CHARACTERISATION OF *BIFIDOBACTERIUM* INDUCED FROM PREBIOTIC (GALACTO-OLIGOSACCHARIDE) SUPPLEMENTED PIGS

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Abstract

Pork is the second most consumed meat worldwide and due to its increasing global demand, there is a requirement for the commercial pig farming industry to maintain efficient production while maintaining good animal welfare standards. Since the use of antibiotics as growth promoters was banned in the EU, the use of dietary fibres as prebiotics for growth promotion in livestock is a rapidly expanding area of scientific research. As such, bacteria that are significantly affected following the addition of prebiotics to animal feed are of great interest to understand the microbe-host relationship. *Bifidobacteria* inhabit the mammalian gastrointestinal tract and confer several host health benefits such as increased luminal short-chain fatty acid (SCFA) concentrations, lower pathogenic colonisation in the gut and reduced intestinal inflammation. Prebiotic galacto-oligosaccharides (GOS) have been shown to enrich *Bifidobacterium* populations in the hindgut, which has been correlated with increased acetate, butyrate and propionate production. Such SCFA are also suggested to improve fat and protein retention. This study aims to sequence *Bifidobacterium spp*. isolated from pigs fed a GOS supplemented diet and identify coding regions responsible for GOS metabolism and SCFA synthesis. Bacteria isolated from pig caecal and colonic samples were sequenced using the Illumina MiSeq sequencing platform. The complete genome sequences of *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium pseudolongum* and *Lactobacillus reuteri* are reported. The assembled circular genomes were 1.96 (*B. animalis* subsp. *lactis*), 1.94 (*L. reuteri*) and 1.97 (*B. pseudolongum*) Mb and comprised of 1542, 1772-1774 and 1570-1572 protein coding genes, respectively. *B. animalis* subsp. *lactis* possessed genes for both the LacS/LacZ and LacEF/LacG pathways to fully metabolise GOS, whereas *L. reuteri* and *B. pseudolongum* possessed only the LacS/LacLM and LacS/LacZ, respectively. Furthermore, both *Bifidobacteria* possess *ackA* and *tesB*, genes responsible for producing proteins involved in acetate and butyrate synthesis, whereas *L. reuteri* only possessed *ackA*.

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COVID-19 disruption statement

Due to the disruption caused by the COVID-19 pandemic, the work undertaken here may be shorter than what is usually presented in an MRes thesis. Laboratory experiments took longer to perform than usual due to restrictions that were put in place to keep students and staff safe during the pandemic. This impacted the time available for true in-depth analysis of the genomes sequenced during this project.

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List of abbreviations

bp	Base pairs
da	Days of age
DNA	Deoxyribose nucleic acid
dsDNA	Double stranded DNA
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union
g	Grams
Gal	Galactose
GIT	Gastrointestinal tract
Glu	Glucose
GOS	Galacto-oligosaccharide
h	Hours
HS	High sensitivity
IleS	Isoleucyl-tRNA synthetase
min	Minutes
mM	Milli molar
MRS	De Man, Rogosa and Sharpe
NaOH	Sodium hydroxide
ng	Nano grams
NGS	Next generation sequencing
nM	Nano molar
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PWD	Post-weaning diarrhoea
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription PCR
s	Seconds
SCFA	Short-chain fatty acid
SNP	Single nucleotide polymorphism
TOS	Transgalctosylated oligosaccharide
UK	United Kingdom
µL	Micro litres
µg	Micro grams
µM	Micro molar
v	Volts
w/w	Weight for weight
WGS	Whole genome sequencing
ZnO	Zinc oxide

1 Introduction

The mass of pigs slaughtered in the UK for human consumption rose from 720 to 960 thousand tonnes from 2009 to 2019 (EUROSTAT, 2021). Pork is the second most consumed meat worldwide and global consumption is predicted to increase 17% to 127.28 thousand tonnes by 2029 (OECD-FAO, 2021). Although production efficiency from the world's leading pork producers continues to improve, persistent and ever-evolving constraints limit the UK and EU industry to develop robust strategies for sustainable production (Young, 2005; Oh and Whitley, 2011; Uehleke, Seifert and Hüttel, 2021).

Approximately 10^{14} microbiota reside in the mammalian gastrointestinal tract (GIT) which influence how food is digested, the catabolism of toxins, host immune response, short-chain fatty acid (SCFA) production and vitamin synthesis (Gustafsson, 1959; Savage 1977; Swanson *et al*., 1987; Ley, Peterson *et al.,* 2006; Brestoff and Artis, 2013; Kim and Isaacson, 2015). The establishment of intestinal microbiota in neonatal pigs is gradual, and colonisation is modulated by microbial exposure from several sources (Inoue *et al.,* 2005). Directly postpartum, the microbial community is dominated by microbiota from the sow but this changes with the introduction of the piglet to new environments and a change in diet post-weaning (Katouli *et al*., 1997; Konstantinov *et al.*, 2006; Thompson, Wang and Holmes, 2008).

One of the most stressful events in a pig's life is when it is weaned from the sow, moving from a liquid milk diet to a complicated and dry plant-based diet which is harder to digest (Dunshea *et al*., 2002b; Tokach *et al.,* 2003). During this time, piglets encounter additional significant challenges such as abrupt separation from the sow, mixing with pigs from other litters or farms, adapting to new social hierarchy and acclimatising to a new environment. Consequently, piglets display reduced food intake immediately post-weaning, with calorie intake dropping to 60% of pre-weaning consumption, resulting in sub-optimal nutrition, reduced growth, and lower production profits (Spreeuwenberg *et al*., 2001). Further consequences of the challenges that neonatal pigs face include perturbed intestinal

morphology, structure, and immune response (McCracken *et al.*, 1999; Lallès *et al*., 2004; Lallès *et al.*, 2007). These stressors can contribute to post-weaning diarrhoea (PWD), frequently caused by *Escherichia coli*, which is characterised by abrupt diarrhoea, dehydration and impeded growth, and can result in premature death (Amezcua *et al*., 2002; Fairbrother, Nadeau and Gyles 2005). Enterotoxigenic *E. coli* (ETEC), in particular ETEC O149, are typically associated with intestinal dysfunction causing PWD via production of adhesins and enterotoxins (Fairbrother, Nadeau and Gyles 2005; Nagy and Fekete 2005; Luppi *et al.*, 2016).

The use of antibiotics as growth promoters was introduced in the 1950s to reduce the incidence of microbial infection in livestock (Cromwell, 2002; Wijtten, Meulen and Verstegen, 2011). Antimicrobials improve growth performance of animals by eliminating microorganisms which may perturb the gut epithelial surface, thus stimulating an immune response; antibiotics reduce the amount of energy required by host to protect against pathogens and maintain a healthy gut (Gaskins, Collier and Anderson, 2006). However, their use in livestock feed for growth promotion is now banned in the EU due to emerging antibiotic resistance among gut microorganisms ending up in animal excrement and human food products, as well as reduced intestinal microbial diversity (Teillant, Brower and Laxminarayan, 2015; Neuman *et al*., 2018). Since the antibiotic ban in 2006, most EU countries started using veterinary pharmaceutical products containing zinc oxide (ZnO) to improve farm animal growth efficiency.

Zinc is an important ingredient of the mammalian diet; it is a cofactor for more than 300 enzymes assigned to various functions including synthesis of nucleic acids, hormones and their receptors, cell proliferation and apoptosis, regulation of enzyme activity, improving immune system function and regulating oxidative stress (Brown, Wuehler and Peerson, 2001; Chasapis, *et al*., 2012). As such, animal feed supplemented with zinc was shown to improve growth performance and reduce prevalence of PWD in pigs (Hahn and Baker, 1993; Poulsen, 1995). However, the widespread use of ZnO as a feed supplement leads to more zinc excreted into the environment which poses numerous environmental concerns due to the impact of zinc on human health such as reduced immune function and infertility (Zhang *et al*., 2012; Duan *et al*., 2015; Jensen, Larsen and Bak, 2016). Therefore, the

European Medicines Agency commissioned the withdrawal of marketing authorisation of veterinary products containing ZnO in the EU by June 2022 (European Medicines Agency, 2017). This is particularly troubling for the UK as the majority of pig starter diets are supplemented with pharmaceutical ZnO (National Pig association, 2021). This resulted in a requirement for the pig production industry to find an alternative growth promoter to improve production performance while maintaining animal health to improve farm efficiency. Consequently, prebiotics were suggested as replacements for antibiotics and ZnO in the production of livestock due to their effect on gut microbiota which in turn can aid protection against infectious disease, diarrhoea and cancers, and improve immune function and digestion (Saad *et al*., 2013).

Prebiotics are defined as substrates that are selectively utilized by host microbes to improve host health (Gibson *et al.*, 2017). This definition encompasses non-digestible carbohydrates like oligosaccharides which resist digestion in the upper GIT and reach the hind gut where they act as fermentable substrates for resident microbiota. Prebiotics display direct and indirect mechanisms of inhibiting colonisation and proliferation of enteropathogens in the gut (Bindels *et al*., 2015; Tran, Everaert and Bindelle, 2016). Prebiotics can coat the gut epithelial surface and saturate pathogenic binding domains, thus blocking pathogen access to mucus membranes and improving host resistance to invasion (Molist *et al.,* 2014). An example of this is galacto-oligosaccharides (GOS, or β-GOS) demonstrating anti-adhesive activity of ETEC and EPEC (enteropathogenic *E. coli*) to porcine ileal cells *in vitro* (Shoaf, *et al*., 2006). Such dietary fibres have also shown to benefit the host by enhancing activity and/or growth of favourable flora in the GIT of mice (Li, Lu and Yang, 2013), pigs (Konstantinov *et al.,* 2004), chickens (Xu *et al.,* 2003; Jung *et al.,* 2008; Pourabedin, Guan and Zhao, 2015; Richards *et al.,* 2020), rats (Bovee-Oudenhoven *et al.,* 2003; Parnell and Reimer, 2012) and humans (Langlands *et al.,* 2004; Vulevic *et al*., 2015). Therefore, GOS are widely used as mammalian dietary supplements due to their beneficial effect on gut health (Schley and Field, 2002; Macfarlane, Macfarlane and Cummings, 2006).

GOS are commercially synthesised by the cleavage of lactose, a β-galactoside, into monosaccharides using β-galactosidases as biocatalysts (Gänzle, Haase and Jelen, 2008; Otieno,

2010). During synthesis, a galactose unit is transferred from a β-galactoside to an acceptor possessing a hydroxyl group, usually glucose (Martins *et al.,* 2019). This hydrolysis and transglycosylation of lactose by β-galactosidase produces oligosaccharides with degrees of polymerisation ranging from 2-8 and β-glycosidic bonds linking saccharides; Gal-β(1→ 3)-Gal, Gal-β(1→ 4)-Gal, Gal-β(1→ 6)-Gal, Gal-β(1→ 4)-Glc and Gal-β(1→ 6)-Glc, where Gal and Glc are galactose and glucose monomers, respectively (van Leeuwen *et al*., 2016).

Probiotics are species specific, live microorganisms that, when consumed, confer host health benefits by improving gut flora. Probiotics demonstrate various mechanisms to protect host from intestinal dysfunction from enteropathogens such as releasing peptidic toxins to inhibit growth of other bacteria, competing for adhesion onto the gut epithelial surfaces, thus preventing colonisation and invasion of pathogenic bacteria, and improving gut immunity (Conway *et al*. 1987; Lee *et al,* 1999; Rolfe, 2000; Alizadeh *et al*., 2015).

A synbiotic is a mixture of prebiotics and probiotics which aid host health by modulating gastrointestinal microbiome diversity and activity (Gibson *et al*., 2017). As a result, synbiotics (*Bifidobacterium*, *Lactobacillus* and fructooligosaccharides) have been used to treat patients with diarrhoea and irritable bowel syndrome. *Bifidobacteria* are gram-postive, non-motile, anaerobic bacteria that can be found in the vagina, mouth and GIT of mammals (Rasic, 1983; Schell *et al.,* 2002). Originally isolated from the faeces of breast-fed infants (Tissier, 1900), *Bifidobacteria* have proved to be a main genus of bacteria residing in the mammalian GIT, with *B. pseudolongum* the dominant species in pigs. Early investigations into the use of *Bifidobacterium* strains as probiotics in pigs at different stages of life have produced mixed results (Kimura *et al.,* 1983; Ervolder 1989; Apgar 1993). More recent data suggests that supplementary GOS increases alpha diversity (the number of different bacterial species present) in pig hind guts, in particular the enrichment of *Bifidobacterium spp.* (Tzortzis *et al.,* 2005; *Jung et al.,* 2008; Tanner *et al.,* 2014). In the gut, greater *Bifidobacterium* populations stimulated by GOS supplementation is coupled with improved bioavailability of SCFA such as acetic, butyric and propanoic acid, which have the potential to improve fat and protein retention in pigs, as well as inhibit bacterial pathogenicity (Jørgensen *et al.,*

1997; Tanner *et al.,* 2014). For example, propionate and butyrate are shown to suppress activity of *Salmonella* pathogenicity islands in *S. Typhimurium* and adhesin fimbriae in enterohemorrhagic *E. coli* (Spring *et al.,* 2000; Lawhon *et al.,* 2002; Sun and O'Riordan, 2013).

It is hypothesised that microorganisms stimulated by prebiotics can be isolated, cultured and purified for use as a probiotic to confer host health benefits. Additionally, the addition of prebiotic with probiotic (synbiotic) to animal feed may improve growth performance better than when using one such treatment. The aim of the present study is to characterize *Bifidobacterium spp.* isolated from the GIT of pigs fed a GOS-supplemented diet and investigate their potential as a synbiotic treatment with GOS in pig farming. The overall impact of this study is to improve the intestinal health of pigs while maintaining production efficiency.

2 Materials and methods

2.1 Animal welfare

This study was conducted according to the requirements of the Animals (Scientific Procedures) Act 1986, Amended 2012, and as far as reasonably possible, according to the principles of GCP (2000) Guidelines on Good Clinical Practice for Clinical Trials for Registration of Veterinary Medicinal Products (VICH); and will as far as reasonably possible (where applicable), meet appropriate current quality standards indicated by the EU (Reg. Nº 429/2008) and EFSA Administrative and Technical Guidance to Applicants on the Preparation of Dossiers for Zootechnical Additives (2012), Assessment of the Efficacy of Feed Additives (2018) and Assessment of the Safety of Feed Additives for the Target Species (2017). All animal study procedures were approved by the Harper Adams Ethics Panel.

