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The potential of non-starter lactic acid bacteria from Cheddar cheese to colonise the gut

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ABSTRACT

This study was undertaken to assess the potential of Non-Starter Lactic Acid Bacteria (NSLAB) from Cheddar cheese to survive gastric transit and display probiotic-related traits including bile salt hydrolase activity, the ability to adhere to the gut epithelium and inhibition of enteropathogen binding. Populations of NSLAB, up to 10^7 CFU/g per cheese were recovered following exposure of cheese to Simulated Stomach Duodenum Passage (SSDP) conditions. A total of 240 isolates were randomly selected from twelve Cheddar cheeses and assessed probiotic traits. Two strains *Lactobacillus paracasei* DPC 7150 and *Lactobacillus rhamnosus* DPC 7102 showed the most probiotic potential. The *Lb. paracasei* and *Lb. rhamnosus* strains displayed adhesion rates of 64% and 79%, respectively and inhibited binding of pathogenic *Escherichia coli* by >20%. This research demonstrates that Cheddar cheese harbours potentially beneficial bacteria, a large portion of which can survive simulated digestion and potentially exhibit health beneficial effects once ingested.

1. Introduction

Cheese is manufactured using a mixture of four major ingredients (milk, rennet, microorganisms and salt), with the assortment of cheese types available being a result of variations in this mix, such as the concentration of salt used, the source of the milk (cow, sheep or goat's milk) or the actual species of microbes added, together with variations in the manufacturing and ripening protocols (Beresford, Fitzsimons, Brennan, & Cogan, 2001). While specific microbial cultures, referred to as 'starter' bacteria, are introduced during cheesemaking for the necessary production of acid, this is not the case for all bacteria present (Fox, McSweeney, Cogan & Guinee, 2004). Non-starter lactic acid bacteria (NSLAB) are microorganisms that grow within cheese during ripening but are not deliberately added and are not required for acid production in the initial cheese manufacturing process (Beresford, 2003). The sources of NSLAB include autochthonous milk microbes capable of surviving the pasteurisation process and those found in the manufacturing plant, with NSLAB populations eventually outcompeting the starter cultures and typically reaching between 10^7 and 10^9 colony forming units (cfu) per gram of cheese at the end of the ripening period (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). The biochemical reactions undertaken by these NSLAB are responsible for various flavour compounds present in maturing cheeses and are, therefore, responsible for important organoleptic characteristics of cheese (Settanni & Moschetti, 2010).

As cheese may contain high NSLAB populations upon ingestion, the fate of these microorganisms is of interest due to their ability to potentially impact human health or the human gut microbiome. Therefore, the capacity of cheese NSLAB populations to survive upper gastrointestinal environments should be taken into consideration when evaluating health implications of cheese intake. The cheese matrix itself has been found to promote survival of deliberately added strains of potential probiotic bacteria, providing a buffering effect against acid stresses encountered during digestion and supplying a dense, high-fat protective barrier for the probiotic strains against the unfavourable digestive tract environment (Gomes da Cruz, Alonso Buriti, Batista de Souza, Fonseca Faria, & Isay Saad, 2009). Particular attention has been focussed on the *Lactobacillus* genus, which are the dominant NSLAB found in Cheddar and most other varieties of long ripened cheese and which have also had several strains granted probiotic status (Fijan, 2014; Swearingen, O'Sullivan, & Warthesen, 2001). Probiotics have been defined by the Food and Agricultural Organisation of the United Nations (FAO)/World Health Organisation (WHO) as being 'live microorganisms, which when consumed in adequate amounts, confer a

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health benefit on the host' (Araya, Morelli, Reid, Sanders, & Stanton, 2002).

Various *in vitro* and *in vivo* assays have been used to determine the capability of selected NSLAB strains to survive gastric transit, most of which involve the ability to survive acid stress and bile tolerance, as well as their potential to express characteristics once within the gut that have health benefits for the host (Haller, Colbus, Gänzle, Scherenbacher, Bode & Hammes, 2001; Maragkoudakis, Zoumpopoulou, Miaris, Kalantzopoulos, Pot & Tsakalidou, 2006; Papanikolaou, Hatzikamari, Georgakopoulos, Yiangou, Litopoulou-Tzanetaki & Tzanetakis, 2012). As well as bile tolerance, the ability of strains to deconjugate bile via bile salt hydrolase (BSH) enzymes is a desirable trait as, as well as potentially conferring a competitive advantage to these organisms within gut environment, BSH enzymes have also been linked to reduced serum cholesterol levels in the host organism (Begley, Hill, & Gahan, 2006). The ability of the surviving strains to colonise the gut, either transiently or on a more long-term basis, is also considered a useful quality as it allows longer exposure of the host to any beneficial effects that these strains may express. The ability of strains to adhere to the intestinal lining once ingested is, therefore, a highly sought-after quality, with either whole cell lines or intestinal mucus used as the binding surface (Servin & Coconnier, 2003). Additionally, the ability of strains to inhibit the binding of food-borne pathogens can also be tested for *in vitro*, with *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* being typical antagonistic targets (Balamurugan, Chandragunasekaran, Chellappan, Rajaram, Ramamoorthi, & Ramakrishna, 2014; Yu, Wang, & Yang, 2011). In addition, some bacteria, including specific strains of NSLAB are also capable of producing exopolysaccharides (EPS), high molecular-weight polymers produced from sugars, which can affect their host by modulating immune responses (Ryan, Ross, Fitzgerald, Caplice, & Stanton, 2015).

To the best of our knowledge, all studies to date that have sought to elucidate the potential of NSLAB isolates to survive gastric transit and display established probiotics traits have started with individual isolates selected from cheese following conventional plating techniques which do not include any step to select for the capacity to survive gastric exposure. These techniques start by separating the bacteria from the cheese matrix by blending with a buffer followed by serial dilution and plating. As the aim of this study was to evaluate the ability of NSLAB of the *Lactobacillus* genus isolated from Cheddar cheeses to survive gastric passage and to determine if strains capable of doing so expressed traits beneficial to the host, the first step in our selection involved exposure of NSLAB populations separated from the cheese matrix to simulated stomach duodenum passage (SSDP) that includes exposure to high acidic conditions and bile acids, prior to selective plating. Strains surviving this selection procedure were then investigated via subtractive screening for their probiotic potential using the *in vitro* tests listed in the Food and Agriculture Organisation (FAO) guidelines (2002) for screening of potential probiotics (Food and Agriculture Organization Of The United Nations, 2006).

2. Materials and methods

2.1. Bacterial strain source and storage

Twelve Irish Cheddar Cheeses were selected for this study produced from both raw and pasteurised bovine milk. Nine of these were commercial brands and three were sampled from Cheddar produced at pilot scale (Moorepark Technology Limited, Teagasc Moorepark) for a separate study. All NSLAB isolated from these cheeses were stored in MRS broth (BD Difco™) with 25% Glycerol (Sigma Aldrich). Long-term stocks were stored at −80 °C. Working stocks were kept at −20 °C, and were propagated twice in MRS broth, then twice on MRS plates for activation and to ensure purity prior to testing. All MRS broth/agar was prepared with 0.05% (w/v) L-Cysteine-HCl.

Enteropathogenic *E. coli* (EPEC) 0111:H2 (strain NCTC 8007) was obtained from the National Collection of Type Cultures (NCTC; London, UK) and was stored at −20 °C in BHI broth with 50% glycerol (v/v). Prior to the Pathogen Exclusion Assays (2.10 below), *E. coli* was grown overnight in BHI broth at 37 °C, aerobically.

