thus bacterial metabolic activity, was markedly affected by increasing concentrations of COS when it exceeded 0.5 % w/w. The viable counts of LAB, however, remained in the order of 10^8 CFU/g at 0, 0.05, 0.1 and 0.5 % w/w COS. Water content decreased when COS was added. This suggests that COS, like other oligosaccharides, is able to retain water. Other nutritional characteristics of yoghurts such as fat and protein content were not altered.

Table 1. Composition of yoghurts supplemented with COS.^a

	acidity (%lactic acid)	Fat (g%)	Water content (g%)	Protein (g%)	Ash (g%)	CFU/g 24 h
Control	1.57±0.02	1.45±0.05	83.70 ± 1.45	3.10 ± 0.15	0.86 ± 0.01	8.3 10 ⁸ ± 0.04
0.05%	1.60±0.02	1.50±0.01	80.80 ± 0.01 ^b	3.00 ± 0.15	0.87 ± 0.01	8.4 10 ⁸ ± 0.09
0.1%	1.49±0.05	1.55±0.05	80.85 ± 0.15 ^b	2.97 ± 0.17	0.85 ± 0.01	8.3 10 ⁸ ± 0.10
0.5%	0.68±0.01 ^b	1.55±0.05	79.50 ± 0.50 ^c	3.40 ± 0.10	0.87 ± 0.01	2.2 10 ⁸ ± 0.10

^aMean values ± S.E.M., ^b $p < 0.05$ compared with control; ^c $p < 0.001$ compared with control

Minor changes in bacterial morphology were recorded. Bacteria growing in yoghurts supplemented with 0.1 % COS appeared to be larger. Cocci growing in control yoghurts showed an average diameter of 630 nm, while the diameter of cocci growing in supplemented yoghurts (0.1 %) was 800 nm. For bacilli the diameters were 580 nm and 770 nm, respectively. The effect of 0.1% COSs on cell size could be related to their harmful effects on the cell wall,¹⁸ since its integrity is necessary for the maintenance of cell shape.⁴⁴

Besides, a reduction on the number of cells per field in yoghurt supplemented with 0.5 % COS was clearly noticed when compared with the other groups (Fig. 1, 4000X). Probably, lactobacilli and streptococci grown in the presence of 0.5 % COS were less resistant to the treatment with polylysine used to fix cells for scanning electron microscopy. At this concentration, a significant decrease on lactic acid production also evidenced a clear inhibition on bacterial metabolic activity (Table 1).

Fernandes *et al.*¹⁶ found that COS (1.7 KDa, DD 65 %) affected the growth of probiotic bacteria, although cell dimensions

were not affected. In accordance with previous studies on different species,¹⁸ they suggested the prevention of nutrient uptake as a mechanism of growth inhibition. Other COS effects regarding water content were observed in the present study. This could also play a role in the mechanisms that cause bacterial metabolic changes. Considering these preliminary results, concentrations of 0.1 % and 0.05 % were selected for further analyses.

Titrateable acidity and viable counts were monitored for a storage period of 28 days at 4 °C (Fig. 2). The acidity tended to increase from day 1 to day 21, and decrease at day 28. However, these variations were not statistically significant, excepting for titrateable acidity between control and 0.1 % COS yoghurts at day 1 ($p < 0.05$). For viable counts the contrary tendency was observed. Viable counts remained above 10^7 CFU/g over the period and acidity was in the range of 1.2 to 1.8 g lactic acid/100g. These results were in accordance with the standards defined for yoghurts.²⁷

