**Effect of milk protein glycation and gastrointestinal digestion on
the growth of bifidobacteria and lactic acid bacteria**

¹Marta Corzo-Martínez, ²Marta Ávila, ¹F. Javier Moreno, ¹Teresa Requena and
¹Mar Villamiel

¹Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM),
Nicolás Cabrera 9, 28049, Madrid, Spain.

²Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA),
Carretera de La Coruña km 7, 28040, Madrid, Spain.

*Author to who correspondence should be addressed

Tel +34 910017951; Fax +34 910017905

E-mail: m.villamiel@csic.es

Current address: Instituto de Investigación en Ciencias de la Alimentación, CIAL
(CSIC-UAM), Nicolás Cabrera 9, 28049, Madrid, Spain.

Abstract

In this paper, β -lactoglobulin (β -Lg) and sodium caseinate (SC) have been glycosylated via Maillard reaction with galactose and lactose and, subsequently, the effect of glycoconjugates hydrolyzed under simulated gastrointestinal digestion on the growth of pure culture of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* has been investigated. Glycopeptides were added to the growth media as the sole carbon source. None of the bacterial strains was able to grow in hydrolysates of native and control heated β -Lg and SC. However, glycopeptides were fermented, in different degree, by *Lactobacillus* and *Bifidobacterium* and hardly any effect was detected on the growth of *Streptococcus*. Digested β -Lg glycoconjugates showed a strain-dependent effect whereas growth profiles of bacteria when hydrolysates of SC glycoconjugates were used as substrates were very similar, regardless of the strain. A general preference towards peptides from β -Lg/SC glycosylated with galactose, particularly at the state of the reaction in which the highest content in the Amadori compound tagatose-lysine is present, was observed. SC glycoconjugates were quickly fermented by some strains, promoting their growth in a greater extent than β -Lg complexes or even glucose. Therefore, from the results obtained in this work it can be concluded that conjugation of both milk proteins with galactose and lactose via the Maillard reaction could be an efficient method to obtain novel food ingredients with a potential prebiotic character.

Keywords: Tagatose-lysine; Lactulose-lysine; β -lactoglobulin; Sodium caseinate; *Bifidobacteria*; Lactic acid bacteria.

1. Introduction

Nowadays, it is possible to dispose of products which afford not only the basic nutritional value but also a wide range of benefits that can contribute to improve consumer well-being. In this respect, due to the fact that severe health disorders can be related to the gastrointestinal function, some of the functional ingredients more demanded by consumers are those with a positive effect on gut microbiota (Saaraela et al., 2002). These ingredients are non-digestible carbohydrates with well-known properties, being lactulose (Tuohy et al., 2002), tagatose (Laerke and Jensen, 1999; Laerke et al., 2000; Jensen et al., 2001), fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (MacFarlane et al., 2008) some of the main oligosaccharides considered as prebiotics.

On the other hand, deliberated glycosylation via the Maillard reaction (MR) or glycation has been largely investigated during the last years as a promising approach to alter the functional properties of proteins for food purposes (Oliver et al., 2006a). Concretely, it has been demonstrated that milk proteins (caseins and whey proteins), usually obtained as by-products in the dairy industry, can increase their degree of applicability by means of glycation. Thus, controlling the reaction conditions it is possible to obtain neoglycoconjugates with different glycation and aggregation degrees, being preferred, in general, the early steps of the reaction (Jimenez-Castaño et al., 2005a, 2005b; Kato, 2002; Oliver et al., 2006a). Particularly interesting could be the case of the conjugation of milk proteins with galactose and lactose, which are isomerised to tagatose and lactulose, respectively, giving rise to the corresponding Amadori compounds, tagatosyl- and lactulosyl-lysine. Taking into account the prebiotic properties of tagatose and lactulose, it could be expectable some effect of these glycoconjugates on gut microbiota.

In spite of the number of works on protein glycation, any information about intestinal absorption and endogenous metabolism of Amadori compounds is still fragmentary. According to several authors, it seems that these initial products of the MR are scarcely digested and excreted, being fermented in the distal colon (Erbersdobler and Faist, 2001; Finot, 1973; Finot, 2005; Lee and Erbersdobler, 1994; Sanz et al., 2007). In this sense, the study of the effect of the Amadori compounds tagatosyl- and lactulosyl-lysine could be of great interest, since such compounds might reach more distal areas of gut than tagatose and lactulose and be fermented by the present microbiota. This is particularly relevant since the incidence of certain chronic gut disorders is much higher in the descending colon and rectum than in the first sections of the large gut (Gibson et al., 2004). Mills et al. (2008), using *in vitro* assays with faecal slurries, pointed out that glycated protein bovine serum albumin modulated the colonic microbiota of volunteers with ulcerative colitis towards a more detrimental composition with significant increases in putatively harmful bacteria and decreases in dominant and putatively beneficial bacterial group. This trend was not completely confirmed in non-ulcerative colitis volunteers.

Thus, the purpose of this work has been to study the effect of glycoconjugates obtained by glycosylation via the Maillard reaction of milk proteins (β -lactoglobulin and sodium caseinate) with galactose and lactose, and subsequently subjected to simulated gastrointestinal digestion, on the growth of twelve potential probiotic strains of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* to broaden the knowledge on the role of Amadori compounds in the modulation of the human gut microbiota.

2. Materials and Methods

2.1 Materials

Glucose (Glu), galactose (Gal), lactose (Lac), tagatose (Tag), lactulose (Lu) and bovine β -lactoglobulin (β -Lg) (mixture of A and B variants) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and sodium caseinate (SC) (Rovita FN 5) was obtained from Proveedora Hispano Holandesa, S.A. (Barcelona, Spain).

Streptococcus salivarius ZL50-7, *Lactobacillus reuteri* R13, *Lactobacillus plantarum* CLC17, *Lactobacillus delbrueckii* ZL96-27, *Lactobacillus brevis* CLC23, *Lactobacillus gasseri* Lc9 and *Bifidobacterium breve* 26M2 belonged to the culture collection of the Nutrition, Bromatology and Food Technology Department of the Faculty of Veterinary Science (Universidad Complutense de Madrid). *Streptococcus thermophilus* STY-31, *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB12 and *Lactobacillus casei* LC-01 strains had been previously purified from a commercial symbiotic product (Simbiotic Drink; Priégola, Madrid, Spain) at our laboratory (Tabasco et al., 2007). *Lactobacillus plantarum* IFPL722, isolated from cheese, was taken from our IFPL culture collection. All cultures were maintained at -80°C in MRS broth (Pronadisa, Madrid, Spain) or, in the case of *S. salivarius* ZL50 and *S. thermophilus* STY-31, in M17 broth (Pronadisa), both supplemented with glycerol (40% v/v) and subcultured in MRS or M17 broth before use in experiments.