2.2. Screening for NSLAB that survive simulated stomach duodenum passage

The ability of NSLAB to survive passage through the upper gastrointestinal tract was tested *in vitro* via Simulated Stomach Duodenum Passage (SSDP), as reported by Pisano et al. (2014). This protocol exposes the NSLAB to the acidic conditions of the stomach (pH 3.0) followed contact with bile acids as would be experienced duodenum, the two primary tests identified by the FAO for the selection of strains with probiotic potential. 5–10 g of each cheese (obtained with a sterile cheese trier) was diluted 1:10 in sterile 2% (w/v) trisodium citrate (VWR™ 27833.260), followed by 5 min of maceration with a stomacher (Bag-Mixer® 400P, Interscience). 10 mL of the resultant slurry was centrifuged at 4000 rpm for 5 min, followed by aspiration of the supernatant. The pellet was resuspended in 10 mL of simulated gastric juice (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$, 0.3% pepsin, at pH 3.0) and incubated for 90 min at 37 °C, with shaking for peristalsis simulation. 17.5 mL of synthetic duodenum juice (6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, and 0.1% pancreatin, at pH 7.4) and 4 mL of 10% (w/v) Ox gall powder (Sigma) containing bile acids were then added to the cell suspension, simulating passage into the upper intestinal tract, and incubation was continued for a further 90 min at 37 °C, with shaking. The suspension was then centrifuged, the supernatant discarded, and the pellet was resuspended in maximum recovery diluent (MRD) (Oxoid CM0733). Survival rates of the bacteria following SSDP were determined via plating serial dilutions in MRD of the samples in duplicate prior to (Time 0, before addition of simulated gastric juice) and following (Time 180, following the full 180 min of exposure to both simulated gastric and duodenum juice) exposure to SSDP conditions. Samples were plated on MRS, in duplicate, and incubated for 48 h at 37 °C, anaerobically. Screening on each cheese was performed in triplicate.

2.3. Pulsed field gel electrophoresis

Following SSDP, 20 colonies from countable MRS plates for each cheese were randomly selected, purified and DNA fingerprint profiles were generated using Pulsed Field Gel Electrophoresis (PFGE) as described by Stefanovic *et al* (Stefanovic, Kilcawley, Rea, Fitzgerald, & McAuliffe, 2017), with modifications. Bacterial cultures were inoculated at 1% and grown overnight in 10 mL MRS broth containing 0.02 M threonine, anaerobically. 0.5 mL of each overnight culture was centrifuged at 12000 rpm for 5 min, the supernatant aspirated and the pellet resuspended in 0.5 mL of Buffer 1 (0.01 M Tris-HCl, 1 M NaCl, pH 7.6). The cell suspension was re-centrifuged, the supernatant aspirated and the pellet resuspended in 0.3 mL Buffer 1, which was then mixed with an equal volume of 2% low melting point agarose in 0.125 M EDTA (pH 7.6) and pipetted into PFGE plug moulds and allowed to solidify at room temperature. Plugs were left overnight (16–24 h), rocking, at 37 °C in 1 mL of previously prepared EC Buffer (0.1 M EDTA, 1. 0 M NaCl, 1% [w/v] N-Lauroylsarcosine sodium salt, 0.006 M Tris-HCl, pH 7.6) with 10 mg/mL Lysozyme and 20 Units/mL Mutanolysin. Subsequently, the EC buffer was aspirated and replaced with 1 mL of Buffer 2 (0.5 M EDTA, 1% (w/v) N-Lauroylsarcosine sodium salt, pH 8.0) with 0.5 mg/mL Proteinase K, and left overnight (stationary) at 55 °C. Plugs were washed twice in TE 10/1 Buffer (0.001 M EDTA, 0.01 Tris-HCl, pH 8.0) with 0.001 M phenylmethylsulphonyl fluoride (PMSF) for 1 h each at 37 °C (stationary) and were then stored in Eppendorf tubes containing 1 mL TE 10/100 Buffer (0.1 M EDTA, 0.01 Tris-HCl, pH 8.0) at 4 °C until required. Plugs were then cut into 1–2 mm slices and washed thrice in 1 mL TE 10/0.1 (0.0001 M EDTA, 0.01 Tris-HCl, pH 8.0) at room

temperature for 30 min each with gentle rocking. Slices were subsequently transferred to Eppendorf tubes containing 0.1 mL 1X Buffer Tango (Thermo Scientific) for minimum 30 min at 4 °C, followed by the addition of 0.4 μL of *Asc*I restriction enzyme (Thermo Scientific) and incubation continued for 24 h at 37 °C (stationary). 0.5 mL of 0.5 M EDTA was added to the slices to deactivate the enzyme, followed by loading of the slices into wells of a 200 mL 1% agarose Pulsed Field Certified™ (Bio-Rad) gel prepared with 0.5X dilution Tris-borate EDTA (TBE) buffer (55 g/L Boric Acid), 40 mL/L 0.5 M EDTA [pH 8.0], 108 g/L Tris). Gels were run using a CHEF-DR® II PFGE apparatus (Bio-Rad) in 2.3 L of the same 0.5X TBE Buffer using the following parameters: Initial Switch Time 1 Second, Final Switch Time 20 Seconds, 6 V/cm, for 16 h, at 14 °C. Resulting gels were stained with 10 mg/mL ethidium bromide (E1510 Merck) for 1 h, followed by 2 destaining washes in distilled water for 40 min each. Gels were photographed using an Alpha Imager® 3400 (Alpha Innotech Corp). Lambda PFG Ladder (New England BioLabs® Inc.) was loaded with every gel as a reference marker.

2.4. PFGE fingerprint profile analysis and dendrogram assembly

BioNumerics® 7.5 Software (Applied Maths) was used to analyse the PFGE images, as per the method described by Stefanovic et al. (2017). Dendrograms were assembled using the Unweighted Pair Group Method Using Average Linkage (UPGMA) distance matrix method and curve-based Pearson correlation. Profiles that had $\geq$ 95% similarity were grouped as the same strain (Shutt, Pounder, Page, Schaecher, & Woods, 2005).

2.5. 16S rRNA gene sequencing

Cultures were sent to GENEWIZ (Hope End, Takeley, Essex, CM22 6TA, United Kingdom) for DNA extraction, amplification and 16S rRNA gene sequencing. Isolated colonies previously grown on MRS agar for 48 h at 37 °C were transferred to cryotubes (Thermo Scientific) containing 1 mL MRS agar via an inoculation needle. Following 24 h growth at 37 °C, the cultures in the cryotubes were sent to GENEWIZ. Universal 16S rRNA primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (forward) and 5′-ACGGCTACCTTGTTACGACTT-3′ (reverse) were used to generate PCR products of approximately 1.4 KB, the DNA sequence of which were then obtained and sequenced following GENEWIZ DNA sequencing instructions. FASTA sequencing data was analysed using Lasergene 8 software (DNAStar Inc., Madison, WI), specifically the SeqMan and EditSeq tools, and resulting sequences were compared with pre-existing genomic data using the nucleotide basic local alignment search tool (BLASTn Suite) on the National Centre for Biotechnology Information (NCBI) server.

