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RESEARCH ARTICLE

An update on the taxonomy and functional properties of the probiotic *Enterococcus faecium* SF68

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Abstract

Enterococcus faecium SF68 (SF68) is a well-known probiotic with a long history of safe use. Recent changes in the taxonomy of enterococci have shown that a novel species, *Enterococcus lactis*, is closely related with *E. faecium* and occurs together with other enterococci in a phylogenetically well-defined *E. faecium* species group. The close phylogenetic relationship between the species *E. faecium* and *E. lactis* prompted a closer investigation into the taxonomic status of *E. faecium* SF68. Using phylogenomics and ANI, the taxonomic analysis in this study showed that probiotic *E. faecium* SF68, when compared to other *E. faecium* and *E. lactis* type and reference strains, could be re-classified as belonging to the species *E. lactis*. Further investigations into the functional properties of SF68 showed that it is potentially capable of bacteriocin production, as a bacteriocin gene cluster encoding the leaderless bacteriocin EntK1 together with putative *Lactococcus lactis* bacteriocins LsbA, and LsbB-like putative immunity peptide (LmrB) were found located in an operon on plasmid pF9. However, bacteriocin expression was not studied. Competitive exclusion experiments in co-culture over 7 days at 37 °C showed that the probiotic SF68 could inhibit the growth of specific *E. faecium* and *Listeria monocytogenes* strains, while showing little or no inhibitory activity towards an entero-invasive *Escherichia coli* and a *Salmonella* Typhimurium strain, respectively. In cell culture experiments with colon carcinoma HT29 cells, the probiotic SF68 was also able to strain-specifically inhibit adhesion and/or invasion of enterococcal and *L. monocytogenes* strains, while such adhesion and invasion inhibition effects were less pronounced for *E. coli* and *Salmonella* strains. This study therefore provides novel data on the taxonomy and functional properties of SF68, which can be reclassified as *Enterococcus lactis* SF68, thereby enhancing the understanding of its probiotic nature.

Keywords

Enterococcus faecium – *Enterococcus lactis* – probiotic – SF68 – taxonomy

1 Introduction

Enterococcus faecium is a lactic acid bacterium (LAB) occurring in a wide variety of environments, probably because of its ability to tolerate a wide range of pH and temperatures, in addition to its capacity to metabolise a wide spectrum of carbohydrates (Franz *et al.*, 1999; Wessels *et al.*, 1990). Some strains of *E. faecium* are important as starter cultures for fermented foods and feeds, while others have been used for many years as human and animal probiotics (Franz *et al.*, 2011; Hanchi *et al.*, 2018; Im *et al.*, 2023). *E. faecium* occurs in the gut of humans and animals (Devriese and Pot, 1995; Devriese *et al.*, 1994; Leclercq *et al.*, 1996) and some strains were shown to cause nosocomial infections such as bacteraemia and endocarditis (Chenoweth and Schaberg, 1990; Im *et al.*, 2023; Morrison *et al.*, 1997; Murray, 1990; Selleck *et al.*, 2019).

Currently there are 61 known *Enterococcus* species organised in so-called 'species groups' of genetically closely related species. The *E. faecium* species group contains the related species *Enterococcus faecium*, *Enterococcus canis*, *Enterococcus durans*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus phoeniculicola*, *Enterococcus ratti*, *Enterococcus villorum* and *Enterococcus thailandicus* (Svec and Franz, 2014). Morandi *et al.* (2012) described a novel species named *Enterococcus lactis* showing a high degree of relatedness to *E. faecium*, *E. durans* and *E. hirae* and thus also considered to belong to the *E. faecium* group.

Since many years, a certain degree of genetic heterogeneity has been noted for the species *E. faecium*. Already in 2002, a study by Vancanneyt *et al.* (2002) showed the presence of two genomic subgroups within the species. Interestingly, all human clinical isolates or strains showing vancomycin resistance or haemolytic activity were located in genomic subgroup I which also contained the *E. faecium* type strain LMG 11423^T (= DSM 20477^T). Subsequently, with the emergence of more powerful, genome-level investigations a more detailed picture of the intra-species population structure of *E. faecium* emerged. Accordingly, Lebreton *et al.* (2013) showed that *E. faecium* strains can be grouped into two distinct clades, of which clade A contains hospital-associated (HA) while clade B contains community-associated strains (CA) strains. A further split in clade A gave rise to the subclades A1 and A2 and subclade A1 now contains mostly animal-associated strains, while subclade A2 contains human clinical isolates (Belloso Daza *et al.*, 2021; Lebreton *et al.*, 2013). The *E. faecium* sequence type 17 (ST17) was identified to be ancestral for the hospital-associated subclade A1 and forms the clonal complex 17 (CC17). Subsequently, most HA strains were found to be members of CC17 (Lee *et al.*, 2019). Distinguishing characteristics between class A and class B strains are that clade A strains frequently show resistance to antibiotics (vancomycin, ampicillin and high levels of aminoglycosides) and contains virulence factors and mobile genetic elements (Belloso Daza *et al.*, 2021; Gorrie *et al.*, 2019; Lee *et al.*, 2019). Also, clade A strains were found to be ampicillin resistant, while clade B strains are generally ampicillin sensitive (Belloso Daza *et al.*, 2021; Montealegre *et al.*, 2017).

In 2021, Belloso Daza and co-workers investigated the whole genomes of *E. lactis*, its heterotypic synonym *E. xinjiangensis* (Li and Gu, 2021; Ren *et al.*, 2016) and *E. faecium*. Besides the *E. faecium* type strain NCTC 7171^T (= DSM 20477^T), they also considered strains from diverse lineages of *E. faecium*, including HA (clade A) and CA (clade B) strains. In addition, the authors used 16S rRNA gene sequence analysis, phylogenomics based on the core pangenome, multi-locus sequence typing, average nucleotide identity and digital DNA-DNA hybridization to confirm the genetic and evolutionary difference between clade A and the clade B and *E. lactis* group, the latter which contains isolates at that time identified as *E. faecium* clade B and *E. lactis* intertwined in the same cluster. This supported the reassignment of the strains of *E. faecium* clade B to the species *E. lactis*. They also confirmed the absence of virulence gene markers *IS16*, hyl_{Efm} and *esp*, and the absence of the PBP5 allelic profile associated with ampicillin resistance in the species *E. lactis*.