2.2 Preparation and purification of glycoconjugates

Carbohydrates, Gal or Lac, and β -Lg in a weight ratio of 1:1 or 2:1, respectively, were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (Merck, Darmstadt, Germany), and lyophilized. The β -Lg-Gal powders were kept at 40 and 50 $^{\circ}\text{C}$ for 24

and 48 h, respectively (Corzo-Martínez et al., 2008), whilst the β -Lg-Lac powders were kept at 60 °C for 8 and 48 h (Table 1) (Fenaille et al., 2004), under vacuum in a desiccator equilibrated at an a_w of 0.44, achieved with a saturated K_2CO_3 solution (Merck). In addition, control experiments were performed with β -Lg stored at 40, 50 and 60 °C without reducing sugars during the same periods (control heated β -Lg). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

Carbohydrates, Gal or Lac, and sodium caseinate (SC) in a weight ratio of 0.2:1 were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (Merck) and lyophilized (Corzo-Martínez et al., 2010b). The SC-Gal powders were kept at 60 °C and 50 °C for 4 and 72 h, respectively, whilst the SC-Lac powders were kept at 60 °C for 8 and 24 h (Table 1), under vacuum in a desiccator equilibrated at an a_w of 0.67 (Oliver et al., 2006b), achieved with a saturated solution of $CuCl_2$ (Sigma-Aldrich). In addition, control experiments were performed with SC stored at 50 and 60 °C without reducing sugars during the same periods (control heated SC). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL. To remove free carbohydrate, 2 mL portions were ultrafiltered through hydrophilic 3 kDa cut-off membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1548g for 2 h. After removal of free Gal or Lac, samples were reconstituted in distilled water at a concentration of 2 mg/mL for further analysis (Corzo-Martínez et al., 2008).

Taking into account the analyses of structural characterization previously carried out in our laboratory (Corzo-Martínez et al., 2008; Corzo-Martínez et al., 2010a; Laparra et al., 2011), two types of glycoconjugates were obtained at different stages of

the Maillard reaction after incubation of every combination of carbohydrate and protein under conditions indicated above (Table 1). One of them, in early stages of the MR (β -Lg:Gal [24 h, 40 °C], β -Lg:Lac [8 h, 60 °C], SC:Gal [4 h, 60 °C] and SC:Lac [8 h, 60 °C]), consisted primarily of complexes with a high content of the Amadori compound tagatosyl- or lactulosyl-lysine and a low aggregation level, while those glycoconjugates incubated under more severe conditions, in advanced stages of the MR (β -Lg:Gal [48 h, 50 °C], β -Lg:Lac [48 h, 60 °C], SC:Gal [72 h, 50 °C] and SC:Lac [24 h, 60 °C]), exhibited a high glycation degree, considerable amounts of advanced glycation products (AGEs and melanoidins), and a high aggregation level.

2.3 *In vitro gastrointestinal digestion*

All SC and β -Lg glycoconjugates, as well as the control heated SC/ β -Lg samples were digested *in vitro* by following the simplified procedure described by Moreno et al. (2005). This digestion model was based on *in vivo* data obtained by gastric and duodenal aspiration and from collection of effluent from ileostomy volunteers at the Institute of Food Research (Norwich, UK).

For the gastric digestion step, glycoconjugates (3 mg) were dissolved in 1 mL of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5). The pH was adjusted to 2.5 with 1 M HCl if necessary. A solution of 0.32% (w:v) porcine pepsin (EC 3.4.23.1) in SGF (pH 2.5) (Sigma, activity of 3,300 units/mg of protein) was added at an approximately physiological ratio of enzyme to substrate (1:20, w:w). The digestion was performed at 37 °C for 2 h. For the intestinal digestion step, the pH was increased to 7.5 with 40 mM NH_4CO_3 (Panreac, Barcelona, Spain) dropwise to inactivate pepsin, and the following was added to adjust the pH to 6.5 and simulate a duodenal environment: (i) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and

glycodeoxycholic acid (Sigma), (ii) 1 M CaCl₂ (Panreac), and (iii) 0.25 M Bis-Tris (pH 6.5) (Sigma). Solutions of porcine trypsin (EC 3.4.21.4; 0.05%, w:v, Sigma, type IX-S, activity of 14,300 units/mg of protein) and bovine α -chymotrypsin (EC 3.4.21.1; 0.1%, w:v, Sigma, type I-S, activity of 62 units/mg of protein) in water were prepared and added at approximately physiological protein:trypsin:chymotrypsin ratios [1:(1/400):(1/100) (w:w:w)]. Simulated intestinal digestion of β -Lg was carried out at 37 °C for 15 min. After protein hydrolysis, trypsin and chymotrypsin were inactivated by heating at 80 °C for 5 min. Digestions were performed without any derivatization of the sulfhydryl groups of cysteine residues in order to remain as close as possible to physiological conditions.

2.4 Analysis of digested samples

Analysis of digested samples was carried out using a Beckman HPLC system equipped with a Phenomenex Jupiter Proteo column (250 mm $\times$ 4.6 mm, 4 mm particle size, 90 Å pore size). Samples were eluted using 0.1% (v/v) TFA in double-distilled water as solvent A and 0.1% (v/v) TFA in double-distilled water and acetonitrile of HPLC grade (Scharlau Chemie, Barcelona, Spain) (1:9, v/v) as solvent B, following the method described by Moreno et al. (2004).

