Gut commensal bacteria show beneficial properties as wildlife probiotics

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Probiotics are noninvasive, environmentally friendly alternatives for reducing infectious diseases in wildlife species. Our aim in the present study was to evaluate the potential of gut commensals such as lactic acid bacteria (LAB) as wildlife probiotics. The LAB selected for our analyses were isolated from European badgers (Meles meles), a wildlife reservoir of bovine tuberculosis, and comprised four different genera: Enterococcus, Weissella, Pediococcus, and Lactobacillus. The enterococci displayed a phenotype and genotype that included the production of antibacterial peptides and stimulation of antiviral responses, as well as the presence of virulence and antibiotic resistance genes; Weissella showed antimycobacterial activity owing to their ability to produce lactate and ethanol; and lactobacilli and pediococci modulated proinflammatory phagocytic responses that associate with protection against pathogens, responses that coincide with the presence of immunomodulatory markers in their genomes. Although both lactobacilli and pediococci showed resistance to antibiotics, this was naturally acquired, and almost all isolates demonstrated a phylogenetic relationship with isolates from food and healthy animals. Our results show that LAB display probiotic benefits that depend on the genus, and that lactobacilli and pediococci are probably the most obvious candidates as probiotics against infectious diseases in wildlife because of their food-grade status and ability to modulate protective innate immune responses.

Keywords: lactic acid bacteria; probiotics; wildlife; genome; antimicrobials; immunomodulation

Introduction

Preventing the transmission of infectious diseases in a context where wildlife populations exist is challenging, as it comprises complex issues associated with economics and practicality, conservation ecology, and public perception.1,2 In Europe, monogastric mammals, such as wild boar (Sus scrofa) and badgers (Meles meles), are the greatest sylvatic reservoir of a considerable number of zoonotic infectious diseases, including bovine tuberculosis (bTB).3 Mycobacterium bovis, the causative agent of bTB, infects a wide range of domestic and wild mammals, as well as humans, with devastating consequences to wildlife diversity, global economy, and public health.4,5 In addition, the risk of bTB transmission increases when coinfections with viruses occur,6 and other zoonotic bacterial pathogens associated with gastrointestinal infections may also be spread.7 Current strategies to reduce the transmission of microbial pathogens between domestic and wild animals include biosecurity measures, culling, and vaccination;8 however, these control measures are limited in scope, longevity, practicality, and acceptability.9 In this respect, the use of beneficial microbes as probiotic therapy is emerging as a sustainable alternative in the field of wildlife infectious diseases.10

Lactic acid bacteria (LAB) are gut commensals that contribute to the maintenance of gut homeostasis through maintaining a beneficial microbial...
balance. On the one hand, these bacteria defend against colonization of opportunistic pathogens because of the production of antimicrobial compounds, such as organic acids, hydrogen peroxide (H₂O₂), ethanol, and bacteriocins.¹¹ On the other hand, the gut immune system responds to LAB via the interaction of microbe-associated molecular patterns (MAMPs) with pattern recognition receptors, such as Toll-like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which are expressed on immune and epithelial cells.¹² Upon MAMP recognition, pattern recognition receptors activate an intracellular signaling cascade converging on the proinflammatory transcription factor nuclear factor-κB (NF-κB), which is crucial in regulating immunological and antimicrobial responses in the intestine.¹³ In this context, the activation of interferon regulatory transcription factors has also been suggested to be critical in maintaining gut homeostasis.¹⁴ Some studies have reported that LAB activate the production of type I interferon (IFN-I) in immune cells via endosomes through TLR2 and TLR3.¹⁵,¹⁶

Recently, we have published that commensal LAB isolated from feces of badgers and wild boar have significant antimycobacterial activity, and influence the innate immunological interaction of macrophages with bacillus Calmette–Guerin (BCG),¹⁷,¹⁸ suggesting a role for gut-resident bacteria in the host response to mycobacterial infection. Furthermore, our results raise the interesting possibility that LAB with significant antimycobacterial activity may be able to reduce the gut burden of M. bovis and other pathogenic microbes. As direct contact between cattle and wildlife is difficult to observe,¹⁹ infectious fecal material from wild mammals is likely to be one of the principal routes of transmission to cattle via contamination of the environment or feed.²⁰ Therefore, use of LAB could be a safe way to reduce the risk of TB and other bacterial infections in wildlife since these bacteria have widely been used as probiotics in animals.²¹ Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host,”²² with some species, such as lactobacilli, considered to be safe. Nevertheless, a detailed in vitro characterization of LAB could help validate their use as wildlife probiotics before in vivo application is considered.

The aim of our study here was to evaluate the potential of individual LAB isolated from a bTB wildlife reservoir as safe probiotics for disease intervention. The selected LAB strains comprised four different genera, (1) Enterococcus, (2) Weissella, (3) Pediococcus, and (4) Lactobacillus, and their therapeutic potential was determined on the basis of functional properties that were predicted following in vitro immunobiological tests and comparative genomics. Both approaches targeted three bacterial markers (or profiles)—(1) safety, (2) antimicrobial activity, and (3) immunomodulation—to correlate and validate the predicted functional probiotic properties in the genomes and biological phenotypes of the LAB isolates. We monitored the safety of the LAB isolates using markers that included genes related to antimicrobial resistance (AMR) and virulence, tolerance to antibiotics, and phylogenetic proximity to food-grade LAB; we then evaluated the LAB on the basis of their antibacterial activity and the presence of bacteriocin gene clusters, and estimated their immunomodulatory potential for (1) the presence of genes associated with immune stimulation; (2) capability to activate NF-κB and IFN-I production in challenged macrophages; and (3) phagocytic intake by monocytes.