Some enterococcal strains are utilised as probiotics, like the extensively studied SF68, which is used in pharmaceutical preparations as well as in animal nutrition. These probiotics have been used mainly for the treatment of diarrhoea, antibiotic-associated diarrhoea, irritable bowel syndrome, for lowering cholesterol levels or for immune regulation (Bellomo *et al.*, 1980; Borgia *et al.*, 1982; Bruno and Frigerio, 1981; Buydens and Debeuckelaere, 1996; Chen *et al.*, 2010; D'Souza *et al.*, 2002; Franz *et al.*, 2011; Gade and Thorn, 1989; Greuter *et al.*, 2020; Im *et al.*, 2023; Lewenstein *et al.*, 1979; McFarland and Dublin, 2008; Wunderlich *et al.*, 1989). In the light of recent insights into the population structure of *E. faecium* strains and the finding that many clade B strains may actually belong to the species *E. lactis* (Belloso Daza *et al.*, 2021), this study aimed to re-appraise the taxonomy of probiotic *E. faecium* SF68. On the other hand, the availability of genomic data and further investigation

TABLE 1 Characteristics and sources of bacteria used in this study

Strain	Characteristic[1]	Reference
Enterococcus lactis SF68 (previously *Enterococcus faecium* SF68), originally deposited as *E. faecium* NCIMB 10415	Probiotic strain, Rif^R	Cerbios Pharma
E. faecium DSM 13590	Van^R	DSMZ
E. faecalis FAIR-E 329	Em^R, Cm^R, Gm^R, Sm^R, Tet^R, *ace*$^-$, *as*$^+$, *cyl*$^+$, *esp*$^+$, *gel*$^+$	FAIR-E collection, isolated from semi-hard cheese
Enterococcus faecalis UKF 210	Em^R, Gm^R, Sm^R, *ace*$^+$, *gel*$^+$	Freiburg University Clinic, catheter isolate
E. faecium UKF 207	Em^R, Pen^R, Sm^R, *acm*+, *efaAfm*+, *esp*+	Freiburg University Clinic, catheter isolate
Listeria monocytogenes EJDe WSLC 1993	Serotype 1/2b, invasive pathogen	University Hohenheim
Enteroinvasive *Escherichia coli* EIEC 12860	Invasive pathogen	University Hohenheim
Salmonella enterica serovar Typhimurium S.TM S1 1344	Invasive pathogen	*Salmonella* Reference Laboratory Robert Koch Institute
S. enterica serovar Enteritidis S.E SE 147 Nx	Invasive pathogen	*Salmonella* Reference Laboratory Robert Koch Institute

[1] Rif = rifampicin; Van = vancomycin; *gel* = gelatinase; *cyl* = cytolysin; *as* = aggregation substance; *esp* = enterococcal surface protein; *ace* = adhesin to collagen from *Enterococcus faecalis*; *efaAfm* = *E. faecium* endocarditis antigen A; *acm* = collagen binding adhesin; Cm = chloramphenicol; Te = tetracycline; Em = erythromycin; Sm = streptomycin; Gm = gentamicin.

into its competitive nature is aimed to increase knowledge on the functional properties of this strain.

2 Materials and methods

Strains, media and culturing conditions

The probiotic *E. faecium* SF68 from Cerbios-Pharma SA (Holzapfel *et al.*, 2018) was used for taxonomic and antagonistic activity investigations. All other bacterial strains used in competitive exclusion experiments are shown in Table 1. All enterococci strains were grown in De Man, Rogosa and Sharpe (MRS) agar (VWR International, Darmstadt, Germany) medium aerobically at 30 °C for routine pre-culturing of the strains, while all experiments were conducted at 37 °C.

Taxonomic considerations based on chromosome comparison

The unannotated SF68 chromosomal sequence was obtained from Cerbios-Pharma SA under a confidentiality agreement, while other chromosomal sequences of *E. faecium* and *E. lactis*, including the type strains DSM 20477^T and LMG 25958^T, respectively, were downloaded from the NCBI website (Table 2). The software used for the comparison was either the BioNumerics software (Applied Maths, a BioMerieux company, Belgium) version 7.6 to compare strains by phylogenomic tree analysis or the Orthologous Average Nucleotide Identity Tool (OAT) (Lee *et al.*, 2016) to calculate average nucleotide identity between investigated strains, both run on a local Windows computer.

In the genome comparison with BioNumerics 7.6, the following parameters were used: minimum of 15 matches in a window size of 25 bases and minimum of 6 matches in a window size of 10 amino acids; minimum stretch length of 30.

Studies on antagonistic activity of SF68 by in silico *analysis for bacteriocin genes*

The presence of bacteriocin genes was determined from the genome sequence of SF68 using BAGEL4, a web-based bacteriocin mining tool (Van Heel *et al.*, 2018) capable of identifying genes encoding bacteriocins. In addition, the software investigates whether genes encoding proteins involved in bacteriocin biosynthesis, transport, regulation and/or immunity occur in the vicinity of the bacteriocin structural gene.

Competitive exclusion of pathogens

The influence of the probiotic SF68 on the growth of pathogenic enterococci was determined in co-culture. For this, the growth kinetics of individual strains were first determined *in vitro* in three technical replicates, as

TABLE 2 Details of the chromosomal sequences used (besides the SF68 chromosome)[1]

#	Species name	Strain number	Assembly	Genome Size (Mbp)	GC%	Scaffolds	CDS
1	*E. lactis*	CX 2-6_2	GCA_019343125.1	2.73	38.4	2	2,474
2	*E. lactis*	KCTC 21015 (T)	GCA_015767715.1	2.72	38.4	1	2,470
3	*E. lactis*	E843	GCA_019880345.1	2.88	38.13	3	2,644
4	*E. lactis*	DH9003	GCA_020268645.1	3.07	38.04	4	2,826
5	*E. lactis*	HJS001	GCA_019967715.1	2.79	38.32	2	2,529
6	*E. lactis*	JDM1	GCA_019203145.1	2.78	38.29	2	2,464
7	*E. lactis*	LMG 25958 (T)	GCA_015904215.1	2.92	38	236	2,655
8	*E. faecium*	LMG 8148 (T)	GCA_001576665.1	2.7	38.3	366	2,506
9	*E. faecium*	SRR24	GCA_009734005.2	2.92	37.86	2	2,701
10	*E. faecium*	ISMMS_VRE_1	GCA_001720945.1	3.26	37.67	6	2,994
11	*E. faecium*	VRE	GCA_009697285.1	3.35	37.67	6	3,146
12	*E. faecium*	AA622	GCA_019977575.1	3.27	38.26	7	2,947
13	*E. faecium*	E8202	GCA_900639535.1	3.28	37.69	7	3,062
14	*E. faecium*	2014-VREF-41	GCA_002007625.1	3.28	37.42	5	3,311
15	*E. faecium*	E8414	GCA_900639715.1	3.44	37.49	9	3,270
16	*E. faecium*	E7240	GCA_900639485.1	3.3	37.65	10	3,066
17	*E. faecium*	2014-VREF-268	GCA_002025045.1	3.39	37.55	4	3,137
19	*E. faecium*	Dallas 144_1	GCA_016415405.1	3.25	37.58	8	3,000
20	*E. faecium*	AUS2002	GCA_907163315.1	3.24	38.03	5	2,989
21	*E. faecium*	6.00E+06	GCA_001518735.1	3.4	37.6	3	3,183
22	*E. faecium*	Dallas 100_1	GCA_016415285.1	3.24	37.67	7	3,002
23	*E. faecium*	VRE001	GCA_001895905.1	3.24	37.81	4	2,973
24	*E. faecium*	E1	GCA_001886635.1	3.21	37.74	5	2,990
25	*E. faecium*	E7429	GCA_900639465.1	3.29	37.83	10	3,075
26	*E. faecium*	E7933	GCA_900639545.1	3.33	37.75	10	3,107
27	*E. faecium*	E8328	GCA_900639615.1	3.18	37.69	5	2,959
28	*E. faecium*	E8377		3.25	37.8	7	3,036
29	*E. faecium*	E8284	GCA_900639625.1	3.24	37.81	6	3,030
30	*E. faecium*	Dallas 51_4	GCA_016406465.1	3.35	37.57	13	3,093
31	*E. faecium*	WGS1811-4-7	GCA_016864255.1	3.21	37.93	5	3,003
32	*E. faecium*	E6975	GCA_900639395.1	3.29	37.62	9	3,101
33	*E. faecium*	Dallas 97_1	GCA_016415025.1	3.23	37.74	8	2,948
34	*E. faecium*	Dallas 53_2	GCA_016406545.1	3.19	37.7	6	2,922
35	*E. faecium*	UAMSEF_09	GCA_005886715.1	3.3	37.76	6	2,975
36	*E. faecium*	UAMSEF_20	GCA_005886735.1	3.3	37.76	6	2,968

[1] Preference was for high quality genomes, as judged by NCBI.

to evaluate whether any competitive effects were due to a result of better substrate utilisation. For this, SF68, *E. faecalis* FAIR-E 329 (potential pathogen based on virulence determinants) and the human pathogenic *E. faecalis* UKF 210 and *E. faecium* UKF 207 associated with infections and isolated from catheters (Table 1), were inoculated into 50 ml MRS broth (pH 6.9) with 1% of an overnight culture and then grown for 16 h at 37 °C. The enterococcal counts were determined by plating suitable dilutions in triplicate onto MRS agar and growth parameters such as growth rate and generation time were determined.