For SDS-PAGE analysis, 32.5 mL of the samples taken at different stages of the digestion were added to 12.5 mL of 4 $\times$ NuPAGE[®] LDS Sample buffer (Invitrogen, CA, USA) and 5 mL of 0.5 M dithiothreitol (DTT, Sigma-Aldrich), and heated at 70 °C for 10 min. Samples (20 mL) were loaded onto a 12% polyacrylamide NuPAGE[®] Novex Bis Tris pre-cast gel and a continuous 2-(N-morpholino)ethanesulfonic acid (MES) SDS

running buffer was used. Gels were run for 40 min at 120 mA gel⁻¹ and 200 V and stained using the Colloidal Blue Staining Kit (Invitrogen).

2.5 Bacterial growth on digested glycoconjugates

For experiments, strains were grown into MRS fermentation broth (Pronadisa), which does not contain either Glu or meat extract (De Man et al., 1960), enriched with 0.2% Tween 80, 0.8% casein acid hydrolysate and 0.05% cysteine, or ESTY broth (Pronadisa), without any carbon source. These media were supplemented with digested β -Lg and SC glycoconjugates at a final concentration of 0.3% (w/v) of the sugar moiety of the molecules. Glu, Tag, Lu, bovine native β -Lg and SC, and β -Lg and SC heated without reducing sugars were used as controls, at the same final concentration that corresponding glycoconjugates. Each substrate was weighed into sterile tubes, then the corresponding autoclaved basic medium was added, and the mixtures were sterilized by filtration (0.22 μ m).

Bacterial strains were cultured overnight at 1% in MRS or M17 broth and grown cultures were diluted 1:10 in MRS fermentation broth or in ESTY broth without any carbon source. Culture dilutions were used as inocula to evaluate the growth of bacterial strains on galactosylated and lactosylated peptides derived from *in vitro* gastrointestinal digestion and on controls. Basic media plus the tested substrates were inoculated with 1% of diluted inocula of each strain and pipetted into 300 μ l wells of sterile 96-well microplates with lid (Sarstedt Inc., Newton, USA). All strains were grown in aerobic conditions at 37 °C for 24 h, in triplicate, excepting *L. acidophilus* LA-5, *L. gasseri* Lc9, *B. breve* 26M2 and *B. lactis* BB12, which were incubated under anaerobic conditions (Gas-Pack, Anaerogen; Oxoid Ltd., Hampshire, England), also in triplicate. The optical densities (OD₆₀₀) of the aerobic strains were recorded at 20-60 min intervals with an

automated microplate reader (Varioskan Flash, Thermo Electron Corporation, Vantaa, Finland) at 600 nm. Maximum growth rates ($\mu_{\max}$) and lag parameters (lag) were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v. 2.1 (Barayni and Roberts, 1994) (available at <http://www.ifr.ac.uk/safety/DMfit/default.html>). In the case of the anaerobic strains, OD₆₀₀ was recorded at 0 h and 24 h. Blanks with medium and carbon source added but without bacteria were carried out.

2.6. Statistical analysis

Statistical analysis was performed using the Statgraphic 5.1 Program (Statistical Graphics Corporation, Rockville, MD, USA) for Windows. One-way analysis of variance (ANOVA) (least significant difference, LSD, test) was used for the statistical evaluation of results derived from the measurement of maximum optical density (OD₆₀₀) reached for each bacterial culture with each substrate studied. Differences were considered significant when $P < 0.05$.

3. Results and Discussion

3.1 Characterization of hydrolysates obtained after *in vitro* gastrointestinal digestion of sodium caseinate and β -lactoglobulin glycoconjugates

To simulate the physiological conditions, glycoconjugates were subjected to an *in vitro* gastrointestinal digestion process consisting of a first stage of gastric digestion with pepsin for 2 h at 37 °C and a second stage of duodenal digestion with trypsin/chymotrypsin for 15 min at 37 °C; then, the digested glycoconjugates were analysed by SDS-PAGE. As observed in Figure 1, control heated β -Lg (lanes a and c)

was rapidly broken down during simulated duodenal digestion, and only traces of intact protein could be observed after 15 min of incubation with trypsin/chymotrypsin. However, in the glycated protein, particularly in β -Lg:Gal [48 h, 50 °C], two bands corresponding to its monomeric and dimeric forms could be clearly visualized after digestion. Glycation with Lac had a similar influence on β -Lg digestibility (data not shown). These results confirmed that glycation and glycation-induced aggregation of β -Lg during the advanced stages of the MR, protects β -Lg from proteolysis during *in vitro* gastrointestinal digestion. In fact, in a previous work carried out in our laboratory (Laparra et al., 2011), by means of SEC analysing of digested β -Lg:Gal/Lac conjugates, we observed an increase in the area of the less retained peaks (corresponding to the formation of aggregated and larger peptides) after incubation under more severe conditions, i.e. 48 h at 50 °C (in the case of glycation with Gal) and 48 h at 60 °C (in the case of glycation with Lac).

SC glycoconjugates were more efficiently digested than complexes derived from β -Lg, regardless the employed glycation conditions (Figure 2). This result can be attributed to the fact that caseins have a flexible and linear conformation rather than a rigid and compact structure (Kaminogawa, 2000), facilitating their enzymatic digestion. In contrast, β -lactoglobulin presents a high structural stability at acid pH, having its peptic cleavage sites (hydrophobic or aromatic amino acid side chains) buried inside its characteristic β -barrel structure, forming a strong hydrophobic core and preventing hydrolysis (Dalgalarondo et al., 1995; Reddy et al., 1988). Thus, as observed in the chromatographic profiles shown in Figure 2, both unglycated and glycated SC were quickly proteolyzed, even in the case of SC glycated under the conditions that promote high protein aggregation levels. Nevertheless, SEC analysis also indicated the higher size of peptides contained in hydrolysates of SC glycoconjugates obtained under more

severe conditions, particularly when SC was glycated with Gal at 50 °C for 72 h (Laparra et al., 2011).

3.2 Bacterial growth on digested β -lactoglobulin:galactose/lactose conjugates

Growth curves for representative strains grown aerobically on peptides from native, control heated and glycated β -Lg, and on Tag, Lu and Glu as sole carbon sources are shown in Figure 3. None of the bacterial strains was able to grow in hydrolysates of native and control heated β -Lg (data not shown). However, in general, the sugar moiety from the digested glycoconjugates was fermented, in different degree, by all microorganisms assayed, except for *S. thermophilus* STY-31 and *S. salivarius* ZL50-7. As observed in Figure 3, the strains showed very different metabolic activity for the same substrate (behaviour strain-dependent), giving raise to different growth profiles. A general preference towards peptides from β -Lg glycated with Gal, particularly at 40 °C for 24 h, over those from β -Lg:Lac conjugates was observed, the former being efficiently metabolized by all strains of *Lactobacillus*.

