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VIRULENCE GENES IN A PROBIOTIC *E. COLI* PRODUCT WITH A RECORDED LONG HISTORY OF SAFE USE

Trudy M. Wassenaar[1,*], Anke Zschüttig[2,7], Claudia Beimfohr[3], Thomas Geske[4], Christian Auerbach[2], Helen Cook[5], Kurt Zimmermann[6] and Florian Gunzer[2]

[1]Molecular Microbiology and Genomics Consultants, Zotzenheim, Germany
[2]Institute of Medical Microbiology and Hygiene, TU Dresden, Dresden, Germany
[3]vermicon AG, München, Germany
[4]Berlin, Germany
[5]Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark
[6]SymbioPharm GmbH, Herborn, Germany
[7]GlaxoSmithKline Biologicals, Dresden, Germany

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The probiotic product Symbioflor2 (DSM 17252) is a bacterial concentrate of six different *Escherichia coli* genotypes, whose complete genome sequences are compared here, between each other as well as to other *E. coli* genomes. The genome sequences of Symbioflor2 *E. coli* components contained a number of virulence-associated genes. Their presence seems to be in conflict with a recorded history of safe use, and with the observed low frequency of adverse effects over a period of more than 6 years. The genome sequences were used to identify unique sequences for each component, for which strain-specific hybridization probes were designed. A colonization study was conducted whereby five volunteers were exposed to an exceptionally high single dose. The results showed that the probiotic *E. coli* could be detected for 3 months or longer in their stools, and this was in particular the case for those components containing higher numbers of virulence-associated genes. Adverse effects from this long-term colonization were absent. Thus, the presence of the identified virulence genes does not result in a pathogenic phenotype in the genetic background of these probiotic *E. coli*.

Keywords: probiotic, *Escherichia coli,* genome sequence, virulence, colonization fitness

Introduction

For producers and legislators alike, the safety aspects of starter cultures, probiotic products and pharmaceuticals are of utmost importance. A safety evaluation should even have priority over a functional evaluation. In addition to an array of microbiological and phenotypical assays that are available to demonstrate the absence of virulence potential in a strain earmarked for probiotic use, a genome sequence can nowadays be considered an essential part of a thorough safety evaluation. Preferably, virulence genes should be absent from the genome of probiotic and starter culture bacteria; the absence of transferable genes providing resistance to clinically relevant antibiotics is also desired. However, the interpretation of genomic data is not without difficulty. Notably, genes present in pathogens are often conserved in commensals [1] and probiotic species as well [2]. This applies in particular to Gram-negative species, where a large part of the proteobacteria for which genome sequences are available is derived from pathogens [3]. This overrepresentation of pathogens and their genes in public databases increases the chance of finding a hit for a query gene from a probiotic genome to a gene detected in a proteobacterial pathogen. Even genes that have been proven to add to the virulence potential of a given pathogen can sometimes be demonstrated in the genome of probiotic strains [2, 4].

In case a long history of safe use can be demonstrated for a given probiotic strain, the presence of genes with high similarity to virulence genes in its genome can be ignored. An example is *Escherichia coli* Nissle 1917, which has been used in probiotic applications for decades [5]. When its genome was analyzed, of all *E. coli* genomes then available, it showed most similarity to that of strain *E. coli* CFT073, a pathogenic *E. coli* causing urinary tract infections [6, 7]. Nevertheless, its documented history of safe use suggests that the presence of genes that, in a different genomic content, have been shown to contribute

* Corresponding author: Trudy M. Wassenaar; Molecular Microbiology and Genomics Consultants, Tannestrasse 7, 55576 Zotzenheim, Germany; Phone: +49 6701 8531; Fax: +49 6701 901803; E-mail: trudy@mmgc.eu

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to virulence, can be considered as nonproblematic, when found in the context of the Nissle genome.

This argument cannot be used for strains for which a long history of safe use is not, or not yet, available. The precautionary principle dictates to mistrust the presence of virulence genes in the genome of a strain that is targeted to be consumed on purpose. Understandably, legislators have preferentially allowed novel probiotic products or starter culture strains that are closely related (for instance, belonging to the same species) to strains with a proven history of safe use. This, however, hampers the development of novel probiotic and starter culture products, as it restricts novel discoveries to a limited number of species only.

The discussion which genes, or gene combinations, can be considered "safe" and which play a role in virulence is complicated because many gene homologs can function in both benign and pathogenic host–microbe interactions. Many gut commensals use the same strategy to enhance their colonization (for instance by means of motility or attachment) that enteric pathogens also use. As a consequence, genes encoding for attachment or motility can enhance virulence of pathogens (and are often assigned a virulence function), while their equivalents in a commensal strain or species enhance their colonization without pathogenicity, and in a probiotic strain these same homologs might actually promote the beneficial effects of these bacteria.