For the co-culture experiments, the probiotic, with one pathogen strain in each experiment, were cultured together in 50 ml of bacterial growth medium. MRS broth was used for enterococci, while Standard One (ST1) broth was used when co-culturing SF68 with non-enterococcal pathogens (Table 3).

Competitive exclusion was studied in three replicates of growth experiments with the co-culture of both pro-

TABLE 3 Strains, inoculum densities and culture media used in co-culture and cell culture experiments

Strain	Inoculum density (cfu/ml)	Medium for co-culture experiment	Selective agar medium
SF 68	1×10^7	Depending on pathogen, either MRS or ST1 broth	MRS or MRS with rifampicin 64 μg/ml
E. faecium DSM 13590^T	1×10^4	MRS broth	MRS with vancomycin (64 μg/ml)
E. faecalis FAIR-E 329	1×10^7	MRS broth	MRS with erythromycin (64 μg/ml)
E. faecalis UKF 210	1×10^7	MRS broth	MRS with erythromycin (64 μg/ml)
E. faecium UKF 207	1×10^7	MRS broth	MRS with erythromycin (64 μg/ml)
L. monocytogenes EJDe WSLC 1993	1×10^4	STI broth	PALCAM with supplement (Unipath)
Enteroinvasive *E. coli* EIEC 12860	1×10^3	STI broth	McConkey (Unipath)
S. enterica serovar Typhimurium S.TM S1 1344	1×10^3	STI broth	Salmonella-Shigella (Unipath)
S. enterica serovar Enteritidis S.E SE 147 Nx	1×10^3	STI broth	Salmonella-Shigella (Unipath)

biotic and pathogen strain. The growth of pathogens in coculture was compared to that of the pathogen growing in culture alone. The growth of the bacteria was followed over 7 days at 37 °C and each culture was transferred (1%) on a daily (24 h) basis into fresh 50 ml co-culture medium. Seven days was arbitrarily chosen as a time frame mimicking a probiotic intervention where bacteria had to compete over a time period of a week to see if that would suffice to allow the dominant strain to establish itself. For SF68, 1×10^7 cfu/ml were used for inoculation, while the amount of pathogen inoculated was related to its infective dose as described in the literature (Krämer and Prange, 2023; Table 3). The numbers of bacteria inoculated were confirmed by plate counting. Microbial counts were furthermore determined daily, ca. 8 h after re-inoculation of fresh medium with 1% of the previous batch culture. Selective media were used, which permitted only the growth of either SF68 or the pathogen (Table 3). For the enterococci, MRS agar with antibiotics was used to discriminate between the different strains (Table 3). The minimum inhibitory concentrations (MIC values) for the pathogenic enterococci towards erythromycin were determined using the E-test. All plates and co-cultures were incubated aerobically at 37 °C. The mean of the three replicate colony counts was calculated for each co-culture.

Prevention of pathogen adhesion and invasion in cell culture

Human colorectal adenocarcinoma HT29 cells were grown in DMEM (Dulbecco's modified Eagle medium) with foetal calf serum (FCS) (1%) and antibiotics (1% penicillin and streptomycin). HT29 cells were incubated at 37 °C in an atmosphere containing 5% CO_2. For adhesion and invasion assays, 24 well plates were used, and each well was seeded with 5×10^5 HT29 cells as described previously (Vizoso Pinto *et al.*, 2007). These were grown for 4 days until a confluent monolayer was obtained, and washed twice to remove antibiotics and FCS. For further experimentation, DMEM without FCS and antibiotics was used. Fresh overnight cultures of probiotic and pathogen strains were centrifuged at 7,200×*g* for 5 min, washed twice with sterile phosphate buffered saline (PBS), and finally resuspended in DMEM without additives (antibiotics, FCS). The optical density was measured at OD_{580} and the cell count was adjusted to approximately 2×10^9 cfu/ml for the probiotic and 2×10^6 cfu/ml for the pathogen, based on previous OD/cell count calibration curves. A 24-well plate was divided into 6 columns with 4 wells each. The first column served as negative control for invasion and the non-invasive, probiotic SF68 was used for this purpose. The second column was the positive control, to test whether the experimental conditions were suitable to detect invasion. Two invasive *Salmonella* strains were available as positive control (Table 1), but the more invasive *S. enterica* serovar Typhimurium strain was gen-

erally used. The third and fourth columns were used for the adhesion experiment. The wells of the third column were used for evaluation of adhesion of the pathogenic strain alone, while the fourth column was used to evaluate the adhesion of the pathogen after pre-incubation with the probiotic. The last two columns were used for the invasion experiments. The wells of the fifth column were used to test the invasion of the pathogenic strain alone, while the wells of the sixth column were used to test invasion of the pathogen after pre-incubation with the probiotic. The four wells of each column served as replicate determinations. At the beginning of the experiment, the probiotic bacteria were added to the appropriate wells, while DMEM was added to wells in which pathogens were subsequently investigated in the presence of probiotic cells. After 1 h incubation with the probiotic, the control strains and the pathogens were added to the respective wells.

The adhesion data were evaluated after a further hour incubation. For this, the supernatant with non-adherent bacteria was removed. After this, the reversibly bound bacteria were detached from the cells by washing three times with sterile PBS. The eukaryotic cells were then disrupted by using 1.5 ml of Triton-X-100 (1% solution in PBS) and the lysate was diluted and spread-plated onto appropriate medium (Table 3). The number of bacteria counted corresponded to both the adherent and possibly invasive bacteria. The numbers of adherent bacteria were calculated by subtracting the number of invasive bacteria (see below) from this total number. The number of invasive bacteria was determined as follows: first, the non-adherent bacteria were removed by aspirating the supernatant and washing one time with sterile PBS. Subsequently, one ml of DMEM containing the antibiotics gentamicin (500 μg/ml), penicillin (100 μg/ml), ampicillin (100 μg/ml), and streptomycin (1 mg/ml) was added to each well. The DMEM containing antibiotics was left in the wells for 1.5 h to kill all adherent bacteria. After this, 100 μl of supernatant were removed and spread plated to test the killing efficiency. We found that adequate killing led to cell counts of less than 50 cfu/ml. The rest of the supernatant was discarded, and the cells were washed three times with PBS to remove residual antibiotics. The cells were then lysed, similarly to the adhesion assay, and samples were spread plated to determine the numbers of bacteria that invaded the cells. The values obtained for the 4 wells were averaged and the inhibition values were statistically evaluated using ANOVA, followed by a t-test using GraphPad Prism 6, a value of $P < 0.05$ was considered significant. For the adhesion and invasion assays the *Salmonella* strains were used as positive controls, as these were determined in our experiments to adhere to and invade HT29 colon carcinoma cells, while the SF68 was used as a negative, non-invasive control.