In order to relate the bacterial growth to substrate utilization, we calculated the maxima growth rates ($\mu_{\max}$) for each of the samples studied. These values and lag times (lag) are shown in Table 2. Likewise, the corresponding values of maximum DO₆₀₀ are also shown in Table 3.

L. casei LC-01 was able to grow in all the tested substrates, reaching a $\mu_{\max}$ of 0.142 h⁻¹ with Lu as carbon source (Table 2), this being similar to that achieved with Glu and Tag. When *L. casei* LC-01 was grown with the conjugate β -Lg:Gal [24 h, 40 °C] digested, it achieved cell densities similar to Glu and Tag, with maximum OD₆₀₀ of 0.9 (Figure 3 and Table 3). In addition, although *L. casei* LC-01 growth on digested β -

Lg:Gal [24 h, 40 °C] was slower (0.048 h^{-1}) than on Glu and Tag (0.135 and 0.129 h^{-1} , respectively), the lag time of growth on glycoconjugate was zero, whilst on Glu and Tag it was 7 h, approximately (Table 2). Regarding peptides from β -Lg:Gal [48 h, 50 °C], β -Lg:Lac [8 h, 60 °C] and β -Lg:Lac [48 h, 60 °C], they presented lower cell densities, with OD_{600} values below 0.7 (Table 3), and slower growth than Glu, Tag and Lu, although their lag time was reduced, except in the case of β -Lg:Lac [48 h, 60 °C].

L. reuteri R13, *L. plantarum* IFPL722 and *L. plantarum* CLC17 showed a similar behaviour. Tagatose was not efficiently used as carbon source by any of the three strains. However, they were able to grow with Glu and Lu, reaching cell densities particularly high with Lu ($\text{OD}_{600} \sim 1.2$) in the case of *L. plantarum* IFPL722, and with Glu ($\text{OD}_{600} \sim 1$) in *L. reuteri* R13. When digested β -Lg:Gal [24 h, 40 °C] was used as carbon source, OD_{600} and μ_{max} values of these three strains were similar to those observed with Glu (Figure 3, Tables 2 and 3). In addition, the lag time of growth on this compound was reduced to zero. Likewise, although to a lesser extent than peptides from β -Lg:Gal [24 h, 40 °C], hydrolysate of β -Lg glycosylated with Gal at 50 °C for 48 h also promoted the growth of these bacterial strains. In contrast, incubation with peptides from β -Lg:Lac [8 and 48 h, 60 °C] conjugates as substrates resulted in a poor growth of *L. reuteri* R13, *L. plantarum* IFPL722 and *L. plantarum* CLC17, reaching OD_{600} and μ_{max} values markedly lower than with Lu.

L. delbrueckii ZL95-27 and *L. brevis* CLC23 did not utilize Tag or Lu as carbon source. Both strains reached the highest cell densities and the fastest growth rates with digested β -Lg:Gal [24 h, 40 °C], displaying no-lag time period (Table 2). This was particularly noticeable in the case of *L. brevis* CLC23, which fermented this compound even faster than Glu, giving rise to significant differences in the maximum DO_{600} values (Table 3). Digested β -Lg:Gal [48 h, 50 °C] was also efficiently metabolized by

both strains, although its fermentation gave rise to lower cell densities and $\mu_{\max}$ values than that of digested β -Lg:Gal [24 h, 40 °C]. Regarding to digested β -Lg:Lac conjugates, as Lu, they did not promote the growth of *L. delbrueckii* ZL95-27 and *L. brevis* CLC23.

Unlike the previous mentioned strains of *Lactobacillus*, *S. salivarius* ZL50-7 and *S. thermophilus* did not efficiently utilize any of the studied β -Lg glycoconjugates or Tag, only growing on Glu and Lu, being especially noteworthy the fast growth rate of *S. salivarius* ZL50-7 with Glu ($\mu_{\max} = 0.747$, the highest $\mu_{\max}$ of all strains and substrates studied).

With respect to the growth of the anaerobic strains, OD₆₀₀ values for the carbon sources under study are shown in Table 4. *B. breve* 26M2 achieved higher and significantly different cell densities with Lu than with Glu as carbon source. However, no growth of *B. lactis* BB-12 was observed with Lu as substrate after 24 h of incubation. Likewise, none of digested β -Lg:Lac conjugates under study was metabolized by *B. breve* 26M2 and *B. lactis* BB12. However, it is noteworthy the ability of both strains of bifidobacteria to show some growth after 24 h of incubation with β -Lg:Gal conjugates, reaching OD₆₀₀ values of up to 0.38, unlike Tag, which was not used as substrate. *L. gasseri* Lc9 was not able to grow with Tag, Lu or β -Lg:Lac conjugates as carbon sources. The maximum growth of this strain was recorded on hydrolysates of β -Lg galactosylated, particularly at 40 °C for 24 h, with an OD₆₀₀ of 0.522. Finally, *L. acidophilus* LA-5 stood out by its ability to utilize all the digested β -Lg:Gal and β -Lg:Lac conjugates studied, unlike Lu, which was not used as carbon source.

In summary, the bifidobacteria and lactic acid bacteria studied did not ferment efficiently the hydrolysates of β -Lg:Lac conjugates. In contrast, all bacterial strains assayed, excepting streptococci, grew to some extent on hydrolysates of β -Lg:Gal

conjugates, both [24 h, 40 °C] and [48 h, 50 °C], observing high $\mu_{\max}$ values and no-lag time periods. This was particularly notable when it was used as substrate the hydrolysate derived from galactosylated β -Lg at 40 °C for 24 h, with the highest content in the Amadori compound tagatosyl-lysine, suggesting that it has a positive effect on the strains studied, which even might be higher than that of Tag. However, other strains should be tested in further studies to confirm these results. In addition, the comparison with other prebiotics could also be interesting.