One of the most difficult species for which to predict, from a genome sequence, virulent or commensal–probiotic interactions with the human host, is *E. coli*. The genomes of different *E. coli* strains vary considerably in size (the larger *E. coli* genomes are over one thousand kilobases larger than the shortest known genome of this species); consequently, genomes vary considerably in gene content [8]. Moreover, the species includes commensal as well as mild or highly pathogenic strains, whereby genome size is not necessarily correlating with pathogenicity.

We evaluated the genome content of *E. coli* strains that comprise a commercial probiotic product, Symbioflor2, that has been on the market for human consumption in Germany since 1954, followed in Austria, Hungary, and Switzerland (amongst other countries) a few years later. The product is composed of six live *E. coli* components, which are cultured separately and then mixed in equal amounts of G1/2, G3/10, G4/9, G6/7, and half the amount of G5 and G8, to produce the final product (the reason for this composition is historical). The product is marketed to regulate and improve the immune system of the gut; it is specifically recommended in case of irritable bowel disease. Consumers are advised to take 20 drops three times daily for a period up to 6 months, equivalent to a dose of $1 \cdot 10^8$ CFU per day.

The genomes of the components of Symbioflor2 have been sequenced [9], and a comparison of their predicted proteomes is presented here. The genome sequences were also used for a comparison to a subset of other *E. coli* genomes, both pathogenic and commensal strains. The genomes of Symbioflor2 *E. coli* contained a number of putative virulence genes. With the use of specific probes, we determined whether presence of these genes correlated to the colonization properties of the individual Symbioflor2 components, as we hypothesized that putative virulence genes could affect their fitness to compete with residual microflora in a human gut. The functionality of the probiotic properties of this product was not part of this investigation.

Methods

Symbioflor2 components

E. coli G1/2 (DSM 16441, serotype rough), G3/10 (DSM 16443, serotype O:35,129), G4/9 (DSM 16444, rough), G5 (DSM 16445, rough), G6/7 (DSM 16446, rough), and G8 (DSM 16447, O:169) were provided by SymbioPharm (Herborn, Germany). All strains were H^{-}. These *E. coli* isolates originated from the stool of one healthy individual in Germany in 1954, and together comprise the product Symbioflor2 DSM 17252.

Genome comparison

The protein-coding genes (CDS) predicted for the Symbioflor2 genomes were compared between each other and with other *E. coli* strains by means of BLASTP, and visualized in a matrix [10]. Cluster analysis of these genomes and 28 additional published *E. coli* genomes was performed according to published methods [11], resulting in a pan-genome tree. Information on the included reference genomes can be found in Ref. [8]. A BLAST atlas was created with the CMG-Biotools system on a local computer [10]. A phylogenetic tree of concatenated fragments of housekeeping genes *adk*, *fumC*, *icd*, *gyrB*, *mdh*, *purA*, and *recA* was performed by ClustalW alignment and a neighbor-joining tree was constructed with NGplot.

Virulence gene identification

Two databases were used to compare the genome content of Symbioflor2 *E. coli* with known virulence genes: the MvirDB at LLNL (http://mvirdb.llnl.gov) [12], using genes from *E. coli* only, and the database of virulence factors of pathogenic bacteria (VFDB) available at http://www.mgc.ac.cn/VFs/main.htm [13]. In case of VFDB, either the virulence factors (VF) or the "VFs for comparative studies" were used. Genes identified in Symbioflor2 genomes were compared to the genes stored in these databases by BlastP, filtering for *e*-values <0.001, and for >98% similarity. Genes that are normally part of a locus, and only function when a complete locus is present, were checked for presence of that complete locus.

Table 1. Probes used in this study

Probe name	Sequence (5′ → 3′)	Specificity	Tm (°C)
pr G1/2	ACAGGCAAACCAAAGGATTG	G1/2, G6/7, G8	58
pr G3/10	GGCTGAACTCACTGGAAAGC	G3/10	62
pr G4/9	CCCCTTTTGCATTTACCAAC	G4/9	58
prG5	AAAAATGCCCGGTTCTTCTTC	G5	60
pr1037	CGACAAGGAATTTCGCTAC	23S rRNA, bacteria	47