3 Results and discussion

Taxonomic considerations based on whole genome analyses

Comparison of SF68 genome to genomes of other E. lactis *and* E. faecium *strains*

The result of the comparison is shown in Figure 1. The results suggest that both species form separate clusters. It should be noted that by increasing the number of strains, the heterogeneity of the individual clusters also increases and the separation of both species becomes less clear. Figure 1 also illustrates the relatively high heterogeneity of the *E. faecium* species. However, from this chromosome comparison, there is no doubt that SF68 is a member of the species *E. lactis*. *E. lactis* were previously reported to have been isolated from diverse ecological niches, including raw milk cheese, pig and human faeces, dairy products and rice wine koji (Lu *et al.*, 2023).

Comparison of SF68 to the type strains of E. lactis *and* E. faecium *by average nucleotide identity*

Using the software OAT (Lee *et al.*, 2016) the average nucleotide identity (ANI) and the Ortho ANI values (Lee *et al.*, 2016) were also calculated. The results presented in Table 4 and Figure 2 clearly show that the type strain of *E. faecium* can be differentiated from two strains of *E. lactis*, including the type strain and SF68, by at least 5% ANI, i.e. the ANI levels are lower than 96% between the *E. faecium* type strain DSM 20477^T and the *E. lactis* strain CX 2-6-2 and strain SF68, respectively. As the species cut-off level for ANI is established to be 96%, this clearly indicates that *E. faecium* and *E. lactis* are separate and distinct species and that SF68 belongs to the species *E. lactis*, rather than *E. faecium*. On the other hand, the *E. lactis* type strain LMG 25958^T and strain SF68 show 99.58% similarity, indicating these to belong to the same species.

Bacteriocin gene detection

The *in silico* search for bacteriocin genes on the SF68 genome using the web-based BAGEL identification tool revealed a bacteriocin gene located on the plasmid pF9, for which the predicted protein product was identical to the previously reported 37 amino acid enterocin

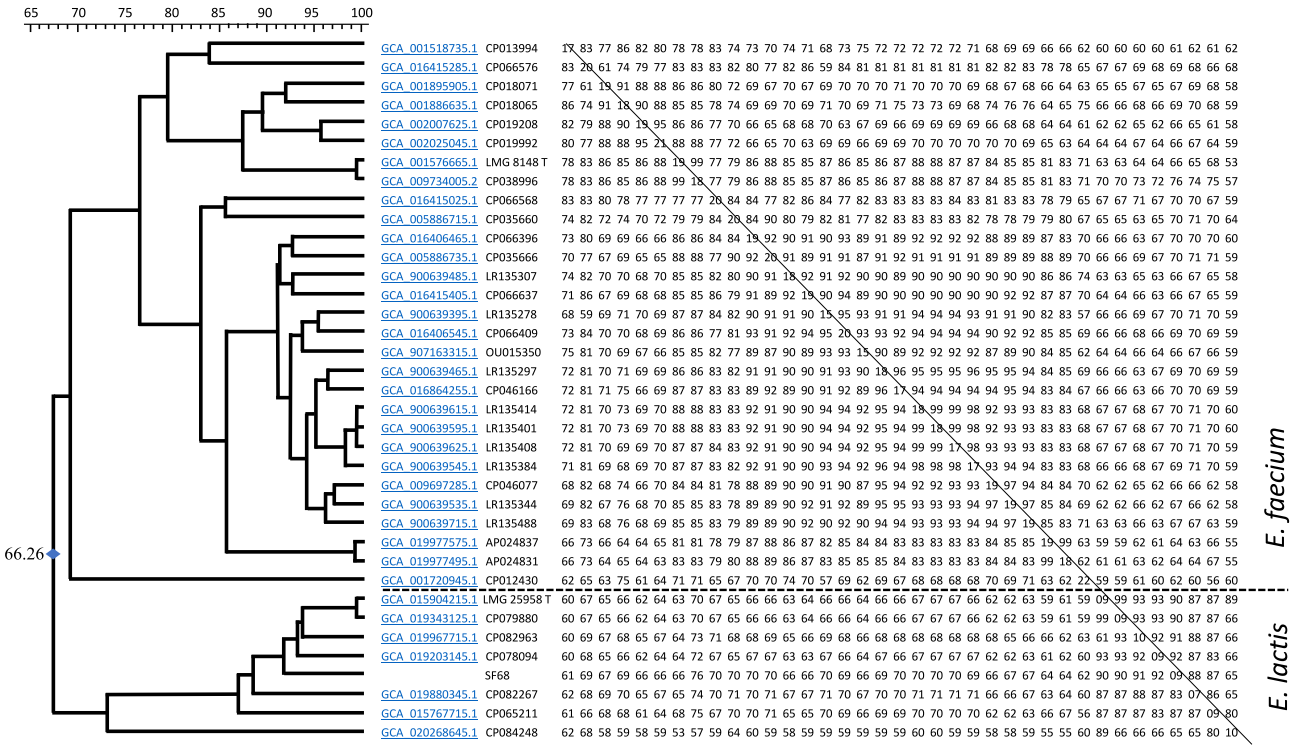

FIGURE 1 Result of the chromosome comparison between chromosomes from *Enterococcus faecium*, *Enterococcus lactis* strains and SF68. See Table 2 for strain details. Values are expressed in arbitrary units of similarity.

TABLE 4 Average nucleotide identity (ANI) and ortho ANI values between strains of *Enterococcus lactis* and the type strain of *Enterococcus faecium*

Genome 1	Genome 2	Ortho ANI value (%)	Original ANI value (%)
E. faecium DSM 20477^T	SF68	94.91%	94.79%
E. faecium DSM 20477^T	*E. lactis* CX 2-6-2	94.82%	94.84%
E. lactis LMG 25958^T	*E. faecium* DSM 20477^T	95.11%	95.09%
E. lactis LMG 25958^T	*E. lactis* CX 2-6-2	99.30%	99.26%
E. lactis CX 2-6-2	SF68	99.35%	99.32%
E. lactis LMG 25958^T	SF68	99.58%	99.60%

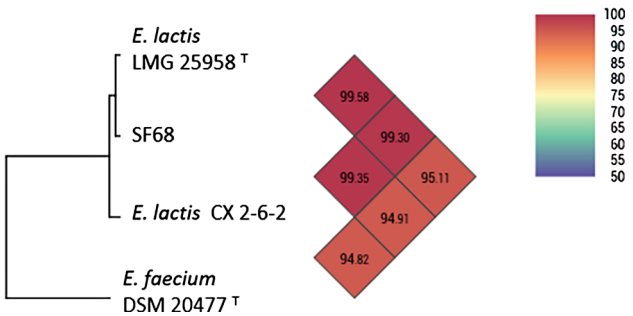

FIGURE 2 Heatmap generated with OrthoANI values calculated from the OAT software (Lee *et al.*, 2016).

