Accepted Manuscript

A glycoproteomic approach reveals that the S-layer glycoprotein of *Lactobacillus kefir* CIDCA 83111 is O- and N-glycosylated

Gustavo J. Cavallero, Mariano Malamud, Adriana C. Casabuono, M. de los Ángeles Serradell, Alicia S. Couto

PII: S1874-3919(17)30127-6
DOI: doi: [10.1016/j.jprot.2017.04.007](https://doi.org/10.1016/j.jprot.2017.04.007)
Reference: JPROT 2823

To appear in: *Journal of Proteomics*

Received date: 19 December 2016
Revised date: 17 March 2017
Accepted date: 10 April 2017

Please cite this article as: Gustavo J. Cavallero, Mariano Malamud, Adriana C. Casabuono, M. de los Ángeles Serradell, Alicia S. Couto, A glycoproteomic approach reveals that the S-layer glycoprotein of *Lactobacillus kefir* CIDCA 83111 is O- and N-glycosylated. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. *Jprot*(2017), doi: [10.1016/j.jprot.2017.04.007](https://doi.org/10.1016/j.jprot.2017.04.007)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**A glycoproteomic approach reveals that the S-layer glycoprotein of
Lactobacillus kefir CIDCA 83111 is O- and N-glycosylated.**

Gustavo J. Cavallero^{1#}, Mariano Malamud^{2#}, Adriana C. Casabuono¹, M. de los Ángeles Serradell², Alicia S. Couto^{1*}

¹Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica - Consejo Nacional de Investigaciones Científicas y Técnicas, Centro de Investigación en Hidratos de Carbono (CIHIDECAR), Buenos Aires, Argentina, Intendente Güiraldes 2160, C1428GA, Ciudad Universitaria, Buenos Aires, Argentina. ²Cátedra de Microbiología, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115, La Plata (1900), Argentina.

*To whom correspondence should be addressed: Tel.: +54 11 4576 3346; fax: +54 11 4576 3346.

E-mail address: acouto@qo.fcen.uba.ar (A.S. Couto)

[#]both authors contributed equally to this work.

ABSTRACT

In Gram-positive bacteria, such as lactic acid bacteria, general glycosylation systems have not been documented so far. The aim of this work was to characterize in detail the glycosylation of the S-layer protein of *Lactobacillus kefir* CIDCA 83111. A reductive β -elimination treatment followed by anion exchange high performance liquid chromatography analysis was useful to characterize the *O*-glycosidic structures. MALDI-TOF mass spectrometry analysis confirmed the presence of oligosaccharides bearing from 5 to 8 glucose units carrying galacturonic acid. Further nanoHPLC-ESI analysis of the glycopeptides showed two *O*-glycosylated peptides: the peptide sequence SSASSASSA already identified as a signature glycosylation motif in *L. buchneri*, substituted on average with eight glucose residues and decorated with galacturonic acid and another *O*-glycosylated site on peptide 471-476, with a Glc₅₋₈GalA₂ structure. As ten characteristic sequons (Asn-X-Ser/Thr) are present in the S-layer amino acid sequence, we performed a PNGase F digestion to release N-linked oligosaccharides. Anion exchange chromatography analysis showed mainly short N-linked chains. NanoHPLC-ESI in the positive and negative ion modes were useful to determine two different peptides substituted with short *N*-glycan structures. To our knowledge, this is the first description of the structure of *N*-glycans in S-layer glycoproteins from *Lactobacillus* species.

Keywords: Glycoproteomics, *L. kefir*, S-layer glycoprotein, *N*-glycosylation, *O*-glycosylation, mass spectrometry.

INTRODUCTION

Surface-layer (S-layer) proteins are ubiquitous in both Bacteria and Archaea. S-layers are arrays of a single protein that constitutes the outermost cell envelope and have been considered to function as protective coats, maintenance of cell shape, and adhesion to specific hosts [1-3]. These proteins are present in some *Lactobacillus* species that are normal inhabitants of the oral and genital cavities and the gastrointestinal tract of humans and animals and also have been isolated from plants and food products.

The functions of bacterial S-layer proteins have been explained only in few cases, and many of them are still hypothetical. These lattices could function as protective agents against a hostile environment, as molecular sieves and ion traps, as structures involved in cell adhesion, surface recognition and inhibition of pathogens [1-3].

In Gram-positive bacteria, such as lactic acid bacteria, general glycosylation systems have not been documented so far. Although, most of the lactobacilli S-layer proteins were determined to be non-glycosylated [1], the glycoprotein nature of the S-layer proteins has been reported for *Lactobacillus buchneri* 41021/251, CD034 and NRRL B-30929 [4,5], *L. helveticus* ATCC12046 [6], *L. acidophilus* NCFM [7], *L. plantarum* 41021/252 [4] and several *Lactobacillus kefir* strains [8]. However, only for *L. buchneri* 41021/251 and CD034, the S-layer glycan structures were studied, finding glucose oligomers attached to serine residues [5]. Further studies have revealed the importance of lactobacillar cell surface glycosylation for adhesion and biofilm formation [9] as well as gastrointestinal persistence [10] and adaptation [11].

The presence of S-layer *L. kefir* strains isolated from kefir, a probiotic fermented milk, was described some years ago [12]. It has been demonstrated that these S-layer proteins are involved in the interaction of bacterial cells with yeasts present in kefir grains [13]. They are also able to inhibit the invasion of *Salmonella enterica* serovar Enteritidis to Caco-2 cells [14], to antagonize the effect of *Clostridium difficile* toxins [15] and to protect bacterial cells against the deleterious effect of lead ions [16]. These glycoproteins show apparent molecular masses ranging from 66 to 71 kDa, and a high heterogeneity among aggregating and non-aggregating strains of *L. kefir* have been demonstrated [8]. However, recent studies have revealed some similarities with the S-layer proteins of other lactobacilli in terms of amino acid composition, such as a high

content of hydrophobic amino acids (34.9-38.4 %) and hydroxylated amino acids (24.6–29.2 %), and the absence of cysteine residues [17].