Materials and methods

LAB isolates

Twelve LAB strains previously isolated from badgers¹⁷ were selected to determine their potential as probiotics. These strains were identified by 16S rRNA sequencing and named as Enterococcus faecalis A7, E. faecalis C34, Lactobacillus reuterii D4, L. plantarum P5, Pediococcus pentosaceus B4, P. acidilactici E24, P. loli F7, P. acidilactici 132, P. acidilactici M17, Weissella cibaria A23, W. paramesenteroides A37, and W. paramesenteroides N43. All displayed extracellular activity against Mycobacterium smegmatis at a low pH and reduced the viability and survival rate of M. bovis BCG when competing together in the same broth for 48 hours.¹⁷ In the present study, further species analysis was carried out using the published Genbank genomes from E. faecalis, L. plantarum, L. reuterii, pediococci, and weissella with feature frequency profiling (FFP).²³ This technique allows both cross-species and cross-genus comparisons between genome sequences to confirm bacterial identification. The isolates previously identified as
P. lolii were confirmed to be P. acidilactici. Cultures from all LAB isolates were maintained in MRS broth/agar (Oxoid) after incubation at 37 °C with no aeration for 24 hours.

Genome sequencing, assembly, and annotation
Twelve LAB isolates were sent as pure isolated colonies on MRS agar plates to MicrobesNG, University of Birmingham, UK, where genome sequencing was carried out using the Illumina MiSeq platform. DNA was extracted using the EZNA® Bacterial DNA kit (Omega Bio-Tek, Norcross, GA), and library preparation was carried out with the 250 Nextera™ XT Library Prep Kit. The DNA from each isolate was sequenced using 2 × 250 bp paired-end reads and put through a standard analysis pipeline. The generated reads were first trimmed using Trimmomatic, and then subjected to quality assessment with in-house scripts. Genomes were assembled from the paired-end reads using Shovill version 1.0.4 (https://github.com/tseemann/shovill) with SPAdes 3.13.0 as assembly module, using default settings. Assembly quality was assessed by N50 and L50, using QUAST 4.5. Genome assemblies were annotated using Prokka 1.13. Sequencing reads, genome assemblies, and metadata have been uploaded to Genbank in BioProject PRJNA517196, with individual accession numbers for genome assemblies and reads provided in Table S1 (online only).

Identification of virulence and AMR genes
Genome sequences were searched for AMR genes with Abricate 0.9 (https://github.com/tseemann/abricate) using the NCBI Bacterial AMR Reference Gene Database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047) and the additional databases, Resfinder and Plasmidfinder. Furthermore, Abricate was used with the virulence factor database (VFDB) to identify putative virulence factors, such as proteinases, gelatinases, hyaluronidases, pathogenesis transcription factors, and biofilm formation proteins.

Antibiotic susceptibility tests
To quantify the susceptibility of the LAB isolates to antibiotics of importance in veterinary and human medicine, a microtiter plate assay (MPA) was used to determine minimum inhibitory concentrations (MIC). The MPA was carried out as previously described but with some modifications recommended by the Clinical and Laboratory Standards Institute. Serial twofold dilutions of selected antibiotics were tested against 2 × 10⁵ CFU/mL of the LAB isolates using Nunc-MicroWell™ 96-well plates (Thermo Fisher Scientific) that were incubated at 37 °C for 18 hours. Growth inhibition was measured at 620 nm with a DTX 880 Multimode Detector microplate reader (Beckman Coulter) to calculate the MIC, which was defined as the lowest concentration of the antibiotic able to inhibit bacterial growth completely. The calculated MICs were then compared with microbiological cutoff values defined by the European Food Standards Agency (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) panel to distinguish resistant from susceptible strains. In this respect, a bacterial strain is defined as susceptible (or resistant) when it is inhibited at a concentration of a specific antibiotic equal or lower (or higher) than the established cutoff value. If the bacterial strain is defined as resistant, a further demonstration of intrinsic resistance is needed to carry out to confirm or rule out the presence of AMR genes. Antibiotics selected for the MPA assay were provided by Apollo Scientific (UK), including gentamycin, kanamycin, streptomycin, ampicillin, chloramphenicol, vancomycin, erythromycin, and tetracycline (as suggested by FEEDAP). Susceptibility to the β-lactams amoxicillin and cephalaxin was determined using Oxoid™ Antimicrobial Susceptibility Disks at a concentration of 30 micrograms.

Comparative genomics to generate phylogenetic trees
Available genomes of Weissella spp., P. acidilactici, P. pentosaceus, L. plantarum, L. reuteri, and E. faecalis and their metadata were downloaded from the NCBI Genome database using ncbi-genome-download version 0.2.9 (https://github.com/kblin/ncbi-genome-download). Owing to the large number of genomes available for L. plantarum, L. reuteri, and E. faecalis, these were first compared with corresponding badger LAB species using purine-pyrimidine FFP (FFPry) and a word length of 15. The resulting genome clusters were then selected for further analysis, together with genomes obtained from LAB used as probiotics and/or found in food and/or used in fermented
food or isolated from healthy individuals. These criteria were also used for isolates of pediococci and weissella. Selected genome sequences were compared with the genomes of the badger LAB using core genome single nucleotide polymorphisms, as identified by ParSNP version 1.2, with settings described previously. Phylogenetic trees were annotated and visualized using Figtree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

**Identification of genes associated with the production of bacteriocins and antimicrobial metabolites**

Genome sequences were searched for gene clusters involved in the biosynthesis of bacteriocins (antimicrobial peptides) using BAGEL3. This software allows for rapid and reliable identification of all classes of bacteriocin clusters, which are usually composed of genes encoding the bacteriocin precursor (prebacteriocin), proteins involved in the transport and processing of the prebacteriocin as an active bacteriocin, posttranslational modification enzymes, and immunity proteins. Furthermore, genome annotations were used to confirm the presence or absence of fructose-6-phosphate aldolase (fsaA) and phosphoketolase, two enzymes that are involved in the two main glycolytic pathways in LAB, the Embden–Meyerhof pathway (EMP) and the phosphoketolase pathway (PKP), respectively. In general, homofermentative LAB convert carbohydrates into lactate using EMP, whereas heterofermentative LAB produce lactate, ethanol, and carbon dioxide as antimicrobial metabolites via the PKP. Genome annotations were also used to identify genes associated with H$_2$O$_2$ production, including genes that encode pyruvate oxidase (box), lactate oxidase (lox), and NADH oxidases. Genes encoding NADH peroxidases were also included in the search for H$_2$O$_2$ production markers, as H$_2$O$_2$ may accumulate in species that lack this H$_2$O$_2$-scavenging enzyme.