K1 produced by *E. faecium* E2039 (Ovchinnikov *et al.*, 2017) (Figure 3A). Together with the enterocins EntQ and EJ97, EntK1 belongs to a relatively new bacteriocin group which is characterised by relatively small peptides (30-50 amino acids) that contain no post-translational modifications, and which are encoded as leaderless peptides lacking a N-terminal leader peptide (Ovchinnikov *et al.*, 2017). Based on Expasy calculations, the encoded EntK1 peptide has cationic (basic) properties with an isoelectric point of 9.73 and a molecular weight of 4,564.39 Da. The target bacterium spectrum for EntK1 was previously reported by Ovchinnikov *et al.* (2017) and was found to be rather narrow, being restricted mostly to strains of the same genus/species. Importantly, Ovchinnikov *et al.* (2017) and Reinseth *et al.* (2021) also found that the bacteriocin was active against nosocomial strains of *E. faecium* from different European hospitals, including vancomycin-resistant strains, at very low concentrations.

A

```
                                    10          20          30
Enterocin K1 (E 2039):      MKFKFNPTGTIVKKLTQYEIAWFKNKHGYYPWEIPRC
                            ::::::::::::::::::::::::::::::::::::
Enterocin K1(SF68):         MKFKFNPTGTIVKKLTQYEIAWFKNKHGYYPWEIPRC
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B

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ATGGCTAAAACAACACCGAATGTCCCTAAAACAATAGGATACACTAATCGTATAATTTTAAAATTATTTTTAG
GTAAAAAAAACATGTTAGCACCCTTTCAAAAATGTATAATATCATGCTACTATTATCTTATCATAATGAAAAG
<-----F  F  M -35    RBS(A)          -10                              RBS(B)
AGGTGATTTCCATGAAATTTAAATTTAATCCCACAGGAACCATCGTAAAAAAATTAACTCAATATGAAATAGC
          M  K  F  K  F  N  P  T  G  T  I  V  K  K  L  T  Q  Y  E  I  A
ATGGTTCAAAAATAAACATGGATACTATCCATGGGAAATTCCTAGATGCTAATCATTGGCACAAGTAAAATGG
 W  F  K  N  K  H  G  Y  Y  P  W  E  I  P  R  C  *  --------------------
GAAACTTGTATAAGTTTCCCATTTTACTTGTGCCAATGATATAATAAATTAAAAATTAAGAAGAGGAATTTTA
------>    <----------------------------
IR1                    IR2
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C

FIGURE 3 (A) Amino acid sequence comparison of enterocin K1 produced by SF68 and by *Enterococcus faecium* E 2039 (Ovchinnikov *et al.*, 2017). (B) The genetic locus of the enterocin B bacteriocin gene showing the putative ribosome binding site (RBS), putative promotor (–35 and –10 sites) sequences (underlined) and inverted repeat sequences (IR1 and IR2, inverted arrows) of a putative rho-independent terminator. (C) The annotated genes of the bacteriocin locus show the LsbB family bacteriocin (EntK1) gene, as well genes for an ABC transporter and a hypothetical protein located upstream of the bacteriocin gene in reverse orientation. Putative promoter sequences are shown as arrows, putative terminators as stem-loop structures, indicating that the bacteriocin gene alone, as well as the ABC transporter gene together with the hypothetical protein gene, occur in operons.

We found the bacteriocin locus to be identical to the open reading frames already present in the GenBank database with accession no MT501398 for the identical bacteriocin structural gene (CDS from position 9,119 to position 9,232) with protein ID QLG04473.1 and the 'ABC transporter gene' which is the putative immunity gene (starting at position 9,047 and ending at position 7,344) with a protein ID QLG04472.1. Sequence analysis of the bacteriocin locus in the pF9 nucleotide sequence clearly showed that the bacteriocin gene did not encode a N-terminal leader or a signal peptide (Figure 3B). An AG-rich potential ribosome binding site (5′-GAAAAGAGG-3′) sequence occurred at an optimum 8 bp spacing upstream of the ATG start codon of the bacteriocin structural gene [RBS (B) in Figure 3B]. Putative –35 (5′-GTTAGCA-3′) and –10 (5′-TATAA-3′) promoter sequences are underlined in Figure 3b. The plasmid pF9 sequence from SF68 also harboured an ORF which encoded a predicted immunity gene, similar to that of the *Lactococcus lactis* bacteriocins LsbA and LsbB (Gajic *et al.*, 2003). This putative immunity gene encodes a 567 amino acid protein and is located immediately upstream of the SF68 EntK1 bacteriocin (Figure 3B,C). The putative immunity protein revealed extensive homology (49.8% identity, 84.6% similarity) to the LmrB immunity protein of *Lc. lactis* over the entire 567 amino acid length. The ORF encoding the putative immunity protein is located on the opposite DNA strand and the ATG start codon occurs 71 bp upstream of the bacteriocin start codon (Figure 3B). A potential ribosome binding site (5′-GAAAGGG-3′, shown in the reverse orientation, i.e. 3′-CCCTTTC-5′; RBS (A) in Figure 3B) could be located 7 bp upstream of the start codon. The first three deduced amino acids

of the immunity protein (MFF) are also shown in Figure 3B. Putative promoter sequences (–35, 3′-TCGTAA-5′; and –10, 3′-ATTTA-5′, both shown in reverse orientation and underlined) are also indicated in Figure 3B. The absence of bacteriocin regulatory genes in the bacteriocin operon could suggest that the bacteriocin is constitutively expressed. The EntK1 bacteriocin was previously determined to be highly active against *E. faecium* strains, including nosocomial, vancomycin-resistant isolates, while no activity could be shown against *E. faecalis* strains (Ovchinnikov *et al.*, 2017).