Since the amino acid sequence of the S-layer glycoprotein from *L. kefir* CIDCA 83111 was recently reported, the aim of this work was to characterize in detail the glycosylation of this protein. To achieve this purpose, HPAEC-PAD and MALDI mass spectrometry were used to study the sugar moieties and nanoHPLC-ESI m.s. for glycopeptides. Interestingly, two *O*-glycosidic and two *N*-glycosidic chains were characterized.

EXPERIMENTAL

Bacterial strain and culture conditions. *Lactobacillus kefir* CIDCA 83111 was used [18].

Bacteria were cultured in deMan-Rogosa-Sharpe (MRS)-broth (DIFCO, Detroit, USA) at 37°C for 48h, under aerobic conditions.

S-layer protein extraction. Extraction was performed using 5M LiCl as described previously [15]. The homogeneity of the protein extract was tested by SDS-PAGE, stained with Colloidal Comassie Blue staining.

In gel- reductive β -elimination. The gel band was excised and then treated with 0.05M NaOH/1M NaBH₄ (0.5 ml) at 50°C during 16h. The solution was separated, acetic acid was added until pH 7 followed by repeated evaporation with methanol. The sample was dissolved in water, desalted in a Dowex 50W (H⁺) (Fluka) column and dried in SpeedVac.

Acid hydrolysis. The β -eliminated sample was further hydrolyzed in 2N TFA for 4h at 100°C. The acid was eliminated by evaporation and the hydrolysate was re-suspended in water for HPAEC-PAD analysis.

Analysis of the sugar composition of the S-layer Glycoprotein by HPAEC (High performance anion exchange chromatography). Analysis was performed in a DX-500 Dionex BioLC system (Dionex Corp.) with a pulse amperometric detector. The following columns and conditions were employed: (a) neutral and aminosugar analysis was performed in a Carbowpack PA-1 column using a 18 mM NaOH isocratic program, flow rate was 0.4 mL/min; (b) acidic monosaccharide analysis was performed in a Carbowpack PA-1 column with a 48 mM NaOH/140mM sodium acetate isocratic program was used, flow rate was 0.4 mL/min; c) for alditols, a CarboPac MA 1 column was used with a 0.4M NaOH isocratic program and a flow rate of 0.4mL/min. d) for oligosaccharides a Carbowpack P-100 microbore column equipped with a P-100 pre-column was

used and a gradient elution with 100 mM NaOH, 70-400 mM sodium acetate for 45 min, with a flow rate of 0.25 mL/min.

Release of N-glycosidic chains by PNGase F treatment: The protein band corresponding to the S-layer glycoprotein was cut out from the gel, frozen for 3 h, and washed (mixing for 30 min) with (a) acetonitrile, (b) 20mM NaHCO₃, pH 7, and (c) acetonitrile. The gel pieces were dried, and the N-glycans were released by incubation with PNGase F (20 milliunits) (New England Biolabs Inc., Beverly, MA) overnight at 37 °C in 20mM NaHCO₃, pH 7 (30 µl). The gel pieces were thoroughly washed, and the supernatants were removed and dried. Glycans were filtered through an Ultrafree McFilter (Mr 5000), dried, resuspended in 0.1% (v/v) formic acid (20 µl), and left at room temperature for 40 min. Finally, the sample was dried and suspended in water.

Glycoprotein Digestion. The protein band corresponding to the S-layer glycoprotein was cut out from the gel and washed with acetonitrile. The gel pieces were reduced with 10 mM DTT in 50 mM NH₄HCO₃ at 55 °C for 30 min. They were further washed with acetonitrile and alkylated with 55 mM IAA in 50 mM NH₄HCO₃ for 20 min at room temperature in darkness. After washing with 50 mM NH₄HCO₃ for 10 min and with acetonitrile for 5 min, they were dried in a SpeedVac. The gel slices were rehydrated with 20 ng/µl trypsin (Sigma) in 40 mM NH₄HCO₃, 9% acetonitrile and incubated at 37 °C overnight. Further digestion was carried upon the addition of 20 ng/µl Glu-C (V8) protease (Promega) in 50mM NH₄HCO₃, pH 8, at 37 °C overnight. After incubation, the supernatant was separated and taken to dryness.

Mass Spectrometry Analysis. Matrices and calibrating chemicals were purchased from Sigma-Aldrich. Oligosaccharide analysis was performed in an Ultraflex II TOF/TOF mass spectrometer equipped with a high performance solid-state laser (λ 355 nm) and a reflector. The system is operated by the Flexcontrol version 3.3 software package (Bruker Daltonics GmbH, Bremen, Germany). Samples were irradiated with a laser power of 25–50% and measured in the linear and the reflectron modes, in positive and negative ion polarities.

Laser-induced Dissociation Tandem Mass Spectrometry (LID-MS/MS) Analysis in the MALDI-TOF/TOF-MS/MS Instrument—The Ultraflex II MALDI-TOF/TOF mass spectrometer was used. For all experiments using the tandem time-of-flight LIFT mode, the ion source voltage was set at 8.0 kV with a precursor ion mass window of 3 Da. Precursor ions generated by LID were accelerated at 19.0 kV in the LIFT cell. The reflector voltage was set at 29.5 kV. 2,5-dihydroxybenzoic acid was used as matrix. The samples were loaded onto a MTP 384 ground

steel target (Bruker Daltonics GmbH) using the sandwich method. Mass spectra were the sum of 100–300 single laser shots, depending on the sample conditions.

Spectrum Calibration: External calibration reagents were used (commercial proteins bradykinin 1–7, *Mr* 757.399; angiotensin I, *Mr* 1296.685; renin substrate, *Mr* 1758.933; insulin β -chain, *Mr* 3494.6506) with β -cyclodextrin (cyclohepta amylose, *Mr* 1135.0) and γ -cyclodextrin (cyclooctaamylose, *Mr* 1297.1) with 2,5-dihydroxybenzoic acid as matrix.

