Development of A Growth Medium Suitable for Exopolysaccharide Production and Structural Characterisation by *Bifidobacterium animalis* ssp. *lactis* AD011

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A B S T R A C T

Exopolysaccharides (EPSs) produced by Bifidobacteria have received considerable attention due to their ability to modify the rheological and physicochemical properties of dairy products. However, the quantification and characterisation of Bifidobacterial EPS is hampered by the presence of EPS-equivalent (EPS-E) substances in complex media such as Reinforced Clostridial Medium (RCM).

This study has developed a media based on RCM which both supports the growth of *Bifidobacterium animalis* ssp. *lactis* AD011 and does not interfere with the quantification and characterisation of the EPS generated. Media development involved the identification of EPE-E containing components via NMR analysis followed by their removal, substitution or pre-treatment. Both beef extract and casein acid hydrolysate required chemical pre-treatment to remove polysaccharide components before the media was free of EPS-E materials. Once EPS-E free components had been identified, lactose, glucose and galactose were evaluated as potential carbon sources. Glucose was found to be the optimum carbon source. The final media composition supported growth to the same extent as RCM providing significant EPS yields and no interferences during analysis.

1. Introduction

Exopolysaccharides (EPS) produced by *Bifidobacterium* sp. have been subjected to considerable investigation, due to their ability to alter the rheological properties and thus the texture of dairy products (Prasanna, Bell et al. 2012) as well as their potential contribution to human health (Ruas-Madiedo, Hugenholtz et al. 2002; Patel and Prajapati 2013). In order to study EPS generation by Bifidobacteria it is necessary to have a growth media that supports both growth and EPS production, whilst at the same time does not contain materials which interfere with EPS analysis.

Bifidobacteria are relatively fastidious organisms that require a complex, nutrient rich media to grow optimally (Roy 2001). A wide range of culture media have been developed for Bifidobacteria primarily aimed at their isolation and enumeration particularly in the presence of other dairy related bacteria such as Lactobacilli and Streptococci. Many of these media are based on commonly available undefined components supplemented with factors that select for Bifidobacteria (Dave and Shah 1996; Roy 2001; Ashraf and Shah 2011). Whilst media such as Tryptone Proteose Peptone Yeast extract medium (TPPY), Reinforced Clostridial Medium (RCM), and De Man Rogosa and Sharpe (MRS) medium are all able to support excellent growth of Bifidobacteria cultures (Dave and Shah 1996; Roy 2001; Ashraf and Shah 2011), the presence of complex undefined components such as whey permeate, beef extract and yeast extract make them unsuitable for EPS studies. This is because these components contain EPS-equivalent (EPS-E) substances which interfere with EPS characterisation and quantification (Gancel and Novel 1994). The removal of these undefined components is problematic since they are known to stimulate the growth of Bifidobacteria (Gyorgy and Rose 1955; Adebayo-Tayo and Onilude 2008). These so called bifidogenic factors have been classified into two groups: protein hydrolysates and oligosaccharides (Biavati and Mattarelli 2006), and have been shown to be retained by dialysis during EPS analysis (Gyorgy, Jeanloz et al. 1974).

The presence of EPS-E containing components in conventional Bifidobacteria growth media has resulted in EPS related studies employing solid rather than broth based culture methods (Ruas-Madiedo and de los Reyes-Gavilan 2005) which prevents large scale bioreactor based cultivation. Since the culture media is known to influence EPS yield and the molecular characteristics of the biopolymers (Ruas-Madiedo and de los Reyes-Gavilan 2005) there is a clear need for a medium able to support growth and EPS production by Bifidobacteria which is free of EPS-E substances. Both defined and semi-defined media suitable for EPS studies have been described for some bacterial strains, such as *Lactobacillus delbrueckii* ssp. *bulgaricus* (Kimmel and Roberts 1998) and *Streptococcus thermophilus* (Levander, Svensson et al. 2001). However, to date no such medium has been described for Bifidobacteria.