It thus appears that SF68 may potentially be capable of producing a bacteriocin with a reported narrow activity spectrum, but which has been reported to be active against nosocomial vancomycin-resistant *E. faecium* strains. This bacteriocin activity may therefore potentially play an important role as a functional property in the suppression of potentially pathogenic VRE strains in the human gut. Nevertheless, no inhibition assays were performed to determine that the bacteriocin genes were actually expressed, and bacteriocin production in the MRS medium used was not investigated. This was only assumed to be possibly the case from the fact that the bacteriocin gene appeared to be constitutively expressed (absence of regulatory genes) and the inhibitory activity for this bacteriocin was demonstrated before by Ovchinnikov *et al.* (2017) and Reinseth *et al.* (2021), using a similarly nutrient rich bacterial growth medium (brain heart infusion) in their studies.

Competitive exclusion of pathogens in vitro

Co-culturing was used in this study to test whether SF68 would be capable of suppressing the growth of pathogenic, competitor enterococci *in vitro*. *E. faecalis* FAIR-E 329 was used as a representative food-borne *Enterococcus* strain that encodes multiple typical enterococcal virulence factors (Table 1), while the *E. faecium* strain DSM 13590 was chosen for co-culturing as it represents a typical vancomycin-resistant strain. Pathogenic *E. faecium* and *E. faecalis* strains were obtained from the Freiburg University clinic to represent typical human pathogens (Table 1). The growth kinetics of these different *Enterococcus* strains was pre-assessed in a pilot study to ensure that the strains exhibit similar growth rates or generation times in order not to influence the co-culture experiment. The co-culture experiment revealed that under these conditions SF68 and *E. faecium* UKF 207 and *E. faecalis* FAIR-E 329 showed a similar generation time of about 50 min, while the generation time of *E. faecalis* UKF 210 was somewhat longer at 67 min (results not shown). Despite this longer generation time, this strain reached a maximum viable count of 2.1×10^9 cfu/ml, which was comparable to that of the other strains (result not shown).

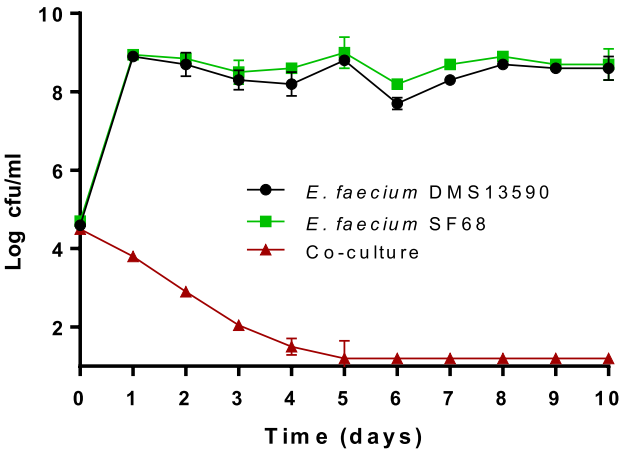

FIGURE 4 Co-culture of probiotic SF68 together with vancomycin-resistant *Enterococcus faecium* 13590 during a pilot experiment performed in MRS broth medium at 37 °C for 10 days. The numbers (log10 cfu/ml) of probiotic *E. faecium* SF68 (RIFREC) were determined using MRS agar containing rifampicin (64 μg/ml), of vancomycin resistant *E. faecium* DSM 13590 (VREC) on agar containing vancomycin (64 μg/ml) and of total enterococci (TEC) were determined on MRS agar without antibiotic. The detection limit reached was log 1.3 cfu/ml.

The inhibitory effect of SF68 on the growth of pathogenic bacteria was clearly strain specific. Nevertheless, it generally appeared to affect Gram-positive more than Gram-negative bacteria, which may be indicative of bacteriocin activity, as Gram-negative bacteria are generally more sensitive to organic acids and low pH. The result of an initial co-culture pilot experiment of the vancomycin-resistant *E. faecium* DSM 13590^T (type strain from human faeces) with the probiotic SF68 is shown in Figure 4. Co-culturing of SF68 with the vancomycin-resistant *E. faecium* DSM 13590^T showed that the probiotic strain grew from ca. log 4.5 cfu/ml to ca. log 9 cfu/ml within one day and stayed at this high level during continued subculturing and incubation. In contrast, the vancomycin-resistant *E. faecium* DSM 13590^T strain steadily declined to below log 2 cfu/ml after day 5, staying at this level up to 10 days of subculturing (Figure 4).

Following this pilot experiment, the effect of the probiotic SF68 was also tested on enterococcal pathogens isolated from infections, as well as other pathogens. The growth of the pathogenic *E. faecium* 207 and *L. monocytogenes* EJDe was also inhibited by SF68 (almost 5 log units decrease in viable numbers for the enterococci and 1.5 log decrease for the *L. monocytogenes* strains,

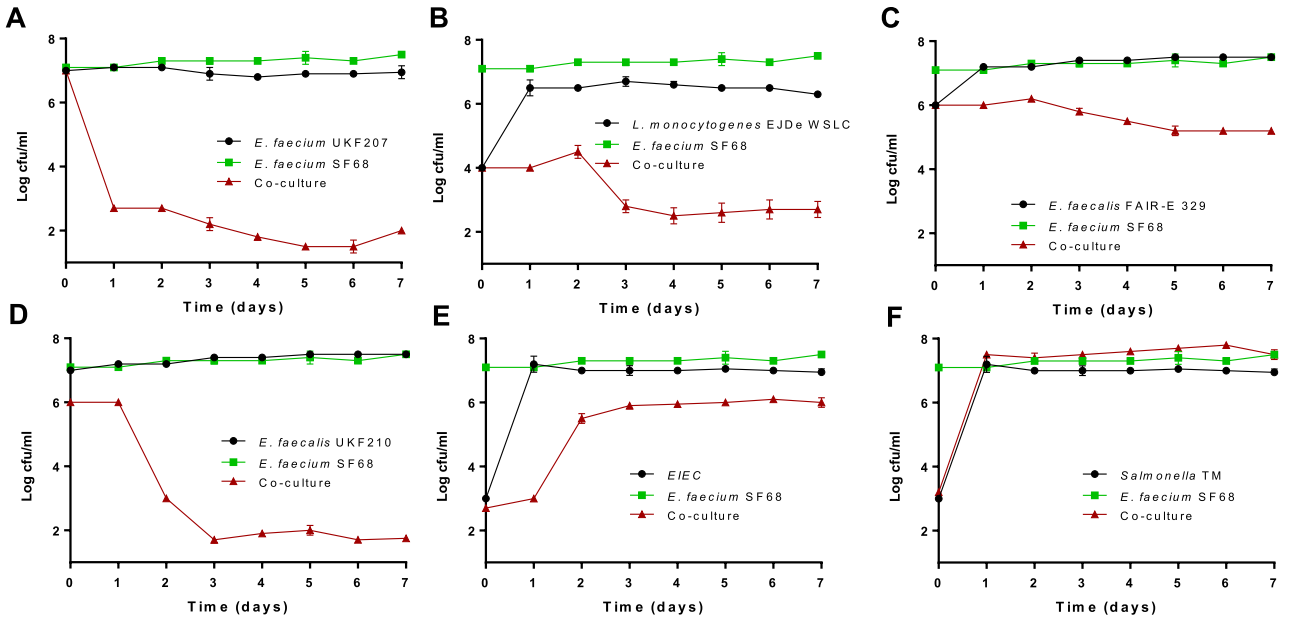

FIGURE 5 Numbers of probiotic *Enterococcus faecium* SF68 (RifR) in co-culture with (A) *E. faecium* strains UKF207 (pathogenic, EryR), (B) *Listeria monocytogenes* EJDe WSLC, (C) FAIR-E 329 (semi-hard cheese, EryR), (D) *Enterococcus faecalis* strain UKF210 (pathogenic, EryR), (E) enteroinvasive *Escherichia coli* EIEC 12860 and (F) *Salmonella enterica* serovar Typhimurium S.TM S1 1344 alone or during co-culturing experiment for 7 days. The detection limit was log 1.3 cfu/ml. Note that in these graphs the red 'coculture' curve indicates the pathogen counts except for (D) where the co-culture curve indicates the SF68 counts.

respectively) (Figure 5A,B). The growth of *E. faecalis* FAIR-E 329 was also reduced by approx. 1 log unit (Figure 5C). One exception was *E. faecalis* UKF 210 that inhibited SF68 by 4 logs (Figure 5D). However, it should be noted that at the starting point SF68 was ca. 1 log less concentrated at ca. 1×10^6 cfu/ml, which may have been an advantage for strain UKF 210 and which could have caused it to outgrow the probiotic SF68. Furthermore, the strain *E. faecalis* UKF 210 was also found to produce cytolysin in a PCR test for the cytolysin gene (results not shown). This compound has cytolytic activity against prokaryotic as well as eukaryotic cells (Coburn and Gilmore, 2003) and may explain why the *E. faecalis* UKF 210 strain was able to inhibit the probiotic SF68 in co-culture. Thus, while SF68 clearly exhibited a strong inhibitory effect against pathogenic and vancomycin-resistant *E. faecium* strains, as well as against pathogenic *L. monocytogenes*, competitive exclusion of *E. faecalis* strains clearly showed different outcomes.