Glycopeptide analysis: the glycopeptide mixtures obtained after protease digestions were purified by cotton HILIC SPE micro tips [19]. The enriched glycopeptide mixtures were re-suspended in 50% ACN -1% formic acid in water, 1:1.

The digests were analyzed in a nanoLC 1000 coupled to an EASY-SPRAY Q Exactive Mass Spectrometer (Thermo Scientific) with a HCD (High Collision Dissociation) and an Orbitrap analyzer. An Easy Spray PepMap RSLC C18 column (50 μ m x 150 mm, particle size 2.0 μ m, pore size: 100 Å) at 40°C was used for separation. Separation was achieved with a linear gradient from 5% to 35% solvent B developed in 75 min, at a flow of 300 nl/min (mobile phase A: water-0.1% formic acid; mobile phase B: ACN-0.1% formic acid). Injection volume 2 μ l. Spray voltage (+): 3.5 kV; (-): 3.0 kV. A full-scan survey MS experiment (*m/z* range from 400-2000; automatic gain control target 3×10^6 ; maximum IT: 200 ms, resolution at 400 *m/z*: 70000. Data Dependent MS² method was set to the centroid mode, resolution 17500; maximum IT 50 ms; automatic gain control target 10^5 ; fragment the top 15 peaks in each cycle; NCE: 27.

Data interpretation

Data from de nanoHPLC-ESI-Orbitrap experiments were manually evaluated. Automatic search of peptides and glycopeptides was assisted by SequestHT on Proteome Discoverer 1.4 (Thermo Fisher Sc.)

Trypsin and Glu-C were selected as the enzymes and one missed cleavage was permitted. The mass accuracy tolerance was set to 10 ppm for precursor ions. The static modification was carbamidomethylation in the Cys residues. HexNAc₂, HexNAc₂dHex, HexNAc₂Hex; HexNAc₂Hex₃ and HexNAc₂Hex₃dhex were selected as dynamic carbohydrate modifications on Asn. A maximum of two modifications per peptide was permitted. Deconvolution was assisted by Xtract on Thermo Xcalibur 3.0.63.

RESULTS

2.1 - Sugar components analysis.

The glycosylated nature of S-layer protein extracted from *L. kefir* strain CIDCA 83111 was previously reported by PAS staining [8]. In order to perform a sugar analysis of this S-layer protein, the gel band was excised and subjected to reductive β -elimination. When the β -eliminated sample was further subjected to a total acid hydrolysis and analyzed by HPAEC-PAD (Fig 1A) under conditions where neutral and aminosugars are separated (condition a), the presence of a main peak corresponding to glucose was observed. Minor peaks coincident with glucosamine, galactose and mannose were also evident. The same sample was further analyzed under conditions where acidic sugars are separated (condition b) (Fig 1B). Interestingly, a peak coincident with an authentic standard of galacturonic acid was detected. In addition, when an alditol analysis was performed (condition c) (Fig 1C), a peak coincident with sorbitol was detected indicating that glucose is the sugar that links the O-linked chain to the peptide backbone. To complete the chromatographic analysis, the β -eliminated sample was analyzed under conditions where oligosaccharides are separated (condition d) (Fig 1D). Main peaks corresponding to reduced maltooligosaccharides among 5 and 8 glucose units were determined. Up to this point the presence of O-glycosidic chains bearing glucose units was assured.

2.2 Analysis of O-linked glycans by MALDI-TOF m.s.

In order to get deeper into the O-linked sugar structure, the reduced fraction obtained by β -elimination was subjected to MALDI-TOF m.s. analysis in the negative ion mode using 2,5 dihydroxybenzoic acid as matrix (Fig.2). Major ion at m/z 829.0 (calc. m/z 829.2903 $C_{30}H_{53}O_{26}^-$) corresponds to the reduced oligosaccharide bearing five hexose units. A minor signal at m/z 667.0 (calc. m/z 667.2297, $C_{24}H_{43}O_{21}^-$) corresponds to the reduced oligosaccharide constituted by four hexose units. On the other hand, ion at m/z 991.5 (calc. m/z 992.3432; $C_{36}H_{63}O_{31}^-$) corresponds to six hexose units and ion at m/z 1005.0 (calc. m/z 1005.3151; $C_{36}H_{61}O_{32}^-$), to a Hex₅HexA reduced structure. Two additional signals at m/z 1167.0 (calc. m/z 1167.3680; $C_{42}H_{71}O_{37}^-$) and m/z 1328.4 (calc. m/z 1329.4208; $C_{48}H_{81}O_{42}^-$) corresponding to reduced Hex₆HexA and Hex₇HexA respectively, were also detected.

2.3 Analysis of O-glycosylated peptides by HPLC-ESI

Next step consisted in determining the site of linkage of this oligosaccharide to the protein. Therefore, a sequential enzymatic digestion with trypsin and Glu-C was performed (S1) on the excised gel band corresponding to the S-Layer glycoprotein of *L. kefir* and an enriched glycopeptide fraction was obtained after cotton-hilic chromatography. The glycopeptide enriched fraction was further analyzed by HPLC-ESI. Figure 3 shows the total ion chromatogram (TIC) obtained by LC/MS/MS in the positive ion mode with MS range m/z 400-2000 (panel A). In order to locate the glycopeptide peaks and determine m/z and charge state, the intensity of the oxonium Hex^+ (m/z 163.06) (panel B) that arose by data dependent MS/MS was depicted as an extracted chromatogram. Interestingly, the oxonium Hex^+ ion location in the extracted chromatogram suggested the presence of O-linked structures in the initial minutes of the HPLC run.

Searching for glycopeptides in the nanoHPLC-ESI analysis acquired in the positive ion mode, at 5.64 min, a family of glycopeptides with $z=+3$ (m/z 1544.8824; 1652.9221; 1706.9264; 1760.9561; 1814.9486; 1868.9785; 1922.9988; 1977.0089) were detected (Figure 4A). Deconvolution of these ions leads to peptide $^{147}\text{SASASSASSASSTE}^{160}$ substituted with 21 to 29 hexose units. Accordingly, also a family of ions $z=+4$ were detected (m/z 1564.2961; 1604.5615; 1645.0615; 1685.8400; 1726.0867; 1766.5977; 1807.3851; 1847.6289; 1888.4052). In this case peptide 147-160 substituted with 31-39 hexose units was detected. (Figure 4B). Table 1 shows the glycoform structures corresponding to the peptide, calculated masses, experimental masses and errors. In S2 some examples of MS/MS spectra of the corresponding ions containing the characteristic reporter fragments (m/z 145.05; 163.06 and 325.11) in the low mass region are shown.