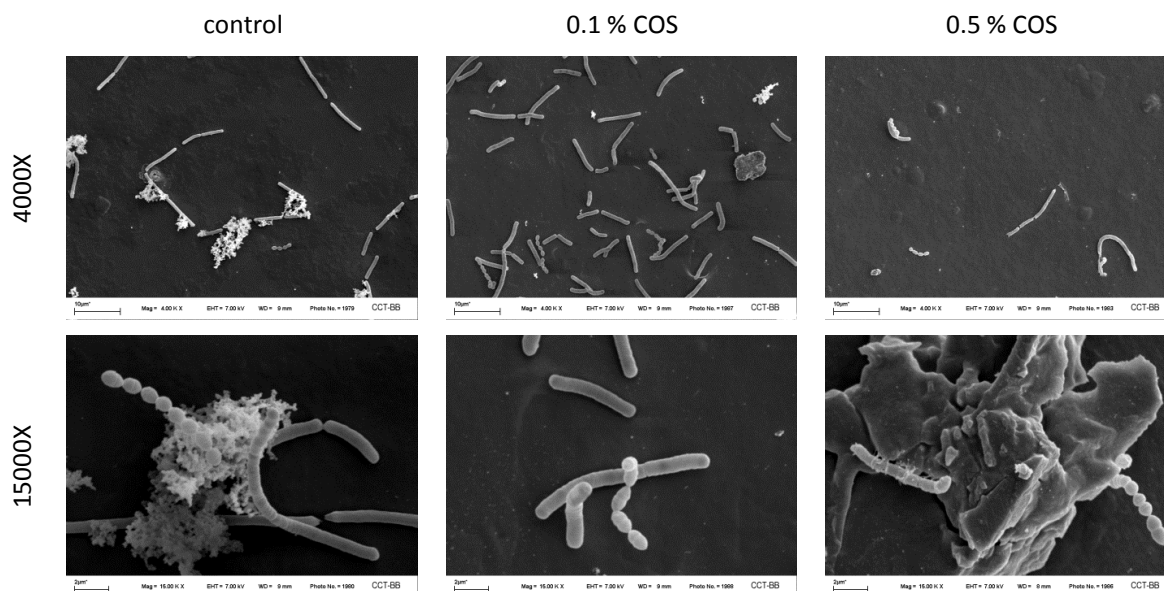


Fig. 1. S.E.M. micrographs of yoghurts supplemented with COS.

Table 2. Fatty acid profiles of yoghurts supplemented with COS over 28 days of storage.

	D1			D21			D28		
	control	0.05%	0.1%	control	0.05%	0.1%	control	0.05%	0.1%
C14:1	0.43 ± 0.25	0.43 ± 0.43	0.00	0.33 ± 0.16	0.42 ± 0.17	0.63 ± 0.31	0.55 ± 0.02	0.43 ± 0.14	0.47 ± 0.14
C14	3.77 ± 3.77	5.17 ± 5.17	2.27 ± 2.07	4.91 ± 2.88	8.09 ± 1.29	5.94 ± 0.36	7.95 ± 0.36	6.74 ± 1.13	7.66 ± 0.99
C15	0.37 ± 0.37	0.67 ± 0.40	0.18 ± 0.18	0.67 ± 0.26	0.45 ± 0.05	0.40 ± 0.12	1.41 ± 0.04	1.21 ± 0.23	1.31 ± 0.24
C16:1	1.48 ± 0.54	1.12 ± 0.39	1.93 ± 0.58	1.06 ± 0.31	1.15 ± 0.42	0.64 ± 0.06	1.77 ± 0.17	1.47 ± 0.09	1.34 ± 0.15
C16:1	-	-	-	-	-	-	0.15 ± 0.15	0.11 ± 0.11	-
C16	27.29 ± 4.05	27.22 ± 5.17	22.96 ± 2.21	26.58 ± 4.81	28.20 ± 2.61	26.11 ± 0.19	30.89 ± 0.74	28.07 ± 0.74	29.16 ± 0.92
C17	1.50 ± 0.56	0.45 ± 0.22	0.70 ± 0.10	1.49 ± 0.25	1.21 ± 0.50	1.26 ± 0.05	1.64 ± 0.04	1.60 ± 0.17	1.55 ± 0.25
LA ^a	4.52 ± 0.26	5.13 ± 0.87	6.10 ± 0.89	6.80 ± 0.81	7.38 ± 0.19	7.40 ± 0.69	6.67 ± 0.56	7.32 ± 0.76	6.84 ± 0.85
OLE ^b	14.79 ± 1.50	19.28 ± 0.91	17.72 ± 1.35	20.13 ± 7.19	16.27 ± 1.05	16.89 ± 0.15	14.67 ± 0.78	19.44 ± 2.93 ^g	15.80 ± 0.60
C18:1	0.62 ± 0.32	0.98 ± 0.24	0.94 ± 0.60	1.25 ± 0.22	1.74 ± 0.33	0.74 ± 0.74	1.52 ± 0.29	1.81 ± 0.44	1.68 ± 0.53
C18:1	1.41 ± 0.32	2.12 ± 0.32	2.31 ± 1.26	2.32 ± 0.83	1.98 ± 0.17	2.06 ± 0.06	1.61 ± 0.21	2.33 ± 0.28	1.95 ± 0.22
C18	33.25 ± 4.16	31.16 ± 8.22	31.49 ± 0.45	30.89 ± 0.13	29.24 ± 3.50	32.10 ± 3.03	28.37 ± 0.94	26.34 ± 1.40	29.19 ± 3.65
CLA ^c	0.46 ± 0.23	0.82 ± 0.09	1.21 ± 0.10 ^f	0.77 ± 0.14	0.67 ± 0.06	0.83 ± 0.20	0.56 ± 0.04	1.13 ± 0.37	0.90 ± 0.03
C20	1.67 ± 1.55	0.26 ± 0.27	1.52 ± 1.09	0.38 ± 0.38	-	0.35 ± 0.35	-	-	-
cyc ^d	-	-	-	-	-	-	0.10 ± 0.10	0.20 ± 0.10	-
NI ^e	8.42 ± 2.50	5.13 ± 0.72	10.64 ± 1.52	2.75 ± 0.39	2.86 ± 0.04	4.58 ± 2.56	2.06 ± 0.15	1.63 ± 0.21	2.20 ± 0.29