2.6. Bile salt hydrolase activity

A modified version of the method described by Pisano et al. (2014) was used for this analysis. NSLAB strains were grown overnight in each of (i) MRS broth, (ii) 0.3% (w/v) bile modified (m) MRS broth and (iii) 0.5% (w/v) mMRS broth. Modified MRS containing two levels of physiologically relevant bile concentrations were used in parallel with regular MRS to determine whether BSH activity could be induced (Ruiz, Margolles, & Sánchez, 2013). Following overnight incubation, BSH activity was screened for by loading 0.025 mL of each of the three variants of overnight NSLAB culture into wells (5 mm in diameter) bored in mMRS agar plates containing 0.03% (w/v) bile (Difco™ Oxgall, BD), 0.375 g/L $CaCl_2$ and 1% agar, in duplicate. The resulting bile plates were incubated for 72 h at 37 °C, anaerobically. The presence of opaque haloes of deconjugated bile surrounding wells identified strains expressing BSH enzyme. *Lactobacillus reuteri* NCBI 30242 cultures were used in parallel as a positive control due to their documented ability to produce BSH enzymes.

2.7. Antibiotic resistance profile

VetMIC™ plates (National Veterinary Institute of Sweden, Uppsala, Sweden) were used to determine the resistance/susceptibility of BSH-producing strains to antibiotics of importance as listed by the European Food Safety Authority in 2012: Ampicillin, Vancomycin, Gentamicin, Kanamycin, Streptomycin, Erythromycin, Clindamycin, Tetracycline and Chloramphenicol (Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, 2012). Fresh *Lactobacillus* cultures were streaked on MRS plates and incubated for 48 h at 37 °C, anaerobically. Colonies were then suspended in MRD, and a portion of this was transferred to 10 mL ISO-MRS broth (90% Iso-Sensitest broth [Thermo Scientific], 10% MRS broth) to achieve a final inoculum of $\approx 5x10^5$ cfu/mL. Wells of both VetMIC™ Lact-1 and Lact-2 plates were seeded with 0.1 mL from the ISO-MRS cultures, sealed with clear film (as provided with the VetMIC™ plates) and incubated anaerobically at 37 °C for 48 h. Growth of *Lactobacillus* cultures in the form of pellets at the bottom of the wells was examined using a backlight (colony counter) and the minimum inhibitory concentration (MIC) was recorded as the lowest concentration able to completely inhibit visible growth. Experiments were repeated 3 times.

2.8. Cell culture and establishment of co-culture

Caco-2 and mucus-producing HT29-MTX cells were grown in tandem and a co-culture was established as an intestinal model using the method reported by Yi *et al.*, with modifications (Li, Arranz, Guri, & Corredig, 2017). Caco-2 and HT29-MTX cultures were obtained from Istituto Zooprofilattico Sperimentale di Brescia (Italy) and Public Health England General Cell Collection (ECACC 12040401), respectively. Both cells lines were resuscitated from Liquid N_2 and maintained separately in Dulbecco's Modified Eagle's Medium (DMEM) medium (Merck D5796) supplemented with 10% Foetal Bovine Serum (FBS) (Merck F7524), 1% non-essential amino acids (NEAA) (M7145), 1% antibiotic solution (100 U/mL penicillin, 100 mg/mL streptomycin) (Merck P0781) and 2 mM L-glutamine (Merck 59202C) and were incubated at 37 °C in 5% CO_2 in a humidified atmosphere. Cells were sub-cultured into 75-cm^2 tissue culture flasks (Merck C7231) every 2–3 days, once a confluence of $\geq$ 80% was reached. For co-cultures, Caco-2 and HT29-MTX were inoculated at a ratio of 3:1 onto the apical membrane of the Corning® Transwell® Polyester Membrane Cell Culture Inserts (Merck CLS3460) with a total of $6x10^4$ cells per well. Co-cultures were allowed to reach confluence, differentiate and (in the case of the HT29-MTX cells) produce mucus for 21 days. Media was changed for both apical and basolateral layers every 2–3 days, and transepithelial electrical resistance (TEER) measurements were taken prior to media changes to monitor the integrity of the developing monolayer. One day prior to Bacterial Adhesion Assays and Pathogen Exclusion Assays, media in the Transwells was replaced with antibiotic-free, complete DMEM.

2.9. Adhesion assays

Mucus adhesion assays were carried out as per Morrin *et al*, Morrin, Lane, Marotta, Bode, Carrington, Irwin, & Hickey (2019) with modifications. Caco-2 cells were used between passages 41–53, while HT29-MTX cells were used between passages 66–79. *Lactobacillus* cultures were grown overnight in MRS broth and the O.D. 600 measurement was taken via a spectrophotometer. This reading was used to determine the necessary volume required for an O.D. 600 reading of 0.4 in 10 mL, followed by centrifugation of the appropriate volume per strain at 5000 rpm for 5 min. Cultures were resuspended in 10 mL unsupplemented DMEM and incubated anaerobically at 37 °C for 2 h, to reach a final O.D. of 0.5 ($\approx 1x10^8$ CFU/ml). Quantification of the exact CFU/mL per bacterial suspension was enumerated via serial dilutions and spread-plating onto MRS, followed by anaerobic incubation for 48 h at 37 °C. Media from apical and basolateral layers of Caco-2/HT29-MTX

co-culture wells were aspirated and two phosphate buffered saline (PBS) (Merck D8537) washes were performed, followed by complete aspiration of the used PBS in both cases. Subsequently, 0.5 mL ($\approx 5 \times 10^7$ CFU/ml) of each bacterial culture was added to separate co-culture wells (in triplicate) and were incubated for 2 h at 37 °C, anaerobically. Post incubation, wells were washed thrice with PBS for removal of loosely or non-adherent bacteria and 0.5 mL 0.1% Triton X100 (Merck T8787) was added to each well and left at room temperature for 15 min to allow for lysis of the eukaryotic cells. Serial dilutions of the lysates were prepared and plated via spread-plating on MRS. Following anaerobic incubation for 48 h at 37 °C, CFU/mL were enumerated and % adherence of each bacterial culture was determined using CFU/mL of the original suspensions calculated previously ([CFU/mL of adherent bacteria ÷ CFU/mL of total initial bacteria] × 100). Adhesion assays were performed in triplicate. *Lb. rhamnosus* GG (ATCC 53103) cultures were used in parallel as a positive control.

2.10. Pathogen exclusion assays

The ability of two NSLAB strains to inhibit binding of an enteric pathogen to cocultured cells was examined using an exclusion model. Pathogen Exclusion Assays were carried using the Adhesion Assay method (2.9 above), with minor modifications. Caco-2 cells were used between passages 39–43, while HT29-MTX cells were used between passages 61–66. Overnight *E. coli* cultures in BHI broth were adjusted to an O.D. 600 of 0.01 ($\approx 8x10^6$ CFU/mL), centrifuged at 10,000 rpm for 5 min and resuspended in DMEM. Quantification of the exact CFU/mL per bacterial suspension was enumerated by serial dilutions and spread-plating onto Eosin Methylene Blue (EMB) Agar (Oxoid 0069), followed by aerobic incubation for 24 h at 37 °C. NSLAB strains were incubated anaerobically at 37 °C with the co-cultures for 1 h before being washed twice with PBS (for removal of free or loosely bound *Lactobacillus*) followed by the addition of 0.5 mL ($\approx 4x10^6$ CFU/mL) of the *E. coli* suspension and another hour of incubation under the same conditions. The cells were lysed using the same procedure as before, with serial dilutions being plated on EMB agar for *E. coli* enumeration. Controls included wells with exclusively *Lactobacillus* or *E. coli* cultures that were incubated for 2 h. Percentage exclusion of *E. coli* was calculated by comparing the CFU/mL of *E. coli* that adhered to the co-culture when exclusively present in the wells versus when present in conjunction with the NSLAB strain (100 – {[CFU/mL of adherent *E. coli* in the presence of NSLAB ÷ CFU/mL of adherent *E. coli* exclusively] × 100}). Pathogen Exclusion Assays were performed in triplicate.