2.2 Experimental animals and treatments

JSR 9T dam x JSR Tempo sire pigs were used to test the effect of prebiotic GOS on GIT microorganisms for the first 14 days post weaning, compared to a basal diet (negative control). Pre-weaned piglets were housed in litters with full access to the sow for 14 days. Post-weaning piglets were housed in 24 pens of 7 pigs (n = 168) with plastic slated floors. Feed and water (the latter via nipple drinkers) were provided *ad libitum.* Environmental enrichment was provided via chew toys and chains. No compressed wood blocks or edible materials were provided to prevent potential effects of these on microbiome diversity. The diet regime was as follows: piglets were fed from the sow from 0 to 14 days of age (da), creep feed for 14 to ~26 da and a basal diet (control) with the addition of GOS (1% w/w; experimental diet only) for 26 to 40 da. At 40 da, pigs were sacrificed for gut microbiota analysis. Diets were provided by Primary Diets Ltd (Yorkshire, UK) and Nutrabiotic® provided by Saputo Dairy (Shropshire, UK). The diet compositions were as per the tables below:

Nutritional composition of creep feed fed from two weeks of age to weaning

Constituent	Amount/kg
Crude protein (g)	240
Crude fibre (g)	26
Crude oil and fats (g)	130
Crude ash (g)	70
Lysine (g)	16
Methionine (g)	3
Ca (g)	6
Na (g)	3.5
P (g)	6
Vitamin A (iu)	12500
Vitamin D3 (iu)	2000
Vitamin E (iu)	95

Nutritional composition of control (T1) and GOS supplemented (T2) diets fed from 26 – 40 da

Treatments	T1	T2
Raw Material Inclusion (%)		
Ground wheat (raw)	37.13	34.69
Ground wheat (micro)	5.00	5.00
Ground Barley (micro)	15.00	15.00
Sweet whey powder	6.94	6.94
Soya (extruded Hi-Pro)	21.25	21.63
Soya (full fat)	3.00	3.00
White fish (Provimi)	6.50	6.50
L-Lysine (HCL)	0.24	0.24
DL-Methionine	0.14	0.14
L-Threonine	0.15	0.15
Tryptophan	0.01	0.01

Soya oil	3.01	3.51
Limestone flour (Tru .270)	0.25	0.25
Dicalcium Phosphate	0.62	0.62
Salt	0.26	0.26
Premix[1]	0.50	0.50
Nutrabiotic® powder	0.00	1.56
Total Inclusion (%)	**100**	**100**

[1]Premix manufactured by Target Feeds (Shropshire, UK) providing (per kg of complete diet as fed): 12,500 IU of vitamin A, 2,000 IU of vitamin D3, 200 IU of vitamin E, 4.2 mg of vitamin B1, 5.6 mg of vitamin B2, 5 mg of vitamin B6, 50 µg of vitamin B12, 4.4 mg of vitamin K, 20 mg of pantothenic acid, 40 mg of nicotinic acid, 150 µg of biotin, 1.0 mg of folic acid, 200 mg of choline, 140 mg of CU (CuSO4), 2.17 mg of Iodine (KI, Ca(IO3)2), 200 mg of Fe (FeSO4), 62 mg of Mn (MnO), 0.30 mg of Se (inc. selplex), 100 mg Zn (ZnO).

At 40 da, all pigs were weighed and the pig of average weight in each pen was sacrificed for collection of digestate post-mortem. Pigs were euthanised by captive bolt gun fired directly into the cranium to induce a state of unconsciousness. This was immediately followed by destruction of the brain using a pith or exsanguination by severing the major blood vessels in the neck, to prevent the return of consciousness. After sacrifice, each pig was cut down the ventral line and the GIT excised from the carcass. Luminal contents were collected from the caecum and colon into 70 ml plastic, screw-top containers (Sarstedt, NC, US) and a 3 ml syringe (Sarstedt) was used to sub sample intestinal contents from these containers. Syringes were sealed with Nescofilm (Bando Chemical Ind, Tokyo, Japan), secured in a sealed Falcon tube (Sarstedt), and kept at 4°C before being transported to the lab for analysis the following day. Caeca and colonic samples were extracted from 10 pigs (5 fed the GOS supplemented diet, 5 fed the control diet). The animal study and dissections were conducted at Harper Adams University. Samples obtained from dissection were transferred to the University of Nottingham laboratory for analysis. Pigs that were not sacrificed for GIT laboratory analysis were transferred from Harper Adams University to an abattoir and entered the human food chain. (Note: The number of pigs used in this animal study were based on the procedure of Berndtson (1991) for production performance to detect a 10 % difference, with 80 % power, at the 95 % level of

probability. The digesta obtained and analysed in this MRes were taken alongside digesta and tissue samples that were acquired and analysed for a larger PhD project which is not discussed in this thesis. Hence, it is described in the Materials and Methods of this thesis that caecal and colonic contents from 10 pigs were analysed despite more animals being used in the study.)

2.3 *In vitro* isolation of *Bifidobacteria*

Once transferred to the lab, samples were immediately placed into an anaerobic chamber (10% CO_2, 10% H_2, 80% N_2). Caecum and colonic contents were dispensed from the syringe into the falcons they were transported in and homogenised by vortex. Caecal and colonic contents from each individual pig were subject to a 10-fold serial dilution series (10^{-1} to 10^{-4}); depending on sample consistency, either 100 µL or 100 mg sample was diluted with 900 µL phosphate-buffered saline (Oxoid Ltd). 100 µL of each dilution was spread plated onto the surface of De Man, Rogosa, Sharpe (MRS) agar (Oxoid Ltd, Hampshire, UK) supplemented with 5% lithium mupirocin supplement (Sigma-Aldrich, Missouri, United States) and L-cysteine hydrochloride (Sigma-Aldrich), and incubated anaerobically for 48 – 72 h at 37°C.

One litre of MRS media contained 10 g of peptone, 8 g of 'Lab-Lemco' powder, 4 g of yeast extract, 20 g of glucose, 1 ml of sorbitan mono-oleate, 2 g of dipotassium hydrogen phosphate, 5 g of sodium acetate $3H_2O$, 2 g of triammonium citrate, 0.2 g of magnesium sulphate $7H_2O$, 0.05 g of manganese sulphate $4H_2O$ and 10 g of agar. Media was sterilised by autoclaving at 121°C for 15 min before 50 ml lithium mupirocin supplement (Sigma-Aldrich) and 10 ml of 5% filter sterilised (0.22 µm filter membrane; Sartorius, Göttingen, Germany) L-cysteine hydrochloride were added.

For each intestinal sample, one plate from the dilution series with 30-300 colonies was selected and up to ten distinct, well-isolated colonies based on *Bifidobacterium* colony morphology on MRS agar (Vinderola and Reinheimer, 1999) were sub-cultured for characterisation and storage in glycerol at -80°C.

2.4 DNA extraction

Genomic DNA was extracted from isolates using a GenElute bacterial genomic DNA kit (Sigma Aldrich) as per manufacturer's instructions, following the gram-positive bacterial preparation protocol. DNA quality and quantity were assessed using the NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Before each sample measurement, the stylus on both arms of the NanoDrop were cleaned with 1 µL RNase free water (Qiagen, Hilden, Germany) using a Kimwipe tissue (Kimtech Science™, Roswell, GA). The stylus was loaded with 1 µL of DNA sample and DNA concentration (ng/µL) and λ260/280 and λ260/230 absorbance ratios measured. The stylus was wiped clean with 1 µL RNase free water and a Kimwipe tissue between each sample. DNA extraction was repeated until λ260/280 and λ260/230 values were within 1.8 – 2.0 and 2.0 – 2.2, respectively, and DNA concentration was >10 ng/µL.

2.5 *Bifidobacterium* 16s rRNA primer design

Based on GeneBank (Benson *et al.*, 2009) availability, the complete 16s ribosomal RNA (rRNA) sequence of 18 *Bifidobacterium* species were subject to multiple sequence alignment using the Clustal Omega online tool (www.Ebi.ac.uk; Madeira *et al.*, 2019). Primers were manually designed (f 5′-GCGAACGGGTGAGTAATGC-3′ and r 5′-TTTCATGACTTGACGGGCG -3′) based on sequence homology between the strains, leading to a region of approximately 1300 bp to be amplified by PCR (Table 1).

Table 1. Details of primer sequences f 5'-GCGAACGGGTGAGTAATGC-3' and r 5'-TTTCATGACTTGACGGGCG -3' within the 16s rRNA gene of 18 *Bifidobacterium* species.

Bifidobacterium species	GenBank accession no.	Location in 16s rRNA gene[a] (forward/reverse)	Amplicon size (bp)	Reference
B. angulatum	D86182	F: 92 R: 1392	1299	(Miyake, Watanabe, Watanabe and Oyaizu, 1998)
B. animalis				
subsp. *animalis*	LC065042	F: 97 R: 1409	1311	Sakamoto, M. and Ohkuma, M (unpublished)
subsp. *lactis*	MN372119	F: 64 R: 1374	1311	Liu *et al.,* 2020
B. breve	AJ311605	F: 86 R: 1393	1308	Vitali, *et al.,* 2003
B. catenulatum	AB437357	F: 94 R: 1394	1299	Watanabe and Makino (unpublished)
B. dentium	MN372123	F: 66 R: 1369	1304	Liu *et al.,* 2020
B. gallicum	D86189	F: 91 R: 1401	1309	(Miyake, Watanabe, Watanabe and Oyaizu, 1998)

B. infantis	M58738	F: 119 R: 1401	1283	Yang and Woese (unpublished)
B. longum	AY675246	F: 94 R: 1391	1298	Jung, Baek and Kim (unpublished)
B. longum				
subsp. *infantis*	D86184	F: 91 R: 1388	1296	(Miyake, Watanabe, Watanabe and Oyaizu, 1998)
subsp. *longum*	AB437359	F: 94 R: 1391	1296	Watanabe and Makino (unpublished)
subsp. *suis*	AB437360	F: 94 R: 1391	1296	Watanabe and Makino (unpublished)
B. pseudolongum				
subsp. *globosum*	D86194	F: 92 R: 1403	1309	(Miyake, Watanabe, Watanabe and Oyaizu, 1998)
subsp. *pseudolongum*	D86195	F: 92 R: 1402	1309	(Miyake, Watanabe, Watanabe and Oyaizu, 1998)
B. thermophilum	AB437364	F: 95 R: 1395	1299	Watanabe and Makino (unpublished)

[a]Based on *E. coli* numbering (Brosius, Dull, Sleeter and Noller, 1981)

Amplicon size (bp), the expected amplified sequence length following PCR.

2.6 Molecular identification of *Bifidobacteria*

PCR was performed using *Taq* PCR Master Mix Kit (Qiagen) according to the manufacturer's instructions with minor adaptations; each PCR reaction contained 12.5 µL Taq PCR Master Mix, 0.25 µM of each oligonucleotide primer, 100 ng template DNA, $MgCl_2$ concentration raised to 1.75 mM and Milli-Q H_2O used to top up to give a reaction volume of 25 µL. The amplification conditions were initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 45-s, 56.3°C for 45-s and 72°C for 2 min. A final extension was performed at 72°C for 10 min. PCR reactions were performed on the T100 Thermal Cycler (Bio-Rad).

Following amplification, 5 µL of each reaction was run on a 1% agarose gel (w/v; 2 g agarose, 198 mL TAE buffer), stained with 8 µL ethidium bromide, at 85v for 60 min. Agarose gels were inspected with Molecular Imager Gel Doc XR+ (Bio-Rad) for fluorescent bands at the ~1300 bp region (Appendix 1). PCR products of presumptive *Bifidobacteria* were cleaned up (Wizard SV gel and PCR clean-up system; Promega, Southampton, Hampshire, UK) and DNA sequenced using dye terminator sequencing (Eurofins Genomics, Ebersberg, Germany). 16s rRNA sequences produced by Eurofins Genomics were subject to Basic Local Alignment Search Tool (BLAST; available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify *Bifodobacteria* species.

2.7 Microbiological and biochemical characterization of *Bifidobacteria*

The bacterial strains identified as presumptive *Bifidobacteria* from 16s rRNA sequencing with Eurofins experienced further microbiology tests before whole genome sequencing (WGS). Candidates proceeded to WGS if they met the following criteria (in addition to assays aforementioned): strains displayed anaerobic growth on TOS-propionate agar medium (Sigma-Aldrich) supplemented with lithium mupirocin supplement (Sigma-Aldrich), oxidase negative, catalase negative and are gram-positive rods.

One litre of TOS-propionate agar medium (Sigma-Aldrich) supplemented with lithium mupirocin supplement (Sigma-Aldrich) contained: 15 g of agar, 3 g of ammonium sulphate, 10 g of

casein enzymic hydrolysate, 0.5 g of L-cysteine hydrochloride monohydrate, 4.8 g of dipotassium hydrogen phosphate, 10 g of galacto-oligosaccharide, 0.2 g of magnesium sulphate heptahydrate, 3 g of potassium dihydrogen phosphate, 15 g of sodium propionate and 1 g of yeast extract. Media was sterilised by autoclaving at 121°C for 15 min before adding 50 ml lithium mupirocin supplement (Sigma-Aldrich) once cooled.

Bacterial isolates were examined in the API 20 A system (BioMérieux, Marcy-l'Etoile, France), following the manufacturer's instructions. The biochemical tests investigated using the API 20 A strips are: indole formation (IND), urease (URE), glucose (GLU), mannitol (MAN), lactose (LAC), saccharose (SAC), maltose (MAL), salicin (SAL), xylose (XYL), arabinose (ARA), gelatin (GEL), esculin (ESC), glycerol (GLY), cellobiose (CEL), mannose (MNE), melezitose (MLZ), raffinose (RAF), sorbitol (SOR), rhamnose (RHA) and trehalose (TRE). All pure *Bifidobacterium* cultures were inoculated onto the surface of MRS agar supplemented with L-cysteine hydrochloride and incubated anaerobically at 37°C for 24 – 36 h. All growth was harvested using a sterile swab, emulsified in the API 20 A medium, before inoculating the API 20 A strip. All of the inoculated API 20 A strips were incubated anaerobically at 37°C for 48 h. Results were recorded (Table 3) according to the reading table provided by the manufacturer and species identification was obtained using the API 20 A database on APIWEB (provided by BioMérieux; https://apiweb.biomerieux.com/identIndex).