Furthermore, at retention time 7.51 min, another cluster of +4 charged ions was shown (m/z 1463.8036; 1504.3110; 1544.8226; 1625.8561; 1666.3723; 1706.8783; 1747.4017; 1787.9008; 1828.4177; 1868.9435; 1909.4424; 1949.9532; 1990.4730). Deconvolution of these ions points out to the peptide $^{147}\text{SASASSASSASSTEQTALTDQK}^{170}$, containing the previous peptide with one miscleavage, substituted with 23-35 hexose units. (Figure 5A). Also minor signals with $z=+5$ were shown. Among them, the largest glycopeptide detected (m/z 1981.9026) bears 47 hexose units (not shown). In S2, MS/MS spectra of major ions containing diagnostic carbohydrate fragments are shown. A similar glycosylation pattern has been described as an O-

glycosylated signature motif S-S-A-S-S-A-S-S-A in the S-layer glycoprotein of *L. buchneri* [20]. When the analogous signals were searched in the HPLC-ESI analysis performed in the negative ion mode (Figure 5B) a similar cluster of ions with $z = -4$ was detected. Main peaks (m/z 1380.7684; 1421.2803; 1461.7980; 1502.3051; 1542.8199; 1623.8507; 1664.3583; 1704.8676; 1745.3832; 1785.895; 1826.4084; 1866.9222; 1907.4371; 1947.9553) correspond to peptide 147-170 carrying from 20 to 34 hexose units. However, in this case, it was interesting to note that among main signals, another cluster of minor ions was shown (inset). Mass differences with the major signals indicate the presence of a Hex₂₈₋₃₀HexA₁₋₃ structures. These results are in accordance with the MALDI-TOF m.s analysis of the β -eliminated *O*- chains described above. Table 1 shows the structures corresponding to each peptide, calculated masses, experimental masses and errors.

In addition, between 5.14-5.45 min of the nanoHPLC-ESI analysis acquired in the positive ion mode another cluster of ions with $z = +2$ was detected (m/z 845.7368; 926.7651; 1007.7856; 1088.8151) (Fig 6A). Deconvolution of these ions pointed out to peptide ⁴⁶⁹DKTTTSAE⁴⁷⁶ glycosylated with a HexA₂Hex₃₋₈ structure. In this case the low abundance of these signals precluded the correct determination of the experimental monoisotopic value, leading to larger mass errors (See Table 1). S2 shows MS/MS spectra of major ions containing in the low mass region, characteristic reporter fragments. In order to verify the presence of this glycopeptide structure, another experiment in the HPLC-ESI acquired in the negative ion mode was performed. Although the glycopeptide described above could not be detected, it was possible to determine in the same region, the presence of a cluster of signals with $z = -2$ (m/z 796.2504; 877.278; 958.2607; 1039.2854; 906.2847; 987.3111) (Fig.6B). In this case, deconvolution of these ions pointed out to peptide ⁴⁷¹TTTSAE⁴⁷⁶, the same peptide determined in the positive ion mode without the miscleavage, glycosylated with a HexA₁₋₂ Hex₅₋₈ structure.

2.4 Analysis of *N*-glycosidic chains by HPLC-ESI.

The peptide sequence of the S-layer glycoprotein (17, S1), shows ten consensus sequences for putative *N*-glycosylation (N-X-S/T). Therefore, as this type of substitution has not been determined in *Lactobacillus* so far, we performed a PNGase digestion on the gel band corresponding to the S-layer glycoprotein in order to search for its presence. Analysis of the released material by HPAEC-PAD (Fig. 7A) showed a main oligosaccharide migrating very near

the classical *N*-glycosidic core (GlcNAc₂Man₃). Minor peaks corresponding to larger structures were also detected. Next step consisted in searching in the glycopeptide HPLC-ESI analysis, for the intensity of the oxonium ions HexNAc⁺ (*m/z* 204.09) (Fig 3, panel C), [HexNAc - 2H₂O]⁺ (*m/z* 168.07) (Fig 3, panel D) and [HexNAc - CH₆O₃]⁺ (*m/z* 138.05) (Fig 3, panel E) characteristic for *N*-glycosidic moieties that arose by data dependent MS/MS. Notably, three defined regions in the HPLC run bearing these reporter ions were observed: 5-8 min; 26-30 min and 48-55 min. Due to the fact that glycopeptides may acquire multiple charges and that in the positive ion mode they may be protonated and/or sodiated making sometimes complicated the mass spectrometry analysis, we decided to perform first a manual inspection of the spectra acquired in the negative ion mode. As expected, at an elution time of 7.58 min, it was easy to detect a signal of *m/z* 1130.5466 with *z* = -1 and the corresponding signal, of *m/z* 564.7698 with *z* = -2, attributed to peptide ²³³IADTN²³⁷ATNGQK²⁴³ containing an *N*-glycosylation site (Fig. 7B). Next to these signals, a mixture of glycosylated forms with *z* = -2 matching with substitution at ²³⁷N were observed. Signal at *m/z* 921.9228 corresponds to the peptide bearing a HexNAc₂HexdHex structure; ion at *m/z* 1296.0752 corresponds to the addition of two hexoses, two HexNAc units plus H₂O to the latter and *m/z* 1319.0042 matches with HexNAc₂Hex₅dHex₂ structure. Interestingly, the fact that the non-glycosylated peptide was also found suggests only a partial occupation of this *N*-glycosylation site. In the HPLC-ESI analysis acquired in the positive ion mode only two signals in this region could be assigned: one corresponding to the naked peptide with *z* = +2 (*m/z* 566.7837) and one corresponding to the same peptide substituted with two HexNAc units plus H₂O (*m/z* 778.8825) (S3). In S4, MS/MS spectra of major ions containing in the low mass region diagnostic 204.09 fragment are shown. However after the use of SequestH algorithm, the peptide ²³³IADTN²³⁷ATNGQKINGWIK²⁴⁹ containing the same *N*-glycosidic sequon bearing HexNAc₂dHex was identified with an elution time of 56.51 min (S5). Manual inspection of this region showed a signal with *z* = +3 at *m/z* 804.7286 matching with the named peptide carrying a HexNAc₂Hex structure and *m/z* 873.4028 assigned to HexNAc₂Hex₂ were detected. (See S4 for MS/MS)