On the other hand, the lower bacterial growth observed with the hydrolysate of galactosylated β -Lg at 50 °C for 48 h could be attributed to its higher aggregation degree, lower digestibility (Figure 1), and, consequently, the higher difficulty to bacteria to metabolize peptides obtained after its digestion, with a higher size and aggregation degree than those derived from β -Lg:Gal [24 h, 40 °C] (Laparra et al., 2011).

3.3 Bacterial growth on digested sodium caseinate:galactose/lactose conjugates

Figure 3 also depicts growth curves for strains grown in aerobic conditions on peptides from digested SC glycoconjugates as sole carbon sources. Maxima growth rates ($\mu_{\max}$), lag times (lag) and maxima DO₆₀₀ values for each of substrates studied are also shown in Tables 2 and 3, respectively.

While none of the microorganisms assayed was able to grow on digests from native and control heated SC (data not shown), peptides from SC glycoconjugates were fermented in a strain-dependent way by all tested bacterial strain, in some cases even faster and leading to higher cell densities than Glu, and promoting bacterial growth in greater extent than peptides from digested β -Lg complexes. This is probably due to the better digestibility of SC glycoconjugates as compared to those of β -Lg, which allows

the formation of smaller peptides (Laparra et al., 2011) that could be, therefore, more easily internalized and metabolized by bacteria than those derived from gastrointestinal digestion of β -Lg glycoconjugates.

Moreover, unlike digested β -Lg glycoconjugates, which showed a strain-dependent effect, growth profiles obtained when hydrolysates of SC glycoconjugates were used as substrates were very similar, regardless of the strain. The growth of most of the lactic acid bacteria assayed resulted particularly favoured in presence of digested SC:Gal [4 h, 60 °C] complex, showing no-lag time periods and reaching the highest OD₆₀₀ (ranging from 0.85 to 1.6) and $\mu_{\max}$ values (ranging from 0.232 to 0.673 h⁻¹) of all the substrates tested, including Tag (Tables 2 and 3). These results support that the Amadori compound tagatosyl-lysine might promote the growth of gut microbiota in greater extent than Tag, according to the previous results showed for β -Lg glycoconjugates.

Moreover, although to a lesser extent than SC:Gal [4 h 60 °C], digested SC:Gal [72 h, 50 °C] was also used more efficiently and faster than Tag as carbon source by all the bacterial strains assayed, with the exception of streptococci. The ability of the latter SC:Gal glycoconjugate to promote the bacterial growth lower than the former could be attributed to the higher size of SC:Gal [72 h, 50 °C] peptides generated after digestion (Laparra et al., 2011), which could present a higher difficulty to be metabolized by bacteria than SC:Gal [4 h, 60 °C] peptides.

On the other hand, all the bacterial strains utilized the digested SC:Lac conjugates particularly that obtained after 8 h of incubation at 60 °C, with the highest content in the Amadori compound lactulosyl-lysine. Using this digested conjugate as substrate, all lactobacilli reached higher maximum OD₆₀₀ values (from 0.85 to 1.5) and faster growth rates (from 0.192 to 0.474 h⁻¹) than with Lu, displaying no-lag time periods (Tables 2

and 3). These results suggest that the Amadori compound lactulosyl-lysine, derived from SC glycation with Lac, might have a greater beneficial effect than Lu on most of the pure cultures studied.

In good agreement with these results, cell densities of anaerobic strains (Table 4) reached higher OD₆₀₀ values after 24 h of incubation with digested SC galactosylated, particularly at 60 °C for 4 h, than with Tag, this being specially noticeable in the case of bifidobacteria, which were unable to grow with Tag as carbon source. Likewise, hydrolysates of lactose conjugates also led to high cell densities, giving rise to maximum OD₆₀₀ values after 24 h of incubation much higher than with Lu. *L. gasseri* Lc9 and *L. acidophilus* LA-5 cell densities increased with SC:Gal [4 h, 60 °C], SC:Lac [8 h, 60 °C] and SC:Lac [24 h, 60 °C] as substrates considerably more than with Glu. Moreover, it is noteworthy that, with bifidobacteria, the highest OD₆₀₀ values were achieved when hydrolysates of SC:Lac conjugates were used as substrates. This suggests that the Amadori compound lactulosyl-lysine, formed during the initial stages of the MR between SC and Lac, might possess a particularly positive effect on bifidobacteria growth.

4. Conclusions

The effect of digested β -Lg glycoconjugates on the growth of the potential probiotic strains assayed was strain-dependent, whereas growth profiles of bacteria when hydrolysates of SC glycoconjugates were used as substrates were very similar, regardless of the strain. Digests of β -Lg:Gal conjugates were notably fermented by all lactobacilli and bifidobacteria assayed, but not by streptococci, a higher bacterial growth than with tagatose being appreciated in most of the cases. In addition, such glycoconjugates promoted bacterial growth more efficiently than β -Lg:Lac complexes.

On the other hand, digests of SC glycated with Gal and Lac favoured in great extent the growth of all the bacterial strains studied, higher cell densities and faster growth with digested glycoconjugates than with tagatose and lactulose as substrates being observed.

Regarding to the glycation degree, a higher bacterial growth was generally detected with glycoconjugates at initial stages of the MR, with the highest content in the Amadori compound tagatosyl- or lactulosyl-lysine, than with those at advanced stages of the reaction.

Therefore, from the findings described in this work we can infer that conjugation of both milk proteins with galactose and lactose via the Maillard reaction could be an efficient method to obtain novel food ingredients with a potential prebiotic character. To corroborate these results, other probiotic strains should be tested in further studies, comparing, moreover, the effect of Maillard conjugates with that of other prebiotics .

In addition, future studies are needed to determine the effect of these Maillard complexes on mixed cultures from human faeces, this representing a model closer to what might occur *in vivo*.

Acknowledgments

This work has been funded by projects Consolider Ingenio 2010 FUN-c-FOOD CSD2007-00063 (MICINN), ALIBIRD S2009/AGR-1469 (CAM), AGL2009-13361-C02-02 (MICINN) and IBEROFUN 110AC0386 (CYTED). M. Corzo-Martínez thanks Danone Institute for a grant.