^alinoleic acid; ^boleic acid; ^cconjugated linoleic acid; ^dlactobacillic acid; ^enot identified; ^fp<0.05 compared with control D1; ^gp<0.001 compared with control D28.

A chemical gastrointestinal digestion of yoghurts supplemented with 0.1 % COS was simulated by an *in vitro* model. Lipids were extracted from the resulting suspension and analyzed by ^1H NMR. Lipolysis was evidenced by the different patterns of the ^1H NMR signals between 3.5 and 4.5 ppm assigned to glyceryl protons (signals Gly2 on Fig. 3). Before digestion, these signals appeared as a system of two double doublets at 4.29 and 4.15 ppm. The chemical shift and multiplicity of these signals are notably affected by the presence or absence of the acyl chains attached to the glyceryl backbone.⁴⁵ Mono- and di-glycerides were evidenced by new signals appearing between 4.17 ppm to 3.60 ppm. Both digested samples, namely control and supplemented yoghurt, showed almost identical lipolytic and fatty acid profiles (Fig. 3 and Table 3, respectively).

As shown on Table 3, no significant differences were found between CLA percentages of control and supplemented yoghurts.

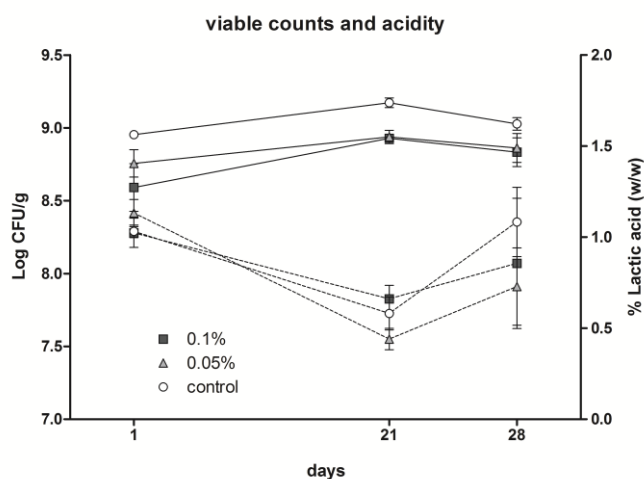


Fig. 2. Titratable acidity (solid line) and viable counts (dashed line) of yoghurts at day 1, 21 and 28 of storage.