This paper outlines the development and validation of a broth medium suitable for EPS production by *Bifidobacterium animalis* ssp. *lactis* AD011 which is able to support significant growth and EPS production whilst being free from material which interferes with both EPS yield determination and EPS characterisation.

2. Material and Methods

2.1. Bacterial strain and starter culture preparation

The strain of *B. animalis* ssp. *lactis* AD011 was isolated from a commercial diary product and was stored on beads (Microbank, Pro-Lab diagnostics Ltd, Neston, UK) at -80°C prior to use. DNA from the culture was extracted using a commercial kit (MO-BIO, Carlsbad, CA, US) and the 16S rRNA gene was amplified using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1510R (5′-GGTTACCTTGTTACGACTT-3′). The PCR product was purified using a Qiagen PCR purification kit (Qiagen, Manchester, UK) and sequenced commercially using a modified Sanger sequencing technique (MWG-BIOTECH, Germany). The sequence was chimera checked using the chimera.uchime component of MOTHUR (Schloss, Westcott et al. 2009) before being compared against the 16S ribosomal RNA sequence database of the NCBI suite, optimised for highly similar sequences (megablast) (Altschul, Madden et al. 1997). The sequence was found to be 100% similar to the 16S rRNA gene sequence for *Bifidobacterium animalis* subsp. *lactis* AD011 (accession number NC_011835.1). Cultures were revived on MRS agar (LabM Ltd, Heywood, UK) incubated anaerobically (80% N_2, 10% CO_2 and 10% H_2), (Don Whitley Anaerobic Workstation, Shipley, UK) at 37^{o}C for 48 hours. Cultures were passaged twice prior to sub culturing into 20 mL of MRS broth (LabM Ltd, Heywood, UK) for 18 hours. The cells were then harvested by centrifugation, washed with maximum recovery diluent (MRD, LabM Ltd, Heywood, UK) and re-suspended in 20 mL of MRD prior to use. Cell growth was monitored at three hourly intervals by measuring the optical density (OD) at 600 nm.

2.2. Determination of glucose and lactate concentrations

Glucose and lactate concentrations were measured using a Picotrace biosensor (TRACE Analytics GmbH, Braunschweig, Germany). 1 mL aliquots of culture medium were collected and filtered through a 0.45 µm syringe filter to remove cells before analysis.

2.3. EPS assay

Broth cultures were centrifuged (13,500 x g, 4°C, 15 min) (Avanti J-26S XPI-Beckman Coulter, Inc, USA) to remove bacterial cells, the resulting supernatant was then mixed with two volumes of absolute ethanol and left at 4°C for 2 days to precipitate the EPS. The precipitate was recovered by centrifugation (25,000 x g, 4 °C, 30 min) and re-dissolved in ultra-pure water (20-25 mL) prior to dialysis (12-14 kDa molecular mass cut-off dialysis tube (Sigma, UK)) for 3 days with three changes of water per day. Finally

samples were freeze-dried, analysed by NMR and quantified by weight and by the phenol-sulphuric acid carbohydrate assay, with glucose (Fisher Ltd, Loughborough, UK) as a standard (Dubois, Gilles et al. 1956).

2.4. Media Development

RCM broth, beef extract and lactose were purchased from LabM Ltd (Heywood, UK). The media (Huddersfield Bifidobacteria media (HBM)) development procedure is outlined in Figure 1. Initially (Step 1) undefined media constituents e.g. beef extract, yeast extract, peptone etc., were subjected to the EPS isolation procedures to evaluate if EPS-E substances were present. Once the EPS-E free, defined constituents had been identified these were used as a starting point for media development (Step 2). The undefined EPS-E containing materials were divided into non-essential and essential cell growth components with non-essential components being omitted (Step 3) and essential components being either replaced with equivalent EPS-E free materials (Step 4) or treated to eliminate EPS-E residues (Step-5).