Symbioflor2-specific oligonucleotide probes and colony lift hybridization

Unique protein-coding genes for each strain were identified by BLASTP comparison [14], and checked for uniqueness in the nonredundant protein database at NCBI (February 2011). For those amino acid sequences that reported no significant hit, or only a single hit (preferentially to a species different from *E. coli*), the corresponding nucleotide sequences were extracted from the sequence files and these were used for a search in the nonredundant nucleotide database at NCBI (March 2011) using BLASTN. This resulted in identification of a sequence unique to the genome of interest, and hybridization probes were designed to target these presumed unique sequences *(Table 1)*. Their specificity was confirmed by Southern blot hybridization using Symbioflor2 components, as well as against a variety of nonrelated *E. coli* isolates and other *Enterobacteriaceae* (data not shown). Probe pr G1/2 could not distinguish between strains G1/2, G6/7 and G8, whose genome sequences were very similar. Probes specific for the latter two could not be identified; thus, probe pr G1/2 was used for detection of these three strains collectively. General bacterial probe pr1037 with binding specificity for the 23S rDNA of bacteria was used as the final hybridization probe and as a reference targeting all lifted *E. coli* colonies. By subtracting all Symbioflor2-specific colony-counts from the pr1037 signal, the number of CFU for Symbioflor2 *E. coli* components was calculated.

Volunteer study for colonization potential

Five healthy human volunteers participated in a colonization experiment; each person took a single high dose of Symbioflor2 after a meal on day 1, while a stool sample had been taken on day zero to provide a baseline. Persons B (male, 46 years) and E (female, 45) took 100 ml of Symbioflor2, containing $2 \cdot 10^7$ CFU/ml, persons A (male, 38) and C (male, 47) received a dose of 50 ml and person D (female, 27) took a dose of 10 ml. Stool samples were taken on days 3 to 7, days 10 to 12, and weekly for a period of 28 weeks thereafter (volunteer E was followed for 36 weeks). The volunteer study was carried out under supervision of an MD and with informed and written consent of all volunteers. The study was registered at the National Association of Statutory Health Insurance Funds (GKV Spitzenverband) Berlin, according to § 67 Abs. 6 AMG (BFarmNr. 147482). Presence of *E. coli* bacteria was determined by culture of serial stool dilutions on MacConkey agar. Symbioflor2 bacteria were detected by colony lift hybridization of these plates, using the Symbioflor2-specific hybridization probes of *Table 1*. Total *E. coli* counts were performed with probe pr1037. The colony lift protocol was performed according to Ref. [15]. Control experiments confirmed the specificity of the probes as shown in *Table 1*.

Safety evaluation following commercial use

The Periodic Safety Update Reports that had been collected in Europe according to BfArM and MedDRA pharmacovigilance regulations (www.meddra.org) were reviewed. These reports, covering a period of 6.6 years (June 2005 to December 2011) were compiled in accordance to European safety regulations and were made available by SymbioPharm GmbH.

Results

Key features of Symbioflor2 E. coli *genome sequences*

Some key features for the six sequenced *E. coli* strains comprising Symbioflor2 not available from Ref. [9] are summarized in *Table 1*. The variation in number of detected coding sequences (CDS) correlated with the differences in genome length (the genome of *E. coli* G4/9 was significantly smaller than that of the others [9]). The genome of strain G3/10 was most completely covered, although it remained in 12 contigs that could not yet be positioned relative to each other.

The presence of plasmids in these strains is noticeable, and their numbers varied between one and six. Based on the plasmid content, strains G1/2, G6/7, and G8 resembled each other, while strains G3/10, G5, and G4/9 were distinct. Four plasmids with sizes below 4 kb contained genes involved in their own replication only. One megaplasmid (pSYM1) present in strain G3/10 contained a gene coding for microcin S [9, 16], as well as conjugational protein and type 3 fimbrial proteins. Plasmid pSYM5 contained a gene for hemagglutinin, while pSYM12 contained a *mob* operon (which was also found in pSYM7) as well as a colicin S4 operon.

Table 2. The six *E. coli* strains comprising Symbioflor2

Features	G3/10	G1/2	G4/9	G5	G6/7	G8
G + C content (%)	50.89	50.74	50.69	50.80	50.73	50.67
Number of chromosomal CDS	4780	4825	4137	4410	5022	4865
Number of rRNAs	22	22	22	22	22	22
Number of tRNAs	87	90	72	84	83	80
Number of plasmid CDS	60 (pSYM1) 3 (pSYM2) 1 (pSYM3) 1 (pSYM4) 4 (pSYM5) 7 (pSYM6)	1 (pSYM10) 10 (pSYM12)	1 (pSYM4)	1 (pSYM3) 5 (pSYM7) 2 (pSYM8) 23 (pSYM9) 1 (pSYM11)	1 (pSYM10) 10 (pSYM12)	1 (pSYM10) 10 (pSYM12)