2.8 Whole genome sequencing library preparation

Once appropriate DNA quality and quantity were achieved (as previously described), sample DNA was quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and Qubit 3.0 Fluorometer (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. Sample DNA was diluted to 0.2 ng/μL in ≥5 μL using RT-PCR grade water (Invitrogen; Thermo Fisher Scientific). DNA libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina Inc., CA, US), Nextera XT Index Kit (Illumina Inc) and MiSeq Reagent Kit v3 600-cycle

(Illumina Inc), according to the manufacturer's instructions with minor adaptations. 5 µL of input DNA was added to 10 of µL Tagment DNA Buffer and 5 of µL of Amplicon Tagment Mix on a 96-well PCR plate. The PCR plate was sealed with adhesive PCR seal (Sarstedt) followed by a non-adhesive sealing film (Microseal 'A' film; Bio-Rad, CA, US) and incubated at 55°C for 5 minutes using the Bio-Rad T100 Thermal Cycler (Bio-Rad). Following incubation at 55°C, 5 µL of Neutralize Tagment Buffer was immediately added to each reaction and incubated at RT for 5 minutes. 15 µL of Nextera PCR Mastermix was added to each reaction, followed by 5 µL of Index 1 primers (N7**) and 5 µL of Index 2 primers (S5**). Note: different combinations of indices are added to each sample reaction as per manufacture's guidelines. The PCR plate was sealed with adhesive PCR seal (Sarstedt) followed by a non-adhesive sealing film (Microseal 'A' film; Bio-Rad, CA, US) and amplified using the Bio-Rad T100 Thermal Cycler (Bio-Rad). The amplification conditions were initial denaturation at 73°C for 3 min, followed by 95°C for 30-s, followed by 12 cycles of 95°C for 10-s, 55°C for 30-s and 72°C for 30-s. A final extension was performed at 72°C for 5 min. DNA quality was assessed on the Agilent 2200 TapeStation system (Agilent, CA, US) using Genomic DNA ScreenTape (Agilent) with Genomic DNA Reagents (Ladder and Sample Buffer; Agilent), according to the manufacturer's instructions, and analysed using 2200 TapeStation Controller Software, and TapeStation Analysis Software (Agilent). 40 µL of each amplified sample was transferred to a fresh 96-well PCR microplate and 24 µL of AMPure XP beads (Beckman Coulter, IN, US) was added to each reaction. The plate was sealed with adhesive PCR seal (Sarstedt), vortexed using the IKA MS 3 Vortexer (Staufen, Germany) at 1800 rpm for 2 mins and incubated at RT for 5 minutes. The plate was placed onto a magnetic stand (Invitrogen; Thermo Fisher Scientific) for 2 minutes before the supernatant was removed from each reaction and discarded. The beads were washed with 200 µL of 80% ethanol, incubated on the magnetic stand for 30 seconds and the supernatant discarded. The wash was repeated once and the beads were left to airdry near a lit Bunsen for 15 minutes. 52.5 µL of Re-suspension Buffer was added to each reaction, the PCR plate sealed with Sarstedt PCR film, vortexed using the IKA MS 3 Vortexer (Staufen, Germany) at 1800 rpm for 2 mins, incubated at RT for 2 minutes, and placed on the magnetic stand (Invitrogen; Thermo Fisher Scientific) for 10 minutes. 50 µL of supernatant from each reaction was transferred to a fresh PCR plate, and DNA quality and quantity

was assessed using the Agilent and Qubit systems, as previously described. The concentration of DNA libraries were converted from ng/µL to nM using the equation nM = [(ng/µL)/(average DNA size (bp) * 660g/mole)] * 10^6, before normalisation of libraries to 4nM in 15 µL using the equation (4nM * 15 µL) = (DNA concentration in nM * V_2), where (15 µL – V_2) is the volume of Re-suspension Buffer to add to make 15 µL of library. 5µL of each 4nM library were pooled together in 1 Eppendorf for denaturing and dilution. In a fresh Eppendorf, 5 µL of 0.2N NaOH was added to 5 µL of pooled library. The mixture was vortexed briefly at 280 x g for 1 minute, incubated at RT for 5 minutes and 990 µL of chilled HT1 was added. 300 µL of the denatured 20pM library was added to 700 µL chilled HT1 (producing a 6pM DNA library). In a fresh Eppendorf, 5 µL of 0.2N NaOH was added to 5 µL of 4nM PhiX Control V3 (Illumina Inc). The mixture was vortexed briefly at 280 x g for 1 minute, incubated at RT for 5 minutes and 990 µL of chilled HT1 was added. 300 µL of the 20pM PhiX solution was added to 700 µL chilled HT1 (producing a 6pM PhiX solution). In a fresh Eppendorf, 50 µL of the 6pM PhiX solution was added to 950 µL of the 6pM DNA library; 600 µL of this mixture was loaded into the sample well of the MiSeq cartridge which was loaded onto the MiSeq for sequencing.

All DNA quality, quantity and fragment size measurements using Agilent and Qubit systems for whole genome sequencing library preparation are provided (Appendix 2).

2.9 Sequence analysis and genomic assembly

FastQ files generated by the MiSeq (Illumina) were subject to downstream processing to construct the genome of each bacterial strain sequenced. Anaconda3 (available at: https://www.anaconda.com/products/individual-d#macos) and Miniconda3 (available at: https://docs.conda.io/en/latest/miniconda.html) contain statistical packages for read analysis, while Terminal (version 2.11; MacOS) was used to input commands for read analysis using the packages in Anaconda3 and Miniconda3. Trimmomatic (version 0.39) was used to remove adapter sequences, remove leading and trailing low quality or N bases, and drop reads shorter than 36 bp (according to

Trimmomatic Manual: Version 0.32). FastQC (version 0.11.7) was used to visualise Phred quality scores from trimmed and paired sequences. SPAdes (version 3.12.0) was used to assemble contigs from trimmed and paired sequences. Quast (version 5.0.2) was used to assess the quality of scaffold assembly.

The 16s rRNA sequence for each sample was identified from contigs and searched into the BLAST database to identify the identity of the strain isolated. The best matched result (when considering the highest query cover and percentage identity), with a full genome sequence on the database, was selected for use as a reference genome. SeqMan Ultra (DNAStar; version 17.3.0) was used to map trimmed and paired sequences (following Trimmomatic processing) for each sample to its respective identified reference genome. In SeqMan Ultra, the Variant analysis and resequencing > NGS-Based > Whole genome workflow was used, and single nucleotide polymorphism filter stringency was set to “high”.

Consensus sequences of all sequenced strains were deposited into the GenBank database (see Table 2 for accession numbers). Prokka (version 1.14.6) was used to annotate the consensus sequences to reveal coding regions and associated gene products.

3 Results

3.1 Microbiota identified using Illumina MiSeq sequencing.

Pig caeca and colonic luminal contents from 10 pigs were spread onto the surface of MRS agar supplemented with L-cysteine hydrochloride and lithium mupirocin supplement. 193 bacterial colonies were isolated from 20 intestinal (10 caecal, 10 colonic) samples, and 7 of these (isolated from 3 pigs) were subject to whole genome sequencing using the Illumina MiSeq platform. Sequence analyses revealed that the bacterial species sequenced were *Bifidobacterium animalis* subsp. *lactis*, *Lactobacillus reuteri* and *Bifidobacterium* pseudolongum (Tanle 2). Inspection of the 16s ribosomal RNA gene of the seven genomes revealed that both *L. reuteri* 16s regions, and all four *B. pseudolongum* 16s regions, were identical, respectively. This is not surprising for isolates 19E3 and 19E6, and isolates 46D2 and 46D6 as they were cultured from the same site of the same animal, respectively. Furthermore, it is also not surprising that *B. pseudolongum* isolated from 46E1 is identical to *B. pseudolongum* isolated from the caecum of pig 46 (46D2 and 46D6) as the digesta moves through the intestinal tract. The API 20 A anaerobic system identified all 7 isolates as *Bifidobacterium spp*., indicating the similar metabolic capacity of both genera (Table 3). This is reflective of their adaptation to the mammalian lower intestinal niche and highlights the limits of the BioMérieux system to identify bacterial *spp*. compared sequencing methods (Ventura *et al*., 2009).

Table 2. Bacterial strains sequenced using the Illumina MiSeq platform and the source from which they were isolated.

Sample ID	Pig ID number [a]	Sample site	Sample site replicate [b]	Organism isolated [c]	Genome accession no.	Reference genome accession no. [d]
19D1	19	Caecum	1	*B. animalis* subsp. *lactis*	CP084315	CP003941
19E3	19	Colon	3	*L. reuteri*	CP084583	CP006011
19E6	19	Colon	6	*L. reuteri*	CP084584	CP006011
46D2	46	Caecum	2	*B. pseudolongum*	CP084312	CP017695
46D6	46	Caecum	6	*B. pseudolongum*	CP085326	CP017695
46E1	46	Colon	1	*B. pseudolongum*	CP084313	CP017695
49D6	49	Caecum	6	*B. pseudolongum*	CP084314	CP017695

[a] Randomly assigned when piglets separated into pens (see Materials and methods)

[b] Up to 10 separate colonies were cultured from each sample site (see Materials and methods)

[c] Identified from WGS using the Illumina MiSeq platform

[d] Genomes were assembled by mapping paired end reads to a reference genome (see Materials and methods)

Table 3. Results of the BioMérieux API 20 A system.

Reaction/enzymes	19D1[a]	19E3[a]	19E6[a]	46D2[a]	46D6[a]	46E1[a]	49D6[a]
Indole formation	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
Glucose[*]	+	+	+	+	+	+	+
Mannitol[*]	-	-	-	+	+	+	+
Lactose[*]	+	+	+	+	+	+	+
Saccharose[*]	+	+	+	+	+	+	+
Maltose[*]	+	+	+	+	+	+	+
Salicin[*]	+	-	-	+	+	+	+
Xylose[*]	+	+	+	+	+	+	+
Arabinose[*]	+	+	+	+	+	+	+
Gelatin[**]	-	-	-	-	-	-	-
Esculin[**]	-	-	-	-	-	-	-
Glycerol[*]	-	-	-	-	-	-	-
Cellobiose[*]	+	-	-	-	-	-	-
Mannose[*]	+	-	-	-	-	-	-
Melezitose[*]	+	-	-	-	-	-	-
Raffinose[*]	+	+	+	+	+	+	+

Sorbitol*	-	-	-	-	-	-	-
Rhamnose*	-	-	-	-	-	-	-
Trehalose*	+	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-
Spores	-	-	-	-	-	-	-
Gram reaction	+	+	+	+	+	+	+
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod

*Acidification of

**Hydrolysis of

[a] See Table 2 for sample ID information

+, positive test result; - negative test result

3.2 General genome characteristics.

The genome features of the isolates characterised in this study are found in Table 4. The *Bifidobacterium* genomes appeared to assemble better than that of *Lactobacillus*, as shown by the number of contigs generated by SPAdes. All isolates characterised in this study were ~1.9 mb, which is consistent with published genome sequences of the genus *Bifidobacterium* and *Lactobacillus* (Hou *et al.*, 2014; Kelly *et al.*, 2016). Additionally, all genomes sequenced in this study have similar guanine and cytosine content to published *Bifidobacterium* and *Lactobacillus* sequences (Lukjancenko, Ussery and Wassenaar, 2012; Hou *et al.*, 2014; Milani *et al.*, 2014; Kim *et al.*, 2018).

The number of SNPs, gaps or insertions between strains sequenced in this study compared to genomes of the same species, respectively, comprises of less than 0.5 % difference, indicating that the identification of these isolates are robust. Confidence in identification of isolates is further supported by there being more than 5 % difference (SNPs, gaps, insertions) when aligning genomes sequenced in this study to other species from their respective genus.

Table 4. General features of the isolates characterised in this study

Organism	*B. animalis* subsp. *lactis*	*L. reuteri*	*L. reuteri*	*B. pseudolongum*	*B. pseudolongum*	*B. pseudolongum*	*B. pseudolongum*
Sample ID	19D1	19E3	19E6	46D2	46D6	46E1	49D6
Number of DNA contigs	42	171	174	31	40	35	24
Genome size (bp)	1,963,057	1,947,745	1,947,772	1,978,443	1,978,372	1,978,382	1,978,400
GC content (%)	61	39	39	63	63	63	63
Number of coding genes	1,542	1,774	1,772	1,571	1,572	1,570	1,570
Number of SNPs in coding regions [a]	1,508	1,272	1,338	7,348	7,426	6,869	6,569