Looking into the second region, at *Rt* = 30.02 min, a signal with *z* = -2 (*m/z* 770.3560) corresponding to peptide ²⁰³TVTDATPYAN²¹²DTFK²¹⁶ was detected. Nearby, a signal with *m/z* 1046.4855 was assigned to peptide 203-219 substituted with a HexNAc₂dHex structure (Fig. 7C). In order to assure the glycopeptide structure, the analysis performed in the positive ion

mode was investigated. As expected, the corresponding signals with $z = +2$ were detected (m/z 772.0980 and m/z 1048.4679) (S3).

Finally, in the third region, at an elution time of 52.77 min, the automatic search (S5) found peptide $^{125}\text{SVTAFAGGIASFNTTTAPAAAK}^{146}$ glycosylated with HexNAc₂Hex but, the low abundance of the corresponding signals precluded the detection of diagnostic fragments in the MS/MS.

DISCUSSION

This is the first detailed report of the *O*- and *N*- glycosylation of the S-layer glycoprotein of *Lactobacillus kefir*. Classical methodology as reductive β -elimination and HPAEC-PAD analysis were useful to characterize the *O*-glycosidic structure. In addition, MALDI-TOF m.s. analysis confirmed the presence of oligosaccharides bearing from 5 to 8 glucose units carrying galacturonic acid. Further nanoHPLC-ESI analysis in the negative and positive ion modes of the glycopeptides obtained after trypsin and Glu-C digestion, showed two *O*-glycosylated peptides (Table 1). The peptide SSASSASSA has been already identified as a signature glycosylation motif in *L. buchneri*, with four serine residues within the sequence S-S₁₅₂-A-S₁₅₄-S₁₅₅-A-S₁₅₇-SA substituted on average with seven glucose residues [5]. In *L. kefir*, the same peptide sequence was determined [17] but, unlike in *L. buchneri*, it is substituted on average with eight glucose residues decorated with galacturonic acid. Furthermore, another *O*-glycosylated site, peptide 471-476, substituted with a Glc₅₋₈GalA₂ structure was also determined. Since the cell envelope is the first target of physicochemical and environmental stress, several studies indicate that there is a correlation between the different structural and chemical characteristics of the S-layer proteins with the surface properties of lactobacilli [1, 20]. Lactobacilli are often exposed to changes in the osmolarity of their environment which can compromise essential cell functions. Changes in solute concentrations in the environment, cause changes in cell turgor pressure which lead to changes in cell volume [21]. In this sense, it can be speculated that a high degree of glycosylation, as shown in *L. kefir*, may be protective against the harsh environmental conditions. In addition, a negative surface charge, in this case due to the presence of GalA, is thought to stabilize proteins in high salt conditions, possibly via the formation of an energetically

favorable protein-water-salt hydration network, as proposed for other S-layer glycoproteins [22]. In a separate procedure, the presence of *N*-glycosidic modifications was studied. Although it has been reported that tunicamycin did not affect the SDS-PAGE pattern of the *L. kefir* S-layer glycoprotein [8] the fact that ten characteristic sequons (Asn-X-Ser/Thr) are present in the S-layer amino acid sequence led us to search for this type of modification. PNGase F treatment of the corresponding gel band followed by HPAEC-PAD analysis showed that mainly short *N*-linked oligosaccharides were released. Therefore, a glycoproteomic approach by nanoHPLC-ESI in the positive and negative ion mode was performed allowing us to determine at least two different peptides substituted with short structures. This result is in agreement with a recent report showing that *L. kefir* JCM 5818 S-layer is effective on preventing *L. acidophilus* binding to hDC-SIGN and also it has strong activity against infection of DC-SIGN expressing cells, but deglycosylation by PNGase F remarkably reduced its activity suggesting the presence of *N*-glycosidic chains and their involvement in the adhesion process [23]. Table 2 shows a summary of the glycosidic structures described.

CONCLUSION

In this work, we provide evidence that the S-layer glycoprotein present in *L. kefir* CIDCA 83111 is *O*- and *N*-glycosylated. In fact, as far as we know, this is the first description of the structure of *N*-glycosidic chains in S-layer glycoprotein from *Lactobacillus* species.

The knowledge of type and site of glycosylation of the S-layer glycoprotein of *L. kefir* gives the exciting possibility to study in depth the structure-activity relationships. Although glycosylation may be only part of the puzzle, it is nowadays evident that in *L. kefir*, carbohydrates play a prominent role as protective coats and as promoters for cell adhesion and surface recognition. Future comparative studies on wild-type strains with different characteristics will help to elucidate the functional significance of the carbohydrate moieties of S-layer glycoproteins.

Acknowledgements

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas Grant PIP-11220110100660, Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) Grants PICT-0736 and PICT 2012-0910, and Universidad de Buenos Aires Grant 20020130100476BA. The Ultraflex II (Bruker) TOF/TOF mass spectrometer was supported by ANPCyT Grant PME 125 and the ESI-Orbitrap by ANPCyT Grant PME 2012. Gustavo J. Cavallero and Mariano Malamud are fellows from ANPCyT and CONICET respectively. Alicia S. Couto and María de los Ángeles Serradell are members of Carrera de Investigador Científico y Tecnológico of CONICET.