Comparison of the six Symbioflor2 strains

The protein-coding genes of the strains were deduced from the genomes, and these were binned into gene families, both within genomes and between them. Since a single gene family can contain more than one gene in a given genome, the number of gene families per genome is lower than its number of genes *(Table 3)*. Genes not finding a homolog in any other genome result in a gene family with a single member (singletons). From this comparison, the Symbioflor-specific pan-genome was defined as comprising all genes and gene families of the six strains combined; similarly, a core genome was defined as those genes and gene families conserved in all six strains *(Table 3)*. Combining all 28,180 genes of the six probiotic *E. coli* gives a pan-genome with 6486 gene families, in which there are 7281 genes extra compared to the conserved core genome of 20,899 genes; this means that only 26% of the genes found in any of the genomes is not present in all the other genomes. This is indicative of a relatively low genetic diversity between these six strains, as a set of six randomly chosen *E. coli* genomes would most likely produce a larger difference between pan-genome and core genome (Ref. [8] and O. Lukjancenko, personal communication). *Table 3* also lists the pan- and core genome after addition of 5 nonrelated *E. coli* genomes (two commensal strains, two pathogenic strains, and an environmental isolate). As expected, this increased the pan-genome more than it affected the core-genome.

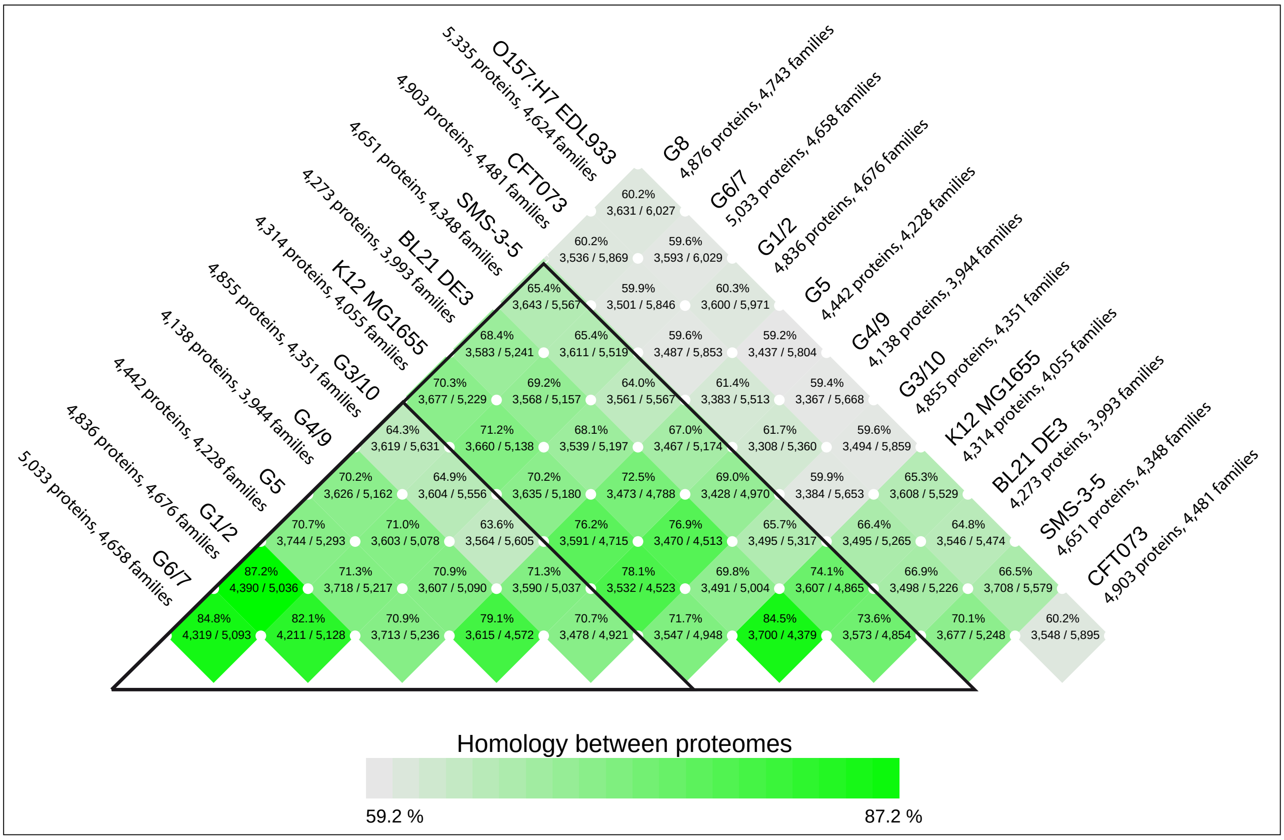
We next compared the protein coding genes of the six sequenced strains by pairwise comparison. Again, this analysis showed a high degree of similarity between the genomes, as summarized in the BLAST matrix of *Fig. 1*. The highest overlap (84.8%) between two proteomes was found between G6/7 and G8, whereas strain G3/10 was most dissimilar with the other probiotic strains, as indicated by the lighter colored row in the matrix. Nevertheless, that strain shares between 63.6% and 71.3% of protein genes with the other strains. Interestingly, these results, based on over 28,000 genes included in the analysis, corroborated the similarities observed in plasmid content summarized in *Table 2*, despite the fact that plasmid con-