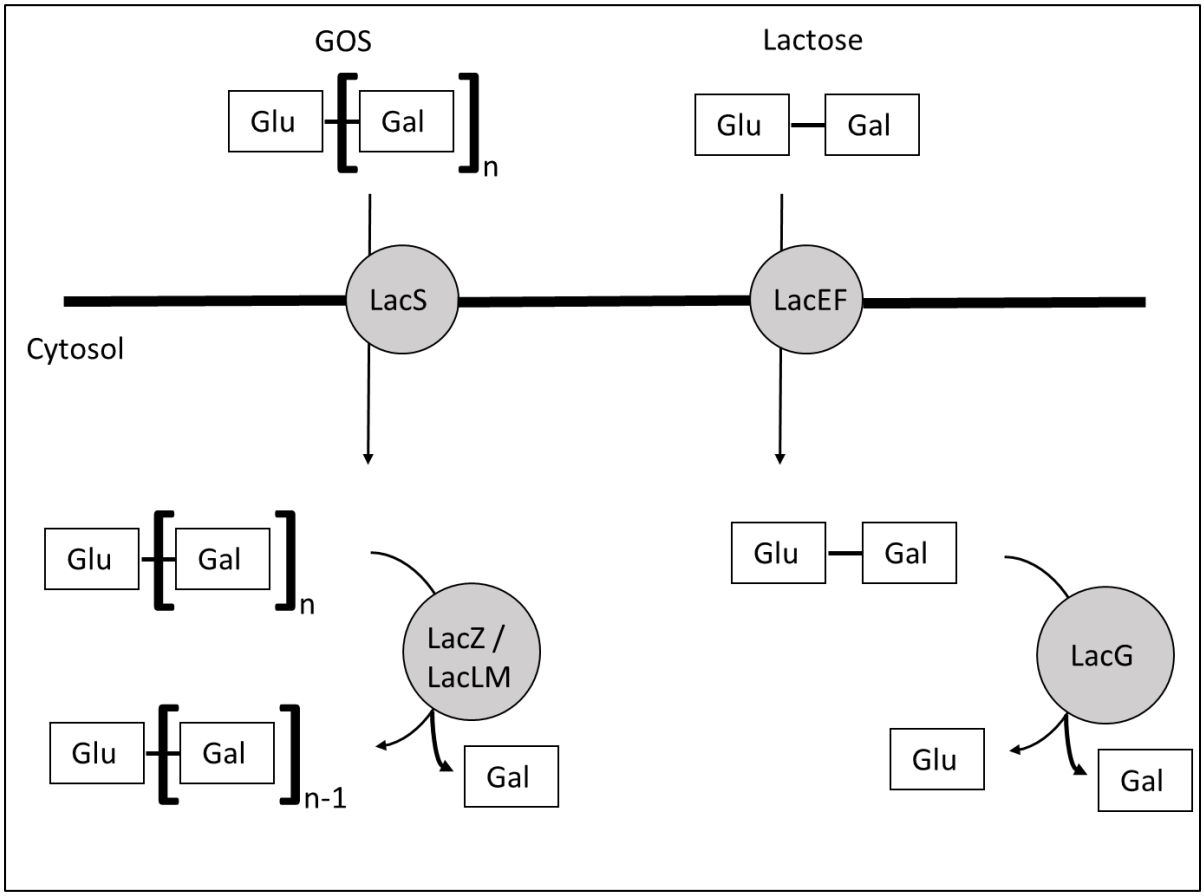
SNPs = Single nucleotide polymorphisms

[a] Compared to reference genome

3.3 Predicted GOS metabolism capabilities of *Bifidobacterium* and *Lactobacillus*.

This study found that *B. animalis* subsp *lactis* possesses genes encoding the LacS and LacZ/LM, and LacEF and LacG, systems (Table 5). This contrasts to both *L. reuteri* and all four *B. pseudolongum* which lacked *LacEF* and *LacG* indicating that they exclusively use the LacS and LacZ/LM route to fully metabolise GOS. This is consistent with other data for *L. reuteri* (Gänzle and Follador, 2012). This might suggest that *B. animalis* subsp. *lactis* could be better equipped for GOS metabolism than the other strains characterised in this study.

Figure 1. Possible pathways of GOS metabolism in *Bifidobacterium* species, adapted from Gänzle and Follador (2012).



GOS, galacto-oligosaccharide; Glu, glucose; Gal, galactose.

Table 5. Presence or absence of genes *Lac* genes encoding proteins involved in β-GOS and lactose import and hydrolysis.

Gene	*B. animalis* subsp. *lactis*	*L. reuteri*	*B. pseudolongum*
LacS	+	+	+
LacZ	+		+
LacLM		+	
LacEF	+		
LacG	+		

+, presence of gene; white background, absence of gene

LacS, membrane bound lactose permease; *LacZ* & *LacLM*, cytosolic β-galactosidases; *LacEF*, membrane bound lactose importer; *LacG*, lactase.

Genomes were annotated with Prokka which identified *Lac* genes in the sequences. To ensure Prokka didn't miss any of these *Lac* genes, all genomes were interrogated for the sequence of each *Lac* gene identified in the other sequences on a low stringency search. For example, Prokka did not find *LacZ* in *L. reuteri*, so the *LacZ* sequences identified in both *Bifidobacterium* strains were searched into *L. reuteri* genome. No *Lac* genes were found in any of the low stringency searches that were not identified by Prokka.

Lac gene sequences detected in this study were subject to sequence alignment using the Clustal Omega online tool (www.Ebi.ac.uk) to detect SNPs in orthologs between species. No SNPs were detected in aligned sequences from isolates of the same species and the same gene in this study. However, SNPs were detected in *LacS* and *LacZ*, respectively, between *B. animalis* subsp. *lactis* and *B. pseuodolongum*, which altered amino acid sequence (Figure 2 & 3).

Figure 2. LacS amino acid sequences of *B. animalis* subsp. *lactis* and *B. pseudolongum* characterised in this study aligned against LacS amino acid sequences of other *Bifidobacterium* species. Highlighted residue sequences are predicted membrane helicases according to software developed by Hallgren *et al*., (2022).

```
B.animalis.subsp.lactis_LacS      MTTNKFQAGAGQTASSAASSDMLRSTTPKTARHGLIRQRAAFAFGNLGQAAFYNAMSTFF   60
B.pseudolongum_LacS               MTT-----------TTPRPTLQSATHQRQTKAKGSARQRIAFAIGNLGQAAFYNAMSTFF   49
B.breve.CP010413.LacS             -------------MSGSNTQRMATSEQPHATPSGNMGQKIAYAFGNLGQAAFYNTMSTFF   47
B.adolescentis.AP009256.LacS      ------------------MSNETTTADGGVRKKGKLGQRIAYACGNLGQAAFYNAMSTYF   42
B.longum.AP010888.LacS            MSVSQ---SPESESQSPNPAGDESGQINRPTKAETLRRRAAYAFGNLGQSAFYNALSTYF   57
                                                                  :: *:* *****:****::**:*

B.animalis.subsp.lactis_LacS      MTYVTTALFARTDKAVAARMIALITGLVVAIRIAEIFLDPILGNIVDNTRTKWGRFRVWQ   120
B.pseudolongum_LacS               MTYTTTALFARSDKAVAARMIALITSLVVAIRIAEIFLDPLLGNIVDNTRTRWGRFRVWQ   109
B.breve.CP010413.LacS             ITFVTTALFIDVDKTLAKRLIAVITGLIVVIRIAEIFLDPLLGNLVDNTNTRWGRFRPWQ   107
B.adolescentis.AP009256.LacS      IVYVTGCLFSGVDKALAAKLIGVITSLVVIIRIAEIFIDPLLGNLIDNTNTKWGRFRPWQ   102
B.longum.AP010888.LacS            VVYVTSVLFVNVEKALATKLIALITSLIVIIRIAEIFLDPLLGNLVDNTNTRFGRFRPWQ   117
                                  :.:.*  **   :*::* ::*.:**.*:* *******:**:***::***.*::**** **

B.animalis.subsp.lactis_LacS      FIGGIVPSVLLVVVFTGLFGLVNVNTTWFITLFIVTFILLDVFYSARDISYWGMIPALSS   180
B.pseudolongum_LacS               FIGGIVPSVLLVVVFTGVFGLVNVNTTWFIIVFVVTFILLDVFYSARDISYWGMIPALSS   169
B.breve.CP010413.LacS             FIGGIVPGILLIMVFTGLFGLVDVNTGVFMVLFVIVFILLDVIYSLRDISYWGMIPAISS   167
```

```
B.adolescentis.AP009256.LacS      FFGGLISAVLLAVIFSGMFGLVNVNTTLFIVLFVITFVVLDVFYSLRDISYWGMIPALSS   162
B.longum.AP010888.LacS            FIGGLVSAVLLVMVYTGLFGLVNVNQTWFIVLFVVVFIVLDVFYSMRDISYWGMIPAISS   177
                                  *:**:: .:** ::::*:****:**   *: :*::.*::***:** ***********:**

B.animalis.subsp.lactis_LacS      DSHERSVYTSLGTLTGSLGYNGITVVVIPIVSYFTFKFTGEQGEGQPGWTAFAIIVALLG   240
B.pseudolongum_LacS               DSHERGVYTSLGTLTGSLGYNGVTVIVIPIVSYFTFRFTGEHAQGQPGWTAFALIIALFG   229
B.breve.CP010413.LacS             DSHERSTYTALGAFTGSIGYNGVTVIVVPVVSWFTWKFTGQWEQGRTGWAAFAIIIAVLG   227
B.adolescentis.AP009256.LacS      DSHERSTYTALGSFTGSIGYNGITVVVIPIVSYFTWTLTGAKGEGQAGWTSFGIIVGLLG   222
B.longum.AP010888.LacS            DSHERSVYTALGTFTGSIGYNGSTIVVVPIVTTFSFMFTGSRAESQSGWTAFGIITALLG   237
                                  *****..**:**::***:**** *:::*:*:*: *:: :**   :.: **::*.:* .::*

B.animalis.subsp.lactis_LacS      LITAWTVAFGTRESSSELRKQD-SHCGPLDAFKAIAHNDQLLWTALSYLLYAIANVATTG   299
B.pseudolongum_LacS               LLTAWTVAFGTREQHNELRGGD-EHCKPLDAFKAIGRNDQLLWMALSYLLYAVSNVATTG   288
B.breve.CP010413.LacS             LLTAWSVAFGTRENQGELRAKAEANGNPIEAFKAIAQNDQLLWVALSYLLYSVANVATTG   287
B.adolescentis.AP009256.LacS      ILTAWTVAFGTKESTNALRAKAQKNGNPLEAFKALFQNDQLLWVALSYLLYAIANVATTG   282
B.longum.AP010888.LacS            ILTAWTVAFGTKENESVLRSRADQSGNPLQAFAAIVKNDQLLWVALSYLLYAVANVATNG   297
                                  ::***:*****:*. . **        *:::** *: :****** *******:::****.*

B.animalis.subsp.lactis_LacS      VLFYQFTYVLGMPQRFAIAGVIPVITGLATTPLYPLLNRVIPRRWLFAGGMGLMILGYAL   359
B.pseudolongum_LacS               VLFYQFTYVLGMPEQFALAGVVPVVTGLLTTPLYPLLNRVVPRRWLFTTGMALMIAGYAM   348
```

```
B.breve.CP010413.LacS                VLFYQFKYVLGAPDSFSIAGVIPVITGLVTTPLYPVLNRHIPRRWLYTAGMALMIAGYTL   347
B.adolescentis.AP009256.LacS         VLLFLFKFVLDNQAAYSMTGVIALVSGLIMAPLYPILNKRIPRRVLYIVGMTSMIVAYIL   342
B.longum.AP010888.LacS               VMFYLFKFVLNLPDAFAITGVIPVIAGLVMAPLYPALNRRIPRRYLFCGGMVLMAIGYVL   357
                                     *::: *.:**.    ::::::: :::**  :**** **: :*** *:  **  *  .* :


B.animalis.subsp.lactis_LacS         FIAAPRNLSVVLVALILFYLPAQTIQMTAILTMTDSIEYGQLKTGQRNEAVTLSVRPMLD   419
B.pseudolongum_LacS                  FIAAPRNLAVVLIALVLFYLPAQTIQMTAILTMTDSIEYGQLKTGRRNEAVTLSVRPMLD   408
B.breve.CP010413.LacS                FIIAPMNLPVVIVALVLFYLPAQTIQMTAILSMTDSIEYGQLKTGKRNEAVTLSVRPMLD   407
B.adolescentis.AP009256.LacS         LGLFSTNMTVVFIALVLFYIPGTLIQMTAILSLTDSIEYGQLKNGKRNEAVTLSVRPMLD   402
B.longum.AP010888.LacS               FILDSASLPVVIVALILFYLPGTFIQMTAILSLTDSIEYGQLKTGKRNEAVTLSVRPMLD   417
                                     :     .: **::**:***:*.  *******::**********.*:**************


B.animalis.subsp.lactis_LacS         KIAGAFSNGIVGFVAVAAGMVGSASAADMTSANIHTFTTWAYIVPSAGIVLSLIVFLLTV   479
B.pseudolongum_LacS                  KIAGAFSNGIVGFVAVAAGMVGSASAADMTARNIHTFTTWAYIVPSVGIVLSLVVFLARV   468
B.breve.CP010413.LacS                KIAGALSNGIVGFVVVAAGMVGNATAADMTAANIRTFKTCAYYLPLAGIVASLVVFLLAV   467
B.adolescentis.AP009256.LacS         KIGGALSNGITGFIAVAAGMTGNATAADMTPSNIHTFEICAFYVPLILIVLSLLVFMFKV   462
B.longum.AP010888.LacS               KIAGALSNGIVGFVAVAAGMVGSATAADMTAANVRTFETFAFYIPLAFIVLSLLVFMFTV   477
                                     **.**:****.**:.*****.*.*:*****  *::**   *: :*   ** **:**:  *
```

```
B.animalis.subsp.lactis_LacS      RIDEKRHAQIVRELETQLADSANTER*------------------   505
B.pseudolongum_LacS               RIDEKRHAQIVEQLEARLADTTR*---------------------   491
B.breve.CP010413.LacS             KIDENMHDHIVEQLEERLASEETNKQ-------------------   493
B.adolescentis.AP009256.LacS      KIDEKMHAKIVKELEAKLASGEIVDDEAQTAETVEAIDEEAKTLTE  508
B.longum.AP010888.LacS            KIDEKMHERIVIELEEKLGVDSLDD--------------------   502
                                  :***: * :** :** :*.
```

*, fully conserved residue. :, residues possess strongly similar properties (> 0.5 in the Gonnet PAM matrix; Dayhoff, Schwartz and Orcutt, 1978). ., residues possess weakly similar properties (≤ 0.5 in the Gonnet PAM matrix; Dayhoff, Schwartz and Orcutt, 1978). Blank, residues share no similar properties, complete mismatch.