2.11. Exopolysaccharide production

Ruthenium red milk agar was used to determine whether *Lactobacillus* strains were capable of producing EPS, as per the method by Stefanovic and McAuliffe (2018). Freshly grown cultures were streaked on ruthenium red milk agar plates (10% reconstituted skim milk, 0.5% yeast extract, 0.08% ruthenium red [Sigma R2751], 1.5% agar), in triplicate, and incubated for 48 h at 37 °C, under anaerobic conditions. White colonies indicated an EPS-producing strain, while colonies that remained pink indicated a negative result. The *Lactobacillus paracasei* DPC1116 strain, an established EPS-producer, was used in parallel as a positive control. EPS production experiments were performed in triplicate.

2.12. Whole genome sequencing and annotation

Genomic DNA from *Lb. rhamnosus* DPC7102 and *Lb. paracasei /casei* DPC 7150 was isolated from overnight cultures grown in MRS broth at 37 °C using the DNeasy UltraClean Microbial Kit (Qiagen), as per the included protocol, and the quantity was measured using the NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific). Contig construction was provided by Microbes NG (http://www.microbesng.uk). Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol, with two modifications: 2 ng of DNA were used as input and PCR elongation time was set to 1 min. Quantification and library preparation of DNA was carried out on a Hamilton Microlab STAR automated liquid handling system. Kapa Biosystems Library Quantification Kit for Illumina was used for quantification of pooled libraries on a Roche light cycler 96 qPCR machine. The Illumina HiSeq was used to sequence genomic libraries using a 250 bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger, Lohse, & Usadel, 2014). *De novo* assembly was performed on samples using SPAdes version 3.7 (Bankevich et al., 2012). Contigs were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Haft et al., 2018). Average Nucleotide Identity (ANI) and OrthoANI values for both strains were calculated using the online tools from Kostas Lab and EZ BioCloud, respectively (Rodriguez-R & Konstantinidis, 2016; Yoon, MinHa, Lim, Kwon, & Chun, 2017). ResFinder 3.2, VirulenceFinder 2.0, and PathogenFinder 1.1 databases were used for *in silico* analysis of potential virulence and acquired antimicrobial resistance genes (Cosentino, Voldby Larsen, Møller Aarestrup, & Lund, 2013; Joensen, Scheutz, Lund, Hasman, Kaas, & Aarestrup, 2014; Zankari et al., 2012). Where necessary, *Lb. rhamnosus* GG (NC_013198.1) and *Lb. paracasei* ATCC 334 (NC_008526.1) were used as reference strains for *Lb. rhamnosus* 7102 and *Lb. paracasei* 7150, respectively.

2.13. Statistical analysis

Statistical analysis and graphs were generated using the IBM SPSS® software platform for Windows (Ver. 26). Adhesion results are expressed as a mean ± SD of the results of three independent assays conducted in triplicate. Tukey's Honest Significant Difference test was used to determine statistically significant differences between the control and test strains, where $p \leq 0.05$ was considered significant.

3. Results

3.1. Selection and identification of NSLAB demonstrating ability to survive SSDP

Screening of NSLAB from 12 Irish Cheddar cheeses was carried out based on the ability of the bacteria to survive SSDP which includes exposure to gastric pH levels and bile acids, the two primary tests identified by the FAO for the selection of strains with probiotic potential. NSLAB populations ranged from 2.26×10^5 to 1.04×10^8 CFU/g prior to SSDP. Exposure to SSDP resulted in population reductions in all cases with log reduction ranging from 0.03 to 2.84 being observed (Table 1).

In an effort to select a representative population of acid resistant, bile

Table 1
NSLAB population numbers in Cheddar cheese pre- and post- SSDP.

Cheese	Milk	NSLAB CFU/g		Log Reduction
		Average CFU/g		
		Pre SSDP	Post SSDP	
1	Raw	6.56E + 06	6.13E + 06	0.03
2	Raw	1.45E + 07	8.66E + 06	0.22
3	Pasteurized	9.10E + 06	5.22E + 06	0.24
4	Pasteurized	2.26E + 05	1.26E + 05	0.25
5	Pasteurized	1.40E + 08	1.19E + 07	1.08
6	Pasteurized	6.30E + 07	5.83E + 07	0.03
7	Pasteurized	6.61E + 07	1.60E + 05	2.62
8	Pasteurized	2.84E + 07	1.75E + 05	2.21
9	Pasteurized	4.23E + 07	7.30E + 04	2.74
10	Pasteurized	5.91E + 07	2.53E + 06	1.37
11	Pasteurized	1.08E + 07	1.55E + 04	2.84
12	Pasteurized	6.18E + 06	2.44E + 06	0.40

acid tolerant NSLAB twenty colonies were randomly selected and purified from the post SSDP MRS plates for each cheese resulting in 240 isolates. These were then subjected to a subtractive screening methodology (Pisano et al., 2014) the first step of which was the generation of PFGE profiles for each isolate in an effort to elucidate how many unique strains were present in each cheese. Dendrograms were generated, one for each cheese, and any isolates with a $\geq$ 95% similarity in the same cheese were grouped as a single strain and only one isolate from each cluster was picked for subsequent analysis. Using this approach, 76 unique strains were identified (Table S1). The number of individual strains identified in each Cheddar cheese varied considerably, with the Bionumerics software identifying up to 12 different bacterial strains in some cheeses. Conversely, Cheddar cheeses 5, 10 and 12 were found to only contain one strain each capable of surviving SSDP. A representative isolate from each strain cluster was selected for further study and each was assigned a unique Teagasc DPC culture collection number (7081–7156, inclusively). 16S rRNA gene sequence analysis identified the strains as constituting *Lb. paracasei/casei* (38 strains), *Lb. curvatus* (13 strains), *Lb. plantarum* (12 strains), *Lb. helveticus* (6 strains), *Lb. rhamnosus* (4 strains), *Lb. coryniformis* (2 strains) and *Streptococcus thermophilus* (1 strain). While a common NSLAB species was not isolated from all cheeses, *Lb. casei/paracasei* isolates were isolated from ten of the twelve cheeses. *Lb. plantarum* was the second most common species of NSLAB capable of surviving SSDP, with isolates being found in three of the twelve cheeses. *Lb. curvatus* and *Lb. helveticus* strains were found in two cheeses, while all the *Lb. coryniformis* and *Lb. rhamnosus* strains originated from cheese 8 and cheese 6, respectively (Table S1).

3.2. Bile salt hydrolase screening

BSH activity was detected in 30 of the 76 strains, although not all tested positive for activity under all three culture conditions (Table 2). All 30 BSH-positive strains produced BSH when incubated overnight in normal MRS, although the diameters of the haloes surrounding the wells differed. None of the *Lb. curvatus* or *Lb. helveticus* strains produced deconjugated bile salt haloes, and neither did the one *S. thermophilus* strain tested. In contrast, only 1 of the 12 *Lb. plantarum* strains was incapable of BSH production. All 11 of the BSH positive *Lb. plantarum* strains were capable of producing deconjugated bile haloes with diameters of $\geq$ 2.0 cm when previously incubated in normal MRS and the majority reproduced haloes of equal size or greater when previously inoculated in MRS broth containing bile (with the exceptions being 7094 and 7095). Of the 38 *Lb. casei/paracasei* strains tested, only 17 exhibited BSH activity. All BSH positive *Lb. casei/paracasei* strains were capable of producing haloes when previously incubated in standard MRS, but this activity was not always retained when previously grown in broth containing bile. A single strain each of *Lb. rhamnosus* (out of a possible 4 strains) and *Lb. coryniformis* (1 of 2 strains) also produced BSH activity, both of which showed consistent BSH activity regardless of which culture conditions were used.