**Antimicrobial properties**

The antimicrobial activity of the LAB isolates was phenotypically characterized using the spot-on-agar test as previously described. Cultures from each of the LAB isolates were spotted as 5 μL aliquots onto agar plates seeded with 10$^8$ CFU/mL of an indicator strain. Spotted plates were then incubated under optimal growth conditions for the indicator strain to evaluate the presence or absence of bacterial inhibition zones. All the bacterial strains employed as indicators and controls and their corresponding growth conditions are shown in Table 1. The controls included the following LAB strains: *L. plantarum* WCFS-1 (a strong acidifier), *P. acidilactici* PA1.0 (a pediocin producer), *Lactococcus lactis* NZ9700 (a nisin producer), and *L. lactis* NZ9800 (negative control of NZ9700).

**Identification of genes associated with immunomodulation**

As indicated above for the virulence factors, Abri- cate was used with the VFDB to identify bacterial markers associated with host immune responses, including proteins involved in mechanisms of evasion, adhesion, and colonization. A search on the genome annotations was also carried out to detect TLR agonists previously described for LAB, including exopolysaccharides (EPS), pili (fimbrial) precursors, lipoteichoic acid (LTA), wall teichoic acid (WTA), and membrane lipoproteins. All these membrane compounds are capable to activate phagocytes via TLR2 and/or TLR5.

**Immune responses in macrophages**

To determine the potential immunomodulatory properties of the LAB isolates, we used macrophages from THP-1 Lucia™ NF-κB (Invivogen) and pIFIT1-GluC THP-1, two different human monocyte cell lines that secrete Gaussia luciferase under the control of promoters NF-κB and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), respectively. Both monocyte cell lines were grown in the Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) supplemented with 15% fetal calf serum (FCS, Seralab) and 1% penicillin/streptomycin (Pen/Strep, Life Technologies) at 37°C in an atmosphere of 5% CO$_2$. Monocytes were differentiated into macrophages by seeding 5 × 10$^4$ monocytes per well in 96-well plates containing RPMI supplemented with phorbol 12-myristate 13-acetate (PMA) at 20 nanograms per milliliter. PMA (Santa Cruz Biotechnology) was dissolved in DMSO at 10 milligrams per milliliter. After 48 h, medium was replaced for RPMI containing 2% FCS and macrophages exposed to 12 LAB isolates for 2 h at a ratio of 20 viable bacteria per macrophage. This ratio is sufficient to activate a significant response of NF-κB and IFIT1 in THP-1 macrophages challenged with the intracellular pathogen *Salmonella enterica* (data not shown).
Table 1. Reference bacterial strains used as indicators and controls in the spot-on-agar test experiments

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Growth conditions</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium smegmatis mc2 155</td>
<td>Fast-growing mycobacterium for rapid antimycobacterial screening, sensitive to nisin, pediocin, and low pH</td>
<td>37 °C on TSB/TSA with aeration</td>
<td>Snapper et al.¹⁰⁰</td>
</tr>
<tr>
<td>Micrococcus luteus ATCC4698</td>
<td>Indicator strain, sensitive to posttranslationally modified bacteriocins (e.g., nisin)</td>
<td>37 °C on TSB/TSA with aeration</td>
<td>American Type Culture Collection (ATCC)</td>
</tr>
<tr>
<td>Listeria innocua ATCC4698</td>
<td>Indicator strain, sensitive to nonposttranslationally modified bacteriocins (e.g., pediocin)</td>
<td>37 °C on TSB/TSA</td>
<td>ATCC</td>
</tr>
<tr>
<td>Streptococcus thermophilus DN-001116</td>
<td>Indicator strain, tolerant to nisin and pediocin</td>
<td>37 °C on M17</td>
<td>Isolated from a Danone Yoghurt</td>
</tr>
<tr>
<td>Lactobacillus sakei Lb790</td>
<td>Indicator LAB strain</td>
<td>30 °C on MRS</td>
<td>Schillinger and Lucke¹⁰¹</td>
</tr>
<tr>
<td>Lactococcus lactis MG1614</td>
<td>Indicator LAB strain</td>
<td>30 °C on MRS</td>
<td>Gasson¹⁰²</td>
</tr>
<tr>
<td>Pediococcus pentosaceus ATCC 33216</td>
<td>Indicator LAB strain</td>
<td>30 °C on MRS</td>
<td>ATCC</td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 33186</td>
<td>Indicator LAB strain</td>
<td>30 °C on MRS</td>
<td>ATCC</td>
</tr>
<tr>
<td>Lactococcus lactis NZ9700</td>
<td>Nisin producer</td>
<td>30 °C on MRS</td>
<td>Kuipers et al.⁴³</td>
</tr>
<tr>
<td>Lactococcus lactis NZ9700</td>
<td>Non-nisin producer, moderate acidifier</td>
<td>30 °C on MRS</td>
<td>Kuipers et al.⁴³</td>
</tr>
<tr>
<td>Pediococcus acidilactici PAC1.0</td>
<td>Pediocin producer</td>
<td>30 °C on MRS</td>
<td>Gonzalez and Kunka⁴²</td>
</tr>
<tr>
<td>Lactobacillus plantarum WCFS-1</td>
<td>Strong acidifier</td>
<td>30 °C on MRS</td>
<td>Kleerebezem et al.⁴¹</td>
</tr>
</tbody>
</table>