Figure 3. LacZ amino acid sequences of *B. animalis* subsp. *lactis* and *B. pseudolongum* characterised in this study aligned against LacZ amino acid sequences of other *Bifidobacterium* species.

```
B.animalis.subsp.lactis.LacZ      MNEETLQEVAEEKATEPPRNDVPSTDGALRSEEPTAAWLTDPRVFAVNRLPGHTDHNCAD   60
B.pseudolongum.LacZ               -----------------------------------MHWLDDPRVFAVNRLPAHTDHMSVD   25
B.breve.CP010413.LacZ             --------------MNTTDDQLKNGDPIVSPSMPTTAWLTDPRVYAVHRLDAHSDHACWS   46
B.adolescentis.AP009256.LacZ      -------------------MADTAELAIVHATTASASWLTDPTVFAANRKPAHSSHRYVI   41
B.longum.AP010888.LacZ            ------------------------MQHPIPTTIASSDWLTDPTVFAVNREPAHSDHRFYD   36
                                                           ** ** *:*.:*  .*:.*

B.animalis.subsp.lactis.LacZ      -----DQSGALKQHLDGEWAVKVVPSHLDRLPTESLDEHWQHAHLPPEFASRSFADGDFT   115
B.pseudolongum.LacZ               -----GASGALRQSLDGVWRARVVPSHLDRLPMESTSERWQTVHIPPAFASDCFDECGYM   80
B.breve.CP010413.LacZ             HAPVNGEGSNLGQSLDGEWRVRVETAPTGRFPDGTSDGPDWINDVPPLFAASDFDDSSFS   106
B.adolescentis.AP009256.LacZ      -----GETREPKQSLDGEWKVRIEQARNV-------------DVESAPFAAVDFEDGDFG   83
B.longum.AP010888.LacZ            HVPQPNETMSLKQNLDGLWNVAVTTAPVFGFPMNDSG-----NAESPDFTATDYDDTGFS   91
                                       .      * *** * . :  :                    *::  : : .:

B.animalis.subsp.lactis.LacZ      RVQVPGCLEMQGLMRPQYVNIQYPWDGHENPQAPSVPTDNLVALYRRAFTADDRVGEALS   175
B.pseudolongum.LacZ               DVNVPGCLETQGLMRPQYVNIQYPWDGHEQPQAPHVPDDNLVALYRRTFDADPRVRQALR   140
B.breve.CP010413.LacZ             RVQVPSHLETAGLLAPQYVNVQYPWDGHEDPKAPAIPEHGHVAVYRREFDADGEVAQAVR   166
B.adolescentis.AP009256.LacZ      AIEVPGHLQMAGYLKNKYVNIQYPWDGHEDPQAPNIPENNHVAIYRRRFALDAQLARTLE   143
B.longum.AP010888.LacZ            RIAVPSTLETKGLLNHKYVNVQYPWDGHSDPKAPNIPTDSHVAIYRRTFETSTPVSAAIE   151
                                   : **. *:  * :  :***:*******.:*:** :* .. **:*** *  .  :  ::
```

```
B.animalis.subsp.lactis.LacZ      RGERVSLTFHGAATAIYVWLNGVFVGYAEDSYTPSEFDVTEALHAG----------ENLL   225
B.pseudolongum.LacZ               AGERVSLTFDGAATAIYVWLNGAFVGYAEDAYTPSEFDVTDALGEE----------GNTL   190
B.breve.CP010413.LacZ             EGRPVTLTFQGAATAIYVWLNGSFVGYAEDSFTPSEFDVTDAIKME----------GNVL   216
B.adolescentis.AP009256.LacZ      NDGTVSLTFHGAATAIYVWLDGTFVGYGEDGFTPSEFDVTEALRNGNGNAADSPEAEHTL   203
B.longum.AP010888.LacZ            NKRRITLTFHGASTAIYVWLNGSFVGYAEDSYTPSEFDVTEALISG----------TNTL   201
                                      ::***.**:*******:* ****.**.:********:*:             : *

B.animalis.subsp.lactis.LacZ      AVACFQYSSASWLEDQDCWRFHGLFRDVELEVRPHAHVRDMLAHADWNVDAQCGELA---   282
B.pseudolongum.LacZ               AVACFQYSSASWLEDQDCWRFHGLFRGVRLDVRPRVHVRDMQATADWDVAAQCGVLD---   247
B.breve.CP010413.LacZ             AVACYEYSSASWLEDQDFWRLHGLFRSVELNARPSAHVADIHADTDWDPATSRGSLS---   273
B.adolescentis.AP009256.LacZ      TVACYEYSSASWLEDQDFWRLHGLFRTVELAAQPHTHVETVQLEADYTAADTAGTADTAE   263
B.longum.AP010888.LacZ            AVACYEYATASWLEDQDFWRMHGLFRSVELTAQPTVHIEDLHITADFEATTHAGTID---   258
                                  :***::*::******** **:***** *.* .:* .*:  :   :*:      *

B.animalis.subsp.lactis.LacZ      --VELDLDGAWCAANVELRLSTWEEHADGAALLWSATVESAP------------------   322
B.pseudolongum.LacZ               --LRLALEGDAAAHSVDVRVCAVDD--EAATPLWEATLDAER------------QSASAD   291
B.breve.CP010413.LacZ             --LDILVDGTPNAATADLVLRD-----KNGTTVWHTSTEATG------------------   308
B.adolescentis.AP009256.LacZ      LNAALTLRNPADAMTIESTLRD-----GDGNVVWESTQACNGE-----------------   301
B.longum.AP010888.LacZ            --AHAVIRNIAGAKQLSAALMD-----ANGTPVWQDSYAMSDIVDTSGVPCCGTADTAID   311
                                        : .   *   .  :         .  :*  :
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B.animalis.subsp.lactis.LacZ      --KIRYATTCEQVLPWSAEQPNLYVLEAVVRDANGRVLETARTRIGFRHVEIRDGVLVLN   380
B.pseudolongum.LacZ               DAVLRGRAAIADVRAWSAEEPNRYRVDVLVYDADGQPVETSSAVVGFRHVEIEHGIFTVN   351
B.breve.CP010413.LacZ             --TLHAEAEIDDASPWSAERPDLYTLSVALLDADGRILEIARTRIGFRRVSIEDGILKLN   366
B.adolescentis.AP009256.LacZ      --IALNSGKMTNIAPWSAESPTLYTLTVRVVGHDGAIIETVTQKIGFRTFRIENGIMTIN   359
B.longum.AP010888.LacZ            STAVQFRANLSNIRPWSAEKPHLYTLTLTVRAADNSIIEVVPQRLGFRHFEIVDGIMRLN   371
                                            :   **** *  * :   :   :.  :*     :*** . * .*:: :*

B.animalis.subsp.lactis.LacZ      GERIVFHGVNRHEFDARRGRSVTEEDMLWDVRFMKRHNINAVRTSHYPNQTRWMELCDEY   440
B.pseudolongum.LacZ               GERIVLRGVNRHEFDARLGRSVTEEDMLWDVRFMKRHNINAVRTSHYPNQTRWLELCDEY   411
B.breve.CP010413.LacZ             GKRLVFRGVNRHEFDCRRGRAITEEDMLWDIRFMKRHNINAVRTSHYPNQSRWYELCDEY   426
B.adolescentis.AP009256.LacZ      GKRIVFKGADRHEFDAKRGRAITREDMLSDVVFCKRHNINAIRTSHYPNQEYWYDLCDEY   419
B.longum.AP010888.LacZ            GKRIIFRGANRHEFDARLGRAITEQEMLTDIITCKRNNINAIRTSHYPNQTRFYELCDEY   431
                                  *:*::::*.:*****.: **::*.::** *:   **:****:********  : :*****

B.animalis.subsp.lactis.LacZ      GLYVIDEANLETHGSWNLPGDTAD-GVSIPGDDVRWQPACVDRVESMVRRDRNHACVVAW   499
B.pseudolongum.LacZ               GIYMIDEANLETHGSWNLPGDVTD-GRSIPGDDPMWLAACVDRVQSMVVRDRNHACVIAW   470
B.breve.CP010413.LacZ             GIYLIDETNLETHGSWNSPGDIPV-GTSVPGDDEAWLGACIDRLDSMIMRDRNHPSVLIW   485
B.adolescentis.AP009256.LacZ      GLYLIDETNMETHGTWVA-NNVERPEDGIPGSRPEWEGACVDRINSMMRRDYNHPSVLIW   478
B.longum.AP010888.LacZ            GLYLIDETNLETHGSWTIPGDVETPETAIPGSNPIWEGPCVDRIASMIGRDRNHPSVLIW   491
                                  *:*:***:*:****:*   .:      .:**.   *   *:**: **: ** ** .*: *
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B.animalis.subsp.lactis.LacZ      SLGNESYAGDVIRAMGNRCRELDPTRPVHYEGVTWNREYDDISDFESRMYAKPDEIREYL   559
B.pseudolongum.LacZ               SLGNESYAGTVIEQMGERCRAWDPTRPVHYEGVQWNLAYSAISDFESRMYARPDDIRDYL   530
B.breve.CP010413.LacZ             SLGNESYAGEVLKAMSAHAHRLDPGRPVHYEGVNWNHAYDEISDFESRMYAKPAEIRDWL   545
B.adolescentis.AP009256.LacZ      SLGNESSAGEVFRAMYRHAHTIDPNRPVHYEGSVHMREFEDVTDIESRMYAHADEIERYL   538
B.longum.AP010888.LacZ            SLGNESYAGEVFRAMYRFAHAADSTRPVHYEGVVHDRPFDDVTDIETRMYAKPAEIEEYL   551
                                  ****** ** *:. *   .:  *  *******      :. ::*:*:****:  :*. :*

B.animalis.subsp.lactis.LacZ      ES-------DPAKPYISCEFMHAMGNSVGGLGEYVALERYPQYQGGFIWDFMDQALWQRL   612
B.pseudolongum.LacZ               EH-------NPAKPYISCEYMHAMGNSLGGLSEYTALERYPHYQGGFIWDFIDQALWQRL   583
B.breve.CP010413.LacZ             EHGDE--RGEASKPFVSCEYMHAMGNSCGGLSEFIDLERYERYSGGFIWDYIDQGLVQRL   603
B.adolescentis.AP009256.LacZ      NDGSPAHTDGPKKPYISCEYMHAMGNSCGNMDEYTALERYPMYQGGFIWDFIDQAIETKL   598
B.longum.AP010888.LacZ            TN-------DPKKPYISCEYMHAMGNSVGGMHLYTELERYPHYQGGFIWDFIDQALWQQL   604
                                            **::***:******* *.:  :  ****  *.******::**.:  :*

B.animalis.subsp.lactis.LacZ      DDGTERLAYGGDFGDRPSDYEFSGDGIVFADRTVSAKAQEVKAQYAGVRLEPDGRGVRVT   672
B.pseudolongum.LacZ               DDGTERLAYGGDFGDRPSDLNFSGDGIVFADRTPSAKAQEVKAQYAPVRISVEPERVLVH   643
B.breve.CP010413.LacZ             PDGCERLSVGGDWGDRPTDYEFVGNGIVFADRTPSPKAQEVKQLYSPVKLTPDGHGVTIE   663
B.adolescentis.AP009256.LacZ      PDGTTRMCYGGDFGDRPSDYEFSGDGLLFADRTPSPKAQEVKQLYANVKIAVSVDEARIT   658