To remove EPS-E from undefined media constituents (i.e. beef extract and casein acid hydrolysate) a concentrated solution of material (20% w/v) was prepared in ultra-pure water and the solution autoclaved. After cooling to room temperature, three volumes of absolute ethanol were added and the solution was left standing at 4°C for 36-48 hours during which time EPS-E materials precipitated. Finally, the mixture was centrifuged twice (25,000 x g, 4°C, 30 min) to obtain a clear supernatant after which the ethanol was removed by distillation. The retained material was centrifuged (25,000 x g, 4°C, 30 min) before being added to other components of the media.

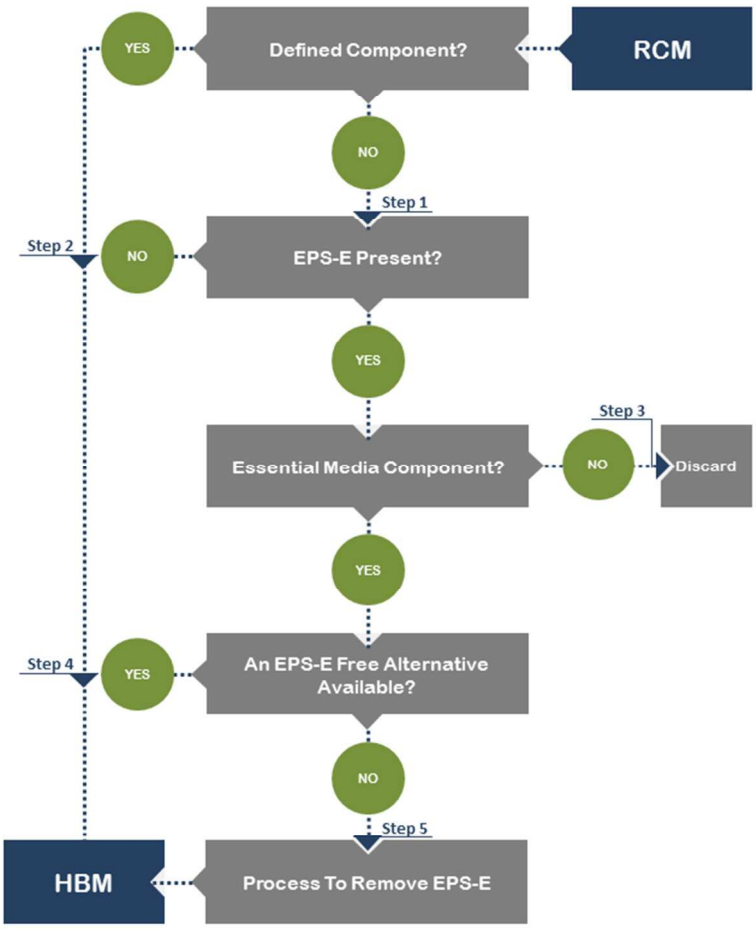

Figure 1. Media development flow chart.

2.5. ^{1}H NMR spectroscopy

For EPS and EPS-E detection, freeze-dried samples (6.5 mg) were dissolved in D_2O (0.65 mL) (99.99% Goss Scientific Instruments Ltd, Essex, UK). NMR spectra were recorded at an elevated probe temperature (70°C) in order to shift the HOD signal into a clearer region of the spectrum. The higher temperature also increased spectral resolution by reducing the sample viscosity. All NMR spectra were recorded on a Bruker Avance 500.13 MHz ^{1}H (125.75 MHz ^{13}C) spectrometer (Bruker-biospin, Coventry, UK) operating with Z-field gradients where appropriate, and using Bruker's pulse programs. Spectra were referenced with respect to internal acetone ($\delta = 2.635$).