Medium was then replaced with RPMI containing 2% FCS and 1% Pen/Strep and macrophages incubated for 20 more hours to monitor the activation of NF-κB or IFIT1. Supernatants were transferred to white-bottom 96-well plates and luciferase activity was measured in the presence of 2 μg/mL of coelenterazine (NanoLigh Technology) using CLARIOstar® plate reader (BMG Biotech). Activation was calculated as a fold increase over the measurements recorded for unchallenged macrophages.

**Phagocytosis with human blood cells**

Whole fresh blood was collected into heparinized tubes from healthy people and diluted in equal volumes of Opti-MEM media and PBS supplemented with 10 mM EDTA (EDTA-PBS). After reaching 37 °C, the blood cells were challenged with PKH2-labeled LAB at a multiplicity of infection of 100 bacteria per one blood cell. The LAB were labeled using PHK2 dye at 2 × 10⁻⁶ M in a solution that was exposed to human serum to facilitate opsonization. Blood cells and bacteria were then incubated in an orbital shaker at 37 °C for 30 min and subsequently lysed with 1 × RBC lysis solution (Biolegend) following incubation at room temperature for 15 minutes. The cells were washed twice with EDTA-PBS, resuspended in PBS, and acquired on the flow cytometer BD FACS Celesta. FACS based on forward (FSC) and side (SSC) scatter was used to distinguish the main blood cell populations on the basis of their size and granularity (lymphocytes versus phagocytes), while the FITC channel allowed measurement of the green fluorescent protein (GFP) levels in blood cells that were bound (e.g., lymphocytes) and/or phagocytized (e.g., phagocytes, including monocytes and polymorphonuclear neutrophils (PMNs)). The resulting SCC/GFP plot was then used to quantify the LAB intake by phagocytes as recently described by our group.¹⁸
**Statistical analysis**
Statistical analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com). Data are mean ± SD of three biological replicates, unless indicated. Differences between isolates were analyzed by a one-way ANOVA followed by Fisher's least significant difference test.

**Results**

**Pediococci show tolerance to antibiotics; enterococci contain genes associated with virulence and AMR**

The following 12 LAB isolates were selected to evaluate their potential as wildlife probiotics: *E. faecalis* A7; *E. faecalis* C34; *L. reuterii* D4; *L. plantarum* P5; *P. pentosaceus* B4; *P. acidilactici* E24; *P. acidilactici* F7; *P. acidilactici* I32; *P. acidilactici* M16; *W. cibaria* A23; *W. paramesenteroides* A37; and *W. paramesenteroides* N43. As illustrated in Figure 1, we first assessed how safe they are on the basis of the identification of genes associated with virulence and AMR, and their tolerance to different classes of antibiotics. The genome sequences of the isolates were obtained and searched for putative virulence factors and AMR genes, while antibiotic tolerance was determined using the MIC test and antimicrobial susceptibility disks. Only the two *Enterococcus* isolates were found to carry genes encoding virulence factors, including a serine proteinase (*sprE*), a gelatinase (*gelE*), a virulence system regulator (*fsrABC*), two hyaluronidases (*EF0818* and *EF3023*), and a biofilm regulator (*bopD*) (Fig. 1A and Table S2, online only). The two enterococci were also the only isolates with AMR genes, including a dihydrofolate reductase (*dfrE*) and three efflux pumps (*emeA*, *lsaA*, and *efrAB*). However, none of the above genes were predicted to be transferable, as they are present in the chromosomal DNA. With regard to antibiotic susceptibility, all LAB isolates were susceptible to amoxicillin, ampicillin, and chloramphenicol (Fig. 1B and Table S3, online only). By contrast, all were resistant to at least one of the
aminoglycosides tested (gentamycin, kanamycin, and streptomycin); and lactobacilli, pediococci, and weissella showed resistance to vancomycin. The Pediococcus isolates were also resistant to cephalaxin, and a few of them displayed resistance to erythromycin and tetracycline, to which enterococci, lactobacilli, and weissella were also sensitive.

Most LAB display phylogenetic relationships with isolates recovered from food and healthy animals and humans

The genomes of the 12 LAB isolates were compared using core genome SNPs with available LAB genomes to assess their phylogenetic relationships. As illustrated in Figure 2, the genomes of the E. faecalis strains (A7 and C34) clustered into two
different major groups that are significantly distant from the cluster comprising most of the isolates of food origin (higher up the tree). Although both strains showed similarities with the genomes of some food isolates, they were grouped in clusters mainly composed of isolates recovered from animals and humans. By contrast, isolates of lactobacilli, pediococci, and weissella were grouped in closer proximity to food isolates (Figs. 3–6). The *L. plantarum* isolate (P5) was placed very close to isolates derived from fermented food (Fig. 3), as observed with the pediococci isolates (Fig. 5), while the genomes of *L. reuteri* and *W. cibaria* clustered with the genomes of isolates that were recovered not only from foods but also from animals and humans (Figs. 4 and 6). With regard to the
W. paramesenteroides isolates, the very low number of available published genomes allowed for no interpretation.