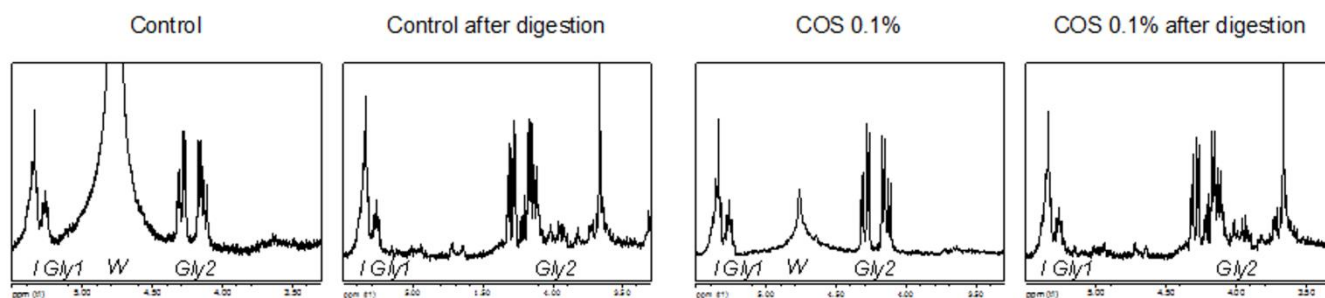


Fig. 3. Section of ^1H NMR spectra of yoghurt lipids. Gly1 and Gly2 refer to glyceryl protons attached to C2 and C1/C3 respectively. I = olefinic protons. W = water signal.

Table 3. Fatty acid profiles of chemically digested yoghurts.

	control	0.1% COS
C14:1	0.54 ± 0.04	0.18 ± 0.18
C14	7.27 ± 0.04	3.36 ± 1.47
C15	1.27 ± 0.06	0.43 ± 0.43
C16:1	2.54 ± 0.43	1.54 ± 1.54
C16:1	0.58 ± 0.14	0.40 ± 0.40
C16	33.85 ± 1.49	30.03 ± 1.53
C17	1.38 ± 0.74	0.23 ± 0.23
LA ^a	7.33 ± 1.77	8.98 ± 3.51
OLE ^b	13.68 ± 0.52	13.88 ± 2.19
C18:1	2.29 ± 0.19	1.46 ± 0.31
C18:1	1.05 ± 0.15	0.54 ± 0.54
C18	25.23 ± 5.0	33.59 ± 12.04
CLA	0.42 ± 0.07	0.17 ± 0.02
NI ^c	2.86 ± 0.89	5.77 ± 0.88

^alinoleic acid; ^boleic acid; ^cnot identified

However, when the fatty acid profiles of Table 3 were compared with data shown in Table 2, it was clear that CLA percentages were reduced after chemical digestion in yoghurts supplemented with COS. During storage, these percentages ranged from 0.6 to 1.2 % in the supplemented groups, but decreased to 0.1 % after digestion. CLA percentages of fresh yoghurts supplemented with COS 0.1% (day 1) were significantly different to CLA percentages after digestion ($p < 0.001$, t -test). In contrast, CLA percentages of controls were not different before and after digestion. This shows that labile substances such as CLA are affected by the presence of COS in chemical environments that mimic gastrointestinal processes.

The application of a sensory methodology affords early results for the food product development, providing prior knowledge with respect to its acceptance on the consumer market and/or specific characteristics. In this study, yoghurt with 0.1 % w/w COS was selected for the affective test since it was the maximum concentration that showed suitable results in performed assays. Good acceptance was obtained by yoghurts with 0.1 % w/w COS, getting scores of 6 to 7 (data not shown).

Moreover, no differences between control and supplemented yoghurts on the studied attributes (overall acceptability, flavor, color and texture) were found in the statistical analysis.

Conclusions

The nutritional and organoleptic properties of yoghurts were not modified at 0.1 % w/w or lower concentrations of COS. Higher concentrations caused metabolic alterations in lactic acid bacteria, evidenced by a decrease in lactic acid production. Consequently, the product obtained did not fulfill the standards defined for yoghurt by the Codex Alimentarius.²⁷ Taking this into account, chitooligosaccharides (MW 2.7 KDa, DD 68 %) could be incorporated into yoghurts at a maximum concentration of 0.1 % w/w with good sensory acceptance. Since the doses required for clinical trials range from 1 % to 5 % per day, it would not be possible to incorporate such quantities into yoghurts without affecting the microbial fermentation process. If a health promoter effect is pursued, the desired amount of COS should be added to yoghurt immediately before consumption. Regarding prebiotics and biopreservatives, doses up to 0.1 % COS could be used without causing metabolic inhibition of starters. Bearing in mind the increasing interest on COS benefits for human health and food industry, the outcomes of the present study will certainly contribute to a better use of these oligosaccharides as food ingredients.