3. Results and Discussion

3.1. Media Evaluation

Two commonly used media (BIM and MRS (Roy 2001; Ashraf and Shah 2011)) for Bifidobacteria were evaluated for their EPS-E content (Figure 2). Both media generated a number of peaks in the proton NMR spectra in the region normally associated with EPS (Figure 2) which would interfere with structural analysis. Both MRS and the basal media for BIM, RCM (Roy 2001) contain peptone, beef extract and yeast extract components (Table 1) likely to contain EPS-E materials. Since RCM is simpler in composition than MRS, it was chosen as a starting point for further study.

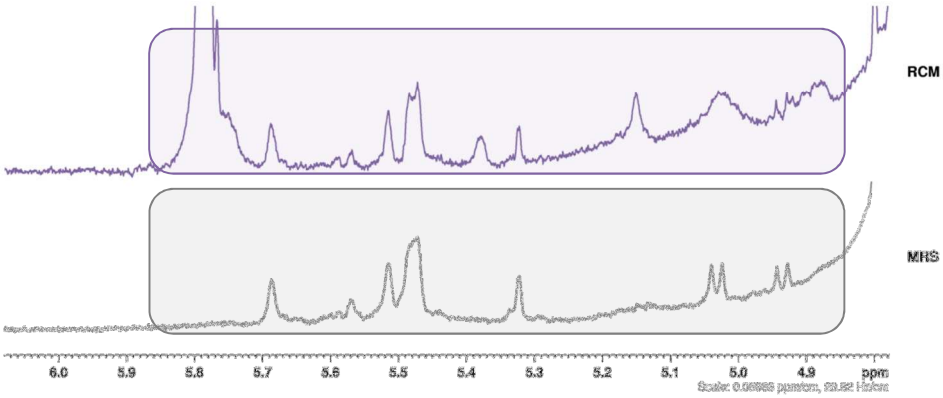

Figure 2. Proton NMR spectra (anomeric region) of EPS-equivalent content of RCM and MRS broth.

Table 1. Media Composition.

Ingredients	Media Composition (g/l)	
	RCM	MRS
Beef extract (BE)	10	10
Yeast extract (YE)	3	5
Peptone (pep)	10	10
Starch (St)	1	-
Glucose	10	20
Sodium chloride	5	-
Sodium acetate	3	5
Potassium phosphate	-	2
Magnesium sulphate	-	0.2
Manganese sulphate	-	0.05
Ammonium citrate	-	2.0
Tween® 80	-	1.1

3.2. Assessment of EPS-E in the undefined components of RCM

RCM produced on average 895.2 mg/L of EPS-E (Table 2), not surprisingly the majority of this originated from the soluble starch (St) 31%, followed by beef extract (BE) 26%, yeast extract (YE) 24% and peptone (Pep) 18%. The ^{1}H-NMR spectra (the anomeric region (δ4.6-5.8)) of RCM with and without EPS-E containing components (Figure 3) show a range of peaks in the region normally associated with EPS which would interfere with structural analysis.

Table 2. Mean EPS-equivalent content of cell-free RCM broth, (three replicates).

Medium	EPS equivalent (mg/L)	Removal of EPS-E Materials (% w/w)
RCM (All)	895.2 ± 3.1	0
RCM without (St)	624.6 ±2.7	30.2
RCM without (St and YE)	402.8 ± 2.4	55.0
RCM without (St and YE and Pep)	241.2 ± 1.9	73.0
RCM without (St and YE and Pep and BE)	3.3 ± 0.3	99.6