REFERENCES

- [1]- Avall-Jääskeläinen S, Palva A. *Lactobacillus* surface layers and their applications. FEMS Microbiol Rev 2005; 29:511–529.
- [2]- Hynönen U, Palva A. *Lactobacillus* surface layer proteins: structure, function and applications. Appl. Microbiol. Biotechnol. 2013; 97:5225–5243.
- [3]- Gerbino E, Carasi P, Mobili P, Serradell MA, Gómez-Zavaglia A. Role of S-layer proteins in bacteria. World J Microbiol Biotechnol 2015; 31:1877–1887.
- [4]- Möschl A, Schäffer C, Sleytr UB, Messner P, Christian R, Schulz G. Characterization of the S-layer glycoproteins of two lactobacilli. In: T.J. Beveridge, S.F. Koval, editors. Advances in bacterial paracrystalline surface layers. Vol. 252. Plenum Press; New York: 1993. p. 281-284.
- [5]- Anzengruber J, Pabst M, Neumann L, Sekot G, Heintl S, Grabherr R, Altmann F, Messner P, Schäffer C. Protein O-glucosylation in *Lactobacillus buchneri*. Glycoconj J. 2014; 31(2):117-31.

- [6]- Mozes N, Lortal S. X-ray photoelectron spectroscopy and biochemical analysis of the surface of *Lactobacillus helveticus* ATCC 12046. *Microbiology* 1995; 141:11–19.
- [7]- Konstantinov SR, Smidt H, de Vos WM, Bruijns SCM, Singh SK, Valence F, Molle D, Lortal S, Altermann E, Klaenhammer TR, van Kooyk Y. S layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105: 19474–79.
- [8]- Mobili P, Serradell MA, Trejo S, Avilés-Puigvert X, Abraham AG, De Antoni GL. Heterogeneity of S-layer proteins from aggregating and non-aggregating *Lactobacillus kefir* strains. *Antonie Van Leeuwenhoek* 2009; 95:363-72.
- [9]- Lebeer S, Verhoeven TLA, Francius G, Schoofs G, Lambrichts I, Dufrene Y, Vanderleyden J, De Keersmaecker SCJ. Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. *Appl. Environ. Microbiol.* 2009; 75:3554–3563.
- [10]- Denou E, Pridmore RD, Berger B, Panoff JM, Arigoni F, Brussow H. Identification of genes associated with the long-gut-persistence phenotype of the probiotic *Lactobacillus johnsonii* strain NCC533 using a combination of genomics and transcriptome analysis. *J. Bacteriol.* 2008; 190:3161–3168.
- [11]- Marco ML, de Vries MC, Wels M, Molenaar D, Mangell P, Ahrne S, de Vos WM, Vaughan EE, Kleerebezem M. Convergence in probiotic *Lactobacillus* gut-adaptive responses in humans and mice. *ISME J.* 2010; 4:1481–1484.
- [12]- Garrote GL, Delfederico L, Bibiloni R, Abraham AG, Pérez PF, Semorile L, De Antoni GL. Lactobacilli isolated from kefir grains: evidence of the presence of S-layer proteins. *J Dairy Res.* 2004; 71:222-30.

- [13]- Golowczyc M, Mobili P, Garrote GL, Serradell MA, Abraham AG, De Antoni GL. Interaction between *Lactobacillus kefir* and *Saccharomyces lipolytica* isolated from kefir grains: evidence for lectin-like activity of bacterial surface proteins. J Dairy Res 2009; 76:111-116.
- [14]- Golowczyc M, Mobili P, Garrote GL, Abraham AG, De Antoni GL. Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar Enteritidis. Int J Food Microbiol 2007; 118:264-273.
- [15]- Carasi P, Trejo FM, Pérez PF, De Antoni GL, Serradell MA. Surface proteins from *Lactobacillus kefir* antagonizes *in vitro* cytotoxic effect of *Clostridium difficile* toxins. Anaerobe 2012; 18:135-142.
- [16]- Gerbino E, Carasi P, Araujo-Andrade C, Tymczyszyn EE, Gómez-Zavaglia A. Role of S-layer proteins in the biosorption capacity of lead by *Lactobacillus kefir*. World J. Microbiol. Biotechnol. 2015; 31:583–592.
- [17]- Malamud M, Carasi P, Bronsoms S, Trejo SA, Serradell MA. *Lactobacillus kefir* shows inter-strain variations in the amino acid sequence of the S-layer proteins. Antonie Van Leeuwenhoek 2016; *accepted manuscript*.
- [18]- Garrote GL, Abraham AG, De Antoni GL. Chemical and microbiological characterization of kefir grains. J Dairy Res 2001; 68:639-652.
- [19]- Selman MH, Hemayatkar M, Deelder AM, Wuhler M. Cotton HILIC SPE microtips for microscale purification and enrichment of glycans and glycopeptides. Anal. Chem. 2011; 83:2492–2499.
- [20]- Francius G, Lebeer S, Alsteens D, Wildling L, Gruber HJ, Hols P, De Keersmaecker S, Vanderleyden J, Dufrêne YF. Detection, localization, and conformational analysis of single polysaccharide molecules on live bacteria. ACS Nano 2008; 2(9):1921– 1929.

[21]- Sengupta R, Altermann E, Anderson RC, McNabb WC, Moughan PJ, Roy NC. The Role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal Tract. *Mediators Inflamm.* 2013; 2013:237921.

[22]- Mengele R, Sumper M. Drastic differences in glycosylation of related S-layer glycoproteins from moderate and extreme halophiles. *J. Biol. Chem.* 1992; 267:8182–8185.

[23]- Prado Acosta M, Ruzal SM, Cordo SM. S-layer proteins from *Lactobacillus sp.* inhibit bacterial infection by blockage of DC-SIGN cell receptor. *Int. J. Biol. Macromol.* 2016, 92:998–1005.

