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Bovine glycomacropeptide promotes the growth of *Bifidobacterium longum* ssp. *infantis* and modulates its gene expression

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ABSTRACT

Bovine milk glycomacropeptide (GMP) is derived from κ-casein, with exclusively *o*-linked glycosylation. Glycomacropeptide promoted the growth of *Bifidobacterium longum* ssp. *infantis* in a concentration-dependent manner, and this activity was lost following periodate treatment of the GMP (GMP-P), which disables biological recognition of the conjugated oligosaccharides. Transcriptional analysis of *B. longum* ssp. *infantis* following exposure to GMP revealed a substantial response to GMP relative to bacteria treated with GMP-P, with a greater number of differentially expressed transcripts and larger fold changes versus the control. Therefore, stimulation of *B. longum* ssp. *infantis* growth by GMP is intrinsically linked to the peptide's *O*-linked glycosylation. The pool of differentially expressed transcripts included 2 glycoside hydrolase (family 25) genes, which were substantially upregulated following exposure to GMP, but not GMP-P. These GH25 genes were present in duplicated genomic islands that also contained genes encoding fibronectin type III binding domain proteins and numerous phage-related proteins, all of which were also upregulated. Homologs of this genomic arrangement were present in other *Bifidobacterium* species, which suggest it may be a conserved domain for the utilization of glycosylated peptides. This study provides insights into the molecular basis for the prebiotic effect of bovine milk GMP on *B. longum* ssp. *infantis*.
Key words: glycomacropeptide, *Bifidobacterium*, bovine milk, transcription

INTRODUCTION

A bifidobacteria-rich microbiome has numerous biological benefits to the host, including maintenance of a healthy gastrointestinal tract, inhibition of microbial infection, and alleviating symptoms associated with digestive illness (Leahy et al., 2005; Picard et al., 2005). Therefore, interest is growing in enhancing the bifidobacterial population of the gastrointestinal tract. Human milk oligosaccharides (**HMO**) represent the main impetus for bacterial colonization of the distal large intestine of the breast-fed infant (Scholtens et al., 2012). The high concentrations of HMO and oligosaccharides either *N*- or *O*-linked to proteins processed after intestinal digestion are thought to be the main contributors to the predominance of *Bifidobacterium* species in the infant gut (Garrido et al., 2013a). Indeed, the genome of one particular strain, *Bifidobacterium longum* ssp. *infantis* ATCC15697, a common member of the gastrointestinal microbiota of breast-fed infants, has revealed particular adaptations for the metabolism of HMO (LoCascio et al., 2007, 2010; Sela et al., 2008, 2011, 2012; Garrido et al., 2011, 2012b; Kim et al., 2013), milk glycoconjugates (Garrido et al., 2013a), and even commercial oligosaccharides such as galacto-oligosaccharides (Garrido et al., 2013b), and fructo-oligosaccharides (Perrin et al., 2001). In particular, the strain consumes these various carbohydrates using a variety of glycosyl hydrolases and ABC transporters (Sela et al., 2008; Garrido et al., 2013a).

However, the large quantities of HMO required for use as functional ingredients are unavailable, whereas commercial oligosaccharides such as galacto-oligosaccharides and fructo-oligosaccharides and individual HMO cannot match the complexity of the HMO pool present in breastmilk in terms of biological benefits. Bovine milk is potentially an alternative source of complex oligosaccharides with associated biological activity. However, the concentration and number of oligosaccharides is much lower than that of human milk, and currently the large quantities required are commercially unavailable. Certain glycoconjugates present in bovine milk have been purified at a large scale, as previously reviewed (O'Riordan et al., 2014), and may offer an

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alternative source of functional oligosaccharides. Karav et al. (2016) recently demonstrated the ability of bifidobacteria to use *N*-linked glycan chains cleaved from bovine milk glycoproteins as growth promoting factors and different bifidobacterial strains selectively consumed varying glycan structures.