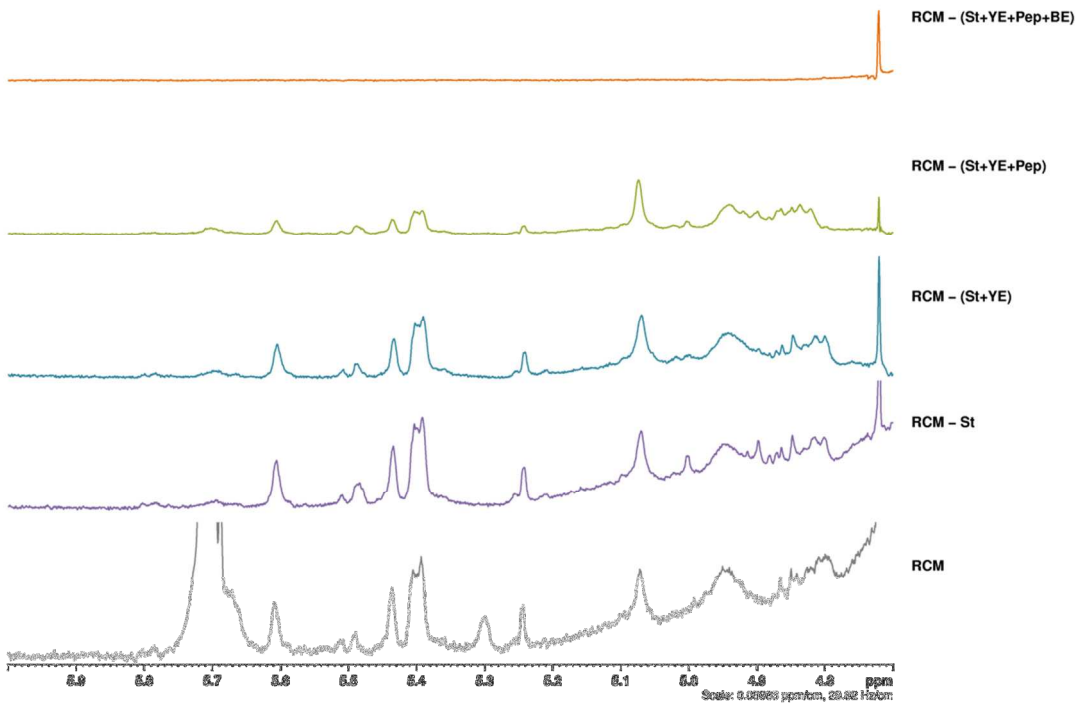

Figure 3. Proton NMR spectra (anomeric region) of EPS-equivalent content of RCM broth.

3.3. Medium development

Sodium chloride and sodium acetate (Fisher Scientific Ltd, Loughborough, UK) where used as a starting point for the media together with L-cysteine-HCl (Fisher Scientific Ltd, Loughborough, UK) which, in addition to improving the anaerobic conditions by reducing the redox potential, is considered as an essential nitrogen source for Bifidobacteria (Shah 1997). Starch was the first component to be eliminated

from the media since it significantly contributed to the EPS-E content (Table 1). It had been previously reported (Gancel and Novel 1994) that yeast nitrogen base (YNB) without amino acids (Sigma-Aldrich Ltd, Gillingham, UK) was a suitable EPS-E free replacement for yeast extract and NMR analysis identified it as being free from EPS-E interferences (Figure 4). YNB also has the advantage of providing a range of trace elements and minerals that support bacterial growth. This was combined with casein-acid hydrolysate (CAH) (Fluka, Louis, USA) which provided a source of amino acids and short peptide fragments. Although CAH does not generate interfering peaks during NMR analysis (Figure 4-top), some of its non-sugar based components are retained by the EPS extraction procedure and subsequently interfere with the quantification and physico-chemical analysis of EPS. Consequently, CAH was pre-treated prior to being added to the media (Treated Casein-acid Hydrolysate-TCAH).