Legends to Figures

Figure 1- HPAEC-PAD analysis of the S-layer glycoprotein subjected to a reductive β -elimination treatment. A) The sample was further hydrolyzed and neutral and aminosugar components were analyzed; B) Idem A, analyzed for acidic components; C) Idem A, analyzed for reduced monosaccharides; D) Analysis of the reduced oligosaccharides. Glc: glucose; Sor: sorbitol; Gal: galactose; Man: mannose; GlcN: glucosamine; GalA galacturonic acid; GlcA: glucuronic acid. M3-M9: reduced maltooligosaccharides containing from 3 to 9 glucose units.

Figure 2- MALDI-TOF mass spectrum in the negative ion mode of the oligosaccharides obtained from the S-layer glycoprotein of *L. kefir* CIDCA 83111 after reduced β -elimination treatment of the corresponding SDS-PAGE gel band.

Figure 3 - Extracted ion chromatograms of *N*- and *O*-glycopeptides from S-layer glycoprotein obtained by C18-nano LC-ESI-Orbitrap -MS/MS analysis after trypsin treatment and diagnostic ions that arose by data dependent MS/MS. a) Total ion chromatogram (TIC) obtained by LC/MS/MS in the positive ion mode with MS range m/z 400-2000; b) Diagnostic oxonium Hex^+ (m/z 163.06) ; c) Diagnostic HexNAc^+ (m/z 204.09); d) Diagnostic $[\text{HexNAc} - 2\text{H}_2\text{O}]^+$ (m/z 168.07); e) Diagnostic $[\text{HexNAc} - \text{CH}_6\text{O}_3]^+$ (m/z 138.05).

Figure 4 – HPLC-ESI analysis of *O*-linked glycopeptides corresponding to peptide 147-160. a) Spectrum performed in the positive ion mode acquired at $R_t = 5.64$ min showing cluster ions with $z = +3$. b) Spectrum performed in the positive ion mode acquired at $R_t = 5.67$ min showing cluster ions with $z = +4$. (See Table 1 for structures)

Figure 5- HPLC-ESI analysis of *O*-linked glycopeptides corresponding to peptide 147-170. a) Spectrum performed in the positive ion mode acquired between $R_t = 10$ -15 min showing cluster ions with $z = +4$. b) Spectrum performed in the negative ion mode showing cluster ions with $z = -4$. The inset is a magnification of range m/z 1700-1900. (See Table 1 for structures)

Figure 6- HPLC-ESI analysis of *O*-linked glycopeptides corresponding to peptide 469-476 and 471-476. a) Spectrum performed in the positive ion mode acquired between $R_t = 5.14 - 5.45$ showing cluster ions with $z = +2$ corresponding to peptide 469-476. b) Spectrum performed in the negative ion mode showing cluster ions with $z = -2$ corresponding to peptide 471-476.

Figure 7- Analysis of *N*-linked glycopeptides. a) HPAEC-PAD analysis of the oligosaccharides released by PNGase F from the SDS-PAGE gel band. b) HPLC-ESI analysis in the negative ion mode obtained at $R_t = 7.58$ min showing signals corresponding to peptide $^{233}\text{IADTNATNGQK}^{243}$ and different glycoforms. c) HPLC-ESI analysis in the negative ion mode obtained at $R_t = 30.02$ min showing peptide $^{203}\text{TVTDATPYANDTFK}^{216}$ and different glycoforms. ■ = HexNAc; ▲ = dHex, ● = Hex

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1- O- glycosylated peptides detected in *Lactobacillus kefir* CIDCA 83111 S-layer glycoprotein by HPLC-ESI.

Peptide	Modification	charge	m/z calc.	m/z exp.	Error (ppm)
¹⁴⁷ SASASSASSASSTE ¹⁶⁰	Hex ₂₀	3+	1490.861 1	1490.8 721	7.37
	Hex ₂₁		1544.878 7	1544.8 824	2.37
	Hex ₂₃		1652.913 9	1652.9 221	4.94
	Hex ₂₄		1706.931 5	1706.9 264	-3.01
	Hex ₂₅		1760.949 1	1760.9 561	3.96
	Hex ₂₆		1814.966 7	1814.9 486	10.51
	Hex ₂₇		1868.984 3	1868.9 785	-3.12
	Hex ₂₈		1923.001 9	1922.9 988	-1.63
	Hex ₂₉		1977.019 5	1977.0 089	-5.38
¹⁴⁷ SASASSASSASSTE ¹⁶⁰	Hex ₃₁	4+	1564.042 875	1564.2 961	161.90
	Hex ₃₂		1604.556 075	1604.5 615	3.38
	Hex ₃₃		1645.069 275	1645.0 615	-4.73
	Hex ₃₄		1685.582 475	1685.8 4	152.78
	Hex ₃₅		1726.095 675	1726.0 867	-5.20
	Hex ₃₆		1766.608 875	1766.5 977	-6.33
	Hex ₃₇		1807.122 075	1807.3 851	145.55
	Hex ₃₈		1847.635 275	1847.6 289	-3.45
	Hex ₃₉		1888.148 475	1888.4 082	137.56
¹⁴⁷ SASASSASSASSTEQT TALTDQK ¹⁷⁰	Hex ₂₀	4+	1382.782 8	1382.7 823	-0.34
	Hex ₂₁		1423.296	1423.2	-2.23

			0	928	
	Hex ₂₂		1463.809 2	1463.8 036	-3.81
	Hex ₂₃		1504.322 4	1504.3 11	-7.56
	Hex ₂₄		1544.835 6	1544.8 226	-8.40
	Hex ₂₆		1625.860 0	1625.8 561	-2.41
	Hex ₂₇		1666.375 2	1666.3 723	-1.73
	Hex ₂₈		1706.888 4	1706.8 783	-5.90
	Hex ₂₉		1747.401 6	1747.4 017	0.07
	Hex ₃₀		1787.914 8	1787.9 008	-7.82
	Hex ₃₁		1828.428 0	1828.4 177	-5.62
	Hex ₃₂		1868.941 2	1868.9 435	1.24
	Hex ₃₃		1909.454 4	1909.4 424	-6.27
	Hex ₃₄		1949.967 6	1949.9 532	-7.37
	Hex ₃₅		1990.480 8	1990.4 73	-3.91
¹⁴⁷ SASASSASSASSTEQT TALTDQK ¹⁷⁰	Hex ₂₀	4-	1380.768 2	1380.7 684	0.16
	Hex ₂₁		1421.281 4	1421.2 803	-0.76
	Hex ₂₂		1461.794 6	1461.7 98	2.34
	Hex ₂₃		1502.307 8	1502.3 051	-1.76
	Hex ₂₄		1542.821 0	1542.8 199	-0.70
	Hex ₂₆		1623.845 4	1623.8 507	3.25
	Hex ₂₇		1664.360 6	1664.3 583	-1.37
	Hex ₂₈		1704.873 8	1704.8 676	-3.62
	Hex ₂₉		1745.387 0	1745.3 832	-2.16
	Hex ₃₀		1785.900	1785.8	-2.90