B.longum.AP010888.LacZ            DDGTERLTYGGDWDDRPCDYEFAGDGLVFADHSPSPKLQEVKRLYAPVVLTVSDHDVTIE   664
                                   **  *:  ***:.*** * :* *:*::***:: * * ****  *: * :  .   . :
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B.animalis.subsp.lactis.LacZ      NTNAFQGTSGTVFVARMLLDGREAWSKSYEFEVAAGSARSFDIGFPDVHSL-PDGG----   727
B.pseudolongum.LacZ               NGNAFVGTGDSVFVARMLVDGREVWSAARTLDVPAGETRALDLVFPPVEDVLPAGGDSAL   703
B.breve.CP010413.LacZ             NRNLFASTDGYVFAARLLEDGREIWHADYRFDVAAGDTQHHDIAFPDIDSDE--------   715
B.adolescentis.AP009256.LacZ      NDNLFVSTGDYRFVLRILADGKPVWSTTRRFDVAAGESASFEVDWPVDDYRS--------   710
B.longum.AP010888.LacZ            NRNLFVSTIDFAFTAKLLADGNAIWQADYRFDVPAGETRTFPIDFPSVDA----------   714
                                  * * * .* .  *. ::* **.  *     ::* **.:    : :*  .

B.animalis.subsp.lactis.LacZ      VMHEVVYEVSQQLAHDTAWAEAGYEIAWGQAVVRPHASGRSGSKMN--------DAFDDD   779
B.pseudolongum.LacZ               RAHEVVYEVSQRLARATAWAEAGHELAWGQCSRALDARALAA--WH--------TPGTAE   753
B.breve.CP010413.LacZ             GTREVTYEVDLLLAEATAWAPAGYELAFGQLTDTLNPEG----------D--ITGNDQDD   763
B.adolescentis.AP009256.LacZ      NAEELVLEVSQQLGNACDWAPAGYELAFGQCVVAGAKTTADA----------VDAAGAP   759
B.longum.AP010888.LacZ            -AGEITYEVDQSLATATPWAPAGYELAFGQHTVQSVVPASAGTDSTACGAVPSTADSDTS   773
                                     *:. **.  *.    ** **:*:*:**

B.animalis.subsp.lactis.LacZ      NLQIVTLGRWNAGVRVGQ--REILLSRTHGGVISLRDGEREYVIRVPKLLTFRPLTDNDR   837
B.pseudolongum.LacZ               SGARVTLGRWNGGMRLGS--REMLLSRTQGGIVSMRDGAREMVSRVPRLITFRPLTDNDC   811
B.breve.CP010413.LacZ             GRATVTLSRWNAGIRRND--EEILMSRTQGGIVSWKRNGREMVIRRPEIVTFRPLTDNDR   821
B.adolescentis.AP009256.LacZ      ADGTVTLGRWNAGVRGQG--REALFSRTQGGMVSYTFGEREFVLRRPSITTFRPLTDNDR   817
B.longum.AP010888.LacZ            DTGTVTVGRWNIGVVSGDGRTEALLSRTQGGMVSFKRDGREMVLRRPAITCFRPLTDNDR   833
                                      **:.*** *:       * *:***:**::*   . ** * * * :  ********
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B.animalis.subsp.lactis.LacZ      GMSSGFDRVQWFGAGRYARVVTGVGQVYRDELTGDLCGEYWYELADGAQTQVPIRYRIDS   897
B.pseudolongum.LacZ               GASSGFDRAQWFGAGRYARVVTGIGQVEWDPDRGELTGEYWYELADGARTTVPVRYSVDS   871
B.breve.CP010413.LacZ             GNRSGYDRAAWFAAGRYAVVTDT---SITQSDDGGLTAAYRYKLADPDHTPVSVTYRVTP   878
B.adolescentis.AP009256.LacZ      GAGHAFERAAWAVAGKYARCVDC---AIANRGENAVEATYTYELAIPQRTKVTVRYVADT   874
B.longum.AP010888.LacZ            GNGSGFDRVRWFGAGRYARIANQ---QFSQTE-TGVIAEYTYTLAEPGETQVAVRYEVDA   889
                                  *   .::*. *  **:**  .       :     : . * * **   .* * : *

B.animalis.subsp.lactis.LacZ      QL-RMHIELEYTG-CA-GAPSLPAFGLEWMLPKQYENLEFYGRGPAETYRDRKR-AKLGI   953
B.pseudolongum.LacZ               AM-RLHVEATWPG-EA-DATSLPLFGLEWVLPVRYSQLEFYGPGPWETYTDRDR-AKVGA   927
B.breve.CP010413.LacZ             DM-RMQLTVEYPGNAA-GAASLPAFGIEWELPGEYQHLRYYGTGPEETYRDRKQGGKLGI   936
B.adolescentis.AP009256.LacZ      AG-LVSLDVEYPGEKNGDLPTIPAFGIEWALPVEYANLRFYGAGPEETYADRRH-AKLGV   932
B.longum.AP010888.LacZ            ASGRVHLAARYAG-AT-DAPTLPAFGLEWTLPKQYENLRFYGLGPEETYRDRLHGGKLGI   947
                                      : :   : *    .  ::* **:** ** .* :*.:** ** *** ** : .*:*

B.animalis.subsp.lactis.LacZ      WNTTAQADMAPYLVPQETGNHEDVRWAYVFDADCHGLLVEAD---DSLALSLLPHSSLEI   1010
B.pseudolongum.LacZ               WRTTAFDDMQPYLVPQETGNHAHVRWARVTDEDGHGLLIESARPGTDLALSLLPYDTLTI   987
B.breve.CP010413.LacZ             WDTTSEASTAPYLMVQETGSHEDVRWLEATDIQGHGLRIIQR-GDRHFTASLLPWNTYTI   995
B.adolescentis.AP009256.LacZ      WSTTAGDDCAPYLLPQETGNHEDVRWAEITDDSGHGVRVKRGAGAKPFAMSLLPYSSTML   992
B.longum.AP010888.LacZ            FERTAAEDNAPYLVPQETGNHEDLRWAEVLDAQGHGMRISQA-GSEHFAASLLPYSSLML   1006
                                  :  *:  .  ***: ****.* .:**    * . **: :        :: **** .:  :
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B.animalis.subsp.lactis.LacZ      ENATHQNELAQPRHMFLRLLAGQMGVGGDDSWGAPVHDRYLLPADEPLKLAVTISML      1067
B.pseudolongum.LacZ               EAATHQDELPKPRHMFLRLLAGQMGVGGDDSWGAPVHDRYQLDAARELTLDVTMLLV      1044
B.breve.CP010413.LacZ             EAARRHEDLPAPRHNYLRLLAAQMGVGGDDSWGAPVHTAYQLPADRPLTLDVNLELI      1052
B.adolescentis.AP009256.LacZ      EEALHQDELPKPRHMFLRLLAAQMGVGGDDSWMSPVHEQYQLPADQPLSLNVQLKLF      1049
B.longum.AP010888.LacZ            EEATHQNELPPVRHTFLRLLAAQMGVGGDDSWGAPVHEQYQLPADRAYTLDVNLELF      1063
                                  * * ::::*   ** :*****.********** :***  * * * .  .* * : :
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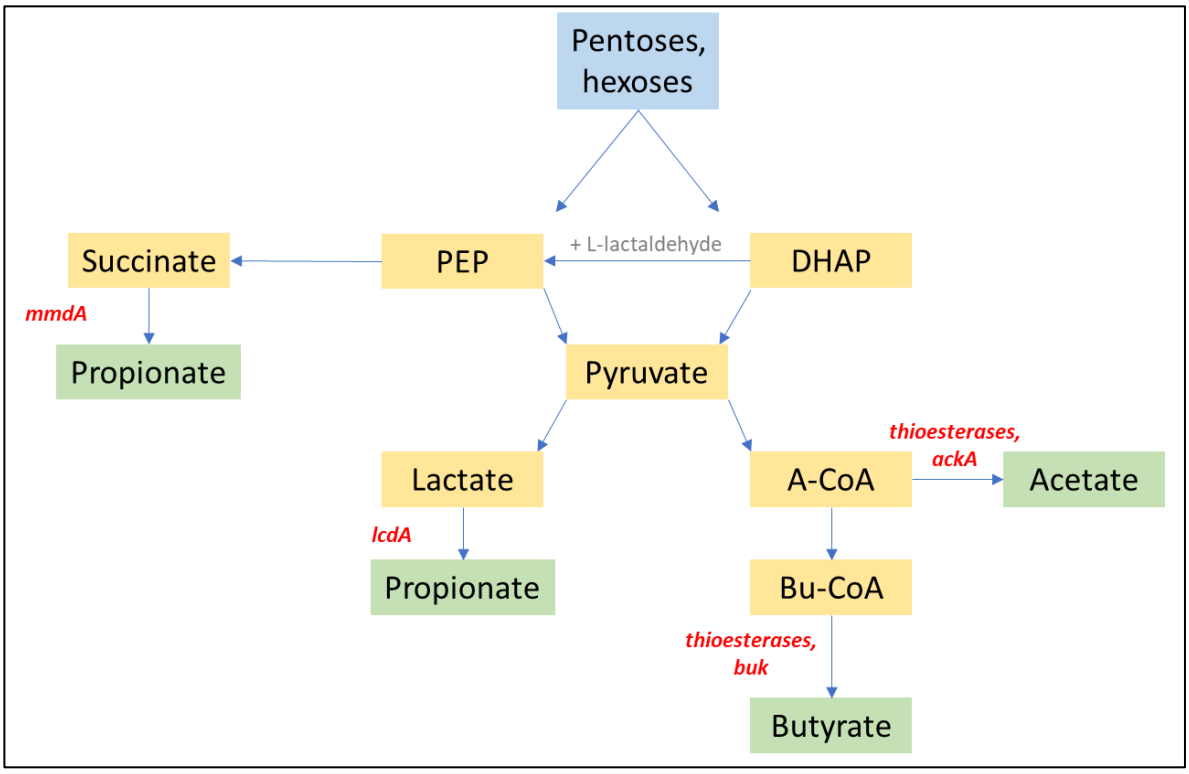
*, fully conserved residue. :, residues possess strongly similar properties (> 0.5 in the Gonnet PAM matrix; Dayhoff, Schwartz and Orcutt, 1978). ., residues possess weakly similar properties (≤ 0.5 in the Gonnet PAM matrix; Dayhoff, Schwartz and Orcutt, 1978). Blank, residues share no similar properties, complete mismatch.

3.4 *Bifidobacterium* are capable of producing more SCFA from GOS than *Lactobacillus*.

All three genomes characterised in this study were inspected for key prokaryotic genes used in the synthetic pathways of acetate, butyrate and propionate (Reichardt *et al.*, 2014; Xu, Bai, Chen and Bai, 2017; Zhao, Dong, Zhang and Li, 2019; Figure 4). Genomes were inspected for the presence or absence of *ackA* (acetate kinase), *buk* (butyrate kinase), *entH, fadM, menI, tesA, tesB, ybgC, ybhC, yciA, yigI* (possible thioesterases), *lcdA* (lactoyl-CoA dehydratase subunit alpha) and *mmdA* (methylmalonyl-CoA decarboxylase α-subunit of Negativicutes; Table 6). ackA, buk, lcdA and mmdA are essential for SCFA synthesis in prokaryotes, while the thioesterases are thought to contribute toward the production of SCFA (Zhao, Dong, Zhang and Li, 2019). Both *Bifidobacterium* strains possessed *ackA* and *tesB*, whereas the *L. reuteri* genome contained only *ackA*. The possession of these acetate and butyrate synthesis genes is consistent with genomic data from other *Bifidobacterium spp.* but different to other *Lactobacillus* species (Zhao, Dong, Zhang and Li, 2019). The two critical genes *mmdA* and *lcdA* required for the succinate and acrylate pathways, respectively, in mammalian gut prokaryotes were not detected in any of the genomes in this study. All genomes characterised in this study were subject to low stringency searches of these gene sequences, but no positive results were returned. This proposes that, although associations between proliferation of *Bifidobacterium* and increased propionate concentration (stimulated by GOS supplementation) have been made (Tanner *et al.*, 2014), either (a) *Bifidobacteria* use an alternative pathway to synthesise propionate; (b) *mmdA* and *lcdA* orthologues are not yet defined in *Bifidobacteria*, thus remained undetected in this study, or; (c) cross feeding occurs where propionate synthesis increases in other intestinal microbes alongside the proliferation of *Bifidobacterium*. Bacteria capable of producing propionate in the mammalian gut are *Lactobacilli* (Pan *et al.*, 2019), *Desulfovibrio* (Wang *et al.*, 2019) and *Propionibacterium* (Pourabedin, Guan and Zhao 2015).

Figure 4. Possible pathways of SCFA synthesis in *Bifidobacterium* species, adapted from Reichardt *et al.*, (2014) and Zhao, Dong, Zhang and Li (2019).