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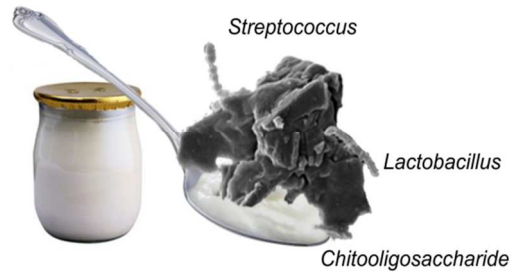
References

- 1 Y-J. Jeon, F. Shahidi and S-K. Kim, *Food Rev. Int.*, 2000, **16**, 159-176.
- 2 B. B. Aam, E. B. Heggset, A. L. Norberg, M. Sørli, K. M. Vårum and V. G. H. Eijsink, *Mar. Drugs*, 2010, **8**, 1482-1517.
- 3 R. K. Baboota, M. Bishnoi, P. Ambalam, K. K. Kondepudi, S. M. Sarma, R. K. Boparai and K. Podili, *J. Func. Foods*, 2013, **5**, 997-1012.
- 4 S. Y. Chae, M-K. Jang and J-W. Nah, *J. Control. Release*, 2005, **102**, 383-394.
- 5 M. Hayes, in *Marine Bioactive Compounds*, ed. M. Hayes, Springer, US, 2012, ch. 4, pp. 115-128.
- 6 European Food Safety Authority, panel on Dietetic Products, Nutrition and Allergies (NDA), Scientific Opinion, EFSA Journal, 2010, **8**, 1687.
- 7 European Food Safety Authority, panel on Dietetic Products, Nutrition and Allergies (NDA), Scientific Opinion, EFSA Journal, 2011, **9**, 2214.
- 8 Food and Drug Administration, GRAS notice GRN0003697, Kitozyme S. A., 2011.
- 9 National Institutes of Health, US, <https://clinicaltrials.gov/ct2/show/study/NCT00468962>, (accessed May 2015).
- 10 H-J. Hwang, J-A. Im and S-H. Kim, *J. Chitin Chitosan*, 2009, **14**, 125-129.
- 11 H. M. Kim, S. H. Hong, S. J. Yoo, K. S. Baek, Y. J. Jeon and S. Y. Choung, *J. Med. Food*, 2006, **9**, 427-430.
- 12 C-R. Choi, E-K. Kim, Y-S. Kim, J-Y. Je, S-H. An, J. D. Lee, J. Howang, S. S. Ki, B-T. Jeon, S-H. Moon and P-J. Park, *Int. J. Food Sci. Nutr.*, 2012, **63**, 103-106.
- 13 M. Roberfroid, G. R. Gibson, L. Hoyles, A. L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watzl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, M-J. Davicco, L. Léotoing, Y. Wittrant, N. M. Delzenne, P. D. Cani, A. M. Neyrinck and A. Meheust, *Brit. J. Nutr.*, 2010, **104**, S1-S63.
- 14 H. W. Lee, Y. S. Park, J. S. Jung and W. S. Shin, *Anaerobe*, 2002, **8**, 319-324.
- 15 T. W. Liang, C. P. Liu, C. Wu and S.L. Wang, *Carbohydr. Polym.*, 2013, **92**, 2141-2148.
- 16 J. C. Fernandes, P. Eaton, I. Franco, O. S. Ramos, S. Sousa, H. Nascimento, A. Gomes, A. Santos-Silva, F. X. Malcata, M. E. Pintado, *Int. J. Biol. Macromol.*, 2012, **50**, 148-152.
- 17 J. C. Fernandes, K. F. Tavaría, S. C. Fonseca, Ó. S. Ramos, M. E. Pintado, and F. X. Malcata, *J. Microbiol. Biotechnol.*, 2010, **20**, 311-318.
- 18 P. Eaton, J. C. Fernandes, E. Pereira, M. E. Pintado and F. X. Malcata, *Ultramicroscopy*, 2008, **108**, 1128-1134.
- 19 S. Wang, J. Wang, H. Mou, B. Luo and X. Jiang, *Foodborne Pathog. Dis.*, 2015, **12**, 360-365.
- 20 J. Šimůnek, I. Koppová, L. Filip, G. Tishchenko and G. Beždecki, *Folia Microbiol.*, 2010, **55**, 379-382.
- 21 J. Šimůnek, V. Brandysová, I. Koppová and J. Šimůnek Jr., *Folia Microbiol.*, 2012, **57**, 341-345.