			2	95	
	Hex ₃₁		1826.413 4	1826.4 084	-2.72
	Hex ₃₂		1866.926 6	1866.9 222	-2.34
	Hex ₃₃		1907.439 8	1907.4 371	-1.40
	Hex ₃₄		1947.953 0	1947.9 553	1.19
¹⁴⁷ SASASSASSASSTEQT TALTDQK ¹⁷⁰	Hex ₂₉ HexA	4-	1789.395 0	1789.3 876	-4.13
	Hex ₂₈ HexA ₂		1792.889 8	1792.8 851	-2.63
	Hex ₂₈ HexA ₂ ⁺ H ₂ O		1797.392 5	1797.3 877	-2.65
	Hex ₃₀ HexA		1829.908 2	1829.8 981	-5.52
	Hex ₂₉ HexA ₂		1833.403 0	1833.3 987	-2.36
	Hex ₂₉ HexA ₂ ⁺ H ₂ O		1837.905 7	1837.9 108	2.79
	Hex ₂₈ HexA ₃ ⁺ H ₂ O		1841.400 5	1841.4 114	5.92
	Hex ₃₁ HexA		1870.421 4	1870.4 252	2.03
	Hex ₃₀ HexA ₂		1878.418 9	1878.4 196	0.39
	Hex ₃₀ HexA ₂ ⁺ H ₂ O		1882.920 8	1882.9 182	-1.36
⁴⁶⁹ DKTTTSAE ⁴⁷⁶	Hex ₃ HexA ₂	2+	845.8219	845.73 68	89.74
	Hex ₄ HexA ₂		926.8386	926.76 51	79.29
	Hex ₅ HexA ₂		1007.865	1007.7 856	78.77
	Hex ₆ HexA ₂		1088.891 4	1088.8 151	70.06
⁴⁷¹ TTTSAE ⁴⁷⁶	Hex ₅ HexA	2-	796.2734	796.25 04	-28.88
	Hex ₆ HexA		877.2998	877.27 8	-24.85
	Hex ₇ HexA		958.3262	958.26 07	-68.35
	Hex ₈ HexA		1039.352 6	1039.2 854	-64.66
	Hex ₅ HexA ₂ Na		906.2717	906.28	14.30

	2		4	47	
	Hex ₆ HexA ₂ Na		987.2981	987.31	13.13
	2		4	11	

Table 2 Summary of the glycoforms found in the S-layer glycoprotein of *L. kefir* CIDCA 8111.

	Glycopeptide		oligosaccharide	Peptide sequence
	Ret. time (min)	charge	modification	
<i>O</i> -glycosylation	5.14-5.45	+2	Glc ₃₋₆ GalA ₂	⁴⁶⁹ DKTTTSAE ⁴⁷⁶
	5.14-5.45	-2	Glc ₅₋₈ GalA ₁₋₂	⁴⁷¹ TTTSAE ⁴⁷⁶
	5.64	+3	Glc ₂₀₋₂₉	¹⁴⁷ SASASSASSASSTE ¹⁶⁰
	5.64	+4	Glc ₃₁₋₃₉	¹⁴⁷ SASASSASSASSTE ¹⁶⁰
	7.51	+4	Glc ₂₀₋₄₇	¹⁴⁷ SASASSASSASSTEQTALTDAQK ¹⁷⁰
	7.51	-4	Glc ₂₀₋₃₄ GalA ₀₋₃	¹⁴⁷ SASASSASSASSTEQTALTDAQK ¹⁷⁰
<i>N</i> -glycosylation	7.58	-2	GlcNAc ₂ MandHex; GlcNAc ₄ Man ₃ dHex; GlcNAc ₂ Man ₅ dHex ₂	²³³ IADTNATNGQK ²⁴³
	7.58	+2	GlcNAc ₂	²³³ IADTNATNGQK ²⁴³
	54.33	+3	GlcNAc ₂ Man; GlcNAc ₂ Man ₂	²³³ IADTNATNGQKINGWIK ²⁴⁹
	30.02	-2	GlcNAc ₂ dHex	²⁰³ TVTDATPYANDTFK ²¹⁶
	30.02	+2	GlcNAc ₂ dhex	²⁰³ TVTDATPYANDTFK ²¹⁶

Conflict of interest

The authors declare that this manuscript has no conflict of interest.

ACCEPTED MANUSCRIPT

Graphical abstract

Significance

A detailed characterization of protein glycosylation is essential to establish the basis for understanding and investigating its biological role. It is known that S-layer proteins from kefir-isolated *L. kefiri* strains are involved in the interaction of bacterial cells with yeasts present in kefir grains and are also capable to antagonize the adverse effects of different enteric pathogens. Therefore, characterization of type and site of glycosidic chains in this protein may help to understand these important properties. Furthermore, this is the first description of *N*-glycosidic chains in S-layer glycoprotein from *Lactobacillus* spp.

Highlights

- Detailed study of the S-Layer protein glycosylation of *L. kefir* CIDCA 83111.
- Presence of two *O*-glycosylated peptides bearing Glc₅₋₈GalA₁₋₂ residues.
- Description of two *N*-glycosylated sites substituted with short *N*-glycan structures.
- First description of *N*-glycosylation in *Lactobacillus* species

ACCEPTED MANUSCRIPT