PEP, phosphoenolpyruvate; DHAP, dihydroxyacetonephosphate; A-CoA, acetyl coenzyme A; Bu-CoA, butyryl coenzyme A. mmdA, methylmalonyl-CoA decarboxylase α-subunit of Negativicutes; lcdA, lactoyl-CoA dehydratase subunit alpha; buk, butyrate kinase; ackA, acetate kinase.

Table 6. Presence or absence of genes involved in prokaryotic short-chain fatty acid synthesis pathways.

Gene	*B. animalis* subsp. *lactis*	*L. reuteri*	*B. pseudolongum*
ackA	+	+	+
buk			
entH			
fadM			
menI			
tesA			
tesB	+		+
ybgC			
ybhC			
yciA			
yigI			
lcdA			
mmdA			

+, presence of gene; white background, absence of gene

ackA, acetate kinase; *buk*, butyrate kinase; *entH, fadM, menI, tesA, tesB, ybgC, ybhC, yciA, yigI,* possible thioesterases *lcdA*, lactoyl-CoA dehydratase subunit alpha; *mmdA*, methylmalonyl-CoA decarboxylase α-subunit of Negativicutes.

3.5 Species-specific primers for measuring absolute abundance of *Bifiodbacterium spp*.

The diversity of microbiota in an environmental sample can be measured by sequencing a hypervariable region of the 16s ribosomal RNA sequence, producing operational taxonomic unit (OTU), comparing reads to a reference database and eventually generating relative abundance metrics (Pruesse E *et al.,* 2007; Caporaso JG *et al.,* 2011). For these workflows, it is common for the hypervariable V4 region of the bacterial 16S rRNA gene to be targeted. However, usually relative abundance data is only gathered for genera and absolute abundance of sub-groups (e.g. species and sub-species) is not revealed. For example, several microbiomic studies have revealed that *Bifidobacterium* are enhanced upon GOS supplementation but these findings do not report data to species level (Tzortzis *et al.,* 2005; *Jung et al.,* 2008; Liu *et al.,* 2017; Slawinska *et al.,* 2019). Therefore, the design of unique primers for use in quantitative PCR is needed to calculate absolute abundance of microbial species from complex samples. In addition to traditional quantitative PCR methods, another way in which this can be achieved is by droplet digital PCR which is a highly sensitive and efficient method for determining absolute abundance of target DNA (Barlow, Bogatyrev and Ismagilov, 2020). While the 16s rRNA sequence is capable of distinguishing bacterial genera, it is inadequate for differentiating between *Bifidobacterial* species due to strong sequence homology within the genus. In *Bifidobacterium,* housekeeping gene *groEL,* encoding heat shock protein 60 (Hsp60, also known as groEL or Cpn60), evolves quickly and has been used for discriminating between *Bifidobacterial spp.* (Zhu, Li and Dong, 2003; Masco *et al.,* 2004; Ventura *et al.,* 2004). Junick and Blaut (2012) aligned *groEL* sequences of *Bifidobacterium* and designed species-specific oligos for quantitative PCR (qPCR) quantification. Their suggested *groEL* sequence primers give a 100% bp match with the *groEL* sequences of the *Bifidobacterium* identified in this study. As such, these primers can be used in qPCR to detect absolute abundance of *B. animalis* and *B. pseudolongum* in future studies to provide a more detailed insight into the effect of GOS on *Bifidobacterial* populations in the porcine GIT

4 Discussion

SCFA are by-products of microbial fermentation and their synthesis is influenced by the configuration of intestinal flora (Hume, 1995; Macfarlane and Gibson, 1995). Absorption of SCFA from the gut lumen are thought to contribute to between 30 and 76 % of energy production in pigs (Engelhardt, 1995). Additionally, SCFA are shown to modulate host health in the GIT via metabolic regulation, improving gut barrier function, maintaining glucose homeostasis and immunomodulation (Chambers *et al.*, 2015; Pingitore *et al.,* 2017; Vetrani *et al*., 2016; Canfora and Blaak, 2017; Mariño *et al*., 2017; Gonzalez *et al*., 2019; Hu *et al*., 2018). Hence, it is advantageous to promote SCFA-producing bacteria in the mammalian gut. This study proposes that the *Bifidobacterium spp*. characterised are better equipped for improving host gut health (compared to the *Lactobacillus reuteri* characterised) since they express genes that make proteins involved in the acetate and butyrate synthesis pathways (*ackA* and *tesB*). This may lead to more acetate and butyrate production in the mammalian gastrointestinal tract which are proposed to improve fat and protein retention, as well as inhibit colonisation of enteropathogens (Jørgensen *et al.,* 1997; Spring *et al.,* 2000; Lawhon *et al.,* 2002; Fukuda et al., 2011; Sun and O'Riordan, 2013). By extension, the inhibition of enteric pathogen adhesion to the gut epithelial surface could reduce PWD in neonatal piglets. Furthermore, SCFA, specifically acetate, butyrate and propionate, are the main energy source for gastrointestinal epithelial cells, and increased concentrations in the gut can help regulate epithelial cell surface (Nepelska *et al*., 2012; Markowiak-Kopeć and Śliżewska, 2020; Oh *et al*., 2021) Therefore, it's clear that increased abundance of bacteria that can synthesise these compounds provide health benefits to the host.

The enzymes involved in the metabolism of oligosaccharides in *Lactobacillus* are well understood but requires further investigation in *Bifidobacterium* (Gänzle and Follador, 2012). In *Lactobacillus spp.,* LacS, a membrane bound lactose permease, imports GOS into the cytoplasm and acts as a galactose-lactose co-transporter (Vos and Vaughan, 1994; Silvestroni *et al.,* 2002; Figure 4). LacZ and LacLM are cytosolic β-galactosidases which hydrolyse terminal galactose units in β-galactosides (Schwab, Sørensen and Gänzle, 2010). LacEF (membrane bound) and LacG (cytosolic)

are enzymes involved in importing and hydrolysing lactose, respectively (Vos and Vaughan, 1994). GOS are transported into the cytosol and hydrolysed by Lac enzymes into simple hexose sugars. These hexose sugars can be further hydrolysed and used for synthesis of SCFAs like acetate, butyrate and propionate (Reichardt *et al.,* 2014; Zhao, Dong, Zhang and Li, 2019;). Therefore, the presence of genes involved in both GOS metabolism pathways may be more advantageous than having access to just one. However, this study does not investigate the extent to which these pathways operate, and so it is suggested that mRNA expression analysis be used to assess whether the expression of one pathway in *L. reuteri* is overexpressed to make up for lacking both pathways as seen in *Bifidobacterium.*