References

- Dalgalarondo, M., Dufour, E., Chobert, J.-M., Bertrand-Harb, C., Haertlé, T., 1995. Proteolysis of β -lactoglobulin and β -casein by pepsin in ethanolic media. *International Dairy Journal*, 5, 1-14.
- Corzo-Martinez, M., Moreno, F.J., Olano, A., Villamiel, M., 2008. Structural characterization of bovine β -lactoglobulin-galactose/tagatose Maillard complexes by electrophoretic, chromatographic and spectroscopic methods. *Journal of Agricultural and Food Chemistry*, 56, 4244-4252.
- Corzo-Martinez, M., Soria, A.C., Belloque, J., Villamiel, M., Moreno, F.J., 2010a. Effect of glycation on the gastrointestinal digestibility and immunoreactivity of bovine β -lactoglobulin. *International Dairy Journal*, 20, 742-752.
- Corzo-Martinez, M., Moreno, F.J., Villamiel, M., Harte, F.M., 2010b. Characterization and improvement of rheological properties of sodium caseinate glycated with galactose, lactose and dextran. *Food Hydrocolloids*, 24, 88-97.
- Erbersdobler, H.F., Faist, V., 2001. Metabolic transit of Amadori products. *Nahrung/Food*, 45, 177-181.
- Finot, P.-A., 1973. Non enzymatic browning. In: Porter, J.W.G., Rolls, B.A. (Eds.), *Proteins in human nutrition*. Academic Press, London, pp. 501-514.
- Finot, P.-A., 2005. The absorption and metabolism of modified amino acids in processed foods. *Journal of AOAC International*, 88, 894-903.
- Gibson, G.R., Probert, H.M., Van Loo, J., Rastall, R.A., Roberfroid, M., 2004. Dietary modulation of the human colonic microbiota: updating the concept of probiotics. *Nutritional Research Reviews*, 17, 259-275.

468 Jensen, B.B., Buemann, B., Bertelsen, H.D., 2001. Tagatose changes the
469 composition of the microbiota and enhances butyrate production by human faecal
470 samples. *American Journal of Clinical Nutrition*, 73, 497S-497S.

471 Jimenez-Castaño, L., López-Fandiño, R., Olano, A., Villamiel, M., 2005a. Study
472 on β -lactoglobulin glycosylation with dextran: effect on solubility and heat stability.
473 *Food Chemistry*, 93, 689–695.

474 Jimenez-Castaño, L., Villamiel, M., Martín-Alvarez, P.J., Olano, A., Lopez-
475 Fandiño, R., 2005b. Effect of the dry-heating conditions on the glycosylation of β -
476 lactoglobulin with dextran through the Maillard reaction. *Food Hydrocolloids*, 19 (5),
477 831-837.

478 Kaminogawa, S., 2000. Food allergens and the mucosal immune system.
479 *Biofactors*, 12, 29-32.

480 Kato, A., 2002. Industrial applications of Maillard-type protein polysaccharide
481 conjugates. *Food Science and Technology Research*, 8, 193-199.

482 Laerke, H.N., Jensen, B.B.D., 1999. Tagatose has low small intestinal digestibility
483 but high large intestinal fermentability in pigs. *Journal of Nutrition*, 129, 1002-1009.

484 Laerke, H.N., Jensen, B.B., Hojsgaard, S., 2000. *In vitro* fermentation pattern of
485 D-tagatose is affected by adaptation of the microbiota from the gastrointestinal tract of
486 pigs. *Journal of Nutrition*, 130, 1772–1779.

487 Laparra, J.M., Corzo-Martínez, M., Villamiel, M., Moreno, F.J., Sanz, Y., 2011.
488 Maillard-type glycoconjugates from dairy proteins inhibit adhesion of *Escherichia coli*
489 to mucin. *Food Chemistry*, 129, 1435-1443.

490 Lee, K., Erbersdobler, H.F., 1994. Balanced experiments in human volunteers
491 with epsilon-fructoselysine and lysinoalanine. In: Labuza, T.P., Reineccius, G.A.,

492 Monnier, V.M., O'Brien, J., Baynes, J.W., (Eds.), Maillard reactions in chemistry, food
 493 and health. The Royal Society of Chemistry, Cambridge, pp. 358-363.

494 MacFarlane, G.T., Steed, H., MacFarlane, S., 2008. Bacterial metabolism and
 495 health-related effects of galacto-oligosaccharides and other prebiotics. Journal of
 496 Applied Microbiology, 104, 305-344.

497 Mills, D.J.S., Tuhoy, K.M., Booth, J., Buck, M., Crabbe, M.J.C., Gibson, G.R.,
 498 Ames, J.M., 2008. Dietary glycated protein modulates the colonic microbiota towards a
 499 more detrimental composition in ulcerative colitis patients and non-ulcerative colitis
 500 subjects. Journal of Applied Microbiology, 105, 706-714.

501 Moreno, F.J., Jenkins, J.A., Mellon, F.A., Rigby, N.M., Robertson, J.A., Wellner,
 502 N., Mills, C.E.N., 2004. Mass spectrometry and structural characterization of 2S
 503 albumin isoforms from Brazil nuts (*Bertholletia excelsa*). Biochimica et Biophysica
 504 Acta e Proteins and Proteomics, 1698, 175-186.

505 Moreno, F.J., Mellon, F.A., Wickham, M.S.J., Bottrill, A.R., Mills, E.N.C., 2005.
 506 Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically
 507 relevant *in vitro* gastrointestinal digestion. FEBS Journal, 272, 341-352.

508 Oliver, C.M., Melton, L.D., Stanley, R.A., 2006a. Creating proteins with novel
 509 functionality via the Maillard reaction: a review. CRC Critical Reviews in Food Science
 510 and Nutrition, 46, 337-350.

511 Oliver, C.M., Melton, L.D., Stanley, R.A., 2006b. Functional properties of
 512 caseinate glycoconjugates prepared by controlled heating in the "dry" state. Journal of
 513 the Science of Food and Agriculture, 86, 732-740.

514 Reddy, I.M., Kella, N.K.D., Kinsella, J.E., 1988. Structural and conformational
 515 basis of the resistance of β -lactoglobulin to peptic and chymotryptic digestion. Journal
 516 of Agricultural and Food Chemistry, 36, 737-741.

517 Saarela, M., Lähteenmäki, R., Crittenden, R., Salminen, S., Mattila-Sandholm, T.,
518 2002. Gut bacteria and health foods-the European perspective. International Journal of
519 Food Microbiology, 78, 99-117.