3.3. Antibiotic resistance profiles

The 30 strains displaying BSH activity were tested for antibiotic sensitivity. Ten were found to be resistant to antibiotics at levels higher than those specified by the EFSA in 2012 (Table 3). All tested strains exhibited resistance to vancomycin (which is common among lactobacilli) at the highest concentration (128 μg/mL) provided in the assay; however, susceptibility levels to this antibiotic are not required by the EFSA and were, therefore, recorded as such (Table 3). The MICs of the other 20 *Lactobacillus* strains were always equal to or lower than the acceptable cut-off values and thus, these were taken forward to the next stage of the subtractive screening protocol. The most prominent resistance phenotype expressed in the resistant lactobacilli was the ability to grow in the presence of high concentrations of chloramphenicol, with 8 of the 10 isolates having MIC values greater than the EFSA cut-off values. Tetracycline resistance was observed in 5 resistant strains, while kanamycin resistance was observed in 3 strains (all of which belonged to the *Lb. casei/paracasei* species). In 6 cases, resistance to chloramphenicol and 1 other antibiotic was observed.

Table 2
BSH activity of selected NSLAB strains in absence of or post exposure to physiological levels of bile. + Deconjugated bile halo with diameter of <1.0 cm, ++ Deconjugated bile halo with diameter of $\geq$ 1.0 cm, +++ Deconjugated bile halo with diameter of 1.0–2.0 cm, ++++ Deconjugated bile halo with diameter of 2.0–3.0 cm, * *Lb. reuteri* NCIMB 30242.

Species	Strain	Bile Salt Hydrolase Activity		
		Incubation Conditions		
		MRS	MRS + 0.3% Bile	MRS + 0.5% Bile
Lb. casei/paracasei	7086	+++	+++	+++
	7087	++	+	-
	7091	++	–	–
	7100	++	++	++
	7104	++	+	++
	7110	++	++	++
	7111	++	++	++
	7143	++	+	++
	7145	+	+	++
	7149	++	++	+
	7150	++	–	–
	7151	++	–	–
	7152	+	–	++
	7153	++	++	–
	7154	++	–	–
	7155	++	–	–
	7156	++	–	–
Lb. plantarum	7081	+++	++++	+++
	7083	++++	++++	+++
	7094	+++	++	++
	7095	+++	+++	++
	7096	+++	+++	+++
	7097	+++	+++	+++
	7126	++++	+++	+++
	7132	++++	+++	+++
	7133	+++	+++	+++
	7134	++++	+++	+++
	7141	+++	++++	++++
Lb. rhamnosus	7102	++	++	++
Lb. coryniformis	7127	++++	+++	+++
Positive Control*	30242	++++	++++	++++

3.4. Lactobacillus adherence to a Mucus-Producing cell model

Seven of the twenty *Lactobacillus* strains that were sensitive to antibiotics were selected, and their ability to adhere to a mucus-producing Caco-2/HT29-MTX co-culture was assessed. The criteria used to select strains for screening were that at least one strain per species was selected and the number of strains chosen per species was based on their overall abundance during the original isolation. *Lb. rhamnosus* GG was used as a positive control due to its established role as a probiotic with excellent adherence to epithelial models (Segers & Lebeer, 2014). Following three independent replicates, *Lb. casei/paracasei* DPC7110, *Lb. casei/paracasei* DPC7149, *Lb. plantarum* DPC7126 and *Lb. plantarum* DPC7096 were found to have adherence values that were statistically significantly different ($p < 0.05$) from and less than the positive control (Fig. 1). The remaining *Lb. casei/paracasei* (7150, 7087) and *Lb. rhamnosus* (7102) strains' demonstrated adherence values that were not statistically significantly different from the positive control. While the control strain exhibited the highest adherence value (90.6 ± 8.2%), *Lb. rhamnosus* strain DPC7102 showed the highest adherence of the strains of interest, at 79.2 ± 8.1%. The other 2 *Lb. casei/paracasei* strains of interest that performed well were strain 7150 and strain 7087, which had adherence values of 64.0 ± 9.1% and 58.0 ± 11.5%, respectively. Adherence to the

Table 3
Antibiotic sensitivity profiles of selected NSLAB strains. * **Va**, Vancomycin; **Am**, Ampicillin; **Gm**, Gentamicin; **Km**, Kanamycin; **Sm**, Streptomycin; **Em**, Erythromycin; **Cl**, Clindamycin; **Tc**, Tetracycline; **Cm**, Chloramphenicol. [1] not required as per EFSA Guidelines. MIC (μg/mL) values in **bold** indicate presence of antibiotic resistance as per the EFSA Guidelines.

Species	Strain	Minimum Inhibitory Concentration (MIC) Values of Resistant Lactobacilli to Antibiotics of Interest								
		Antibiotic* (MIC as μg/mL)								
		Va	Am	Gm	Km	Sm	Em	Cl	Tc	Cm
Lb. casei/paracasei	7086	n.r.[1]	<2	<2	<64	<16	<0.25	<0.25	**<32**	**<16**
	7100	n.r.	<2	<2	<64	<16	<0.25	<0.25	<4	**<16**
	7104	n.r.	<1	<8	**<128**	<64	<0.06	<0.12	<2	<4
	7143	n.r.	<2	<4	**<128**	<16	<0.06	<0.12	<1	**<8**
	7145	n.r.	<2	<4	**<128**	<16	<0.06	<0.25	<1	**<8**
	7152	n.r.	<1	<4	<64	<8	<0.06	<0.12	<2	**<8**
Lb. plantarum	7081	n.r.	<2	<2	<64	n.r.	<0.25	<0.5	**>64**	**<16**
	7083	n.r.	<1	<2	<64	n.r.	<0.25	<0.5	**>64**	**<16**
	7141	n.r.	<1	<2	<32	n.r.	<0.25	<0.5	**>64**	<8
Lb. coryniformis	7127	n.r.	<0.5	<1	<16	<8	<0.12	<0.5	**<32**	**<8**