Weissella show antimycobacterial activity, while enterococci display a phenotype and genotype associated with the production of posttranslationally modified bacteriocins. The antimicrobial characterization of the LAB isolates was first carried out by the spot-on-agar test using different indicator strains, as illustrated in Figure 7A. This cell-to-cell antimicrobial test proved more informative than other tests, such as the agar-well diffusion test. Cell-free supernatants from monocultures showed no differential antibacterial activity among the isolates (data not shown). Simultaneously, genome annotations allowed us to predict the production of potential antimicrobial metabolites (e.g., organic acids, ethanol, and H2O2) to correlate with the observed activity.
antibacterial activity. None of the LAB badger isolates showed antimicrobial activity against other LAB isolates listed in Table 1 as indicator strains. On the contrary, all of them disrupted the growth of *M. smegmatis* (Fig. 7A). In particular, the three *Weissella* isolates displayed a much more prominent antimycobacterial activity when compared with the remainder of the isolates. In this respect, genome
annotations showed that all our isolates harbor genes associated with $\text{H}_2\text{O}_2$ production, such as pox, lox, and/or NADH oxidases, as well as genes that encode NADH peroxidases. However, these annotations suggested that the isolates differ in sugar metabolism and subsequently in the formation of antimicrobial metabolites. The genomes of lactobacilli and pediococci encode fructose-6-phosphate aldolase ($fsa$) and phosphoketolase, while the enterococci isolates encode just $fsa$, demonstrating their role as facultative heterofermenters and obligate homofermenters, respectively. By contrast, the *Weissella* isolates are obligate heterofermenters, as they only contain the phosphoketolase gene. In other words, isolates of lactobacilli, pediococci, and enterococci mainly produce lactate, whereas the *Weissella* isolates generate equal levels of lactate and ethanol.

Figure 6. The phylogenetic tree of isolates of *W. cibaria* ($n = 16$) and *W. paramesenteroides* ($n = 3$) recovered from food, animal and environmental sources, humans, and badgers using parSNP analysis. Colored markers indicate the source of each isolate, with more details added to individual names.
With respect to other indicator strains, the two enterococci isolates displayed a strong antimicrobial activity against *Micrococcus luteus*, which is an indicator strain susceptible to posttranslationally modified bacteriocins (e.g., nisin) (Fig. 7A). Neither of these two isolates showed antimicrobial activity against *Listeria innocua*, which is an indicator strain sensitive to unmodified bacteriocins (e.g., pediocin). Furthermore, genome analysis with BAGEL3 revealed that both enterococci carry clusters involved in the hypothetical synthesis of two posttranslationally modified bacteriocins: a sactipeptide in *E. faecalis A7* and a lassopeptide in *E. faecalis C34* (Fig. 7B). A more detailed description of the genes present in the identified clusters is provided in Table S4 (online only). Interestingly, *E. faecalis C34* also displayed antimicrobial activity against a *Streptococcus thermophilus* strain that shows tolerance to bacteriocins, including nisin and pediocin (Fig. 7B).

In addition, BAGEL3 genome analysis identified clusters involved in the hypothetical synthesis of three unmodified bacteriocins: a class Ile bacteriocin in *P. pentosaceus* B4; an S-type pyocin in *Pediococcus acidilactici* I32; and a plantaricin in *L. plantarum* P5 (Fig. 7B and Table S5, online only). However, none of these isolates showed antimicrobial activity against *M. luteus*, *L. innocua*, or *S. thermophilus*.

**LAB isolates contain genes associated with TLR activation and immunomodulation**

To determine the potential of the badger LAB isolates as immunomodulators, we conducted a genome search using annotations and the
Figure 8. LAB contain genes associated with TLR activation and immunomodulation. (A) Genes in the LAB isolates associated with TLR activation are indicated in blue, green, and pink, which correspond to fimbria/pili, cell wall components, and TLR2 agonists, respectively. (B) Genes in the LAB isolates associated with immunomodulation are indicated in orange, yellow, and purple, which correspond to evasion, adhesion, and colonization, respectively.

VFDB.\textsuperscript{30} Genome annotations helped with the detection of TLR agonists, such as fimbrial precursors, LTA, WTA, EPS, and membrane lipoproteins (Fig. 8A); while the VFDB enabled us to identify markers associated with host immune responses, including genes encoding proteins related to evasion, adhesion, and colonization (Fig. 8B). The search of genome annotations showed that all the isolates share genes associated with the biosynthesis of LTA (\textit{ltsA}) and WTA (\textit{tagGH}, D-Ala). Many isolates also carry genes that code for proteins involved in pili formation, in particular the isolates of enterococci, which harbor genes \textit{ebpABC} and \textit{srtC}, and of pediococci and weisella, where genes \textit{comC} and \textit{outO} were identified. Interestingly, we detected only genes associated with the biosynthesis of fimbria in three isolates: \textit{L. plantarum} P5, \textit{P. pentosaceus} B4, and \textit{P. acidilactici} I32. Furthermore, TLR2 agonists were detected in \textit{L. reuteri} D4 (EPS) and the two pediococci isolates E24 and M16, which share a gene encoding an outer membrane lipoprotein (\textit{lolE}). With regard to the VFDB-based immunomodulatory markers, we found that the enterococci, \textit{L. plantarum} P5, and \textit{P. pentosaceus} B4 contain a significant number of genes encoding proteins associated with evasion, adhesion (\textit{ace} and \textit{fss1/2} in enterococci, \textit{cma} in P5, and \textit{sraP} in P5 and B4). With the exception of lactobacilli, we also detected a considerable number of genes related to capsule formation (\textit{cpsAB} in enterococci and \textit{cap8A/cps12A} in nearly all pediococci and weisella). Additionally, genes that code for proteins involved in colonization via survival and cellular aggregation were observed in enterococci (\textit{efaA}, \textit{prgB/asc10}, and \textit{EF0485}) and \textit{P. acidilactici} E24 (\textit{stkP}, \textit{stpI}, and \textit{steT}).