- 22 G. J. Tsai, Z. Y. Wu and W. H. Su, *J. Food Protect.*, 2000, **63**, 747-752.
- 23 J. C. Fernandes, F. K. Tavaría, J. C. Soares, O. S. Ramos, M. J. Monteiro, M. E. Pintado and F. X. Malcata, *Food Microbiol.*, 2008, **25**, 922-928.
- 24 H. Barreteau, C. Delattre and P. Michaud, *Food. Technol. Biotechnol.*, 2006, **44**, 323-333.
- 25 S. F. Ausar, N. Passalacqua, L. F. Castagna, I. D. Bianco and D. M. Beltramo, *Int. Dairy J.*, 2002, **12**, 899-906.
- 26 A. Y. Tamime, *Eur. J. Clin. Nutr.*, 2002, **56**, S2-S15.
- 27 Codex Standard for Fermented Milks, CODEX STAN 243-2003.
- 28 D. A. Savaiano, *Am. J. Clin. Nutr.*, 2014, **99**, 1251S-1255S.
- 29 O. Adolffsson, S. N. Meydani and R. M. Russell, *Am. J. Clin. Nutr.*, 2004, **80**, 245-256.
- 30 F. Guarner, G. Perdigon, G. Corthier, S. Salminen, B. Koletzko and L. Morelli, *Brit. J. Nutr.*, 2005, **93**, 783-786.
- 31 J. Csapó and É. Varga-Visi, in *Advances in Fermented Foods and Beverages*, ed. W. Holzapfel, Woodhead Publishing, United Kingdom, 2014, ch. 4, pp. 75-105.
- 32 N. Fagali and A. Catalá, *Biophys. Chem.*, 2008, **137**, 56-62.
- 33 J. L. Nauss, J. L. Thompson and J. Nagyvary, *Lipids*, 1983, **18**, 714-719.
- 34 M. Beysseriat, E. A. Decker and D. J. McClements, *Food Hydrocolloids*, 2006, **20**, 800-809.
- 35 M. S. Rodríguez, M. Montero, M. DelloStaffolo, M. Martino, A. Bevilacqua and L. Albertengo, *Carbohydr. Polym.*, 2008, **74**, 797-801.
- 36 G. A. F. Roberts and J. G. Domszy, *Int. J. Biol. Macromol.*, 1982, **4**, 374-377.
- 37 M. C. Terrile, A. Y. Mansilla, L. Albertengo, M. S. Rodríguez and C. A. Casalongué, *Pest Manag. Sci.*, 2015, **71**, 668-674.
- 38 M. DelloStaffolo, N. Bértola, M. Martino and A. Bevilacqua, *Int. Dairy J.*, 2004, **14**, 263-268.
- 39 M. Postek, K. Howard, A. Jhonson and K. McMichael, in *Scanning Electron Microscopy a student's handbook*, Ladd Research Industries, Burlington, US, 1980.
- 40 D. Mazia, G. Schatten and W. Sale, *J. Cell Biol.*, 1975, **66**, 198-200.

ARTICLE

Journal Name

- 41 Association of Official Analytical Chemists, Official methods of analysis, 14th edn., Washington, US, 1984.
- 42 Association of Official Analytical Chemists, Official methods of analysis, 13rd edn., Arlington, US, 1995.
- 43 M. O. Ramírez-Sucre and J. F. Vélez-Ruiz, *LWT - Food Sci. Technol.*, 2013, **51**, 233-241.
- 44 G. Misra, E. R. Rojas, A. Gopinathan and K. C. Huang, *Biophys. J.*, 2013, **104**, 2342-2352
- 45 B. Nieva-Echevarría, E. Goicoechea, M. J. Manzanos and M. D. Guillén, *Food Res. Int.*, 2014, **66**, 379–387.



Chitooligosaccharides can be added to yoghurt at low concentrations without affecting nutritional composition and sensory acceptance.