520 Sanz, M.-L., Corzo-Martínez, M., Rastall, R.A., Olano, A., Moreno, F.J., 2007.
521 Characterization and *in vitro* digestibility of bovine β -lactoglobulin glycosylated with
522 galactooligosaccharides. Journal of Agricultural and Food Chemistry, 55, 7916-7925.

523 Tabasco, R., Paarup, T., Janer, C., Peláez, C., Requena, T., 2007. Selective
524 enumeration and identification of mixed cultures of *Streptococcus thermophilus*,
525 *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* and
526 *Bifidobacterium lactis* in fermented milk. International Dairy Journal, 17, 1107–1114.

527 Tuohy, K.M., Ziemer, C.J., Klinder, A., Knöbel, Y., Pool-Zobel, B.L., Gibson, G.
528 R., 2002. A human volunteer study to determine the prebiotic effects of lactulose
529 powder on human colonic microbiota. Microbial Ecology in Health and Disease, 14,
530 165-173.

531

Table 1. Structural features of glycoconjugates used for the bacterial growth study.

Glycoconjugates	Incubation conditions	Structural features	Reference
β -Lg:Gal	40 °C, 24 h	Maximum content in the Amadori compound tagatosyl-lysine and non-aggregated	Corzo-Martínez et al. (2008)
	50 °C, 48 h	Highly glycated and aggregated	Corzo-Martínez et al. (2008)
β -Lg:Lac	60 °C, 8 h	Maximum content in the Amadori compound lactulosyl-lysine and non-aggregated	Laparra et al. (2011)
	60 °C, 48 h	Highly glycated and aggregated	Laparra et al. (2011)
SC:Gal	60 °C, 4 h	Maximum content in the Amadori compound tagatosyl-lysine and non-aggregated	Corzo-Martínez et al. (2010)
	50 °C, 72 h	Highly glycated and aggregated	Corzo-Martínez et al. (2010)
SC:Lac	60 °C, 8 h	Maximum content in the Amadori compound lactulosyl-lysine and non-aggregated	Corzo-Martínez et al. (2010)
	60 °C, 24 h	Highly glycated and aggregated	Corzo-Martínez et al. (2010)

Table 2. Maxima growth rates ($\mu_{\max}$, h^{-1}) and lag parameters (lag, h) of bacteria growing aerobically on glucose, tagatose, lactulose, and hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated and lactosylated β -Lg and SC as substrates.

Sample			<i>L. casei</i> LC-01		<i>L. reuteri</i> R13		<i>L. plantarum</i> IFPL722		<i>L. plantarum</i> CLC17		<i>L. delbrueckii</i> ZL95-27		<i>L. brevis</i> CLC23		<i>S. thermophilus</i>		<i>S. salivarius</i> ZL50-7	
Conjugate	t (h)	T ^a (°C)	$\mu_{\max}$	lag	$\mu_{\max}$	lag	$\mu_{\max}$	lag	$\mu_{\max}$	lag	$\mu_{\max}$	lag	$\mu_{\max}$	lag	$\mu_{\max}$	lag	$\mu_{\max}$	lag
Glucose	0	0	0.135	6.97	0.532	3.25	0.162	7.04	0.167	7.34	0.246	9.45	0.057	4.66	0.112	7.00	0.747	6.46
Tagatose	0	0	0.129	7.13	NG*		NG		NG		NG		NG		NG		NG	
Lactulose	0	0	0.142	7.52	0.210	4.17	0.177	7.95	0.113	7.91	NG		NG		0.177	8.96	0.492	6.60
β -Lg:Gal	24	40	0.048	0	0.132	0	0.191	0	0.102	0	0.102	0	0.114	0	NG		NG	
	48	50	0.048	3.47	0.043	0.44	0.056	0	0.038	0	0.045	0	0.044	0.44	NG		NG	
β -Lg:Lac	8	60	0.020	1.21	NG		NG		NG		NG		NG		NG		NG	
	48	60	0.037	8.79	NG		NG		NG		NG		NG		NG		NG	
SC:Gal	4	60	0.541	0	0.516	0	0.539	0	0.591	0	0.536	0	0.673	0.31	0.232	0	0.253	0
	72	50	0.103	0	0.130	2.84	0.118	0	0.103	0	0.140	1.61	0.135	3.18	NG		NG	
SC:Lac	8	60	0.445	0	0.410	0	0.410	0	0.428	0	0.474	0	0.323	0	0.192	0	0.373	0
	24	60	0.232	0	0.254	0.83	0.198	0	0.258	0.51	0.251	0	0.265	1.06	0.088	0	0.091	0

*NG: no growth ($\text{OD}_{600} < 0.2$)

Table 3. Values of maximum DO₆₀₀ reached by aerobic strains incubated with glucose, tagatose, lactulose, and hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated and lactosylated β -Lg and SC as substrates.

Sample			<i>L. casei</i>	<i>L. reuteri</i>	<i>L. plantarum</i>	<i>L. plantarum</i>	<i>L. delbrueckii</i>	<i>L. brevis</i>	<i>S.</i>	<i>S. salivarius</i>
Conjugate	T (h)	T ^a (°C)	LC-01	R13	IFPL722	CLC17	ZL95-27	CLC23	<i>thermophilus</i>	ZL50-7
Glucose	0	0	0.881 ^a	1.076 ^a	0.916 ^a	0.884 ^a	0.912 ^a	0.793 ^a	0.663 ^a	1.304 ^a
Tagatose	0	0	0.902 ^a	NG	NG	NG	NG	NG	NG	NG
Lactulose	0	0	0.997 ^b	0.974 ^b	1.195 ^b	0.895 ^a	NG	NG	0.986 ^b	1.314 ^a
β -Lg:Gal	24	40	0.875 ^a	0.833 ^c	0.962 ^c	0.886 ^a	1.068 ^{ae}	0.946 ^b	NG	NG
	48	50	0.691 ^c	0.678 ^d	0.695 ^d	0.663 ^b	0.682 ^b	0.642 ^c	NG	NG
β -Lg:Lac	8	60	0.390 ^d	NG	NG	NG	NG	NG	NG	NG
	48	60	0.412 ^d	NG	NG	NG	NG	NG	NG	NG
SC:Gal	4	60	1.622 ^e	1.528 ^e	1.592 ^e	1.537 ^c	1.573 ^c	1.493 ^d	0.857 ^c	0.851 ^b
	72	50	1.229 ^f	0.886 ^{bc}	1.201 ^b	0.967 ^{ae}	0.964 ^{ae}	0.741 ^e	NG	NG
SC:Lac	8	60	1.467 ^g	1.419 ^f	1.410 ^f	1.238 ^d	1.376 ^d	1.336 ^f	0.857 ^d	0.946 ^c
	24	60	1.254 ^f	0.919 ^{bc}	1.296 ^g	1.036 ^e	1.110 ^e	0.878 ^g	0.707 ^e	0.738 ^d

*NG: no growth (OD₆₀₀ < 0.2)

^{a-g} Different case letters indicate statistically significant (P<0.05) differences between substrates for each bacterial strain.