\textbf{LAB isolates stimulate phagocytes in a species-dependent manner}

As a complementary screening on the potential immunomodulatory properties of the LAB isolates, we proceeded to monitor their interaction with phagocytes by recording innate immune responses associated with NF-κB/IFN activation and phagocytosis (Fig. 9). To study the effect of LAB on the activation of NF-κB and IFN, we employed pNF-κB-GLuc and pIFIT1-GLuc THP-1 cells, two monocytelines that secrete Gaussia luciferase (GLuc) under the control of promoters NF-κB and IFIT1 (a well-characterized IFN-stimulated gene), respectively (Fig. 9A and B). The phagocytic response to the LAB isolates was determined by FACS analysis using human blood cells and PKH2-labeled bacterial cells (Fig. 9C). This analysis allowed us to quantify the percentage of PMNs (e.g., neutrophils) and monocytes that bind to and/or phagocytose LAB. We first found that exposure to \textit{L. reuteri} D4 and the two \textit{P. acidilactici} isolates E24 and F7 triggered NF-κB activation that was significantly higher than that observed with the other isolates (Fig. 9A). Furthermore, a significant increase in IFN activity was recorded upon exposure to viable cells of \textit{L. plantarum} P5,
Beneficial bacteria for wildlife mammals

Figure 9. Badger LAB isolates stimulate phagocytes in a species- and strain-dependent manner. (A) Response of phagocytes to the LAB isolates as measured by NF-κB activation. The activation of NF-κB is presented as a fold increase over a nonstimulated condition using THP-1 macrophages exposed to viable LAB. (B) Response of phagocytes to the LAB isolates as a measurement of IFN activation. The activation of IFN is presented as a fold increase over a nonstimulated condition using THP-1 macrophages exposed to viable LAB. (C) Response of phagocytes to the LAB isolates as a measurement of phagocytic uptake. The phagocytic uptake is shown as a percentage of primary PMNs (gray bars) and monocytes (orange bars) that are positive for LAB isolates labeled with PKH2. For all experiments, phagocytes were exposed to viable LAB cells at a ratio of 20 bacteria per one phagocyte. Comparative statistical analysis was carried out between isolates by a one-way ANOVA followed by a Fisher’s least significant difference (LSD) test (\(P < 0.05\), \(**P < 0.01\), and \(****P < 0.001\)). Data are mean ± SD of three biological replicates.

P. pentosaceus B4, and the two E. faecalis isolates A7 and C34 (Fig. 9B). We observed that neutrophils and monocytes efficiently bind and phagocytose LAB but only those isolates that activate NF-κB or IFN in THP-1 macrophages (Fig. 9C). In this respect, monocytes were much more responsive than neutrophils.

Discussion

LAB are widely used as probiotics owing to their beneficial effects on human and animal health.\(^{52,53}\) In the food and feed industry, probiotic LAB strains are normally selected using well-established, classical screening methods on the basis of criteria that relate to phenotypic traits and functional properties, such as safety, antimicrobial activity, and host colonization.\(^{54}\) In this context, comparative genome analysis using next-generation sequencing technologies has brought new insights into the probiotic characterization of LAB, including a much better understanding of their interaction with immune cells.\(^{55}\) However, the number of studies using a functional genomic approach that comprehensively correlates the traditional phenotypic features of LAB with genome findings is limited.\(^{56,57}\) In this study, we have combined biological responses and genome sequencing from LAB to determine their potential as functional probiotics in wildlife.

Wildlife mammals are reservoirs for economically significant diseases affecting farm animals, with subsequent potential implications for pathogen transmission between animals and humans.\(^{5,58}\) We started by evaluating the safety of LAB isolates that were recovered from European badgers, the most significant wildlife reservoir of bTB in the UK.\(^{3}\)

Among all the selected LAB, only the enterococci isolates carry genes associated with virulence and AMR. The virulence factors that we have detected include proteinases, gelatinases, and hyaluronidases that have extensively been described in previous studies,\(^{59–61}\) as well as virulence regulators, such as fsrABC and bopD, that seem to play an important role in pathogenesis.\(^{62,63}\) With regard to AMR genes, we have identified several that are chromosomally located and nontransferable, and associated with resistance to trimethoprim (dfrE), fluoroquinolones (emeA and efrAB), and clindamycin (lsaA) in E. faecalis.\(^{64,65}\) However, the enterococci isolates were sensitive to nearly all the antibiotics tested in our study, including β-lactams chloramphenicol, vancomycin, erythromycin, and tetracycline. And with the exception of vancomycin, isolates of lactobacilli and Weissella were also susceptible to these antibiotics. In this respect, it is well known that many strains of Lactobacillus and Weissella are inherently resistant to vancomycin,\(^{44,66,67}\) a phenotype that has
also been observed in pediococci.\textsuperscript{68,69} In fact, our isolates of pediococci were naturally tolerant not only to vancomycin, but also to cephalaxin, erythromycin, and tetracycline, as previously reported.\textsuperscript{70,71} The only antibiotic that proved to be effective against the \textit{Pediococcus} strains was chloramphenicol. All LAB isolates were resistant to at least one of the aminoglycosides tested. Very recent publications have reported the emerging resistance of LAB to gentamycin, kanamycin, and streptomycin.\textsuperscript{72}

Although LAB, such as lactobacilli and pediococci, are generally considered as safe, their antibiotic susceptibility should be well assessed before their application as probiotics.\textsuperscript{73} Our data demonstrate that some LAB isolates, especially strains of \textit{Pediococcus}, show significant antibiotic resistance. However, most of the selected LAB strains have been identified as inherently resistant to antibiotics and, for all these cases, EFSA clearly state that further investigations are needed to correlate the observed antibiotic tolerance with the genetic basis of that particular resistance.\textsuperscript{74} The fact that the lactobacilli and pediococci isolates harbor no AMR genes should thus not jeopardize their potential use as probiotics. When resistance to an antibiotic is intrinsic (natural) to certain bacterial species that are intended to be used as probiotics, the FEEDAP consider them as acceptable.\textsuperscript{75} Furthermore, the genomes of lactobacilli, pediococci, and \textit{Weissella} possess significant similarities with those of food isolates. By contrast, the enterococci isolates have shown genetic profiles associated with virulence and AMR, and their genomes have been grouped in clusters that mainly comprise isolates recovered from animals and humans. Although enterococci are involved in beneficial food fermentation, they may also be important nosocomial pathogens.\textsuperscript{75} Therefore, their use as probiotics should be considered with caution.