The effect of SF68 on Gram-negative pathogens is also shown in Figure 5. The growth inhibition effect of pathogenic Gram-negative bacteria was poor, if present at all. Only the entero-invasive *E. coli* 12860 was slightly, but significantly ($P < 0.0001$) inhibited by one log unit (Figure 5E). The growth of *Salmonella* TM in co-culture with the probiotic strain was not significantly different from the growth of these pathogens in pure cultures (Figure 5F). Lewenstein *et al.* (1979) showed that SF68 was able to inhibit *E. coli* viable numbers by ca. 1.5 log units and *S.* Typhimurium by more than 5 log units when grown in co-culture over ca. 20 h, respectively. In Lewenstein's study, the strains were co-cultured for ca. 1 day while determining pathogen and probiotic counts at different time intervals, whereas in our study we studied the development of these bacterial counts over one week by daily (24 h) sub-culturing and determining the cell counts ca. 8 h after (re-)inoculation. Despite these differences, it appears that SF68 may exhibit inhibitory potential also against specific Gram-negative bacterial pathogens. However, this clearly appears to be strain dependent. The mechanisms responsible for this inhibition were not determined, but may rely on lactic acid production, better substrate utilization, possibly bacteriocin production, or a combination of these. It should be mentioned also that these experiments were performed in batch culture under aerobic conditions and it would be useful to evaluate if the same inhibitory effects would also be determined under anaerobic conditions.

Competitive exclusion has already been suggested to be based on several modes of action that eliminate pathogens, i.e. (1) direct and indirect competition for nutrients, (2) competition for physical attachment sites, (3) production of antimicrobial compounds (e.g. bacteriocins or volatile fatty acids including lactic acid), (4) enhancement of host immune system activity and (5) a synergistic interaction of two or more of the above

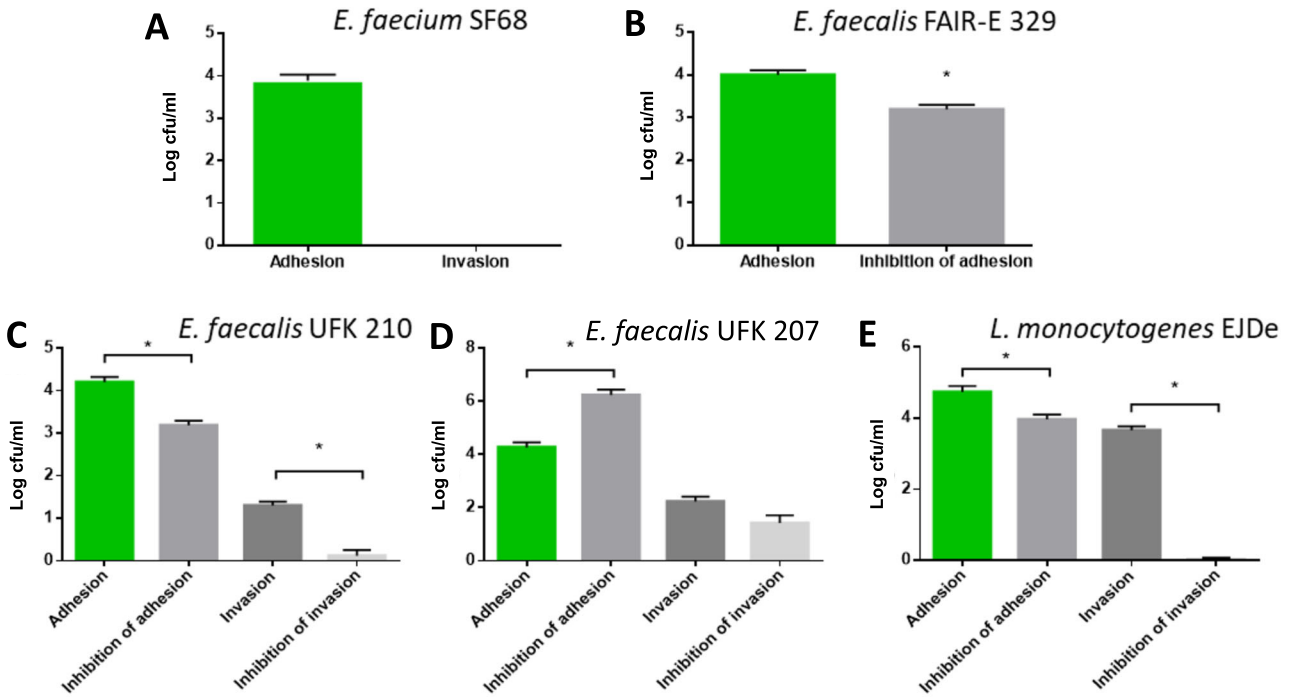

FIGURE 6 Viable counts (log cfu/ml) of (A) adhering or invading *Enterococcus faecium* SF68, (B) adhering or invading Gram-positive pathogens *Enterococcus faecalis* FAIR-E 329, (C) *E. faeca*lis UKF 210, (D) *E. faecium* UKF 207, and (E) *Listeria monocytogenes* EJDe. Shown is the adhesion of pathogens to cells not treated (adhesion) and pre-treated with the probiotic SF68 (inhibition of adhesion), and the invasion of pathogens into HT 29 cells in non-treated (invasion) and cells pre-treated with the probiotic SF68 (inhibition of invasion). All values were determined in quadruplicate and the standard error is shown. A significant inhibition of either adhesion or invasion is indicated with an asterisk (*).

activities (Callaway *et al.*, 2008). In food animals, competitive exclusion was already shown to be effective to control *Salmonella* colonization in poultry or enterotoxigenic *E. coli* in swine (Nisbet *et al.*, 1993; Underdahl *et al.*, 1982, Ushe and Nagy 1985). The probiotic activity of SF68 in prevention of diarrhea (Bellomo *et al.*, 1980; Buydens and Debeuckelaere, 1996; Wunderlich *et al.*, 1989) may be hypothetically at least partially explained by the competitive exclusion properties observed here for the strains *in vitro*, and potentially also by the production of antimicrobial metabolites such as lactic acid and possibly bacteriocin.

Inhibition of pathogen adherence and invasion in HT29 cell culture

The adherence and invasion of SF68 were tested in a pilot study. SF68 adhered well to HT29 cell monolayers, but it was non-invasive (Figure 6A) and could therefore be used as a negative control strain for invasion assays. The results for the competitive exclusion of Gram-positive pathogen adhesion and invasion with the probiotic SF68 are also shown in Figure 6. The inhibition of adhesion and invasion of these pathogens by SF68 was strain specific. Similar to SF68, the *Enterococcus* strain FAIR-E 329 from semi-hard cheese was not invasive under the conditions of our study (result not shown). The adhesion of strain FAIR-E 329 was significantly ($P = 0.002$) reduced by 0.9 log units when cells were pre-treated with the probiotic (Figure 6B). In contrast, the clinical *E. faecalis* strain UKF 210 was invasive, and both the adhesion and invasion were significantly ($P = 0.003$ for adhesion and $P = 0.0009$ for invasion) reduced by 0.9 log units and totally inhibited (1.3 log units), respectively (Figure 6C). An interesting observation was that the adhesion of the *E. faecium* strain UKF 207 to HT29 cells increased significantly ($P < 0.0001$) by ca. 2 log units when cells were pre-treated with SF68, but the invasiveness of this strain was reduced, even though not significantly (Figure 6D). The adhesion of *L. monocytogenes* was also significantly ($P < 0.0001$) inhibited (by approx. 0.8 log units), while the invasion was significantly and completely inhibited (Figure 6E). This inhibition corresponded to a difference of 3.7 log units when comparing to the invasion results of *L. monocytogenes* cells without pre-treatment with SF68 (Figure 6E).