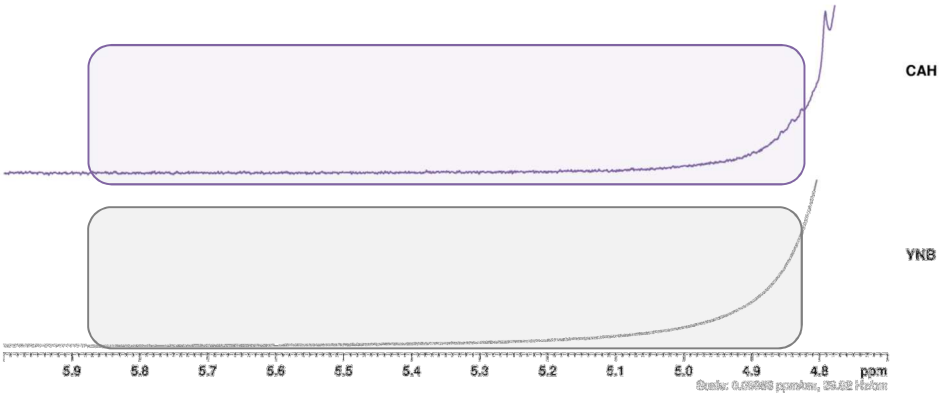

Figure 4. Proton NMR (anomeric region) of casein and yeast nitrogen base.

No effective replacement for beef extract was identified, suggesting that beef extract contains essential growth factors for *B. animalis* ssp. *lactis* AD011 (Figure 5), consequently it was necessary to treat the beef extract to remove EPS-E substances (Treated Beef Extract-TBE). Based on reported preferences of *B. animalis* (Laxmi, Mutamed et al. 2011, Payne, Morris et al. 1998), lactose and galactose were evaluated as alternative sugar sources to glucose.

The final HBM media composition included: TBE 10g/l, TCAH 10g/l, YNB 3 g/l, glucose, galactose or lactose 10g/l, NaCl 5g/l, Na-Acetate 3 g/l and L-cysteine-HCl (0.05%) (Table 3). The impact of individual media components on growth were investigated via single omissions. The omission of TCAH slightly reduced the growth, while more significant impacts resulted from the removal of glucose or TBE (Figure 5), however the amount of EPS produced in the absence of TCAH is reduced (data not shown).

Table 3. HBM Media Composition.

Ingredients	Media Composition (g/l)
Treated beef extract (TBE)	10
Treated casein Acid hydrolysate (TCAH)	10
Yeast nitrogen base (YNB)	3
Glucose	10
Sodium chloride	5
Sodium acetate	3
L-cysteine-HCl	0.5

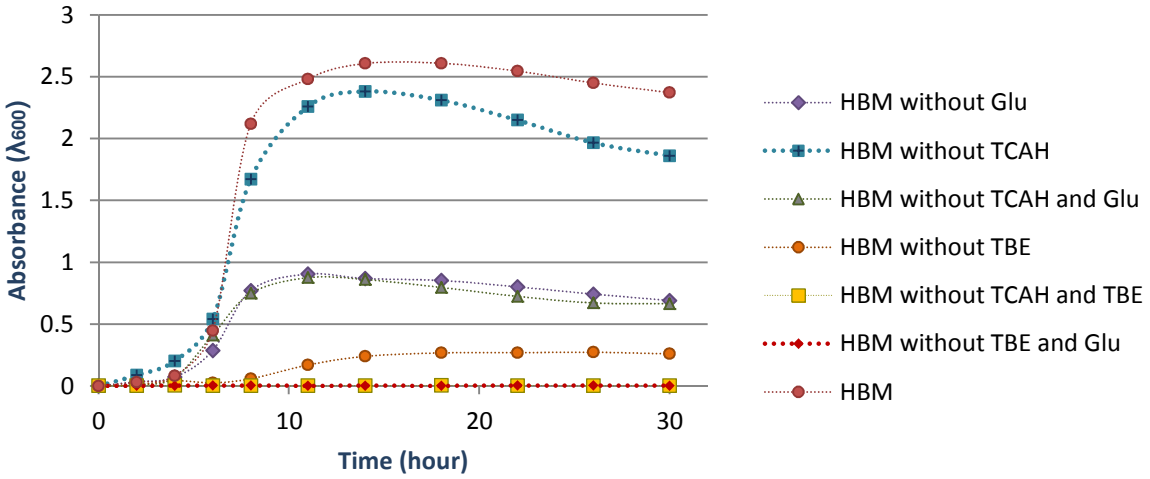

Figure 5. The effect of HBM components on growth of *B. animalis* ssp. *lactis* AD011.
(TCAH: treated casein acid hydrolysate, TBE: treated beef extract, Glu: glucose).