Of the glycosylated proteins present in bovine milk, glycomacropeptide (**GMP**), a 64-AA peptide derived from κ-CN (Delfour et al., 1965), offers great potential as a functional food ingredient. Importantly, GMP can be produced in the quantities required for use as a food ingredient (Tullio et al., 2007) and it is commercially available as an ingredient for application in functional and medical foods, beverages, cosmetics, and supplements (O'Riordan et al., 2014; Ney et al., 2016). Glycomacropeptide has been formulated into medical foods for the treatment of phenylketonuria (Ney et al., 2016; Ney and Etzel, 2017) and has been shown to improve the growth of lactobacilli and bifidobacteria and enhance microbial diversity, both in vitro (Janer et al., 2004; Robitaille, 2013; Ntemiri et al., 2017) and in vivo (Chen et al., 2012; Sawin et al., 2015). Several other biological benefits have also been associated with GMP, including anti-infective (Nakajima et al., 2005; Gustavo Hermes et al., 2013) and antitoxigenic (Kawasaki et al., 1992; Isoda et al., 1989) activities. The carbohydrate content of bovine GMP is known to play a role in many of its associated biological benefits (O'Riordan et al., 2014).

However, reports are conflicting on the importance of the carbohydrate content of GMP in relation to the growth promotion of bifidobacteria, with recent studies suggesting that only the peptide backbone of CMP was essential for prebiotic activity (Robitaille, 2013). In the present work, the growth- and metabolic-related transcriptomic changes of an archetypical infant *B. longum* ssp. *infantis* strain following transient exposure to GMP is reported. The oligosaccharides on GMP were oxidized using sodium metaperiodate to investigate the role of GMP glycosylation. The observed phenotypic changes were subsequently correlated with transcriptional changes as determined by microarray analysis.

MATERIALS AND METHODS

Materials

Glycomacropeptide, with a maximum assayed lactose content of 1%, was provided by Agropur Ingredients, (Eden Prairie, MN). Vivaspin 6 centrifugal filters with a 3-kDa molecular weight cutoff were purchased from Sartorius Stedim Biotech GmbH (Göttingen, Germany). *Bifidobacterium longum* ssp. *infantis* ATCC 15697 was purchased from DSMZ (Braunschweig, Germany). de Man, Rogosa, Sharpe (**MRS**) broth was purchased from Oxoid Ltd. (Basingstoke, UK). The Anaerocult A system was purchased from Merck (Dannstadt, Germany). All other reagents were from Sigma-Aldrich Co. (Dublin, Ireland), unless otherwise stated, and were of the highest grade available.

Periodate Treatment of GMP

Sodium metaperiodate ($NaIO_4$) treatment of GMP (**GMP-P**) was performed as previously described (Alemka et al., 2010). Briefly, GMP (2 mg/mL) was incubated with 0.011 m*M* $NaIO_4$ (Alemka et al., 2010) at 4°C for 30 min. Excess $NaIO_4$ was removed by centrifugal filtration using a 3-kDa molecular weight cutoff with 3 PBS, pH 7.4 washes and the retentate containing GMP-P was lyophilized.

Bacterial Culture and Growth Experiments

Bifidobacterium longum ssp. *infantis* was routinely cultured in MRS supplemented with 0.05% wt/vol L-cysteine (**mMRS**) at 37°C under anaerobic conditions generated using the Anaerocult A system (Kavanaugh et al., 2013). Bacterial culture stocks were maintained in mMRS containing 50% vol/vol glycerol at −80°C and propagated twice in mMRS medium before use. Bacterial growth assays were performed in mMRS supplemented with increasing concentrations of GMP (0.5, 1, 2, 4, and 8 mg/mL) and filter sterilized through a 0.22-μm membrane. The medium was inoculated with a 1% vol/vol inoculation of an overnight culture with an optical density at 600 nm ($\mathbf{OD_{600nm}}$) of approximately 1.0 (corresponding to approximately 2×10^8 cfu/mL). Cultures entered midexponential phase of growth after 16 h, and OD_{600nm} values at this time point were taken for comparison between each treatment. Growth experiments were performed in triplicate and the data presented is the average of 3 independent replicate assays. The OD_{600nm} was measured on a PharmaSpec UV-1700 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan).

RNA Isolation

Bifidobacterium longum ssp. *infantis* was cultured to midexponential phase in mMRS as a control and mMRS supplemented with GMP or GMP-P (2 mg/mL) and bacterial pellets were harvested by centrifugation at 4,500 × *g* for 8 min at 4°C. The supernatant was removed and the bacterial pellets were resuspended in RNAprotect bacteria reagent (Qiagen, Hilden, Ger-