Table 4. Values of DO₆₀₀ after 24 h of incubation of anaerobic strains with glucose, tagatose, lactulose, and hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated and lactosylated β -Lg and SC as substrates.

Sample			<i>B. lactis</i> BB12	<i>B. breve</i> 26M2	<i>L. gasseri</i> Lc9	<i>L. acidophilus</i> LA-5
Conjugate	T (h)	T ^a (°C)				
Glucose	0	0	1.049 ^a	0.624 ^a	0.799 ^a	0.790 ^a
Tagatose	0	0	NG*	NG	NG	0.703 ^b
Lactulose	0	0	NG	0.914 ^b	NG	NG
β -Lg:Gal	24	40	0.382 ^b	0.388 ^c	0.522 ^b	0.522 ^c
	48	50	0.208 ^c	0.169 ^d	0.206 ^c	0.206 ^d
β -Lg:Lac	8	60	NG	NG	NG	0.386 ^e
	48	60	NG	NG	NG	0.386 ^e
SC:Gal	4	60	0.714 ^d	0.700 ^e	1.259 ^d	1.292 ^f
	72	50	0.568 ^e	0.440 ^f	0.673 ^e	0.588 ^g
SC:Lac	8	60	1.100 ^f	1.081 ^g	0.989 ^f	1.233 ^h
	24	60	0.993 ^a	1.136 ^h	1.103 ^g	1.200 ⁱ

*NG: no growth (OD₆₀₀ < 0.2)

^{a-i} Different case letters indicate statistically significant (P<0.05) differences between substrates for each bacterial strain.

Figure captions

Figure 1. SDS-PAGE analysis of hydrolysates of control β -Lg incubated for 24 h at 40°C (a) and 48 h at 50°C (c) and β -Lg glycosylated with Gal for 24 h at 40°C (b) and 48 h at 50°C (d) after *in vitro* gastrointestinal digestion. M: Mw markers.

Figure 2. Chromatographic profiles obtained by RP-LC-UV of: (A) native SC non-digested and control heated SC [4 h, 60 °C] and [72 h, 50 °C] subjected to *in vitro* gastrointestinal digestion, and (B) native SC non-digested and SC glycosylated with Gal at 60 °C for 4 h and at 50 °C for 72 h subjected to *in vitro* gastrointestinal digestion.

Figure 3. Growth curves of aerobic strains of lactic acid bacteria assayed with $\blacktriangle$ glucose, $\triangleleft$ tagatose, — lactulose and hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated ($\blacklozenge$ 24 h at 40 °C and $\blacklozenge$ 48 h at 50 °C) and lactosylated ($\blacksquare$ 8 and $\square$ 48 h at 60 °C) β -Lg and galactosylated ($\blackstar$ 4 h at 60 °C and $\star$ 72 h at 50 °C) and lactosylated ($\bullet$ 8 and $\circ$ 24 h at 60 °C) SC as substrates.

Figure 1.

Figure 2.

Figure 3.

Effect of milk protein glycation and gastrointestinal digestion on the growth of bifidobacteria and lactic acid bacteria
Corzo-Martínez et al.

Reviewers' comments:

Reviewer #1:

The paper is interesting. Few but important recommendations are needed and they should take in account by the authors.

1. Introduction: it seems to long. I think the authors should decrease it.

According to the referee indication, we have decreased the section of Introduction of the revised version of the paper.

2. Material and Methods:

2.1. An important remark is it takes clear for the reader the strain used. Please, add some reference where the commercial starters are used in probiotic dairy food processing. Preferentially, recent IFJM references. Item 2.3 - please, add a reference.

The reference Tabasco et al. 2007 has been added in line 116 to indicate the origin of the commercial starter strains.

Item 2.3: reference Moreno et al. (2005) in line 163 was added for previous description of substrates digestion.

2.2. Include a section of statistical analysis.

Following the referee's suggestion, we have included a section of statistical analysis (Section 2.6, see lines 232-238 in the new version of the manuscript).

3. Results and discussion: I think prudent to include this behaviour is strain-dependent and other strains should be tested in further studies. Additional, the comparison with other prebiotics is interesting. This recommendation should be include in this part and emphasized in the conclusion section.

We are in complete agreement with the reviewer. Thus, we have included in the text that the behaviour observed is strain-dependent (see lines 285 and 368 in the new version of the manuscript) and referee's recommendations (see lines 352-354 in the new version of the manuscript).

4. Conclusion: some recommendation should be included.

Referee's recommendation has been included in the section of Conclusion of the revised version of the paper (see lines 415-418 and 431-433).

Reviewer #2:

The results of the paper with title "Effect of milk protein glycation and gastrointestinal digestion on the growth of bifidobacteria and lactic acid bacteria" demonstrated that conjugation of both milk proteins with galactose and lactose via the Maillard reaction could be an efficient method to obtain novel food ingredients with a potential prebiotic character. The manuscript is well organised and clearly written. It contains a sufficient number of experiments.

For better understand of results I recommend to apply statistical interpretation for Table 2 and 3.

Following the referee's suggestion and also according to reviewer 1, we have carried out the statistical analysis of data (see new tables 3 and 4 of the revised manuscript). In the case of aerobic cultures (Table 2), we have included the statistical analysis in another table (new table 3) with the values of maximum optical density (OD_{600}), since the statistical analysis was unpractical for μ_{max} and lag data (table 2).