3.4. EPS generation

When subjected to the isolation procedure, the uninoculated HBM media, generated 4.3±1.5 mg/L of material which did not generate any anomeric peaks in the proton NMR (Figure 6), a marked improvement on both RCM and MRS (Figure 2). When the HBM media was inoculated with *B. animalis* ssp. *lactis* AD011 and the EPS isolated, its NMR spectra was very similar to EPS isolated from cultures grown on solid media. The NMR signatures of EPS extracted from broth and plate grown biomass were identical when the NaOH treatment step used to extract EPS from plate grown cultures (Salazar, Ruas-Madiedo et al. 2009) was included in the treatment process for broth grown biomass (unpublished results).

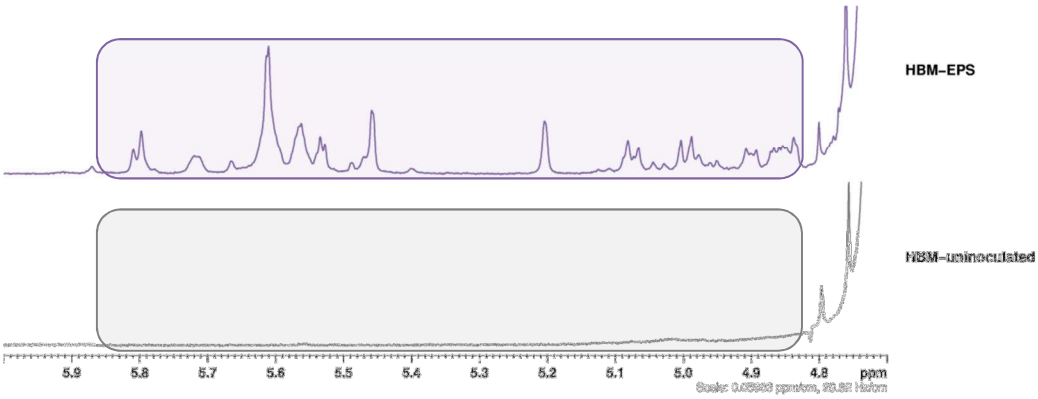

Figure 6. Proton NMR spectra (anomeric region) of EPS-equivalent content of uninoculated HBM broth media vs. Peaks generated by EPS from *B. animalis* ssp. *lactis* AD011.

The ability of glucose, galactose and lactose (HBM-gl, HBM-ga and HBM-l) to support the growth of *B. animalis* ssp. *lactis* AD011 was compared with RCM. The use of glucose as a carbon source generated equivalent levels of growth when compared to RCM (Figure 7), whilst HBM-ga and HBM-l generated reduced growth over the same period; the same results were mirrored in the generation of the fermentation end product lactate (Figure 8). This contradicts reports that some strains of *B. animalis* ssp. *lactis* prefer lactose to glucose and galactose (Van der Meulen, Avonts et al. 2004; Hsu, Yu et al. 2005). However, closely related strain of *Bifidobacterium animalis* ssp. *lactis* are known to differ in their utilization of glucose

(Briczinski, Phillips et al. 2008), with rapid growth on glucose as a lone carbon source having previously been reported (Briczinski, Loquasto et al. 2009).