The antimicrobial screening has revealed that the two enterococci isolates inhibit the nisin-sensitive indicator \textit{M. luteus}. Interestingly, the genomes of both isolates contain clusters associated with the biosynthesis of two posttranslationally modified bacteriocins, a sactipeptide and a lassopeptide, suggesting a direct implication between these bacteriocins and the activity observed against \textit{M. luteus}. Normally, sactipeptides and lassopeptides show a very limited spectrum of antimicrobial activity against phylogenetically related Gram-positive bacteria.\textsuperscript{50} however, our results indicate that the lassopeptide identified in the genome of isolate C34 could have a broader antimicrobial spectrum. This isolate displayed antibacterial activity against the nisin-resistant indicator \textit{S. thermophilus}; and, very recently, we have observed that it antagonizes \textit{Escherichia coli} in cocultures (personal communication). Since most of the characterized lassopeptides do not exhibit significant inhibitory abilities,\textsuperscript{76} the antibacterial role of our lassopeptide-producing \textit{Enterococcus} is worth further investigation. On the other hand, we identified three hypothetical unmodified bacteriocins in the genomes of the pediococci isolates B4 and I32 and \textit{L. plantarum} P5—a class Iic bacteriocin, an S-type pyocin, and a plantaricin. The class Iic bacteriocin shows a high similarity with sakacins by \textit{Lactobacillus} sp.,\textsuperscript{77} while plantaricins have been described extensively in many \textit{L. plantarum} strains.\textsuperscript{49} Although pyocins are more frequently synthesized by Gram negative bacteria,\textsuperscript{51} some examples have also been reported in Gram positive bacteria.\textsuperscript{78} The fact that none of these unmodified bacteriocins display a broad spectrum of antimicrobial activity could explain why we observed no significant antimicrobial activity from the isolates.

The use of \textit{M. smegmatis} as an indicator strain has shown that all the LAB isolates display antimycobacterial activity, in particular, the weissella isolates. LAB produce a wide range of antimicrobial metabolites, some of which have been reported to be active against mycobacteria.\textsuperscript{79} Thus, it is difficult to determine the exact mechanism that the isolates utilize to exert antimycobacterial activity. However, one possible explanation that could help us understand why \textit{Weissella} seem to be more effective than the remainder of the isolates relates to their sugar metabolism. Genome annotations have demonstrated that the weissella isolates are obligate heterofermenters, whereas isolates of lactobacilli, pediococci, and enterococci are obligate homofermenters and/or facultative heterofermenters. Therefore, the combination of lactate and ethanol that \textit{Weissella} generates at equal levels could have a more antimycobacterial effect than a simple abundance of lactate. Furthermore, we have previously reported that lactobacilli and pediococci significantly reduce the survival rate of BCG in cocultures.\textsuperscript{17} In this respect, it is likely that the
production of bacteriocins by *L. plantarum* P5 and the pediococci strains B4 and I32 requires the presence of other bacterial cells, as previously reported.\(^8^0\) In fact, the clusters involved in the biosynthesis of some of these bacteriocins contain genes associated with regulation. Interestingly, none of the LAB isolates displayed any antimicrobial activity against other LAB used as indicator strains (data not shown), suggesting a nondetrimental effect on gut commensal LAB. Taken together with the fact that the LAB isolates display a very specific antibacterial activity against gut-unrelated bacteria, such as streptococci, micrococci, and mycobacteria, this brings a positive outlook with regard to their potential use as probiotics.

We finally determined the immunomodulatory properties of the LAB isolates using human phagocytes. Despite the fact that the phagocytes were not derived from badgers, our human model has proved to be informative at generating preliminary data that show the potential for badger LAB to cause immunomodulation, as previously described.\(^1^7\) *L. reuteri* D4 and the *P. acidilactici* strains F7 and E24 were able to induce significantly the activation of NF-κB in THP-1 monocytes, while the IFN pathway was overstimulated in the same monocytes following exposure to *P. pentosaceus* B4, *L. plantarum* P5, and the enterococci isolates C34 and A7. When blood cells were challenged with all these isolates, we observed a significant phagocytic response, mainly from monocytes, implying the importance of bacterial phagocytosis for the activation of pro-inflammatory mediators via NF-κB and IFN.\(^8^1,8^2\) In this respect, the genome examination showed that the enterococci isolates carry genes coding for proteins associated with internalization by human cells,\(^8^3,8^4\) including the collagen adhesin Ace, the fibrinogen adhesins Fss1 and Fss2, the endocarditis antigen EfaA, and the aggregation substances Asc10 and EF0485. Furthermore, the genomes of *P. pentosaceus* B4, *P. acidilactici* E24, and *L. plantarum* P5 have revealed the presence of genes that express proteins that are critical for adherence and intracellular survival of *Staphylococcus aureus*, such as the serine/threonine kinase/phosphatase system StkP/Stp1\(^8^5\) and the adhesins Cna and SraB.\(^8^6\) *L. plantarum* P5 and *P. pentosaceus* B4 also harbor genes associated with the biosynthesis of fimbrial components, which seem to be important for adhesion and subsequent colonization.\(^8^7\) No unique markers were found in the genomes of *L. reuteri* D4 and *P. acidilactici* F7 to explain their internalization, although the absence of evasion markers could be a factor for consideration, especially for isolate D4. Nearly all isolates of pediococci and *Weissella* carry genes associated with the synthesis of capsule polysaccharides that might provide resistance to phagocytosis, including *cap*\(\delta\)a of *S. aureus*\(^8^8\) and *cps*12 of streptococci.\(^8^9\) In fact, all the *Weissella* isolates and two pediococci (M16 and I32) were not phagocytosed and activated very low levels of NF-κB and IFN.