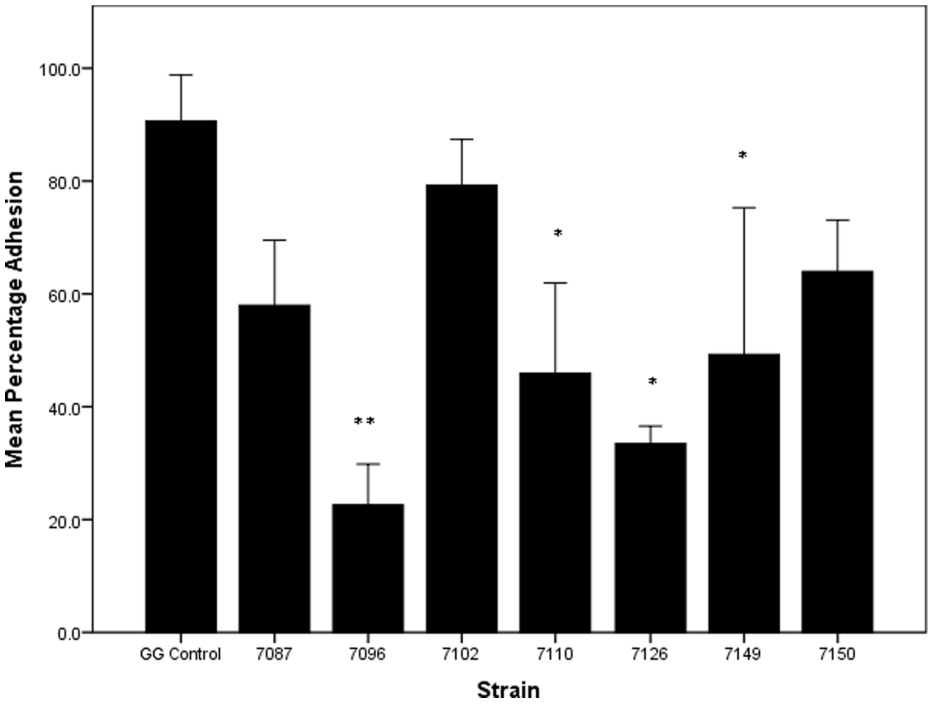

Fig. 1. Ability of *Lactobacillus* strains to adhere to Caco-2/HT29-MTX co-culture. *Lactobacillus rhamnosus* GG was used as a control. Results are expressed as the mean percentage of 3 independent experiments performed in triplicate, with error bars representing standard deviation. * Statistically significant differences occurred compared to the control (GG Control) strain ($p < 0.05$). ** Highly statistically significant differences occurred compared to the control (GG Control) strain ($p < 0.001$).

mucus-producing mammalian co-culture varied greatly among strains, often accompanied with high standard deviations.

3.5. Ability of Lactobacillus strains to inhibit Enteropathogenic E. Coli binding to Mucus-Producing cells

In order to assess further probiotic potential, the two *Lactobacillus* strains with the highest rates of adherence *in vitro*, representing two species of *Lactobacillus* namely *Lb. rhamnosus* DPC7102 and *Lb. casei/paracasei* DPC7150, were tested for their ability to inhibit the binding of a known enteropathogenic *E. coli* (EPEC) strain to mucus (Fig. 2). The experiment performed used an inhibition model whereby the lactobacilli were added to the Caco-2/HT29-MTX co-culture an hour prior to the addition of the pathogen, thereby examining the lactobacilli's ability to 'exclude' binding of the pathogen to the mucins and mammalian cells. *Lb. rhamnosus* DPC7102 was able to exclude 44.2 ± 9.6% of the total *E. coli* from adhering to the mucus co-culture, while *Lb. casei/paracasei* DPC7150 excluded 25.7 ± 2.1% of total *E. coli*.

3.6. Exopolysaccharide production by selected Lactobacillus strains

The ability of lactic acid bacteria (LAB) to produce EPS is seen as a benefit, from both a commercial and health point of view. Therefore, DPC7102 and DPC7150 were grown on skim milk plates containing ruthenium red agar, in parallel with a known EPS-producing positive control (DPC1116). Both strains of interest and positive control strain produced white colonies on the pink plates (Fig. 3), demonstrating that they each produce EPS when grown on skim milk.

3.7. Genome characteristics of Lb. Rhamnosus DPC7102 and Lb. Paracasei DPC 7150

Whole genome sequencing and annotation was undertaken on the two strains *Lb. rhamnosus* DPC7102 and *Lb. paracasei/casei* DPC 7150 in an effort to fully characterise the strains to species level and to determine if either contained antibiotic resistance, virulence or other genes associated with human pathogens. The data obtained (Table 4) demonstrated that both ANI and OrthoANI values were > 95%,

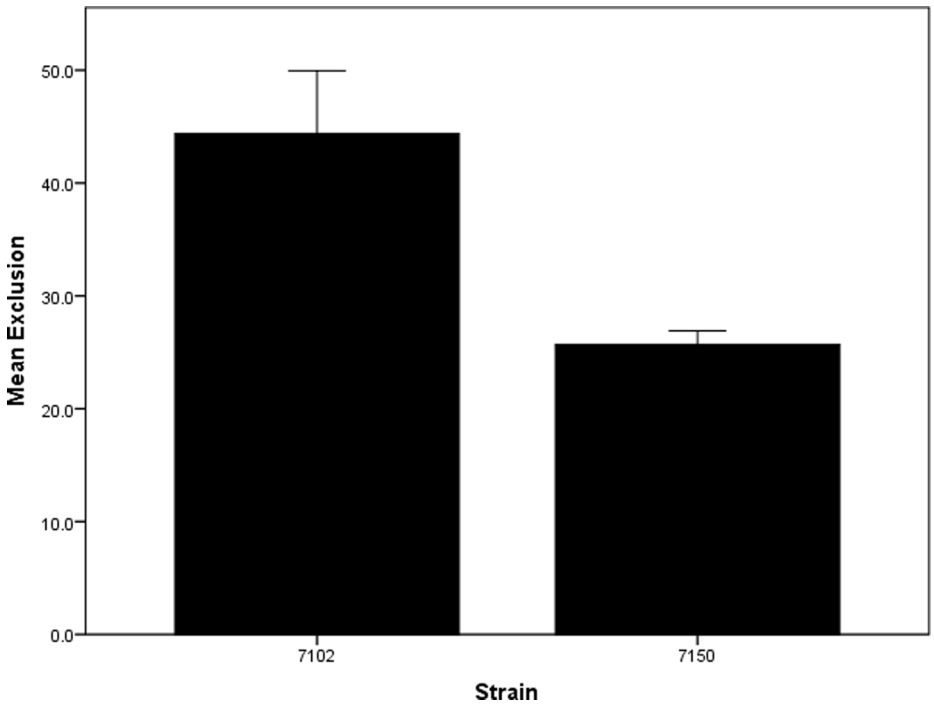

Fig. 2. Ability of *Lactobacillus* strains to inhibit the binding of Enteropathogenic *E. coli* 0111:H2 to Caco-2/HT29-MTX co-culture. Results are expressed as the mean percentage of 3 independent experiments performed in triplicate, with error bars representing standard deviation.

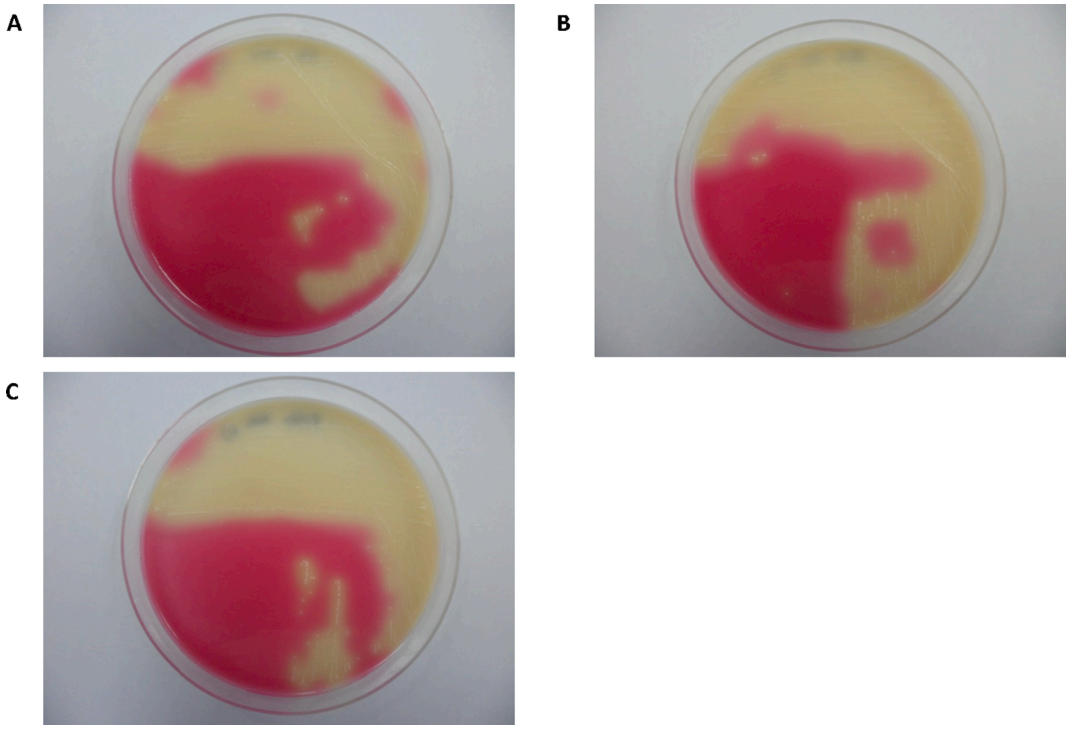

Fig. 3. Growth of white colonies on ruthenium red skim milk agar indicating EPS production. (A) Positive control *Lb. paracasei* DPC1116; (B) and (C) test strains *Lb. rhamnosus* DPC7102 and *Lb. paracasei* DPC 7150 respectively.