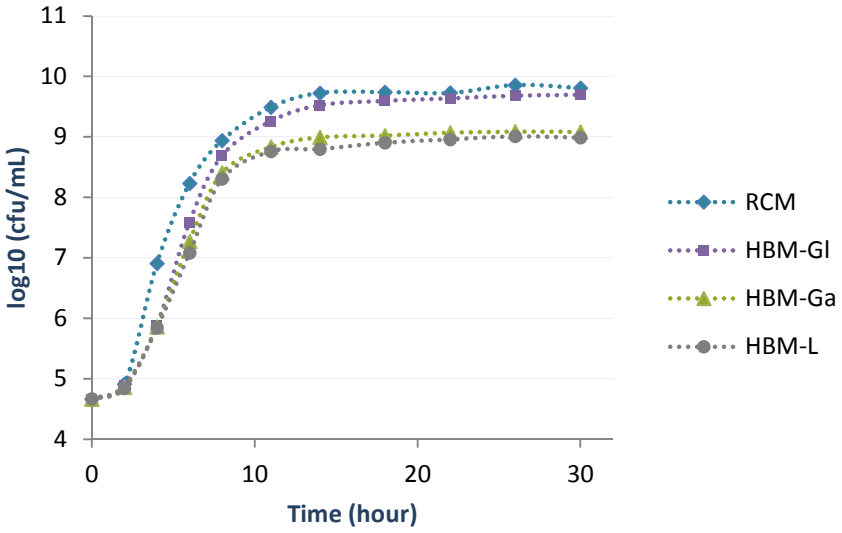

Figure 7. Growth of *B. animalis* ssp. *lactis* AD011 inRCM and HBM cultures.

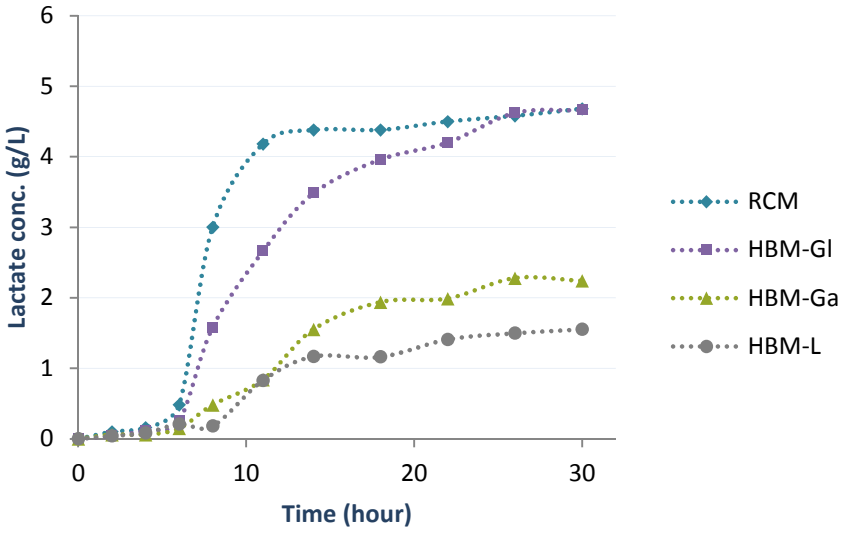

Figure 8. Lactate generation by *B. animalis* ssp. *lactis* AD011 cultured in HBM and RCM cultures.

4. Conclusions

The development of the HBM media provides an EPS-E free broth based culture media that allows the large-scale cultivation and associated EPS production of *B. animalis* ssp. *lactis* AD011. Unlike the available minimal, defined and semi-defined media designed for lactic acid bacteria (Kimmel and Roberts 1998; Levander, Svensson et al. 2001; van Niel and Hahn-Hägerdal 1999), HBM media is based on a range of easily available media constituents treated to remove EPS-E contaminants. The absence of EPS-E compounds simplifies the EPS extraction and purification procedures, prevents interference with many of the analytical techniques traditionally used to quantify EPS production (e.g. using phenol-sulphuric acid test (Dubois, Gilles et al. 1956)) and their structural characterisation (e.g. NMR spectroscopy and High

Performance Anion Exchange Chromatography). However, further work is needed to determine the ability of HBM media to support the growth and EPS production of other bacterial species.

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