Phagocytes generally use TLRs and NLRs to recognize LAB MAMPs\(^1^2\) that include pili, fimbria, LTA, WTA, EPS, and membrane lipoproteins,\(^1^6\) resulting in the activation of intracellular pathways that coincide with the proinflammatory transcription factor NF-κB.\(^1^3\) In this respect, immunomodulatory screening has shown that not only all the LAB isolates possess genes involved in the synthesis of pili, fimbria, LTA, and WTA, but also that only *L. reuteri* D4 and the two *P. acidilactici* strains F7 and E24 were able to stimulate a significant NF-κB response. Therefore, it is likely that other bacterial components, such as EPS and membrane lipoproteins, are being recognized by TLRs and NLRs. It is interesting to see that *L. reuteri* D4 contains three genes associated with the production of EPS,\(^9^0\) while *P. acidilactici* E24 carries a gene encoding the outer membrane lipoprotein LolE.\(^9^1\) We also identified LolE in *P. acidilactici* M16, but this isolate did not display any interaction with phagocytes. Therefore, isolates D4 and E24 could be considered as candidates to boost immune responses as adjuvants and/or to mitigate the negative influence of pathogenic bacteria that interfere with NF-κB activation.\(^9^2\) With regard to *P. acidilactici* F7, this isolate could be another beneficial candidate against bacterial infections, but no differential genes associated with TLR/NLR activation were found in its genome.

Very recent studies have reported that LAB and other commensal bacteria are capable of inducing IFN responses that are dependent on endosomal TRLs\(^1^5,1^6\) and cytosolic nucleic acid—sensing receptors.\(^9^3,9^4\) In our study, we have found that *P. pentosaceus* B4, *L. plantarum* P5, and the two enterococci isolates A7 and C34 significantly activate the IFN pathway. The exact mechanisms...
that trigger that IFN activation are unknown, but the fact that these four isolates express the adhesion factors discussed above could facilitate phagocytosis (and/or endocytosis) and subsequent activation of cytosolic sensors (and/or TLRs). Therefore, isolates A7, B4, C34, and P5 are potential candidates to stimulate IFN responses that may be beneficial against viral infections, in particular, B4 and P5, owing to the lack of virulence and AMR genes in their genomes.

Conclusion

The LAB isolates evaluated in our study have potential probiotic benefits to mitigate the impact of infectious diseases in European badgers, and possibly other species. However, these benefits are diverse and depend on the bacterial genera. The enterococci possess the properties of antibacterial production and immunomodulation, but their genomes contain genes encoding virulence factors and AMR. In this respect, 2001 FAO/WHO Expert Consultation on Probiotics in Food recommends that enterococci are not referred as probiotics and that their use should require a very stringent safety evaluation. Therefore, their exploitation might be limited to their antimicrobial metabolites and immunomodulatory proteins. Furthermore, the Weissella isolates could be considered as an alternative to reduce the burden of mycobacteria as a consequence of their heterofermentative profile. However, lactobacilli and pediococci are probably the most promising probiotics owing to their ability to modulate important proinflammatory markers, such as NF-κB and IFN. Our preliminary results have been generated from in vitro assays and genome analyses, but illustrate the potential for use of LAB as wildlife probiotics. To further explore this possibility, in vivo work will be needed using the isolates in a variety of different animal species, age groups, and diet types. This would help identify the optimal conditions in which they could work, as recommended by the 2016 FAO report on probiotics in animal nutrition. LAB are also ideal candidates for use as safe delivery vectors expressing therapeutic recombinant proteins. Oral administration of probiotics has also been suggested as a convenient and safe way to improve the efficacy of conventional vaccines within communities.

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Author contributions

A.S. planned, performed experiments, and edited the manuscript. A.H.Mv.V. carried out data analysis and aided in preparing and editing the manuscript. M.A.C. aided in preparing and editing the manuscript. J.G.M. designed and performed experiments, aided in data analysis, and prepared and edited the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All procedures with human blood samples have been approved by the Surrey University Animals Ethics Committee in accordance with the Institutional Policy on the Donation and Use of Human Specimens in Teaching and Research and the national guidelines under which the institution operates. The blood protocol approval was under IRAS number 236477, and the sample storage was carried out under HTA license 12365.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Genomes metadata.

Table S2. Safety and immunomodulatory markers.

Table S3. Minimum inhibitory concentrations (MICs) of antibiotics of reference used in human and veterinary medicine against the badger LAB isolates and the microbiological cutoff values (limit) recommended for each of these antibiotics against same bacterial species or closely related species.
Table S4. Genes identified in the clusters associated with the production of the hypothetical ribosomally and posttranslationally modified peptides (RiPPS) in *E. faecalis* A7 (sactipeptide) and *E. faecalis* C34 (lasso peptide).

Table S5. Genes identified in clusters associated with the production of hypothetical unmodified bacteriocins in *Lactobacillus plantarum* P5 (plantaricin), *Pediococcus acidilactici* I32 (pyocin), and *Pediococcus pentosaceus* B4 (class IIc bacteriocin).

**Competing interests**

The authors declare no competing interests.

**References**


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