indicating that they are the same species as the reference strains used confirming that DPC715 is in fact a *Lb. paracasei* (Rodriguez-R & Konstantinidis, 2016). ResFinder 3.2 detected no acquired antibiotic resistance genes in either draft genome. The gene *RRS* (a 16 S ribosomal RNA gene) from *Mycobacterium tuberculosis* was the only exception in *Lb. rhamnosus* 7102, but the coverage was only ≈4% (with 60% being the minimum coverage threshold cutoff) (data not shown). VirulenceFinder 2.0 detected no genes associated with virulence in either genome (data not shown). PathogenFinder 1.1 predicted that neither organism is a likely human pathogen.

Table 4
General genomic features of *Lb rhamnosus* DPC7102 and *Lb. paracasei* DPC7150. *CDS, coding sequence (with protein).

	General Genomic Features of *Lactobacillus* Strains of Interest	
	Lb. rhamnosus DPC7102	*Lb. paracasei* DPC7150
BioSample Accession No.	SAMN12280510	SAMN12280511
No. of Contigs	131	280
GC Content (%)	46.61	46.17
Coverage (X)	149	44
No. of CDS*	2978	2894
ANI/OrthoANI	98.06/97.85	98.56/98.23
Probability of being Human Pathogen (%)	0.095	0.092

4. Discussion

Initially, samples of 12 Irish Cheddar cheeses were subjected to SSDP followed by plating for viable NSLAB on MRS agar. Survivors under SSDP conditions, which include exposure to gastric pH conditions followed by bile acids the two primary criteria identified by the FAO for the selection of strains with probiotic potential (Food and Agriculture Organization Of The United Nations, 2006) provided an indication of potential of NSLAB to transit through the upper intestinal tract and thus, their potential to colonise the host intestines. Apart from Cheddar cheese 4, the NSLAB populations were in excess of 10^6 CFU/g prior to SSDP, which is the accepted minimum level at which probiotics should be present in food, from which a portion can be consumed daily to administer the recommended 10^8-10^9 CFU (Kechagia et al., 2013). Following exposure to SSDP, log reductions of between 0.03 and 2.84 were observed in the NSLAB populations, with $> 10^6$ CFU/g being observed in 7 of the 12 cheeses tested. These data provide direct evidence for the first time to support the hypothesis that Cheddar cheese contains NSLAB populations of which a large proportion are capable of surviving simulated gastric digestion and thus will arrive in the small intestine in a viable state. The NSLAB in this study were extracted from the cheese prior to exposure to SSDP; however, it is expected that under normal conditions of cheese consumption the presence of the cheese matrix would provide additional protection, thus, enhancing NSLAB survival and transit to the gut (Gardiner, Stanton, Lynch, Collins, Fitzgerald, & Ross, 1999). Cheddar cheese may, therefore, serve as an efficient food vehicle for delivery of acceptable numbers of viable NSLAB to the gut where they may exert health benefits. The data presented here indicate that cheese contains beneficial bacteria at high levels, which can then potentially colonise the human intestines and exert further beneficial effects (Settanni & Moschetti, 2010). This is in agreement with the work of other groups who have also confirmed the feasibility of cheese as a competent microbe/probiotic delivery matrix (Gardiner et al., 1999; Gomes da Cruz, Alonso Buriti, Batista de Souza, Fonseca Faria, & Isay Saad, 2009; Karimi, Mortazavian, & Da Cruz, 2011; Sharp, McMahon, & Broadbent, 2008; Zhang et al., 2013).

Initially, 76 isolates that were resistant to simulated digestion and deemed to be unique strains were brought forward for further subtractive screening. The majority of these comprised members of the *Lb. casei/paracasei*, *Lb. curvatus* and *Lb. plantarum* species, but also included *Lb. helveticus*, *Lb. rhamnosus*, *Lb. coryniformis* and a single *S. thermophilus* strain. These species are commonly associated with the NSLAB component of Cheddar cheeses, although *Lb. helveticus* and *S. thermophilus* are often added intentionally as either starter LAB or adjunct cultures (Swearingen et al., 2001; Briggiler-Marcó, Capra, Quiberoni, Vinderola, Reinheimer, & Hynes, 2007; Fitzsimons, Cogan, Condon, & Beresford, 2001; Peterson & Marshall, 2010). These results are in agreement with Sumeri, Adamberg, Uusna, Sarand, and Paalme (2012) who also reported *Lb. casei/paracasei* strains being the most prominent members of the NSLAB population to survived simulated gastric transit; however, that study did not provide data on the overall proportion of the NSLAB population that survived this treatment or whether the surviving strains exhibited other probiotic characteristics. Additionally, many potential probiotics have previously been identified from the above species, although presence of beneficial probiotic traits have been cited as being heavily strain-dependant (Lebeer, Vanderleyden, & De Keersmaecker, 2008). The number and species of individual strains capable of surviving SSDP differed between Cheddar cheeses. While most cheeses were observed to contain multiple strains capable of surviving SSDP, single strains were detected in three cheeses while up to twelve strains were observed in the cheeses containing the most diverse populations. Cheese 7 contained the most variation in terms of species, including isolates of *Lb. casei/paracasei*, *Lb. curvatus*, *Lb. helveticus* and *S. thermophilus*. However, the majority of the cheeses (10 of the 12) contained strains from one or two species only. This is not unexpected as the cheeses used here had different sources, which will effect NSLAB content (Peterson & Marshall, 2010). High diversity of NSLAB can even be seen in cheeses manufactured in the same factory, as seen in a study in 2002 which determined that the number of NSLAB strains had high variability even when produced in the same milk vat during different time points, highlighting that NSLAB variability is very commonplace (Williams, Choi, & Banks, 2002).