The SNPs detected in the *Lac* sequences of the *Bifidobacterium* strains change the amino acid sequence in which they code, which may alter the protein structure and ultimately affect protein function. Figure 2 shows the amino acid sequence of membrane bound LacS from both *Bifidobacterium* isolates characterised in this study, as well as LacS amino acid sequence from other *Bifidobacterium* species. While there are 76 complete amino acid mismatches between (and including) the first and last membrane bound segments (highlighted), 38 complete mismatches are in precited membrane segments of the protein while the other 38 are in the sequences of the predicted protein loops. Figure 3 shows the amino acid alignment of LacZ in both *Bifidobacterium* strains isolated in this study, as well as LacZ amino acid sequences from other *Bifidobacterium* species. 180 complete mismatches were identified between the five aligned sequences, comprising approximately one seventh of the sequence length. These differences identified in LacS and LacZ amino acid sequence may alter protein structure and the proteins efficiency in metabolising GOS. Although the amino acid sequences are compared in this study, the potential change in protein structure that is suggested is not fully analysed. As such, future work may include assessing the efficiency of these isolates in metabolising GOS *in vitro*, and whether this correlates with abundance metrics of GOS-supplemented pigs. This further work might contribute to assessing which of these *Bifidobacterium* are better used with GOS as a synbiotic in the pig production industry.

This study also highlights one limit to supplementing nutrient media with mupirocin to select for *Bifidobacteria* from mammalian digesta. Mupirocin acts as an antimicrobial by competing with isoleucine as a substrate for isoleucyl-tRNA synthetase (IleS), a process responsible for valine, leucine and isoleucine synthesis; inhibition of isoleucyl-tRNA synthetase hinders RNA and protein synthesis and ultimately leads to bacterial death (Poovelikunnel, Gethin and Humphreys, 2015). Several important and conserved amino acid residues (RG1 and RG2) of the IleS protein confer mupirocin resistance in *Bifidobacteria* (Serafini *et al*., 2011). Both *Lactobacillus reuteri* genomes were interrogated for antibiotic resistance genes using the Comprehensive Antibiotic Resistance Database (CARD; Alcock *et al*., 2019). Both *L. reuteri* strains displayed a weak match (<25% sequence identity) to mupirocin-resistant IleS which explains their ability to grow in the presence of the antibiotic.

5 Conclusion

The present study found more key genes required for the synthesis of SCFA in the genome of *B. animalis subsp. Lactis* and *B. pseudolongum* compared to *L. reuteri*. It was also found that *B. animalis* subsp. *Lactis* appears to be equipped for more efficient GOS metabolism due to the possession of more *Lac* genes than other strains isolated. This might suggest that *B. animalis* subsp. *Lactis* is the better species of *Bifidobacterium* for use as a 44ymbiotic with GOS. However, the lack of GIT abundance metrics and transcriptomic data in this study prevents a robust conclusion being made on whether *B. animalis* subsp *lactis* is enhanced upon GOS supplementation, synthesises more acetate, butyrate and/or propionate, or more efficient at metabolising GOS.

It is suggested that an extensive review be carried out for *Bifidobacterium spp.* And their capacity to metabolise oligosaccharides *in vitro* due to their association with improved structure and function of mammalian guts upon dietary supplementation with prebiotic fibres. Future microbiomic studies should identify which *Bifidobacterial spp.* Are enhanced upon prebiotic supplementation, and monitor transcriptomics of the genes discussed in this study. SCFA concentrations should also be measured in this type of study to assess any correlation between *Bifidobacterium* and *Lactobacillus* species. This would provide insight into which *Bifidobacterium* species are best for use in conjunction with GOS as a synbiotic to improve intestinal health in the weaning pig.

6 References

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7 Appendices

Appendix 1. Agarose gel electrophoresis following PCR amplification (using primers f 5′-GCGAACGGGTGAGTAATGC-3′ and r 5′-TTTCATGACTTGACGGGCG -3′) of genomic DNA from up to 10 bacterial species displaying *Bifidobacterium* colony morphology on MRS agar that were isolated from pig intestines.

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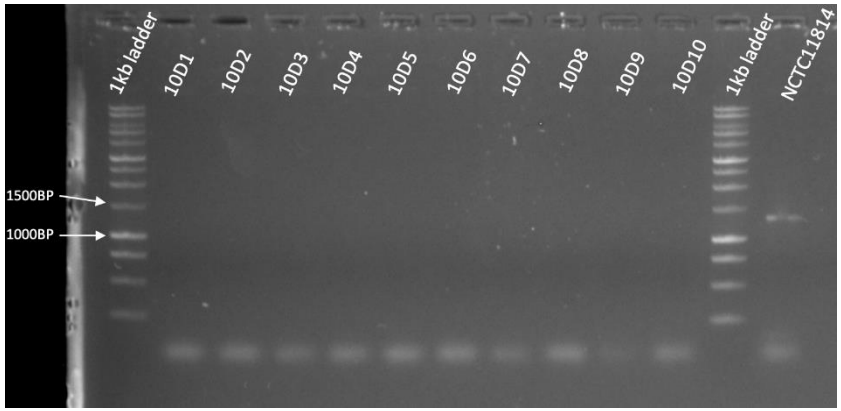

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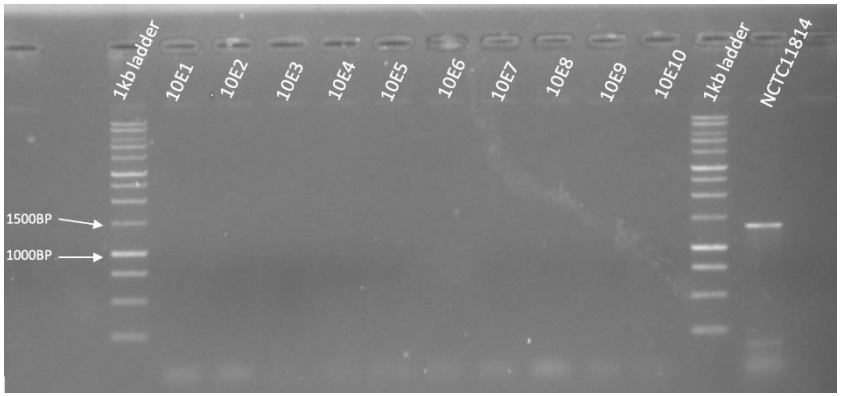

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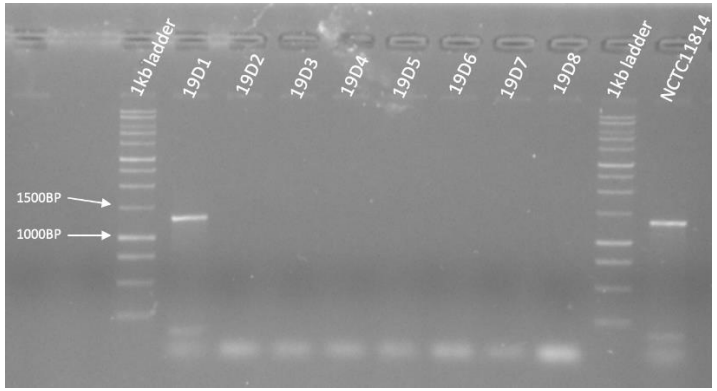
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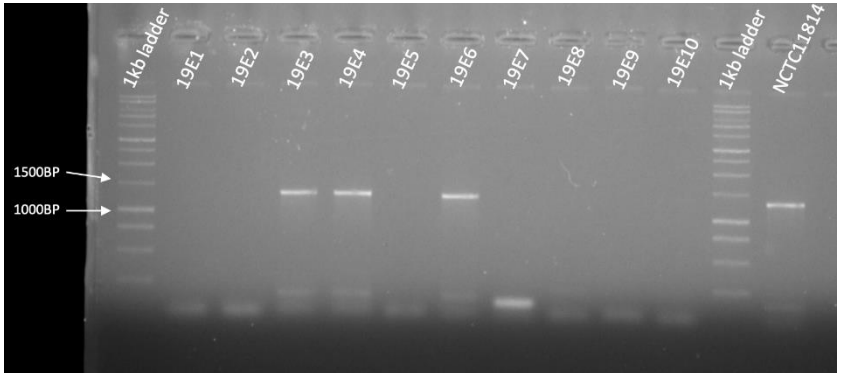
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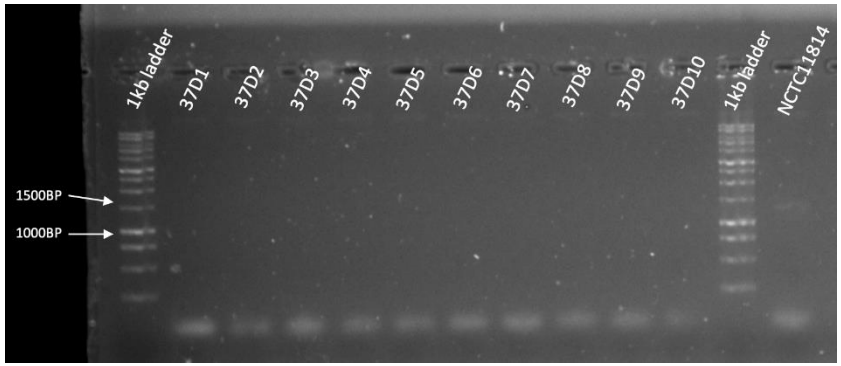
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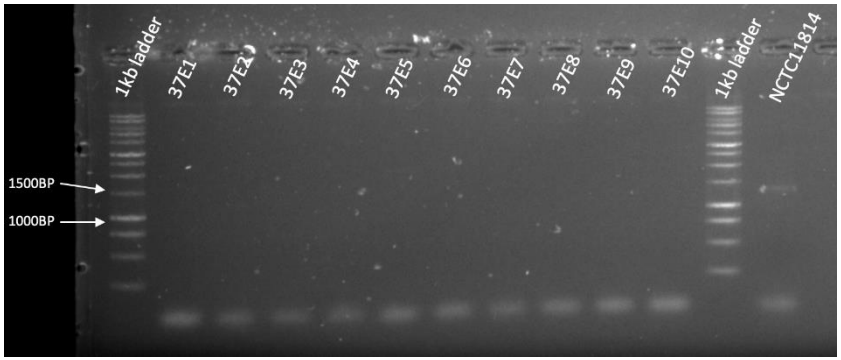
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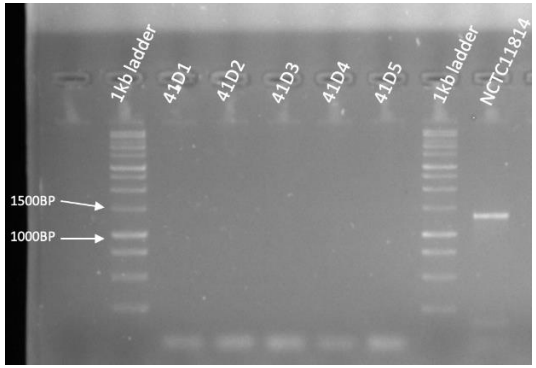
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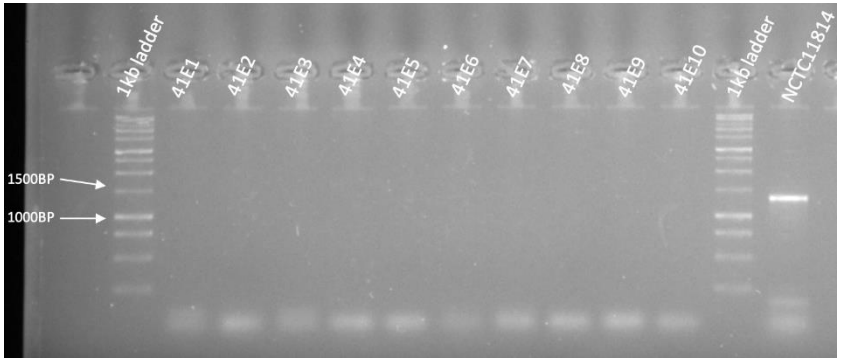
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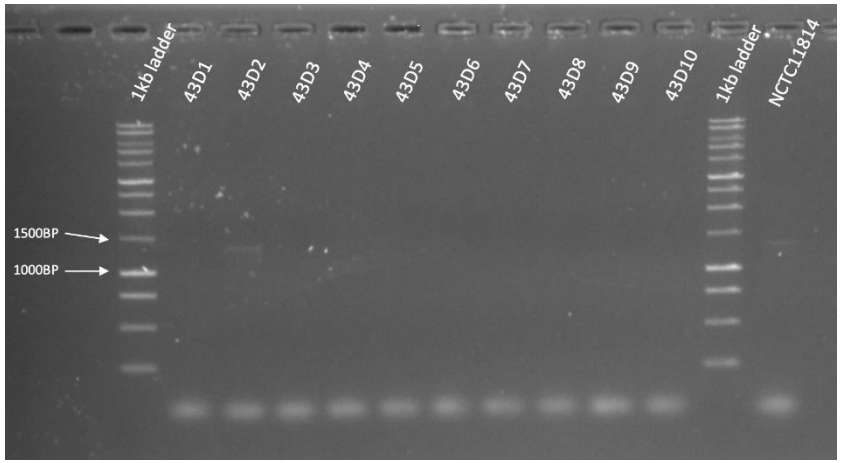
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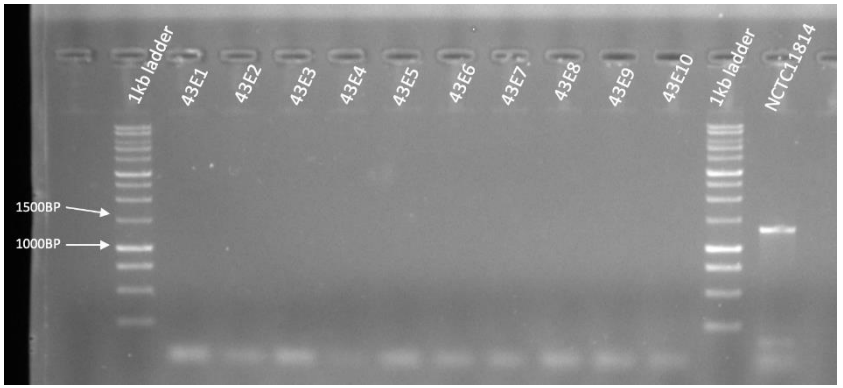
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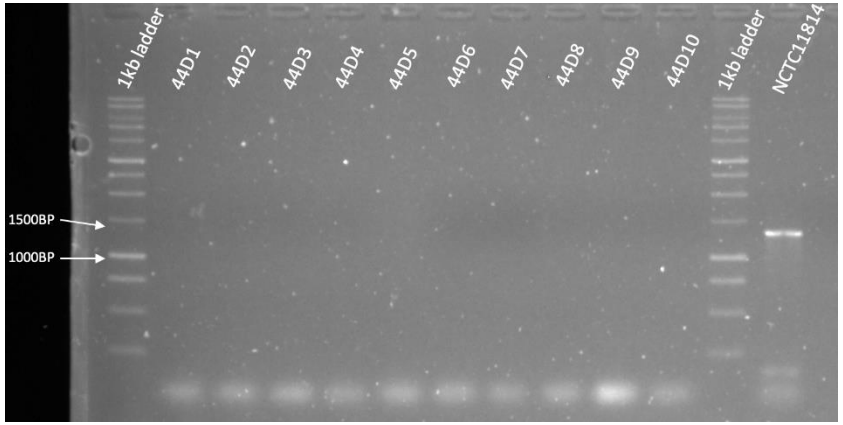
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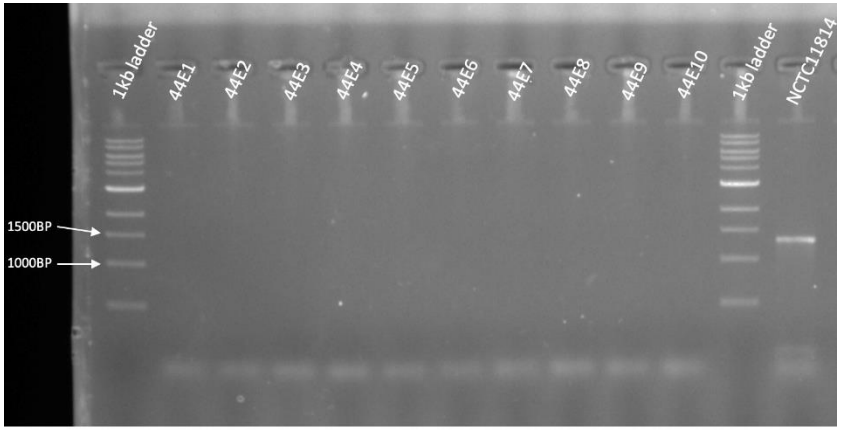
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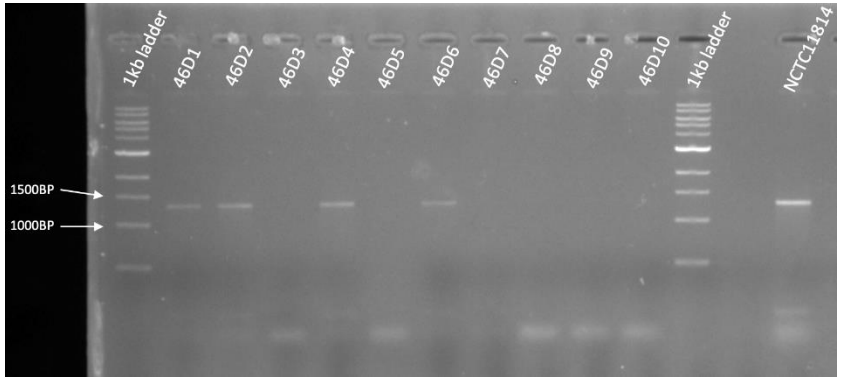
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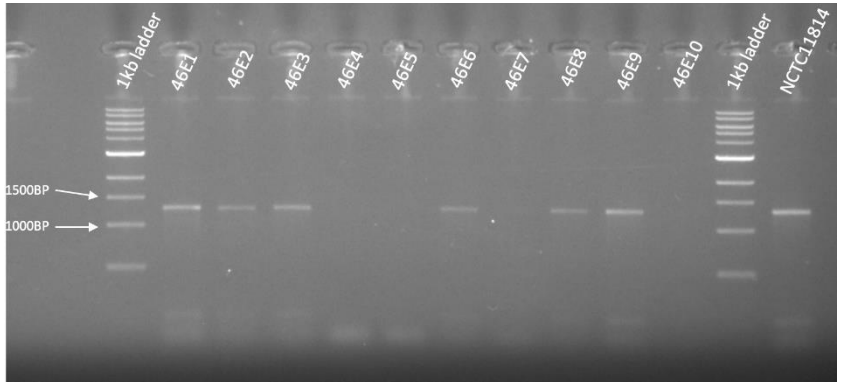
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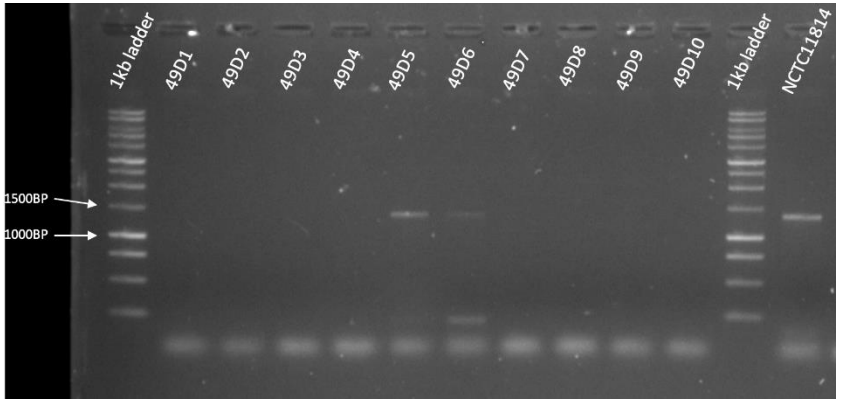
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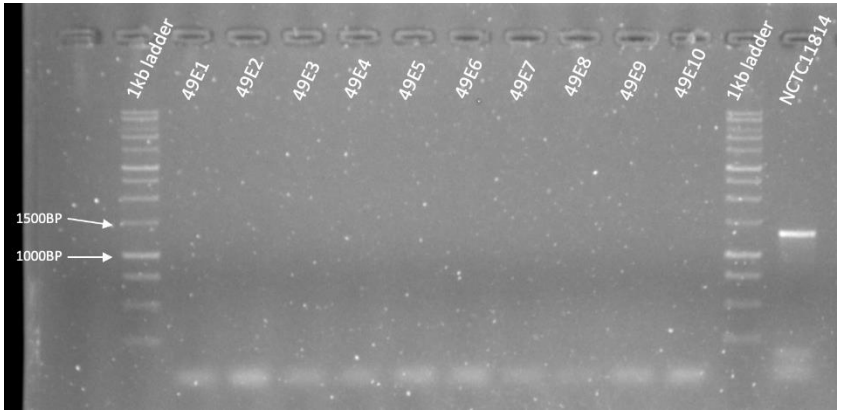
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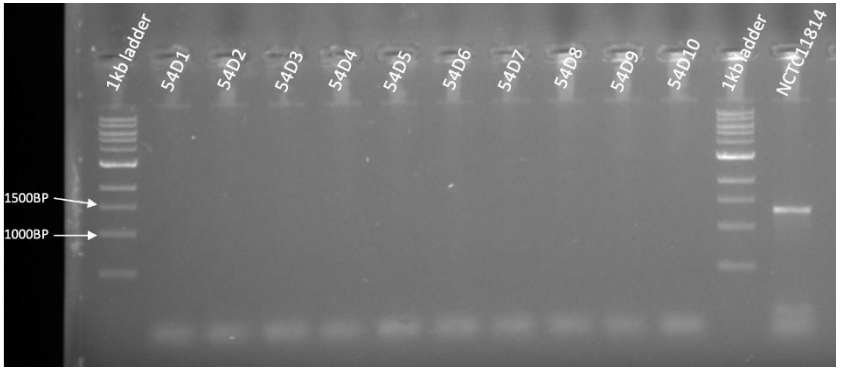
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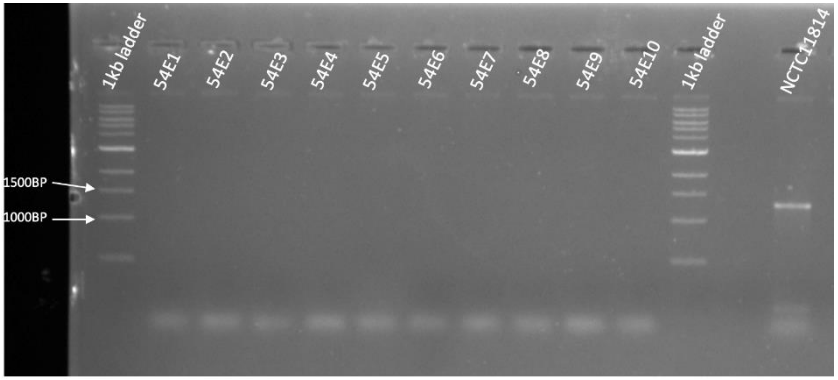
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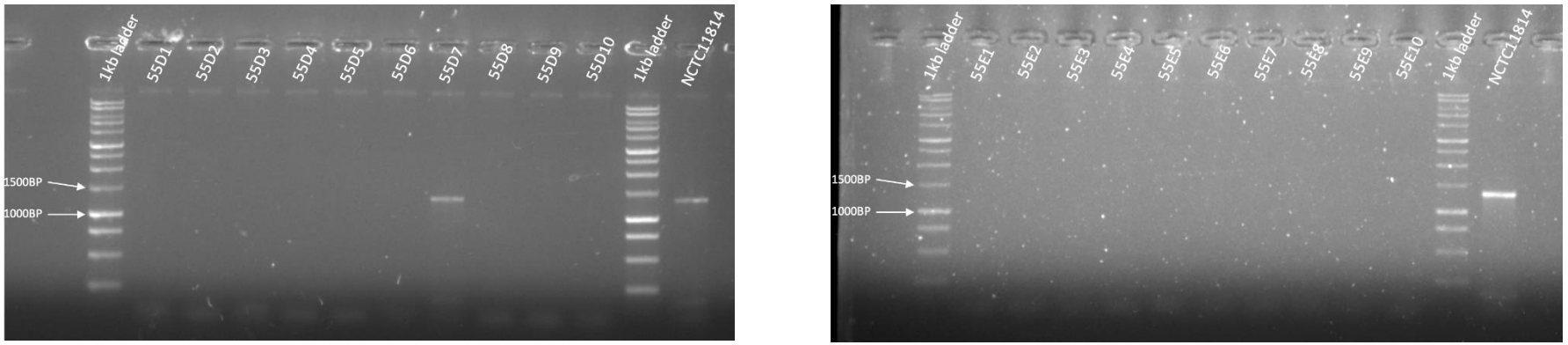

(A) Pig #10, caecum; (B) Pig #10, colon; (C) Pig #19 caecum; (D) Pig #19, colon; (E) Pig #37, caecum; (F) Pig #37, colon; (G) Pig #41, caecum; (H) Pig #41, colon; (I) Pig #43, caecum; (J) Pig #43, colon; (K) Pig #44, caecum; (L) Pig #44, colon; (M) Pig #46, caecum; (N) Pig #46, colon (O) Pig #49, caecum; (P) Pig #49, colon; (Q) Pig #54, caecum; (R) Pig #54, colon; (S) Pig #55, caecum; (T) Pig #55, colon.

NCTC11814, *Bifidobacterium adolescentis.*

Appendix 2. DNA quality, quantity and fragment size measurements using Agilent and Qubit systems for whole genome sequencing library preparation.

Sample ID [a]	Index primer 1	Index primer 2	λ260/280 [b]	λ260/230 [b]	DNA conc (ng/µL)[c]	DNA fragment size (bp) [d]		DNA conc (ng/µL)[e]	DNA fragment size (bp)[f]			nM = [(ng/µL)/(average DNA size (bp) * 660g/mole)] * 10^6	Normalisation to 4nM in 15µL	Re-suspension Buffer to add (µL)
						From	To		From	To	Average size			
19D1	N701	S502	1.60	2.26	6.01	155	815	12.10	269	909	658	27.86	2.15	12.85
19E3	N702	S502	2.02	2.29	7.14	157	914	2.66	175	927	551	7.31	8.20	6.80
19E6	N703	S502	1.84	2.04	3.70	157	970	3.52	171	976	600	8.89	6.75	8.25
46D2	N704	S502	1.85	2.13	36.60	150	1078	6.20	193	976	654	14.36	4.18	10.82
46D6	N705	S502	1.97	2.20	55.00	149	983	4.44	404	956	691	9.74	6.16	8.84
46E1	N706	S502	1.95	2.12	16.30	148	888	2.28	169	948	634	5.45	11.01	3.99
49D6	N701	S503	1.88	2.14	27.80	152	948	8.30	185	909	545	23.07	2.60	12.40

[a] See Table 2 for sample ID information

[b] Absorbance ratios measured using Nanodrop 1000 system

[c] First Qubit measurement

[d] First TapeStation measurement

[e] Second Qubit measurement

[f] Second TapeStation measurement

"First" and "Second" measurements reference to the order of Materials and methods section