Similar to the competitive exclusion in growth medium results, the Gram-negative pathogens were generally less inhibited in their adhesion and invasion potential by the probiotic. The *E. coli* strain 12860 was not

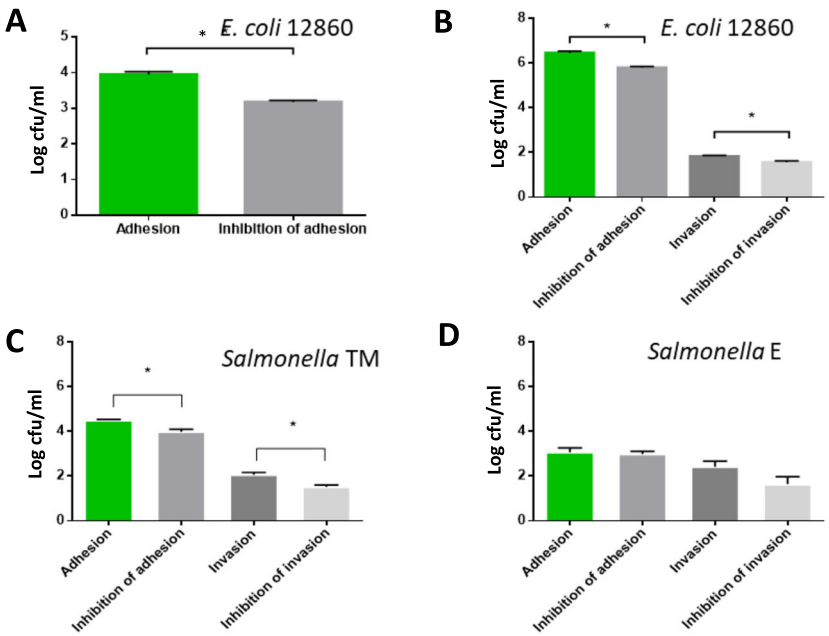

FIGURE 7 Viable counts (log cfu/mL) of (A) adhering or invading Gram-negative *Escherichia coli* 12860 with an inoculum density of 2×10^6 cfu/ml, (B) *E. coli* 12860 with an inoculum density of 2×10^9 cfu/ml, (C) *Salmonella* TM, and (D) *Salmonella* E. Shown is the adhesion of pathogens to cells not treated (first column, green) and pre-treated with the probiotic SF68 (second column, grey), and the invasion of pathogens into HT 29 cells in non-treated (third column, dark grey) and cells pre-treated with the probiotic SF68 (fourth column, light grey). All values were determined in quadruplicate and the standard error is shown. A significant ($P < 0.05$) inhibition of either adhesion or invasion is indicated with an asterisk (*).

invasive when used at a concentration of 2×10^6 cfu/ml under the conditions of this study. However, the adhesion of this strain could be significantly ($P = 0.0002$) inhibited by 0.75 log units when HT29 cells were pre-treated with SF68 (Figure 7A). Even when using higher numbers (2×10^9 cfu/ml) of *E. coli* 12860 in the invasion assay, the invasiveness of the strain was generally low. Nevertheless, a small but significant inhibition of both adherence and invasion (approx. log 0.64 and 0.3, respectively) of *E. coli* 12860 could be noted when HT29 cells were pre-treated with SF68 (Figure 7B). In contrast, the positive control *Salmonella* TM showed good invasion even at an inoculum density of 2×10^6 cfu/ml (Figure 7C). Here again, both a small, but significant inhibition of adhesion and invasion was noted (significance: $P = 0.02017$ for adhesion, $P = 0.0065$ for invasion). However, similar to the case of *E. coli* 12860, this inhibition was rather low (log 0.45 for adhesion, log 0.56 for invasion). In the case of *Salmonella* E, there was no significant inhibition of adhesion by pre-treatment of the HT29 cells (Figure 7D). Although the invasion was reduced by ca. log 0.99 when cells were pre-treated with the probiotic, this effect was not significant ($P = 0.0622$).

The probiotic SF68 showed good adherence to HT29 colon carcinoma cells, while it was clearly non-invasive (Figure 6A). These properties, i.e. to adhere to, but not invade intestinal cells have previously been described as important traits for probiotic strains. In most cases, the probiotic SF68 also showed a significant inhibition of adherence and invasion of bacterial pathogens. The degree of inhibition of pathogens in terms of bacterial counts showed differences at strain level.

4 Conclusions

The results obtained in this study clearly show that the probiotic *E. faecium* SF68 can be unequivocally reclassified as *E. lactis*. Reclassification of bacteria is not uncommon with the advance of modern taxonomic tools based on whole genome comparisons. This was also the case for another lactic acid bacterial genus, i.e. *Lactobacillus*, which was recently re-classified to give rise to even 23 new genera, some of which contain probiotic species (Zeng *et al.*, 2020). The data generated in this study shows that SF68 may have the capacity to produce a bacteriocin enterocin K1, that was previously described in another strain for its activity especially against pathogenic, vancomycin-resistant *E. faecium* strains. Nevertheless, as bacteriocin expression and activity were not determined in this study, we can only imply bacteriocin activity as a potential functional benefit of the strain, but not a confirmed property. Fur-

thermore, our data show that SF68 adheres to intestinal cells and successfully inhibits the adhesion and prevents invasion of several pathogenic bacterial strains in cell culture. These data may serve as basis to explain why SF68 is such a successful probiotic with proven effectiveness and long history of safe use (Holzapfel *et al.*, 2018). Previous reports also indicated that *E. lactis* strains do not contain virulence factors, such as haemolysin and gelatinase (Ben Braiek *et al.*, 2018). This may bring us one step closer to answering the previously asked conundrum (Ferchichi *et al.*, 2021; Franz *et al.*, 2003) on the safety of *E. faecium* strains. These have previously been noted by many authors to be of dualistic nature, i.e. some are clearly pathogenic, whilst some are beneficial as probiotics (Franz *et al.*, 2011), or are even important from a biotechnological point of view in, e.g. cheese ripening (Giraffa, 2003; Litopoulou-Tzanetaki *et al.*, 1993; Sarantinopoulos *et al.*, 2002). The answer may lie in the fact that certain strains may have been misclassified as in the case of *E. lactis*, a species which has low pathogenic but high biotechnological potential (Ben Braiek *et al.*, 2018). Further investigations should focus on this issue in more depth.

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Authors' contribution

Conceptualization: CF, BP, AA, RC, WH; methodology: CF, BP, MV-P, WH; investigations: CF, BP, MV-P, data analysis: CF, BP, MV-P, WH; writing – original draft preparation: CF, BP, MV-P; writing – review and editing: CF, BP, MV-P, AA, RC, WH. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

BP is also employed by Yakult Europe BV., WH is also associated with HEM Pharma Inc., RC and AA are employed by Cerbios-Pharma S.A.

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