The ability of *Lactobacillus* to produce BSH enzymes has been documented by different groups, with high strain variability being observed (Begley et al., 2006; O'Flaherty, Briner Crawley, Theriot, & Barrangou, 2018). Of the NSLAB selected by the initial screen, approximately 40% produced haloes of deconjugated bile when grown on MRS agar with 0.3% cow bile. Various groups have identified beneficial *Lb. plantarum* strains with BSH activity, with evidence suggesting that expression of these enzymes aids persistence and adherence in the intestine (Kumar, Grover, & Batish, 2011; Lambert, Bongers, De Vos, & Kleerebezem, 2008; Nguyen, Kang, & Lee, 2007; Patel, Singhania, Pandey, & Chincholkar, 2010; Yang, Liu, Zhou, Huang, Chen, & Huan, 2019). The haloes produced by all *Lb. plantarum* strains were the largest, along with the positive *Lb. reuteri* strain. Seventeen of the thirty strains that produced deconjugated bile haloes were *Lb. casei/paracasei* strains, although their performance varied depending on previous incubation conditions. While less data exists of BSH positive *Lb. casei/paracasei* strains, certain groups have identified those capable of bile deconjugation such as the *Lb. casei* K17 identified in 2016 and eight *Lb. paracasei* strains in 2006 capable of at least partial hydrolysis of bile salts (Maragkoudakis et al., 2006; Xu, Liu, Radji, Yang, & Chen, 2016). Similarly, a single *Lb. rhamnosus* and *Lb. coryniformis* strain displayed BSH activity. While the probiotic *Lb. rhamnosus* GG strain is capable of BSH production, to date, there is no published data on a *Lb. coryniformis* strain capable of BSH expression, although there is an entry on UniProt for a *Lb. coryniformis* BSH protein (https://www.uniprot.org/uniprot/A0A1P8FCL4).

Of the ten strains that failed the EFSA antibiotic susceptibility profiles, eight were resistant to Chloramphenicol. Genes primarily responsible for Chloramphenicol resistance are *cat* (chloramphenicol acetyltransferase) genes which, when present in *Lactobacillus*, have typically been located on plasmids (Gueimonde, Sánchez, de los Reyes-Gavilán, & Margolles, 2013). This result is surprising as *Lactobacillus* are not traditionally associated with chloramphenicol resistance; however, this can vary between strains and several resistant strains have been identified previously from foods (Guo et al., 2017; Mathur & Singh, 2005). In addition, six of the eight chloramphenicol-resistant strains also showed resistance to a second antibiotic, with four also showing growth in high concentrations of Tetracycline (the remaining two being capable of growth in Kanamycin). A paper published in 2019 examined 182 *Lactobacillus* isolates selected in such a way as to represent all lactobacilli species and found that 31% were resistant to both Chloramphenicol and Tetracycline (Campedelli et al., 2019). It is interesting to observe in both studies that chloramphenicol/tetracycline resistance was observed in some of the investigated species, as seen in this study for one *Lb. casei/*

paracasei, two *Lb. plantarum* and one *Lb. coryniformis* strain. Kanamycin resistance was observed in three different *Lb. casei/paracasei* strains, with two showing dual resistance with Chloramphenicol. *Lb. casei* strains with dual resistance to Kanamycin and Chloramphenicol were also observed in Campedelli et al. (2019), with 10 of the 15 strains tested exhibiting the same dual resistance. Generally, aminoglycoside antibiotics are less efficient against gram positive anaerobes due to membrane impermeability characteristics associated with this group of bacteria (Elkins & Mullis, 2004).

HT29-MTX cells produce both membrane-bound mucin proteins MUC1 and MUC3. While MUC1 is produced early on during differentiation (Day 7) at their apical borders, MUC3 levels peak later (Day 14), with levels remaining consistent in both thereafter (Lesuffleur et al., 1993; Mack, Ahrne, Hyde, Wei, & Hollingsworth, 2003) . More importantly, Days 7–14 of differentiation leads to a marked increase in expression of the MUC5 protein which is secreted and forms the protective gel/mucus layer of the intestinal tract and the initial foothold that allows potential adhesion of ingested microbes (Kleiveland, 2015; Santini et al., 2007) . As adherence varies greatly between strains, it was not surprising that the seven strains investigated here displayed adhesion levels that ranged from 20 to 80%. Bacterial adhesion is an important trait for a potential probiotic as high adherence allows for extended residence times in the gut, during which time any host-specific benefits can take effect. *Lb. rhamnosus* DPC 7102 had the highest adhesion at 79.2%, with the positive control *Lb. rhamnosus* GG showing 90.6% adhesion. Strain DPC 7102 was one of three strains that demonstrated high levels of adhesion that were not significantly different from that of the control strain, with the other two being *Lb. paracasei* DPC 7150 and *Lb. casei/paracasei* DPC 7087 which showed adherence values of 64.0 and 58.0, respectively. *Lb. rhamnosus* DPC 7102 and *Lb. paracasei* DPC 7150 showed the highest adherence values of the seven strains tested and were, therefore, brought forward for additional testing. The other four strains tested showed significantly different and reduced adherence values when compared to the positive control strain and, thus, were not considered for further testing.

Of the two strains tested for pathogen exclusion, *Lb. rhamnosus* DPC 7102 was more efficient at excluding *E. coli* than *Lb. paracasei* DPC 7150 (44.2% and 25.7% exclusion, respectively). These results support our observations in the adhesion assays which demonstrated that the *Lb. rhamnosus* strain was also capable of a higher level of adhesion, leaving less physical space and binding sites for pathogen adhesion once added, leading to greater exclusion of the pathogen. A member of the adhesive EPEC serotype was chosen for this assay. Once attached, they establish compact microcolonies which cause various morphological abnormalities (including destruction of intestinal microvilli and blunt enterocyte borders) and cause diarrhoea (Fagundes-Neto & Scaletsky, 2000). Therefore, the ability of the NSLAB strains to inhibit adhesion of this pathogen highlights their potential as probiotics to protect against similar infectious agents.

The ability of potential probiotic bacteria to produce EPS is desirable as EPS has associated commercial (as an emulsifying agent, improving mouthfeel and texture in dairy products) and health benefits (immunomodulation, inhibition of pathogens and cholesterol-lowering capabilities) (Liu et al., 2017). In this study, both *Lb. rhamnosus* DPC 7102 and *Lb. paracasei* DPC 7150 exhibited white colony growth when streaked onto Ruthenium Red Skim Milk agar, along with the positive control *Lb. casei/paracasei* DPC 1116. This indicates that they are EPS producers as the generation of either capsular EPS or secreted EPS will prevent staining of the cell wall by the ruthenium red agent (Mora et al., 2002).

In an effort to confirm the species information obtained by 16S rRNA gene sequence analysis and to facilitate a more in depth analysis of the two organisms at the genetic level, with particular reference to the presence of additional antibiotic resistance genes or genes associated with virulence or human pathogens, whole genome sequencing and annotation was undertaken. This confirmed the species designations for both isolates but also further classified DPC7150 as *Lb. paracasei.* No genes associated with virulence were detected in either genome and neither organism is predicted to be a likely human pathogen. These are important observations with regard to the potential future use of either of these two strains as probiotics.

5. Conclusion

Of the 76 individual strains of NSLAB with gastric tolerance isolated, 30 of them were also capable of BSH activity, which has been associated with cholesterol-lowering capabilities and indicates the potential health benefits of normal Cheddar cheese microbiomes. From these 30 BSH positive isolates, two *Lactobacillus* strains, DPC7102 and DPC 7150, were further demonstrated to display promising adhesion to and pathogen exclusion from a mammalian co-culture, are capable of EPS production, are also susceptible to antibiotics to an acceptable standard as set by the European Food Safety Authority and do not harbour any genes associated with virulence or human pathogenicity. These two strains are originally from Cheddar cheeses and could potentially be used as Adjunct Cultures in future cheese making. The added protection of the cheese matrix should ensure greater survival of lactobacilli cultures through the harsh gastric environment and into the small intestine, where the bacteria can then adhere and potentially exert their beneficial effects on the host.

6. Ethics statement

There are no Ethical issues/concerns relating to this work.

CRediT authorship contribution statement

N. Leeuwendaal: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft. **C. Stanton:** Conceptualization, Supervision, Writing - review & editing. **P.W. O'Toole:** Conceptualization, Supervision, Writing - review & editing. **T.P. Beresford:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104425.

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