Table 3. Protein genes and gene families of Symbioflor2 strains including their plasmids

	Total no. of genes	Total no. of gene families	Unique no. of genes	Unique no. of gene families
G1/2	4836	4676	211	206
G3/10	4855	4351	565	546
G4/9	4138	3944	177	175
G5	4442	4228	304	301
G6/7	5033	4658	261	261
G8	4876	4743	172	168
Symbioflor2-specific pan-genome	28,180	6486	N.A.	N.A.
Symbioflor2-specific core genome	20,899	3299	N.A.	N.A.
Pan genome after addition of 5 nonrelated *E. coli* genomes*	51,656	8344	N.A.	N.A.
Core genome after addition of 5 nonrelated *E. coli* genomes*	34.296	2,936	N.A.	N.A.

N.A.: not applicable

*For comparison, the following *E. coli* genomes were included: commensal strains K12 MG1655 and BL21 DE3, environmental isolate SMS-3-5, enterohemolytic strain O157:H5 EDL933, and uropathogenic CFT073. For details of these genomes see Ref. [8]



	G8	G6/7	G1/2	G5	G4/9	G3/10	K12 MG1655	BL21 DE3	SMS-3-5	CFT073
O157:H7 EDL933	60.2% 3,631 / 6,027	59.6% 3,593 / 6,029	60.3% 3,600 / 5,971	59.2% 3,437 / 5,804	59.4% 3,367 / 5,668	59.6% 3,494 / 5,859	65.3% 3,608 / 5,529	64.8% 3,546 / 5,474	66.5% 3,708 / 5,579	60.2% 3,548 / 5,895
CFT073	60.2% 3,536 / 5,869	59.9% 3,501 / 5,846	59.6% 3,487 / 5,853	61.4% 3,383 / 5,513	61.7% 3,308 / 5,360	59.9% 3,384 / 5,653	66.4% 3,495 / 5,265	66.9% 3,498 / 5,226	70.1% 3,677 / 5,248	
SMS-3-5	65.4% 3,643 / 5,567	65.4% 3,611 / 5,519	64.0% 3,561 / 5,567	67.0% 3,467 / 5,174	69.0% 3,428 / 4,970	65.7% 3,495 / 5,317	74.1% 3,607 / 4,865	73.6% 3,573 / 4,854		
BL21 DE3	68.4% 3,583 / 5,241	69.2% 3,568 / 5,157	68.1% 3,539 / 5,197	72.5% 3,473 / 4,788	76.9% 3,470 / 4,513	69.8% 3,491 / 5,004	84.5% 3,700 / 4,379			
K12 MG1655	70.3% 3,677 / 5,229	71.2% 3,660 / 5,138	70.2% 3,635 / 5,180	76.2% 3,591 / 4,715	78.1% 3,532 / 4,523	71.7% 3,547 / 4,948				
G3/10	64.3% 3,619 / 5,631	64.9% 3,604 / 5,556	63.6% 3,564 / 5,605	71.3% 3,590 / 5,037	70.7% 3,478 / 4,921					
G4/9	70.2% 3,626 / 5,162	71.0% 3,603 / 5,078	70.9% 3,607 / 5,090	79.1% 3,615 / 4,572						
G5	70.7% 3,744 / 5,293	71.3% 3,718 / 5,217	70.9% 3,713 / 5,236							
G1/2	87.2% 4,390 / 5,036	82.1% 4,211 / 5,128								
G6/7	84.8% 4,319 / 5,093									

Fig. 1. Comparison of the six probiotic *E. coli* and five nonrelated *E. coli* genomes. Protein gene similarity detected between the six Symbioflor2 *E. coli* strains and five other *E. coli* genomes is shown in a matrix, based on pairwise BLASTP comparisons of predicted coding sequences. The degree of similarity is shown by color intensity. Numbers within the cells represent the percentage identity between each compared pair, given by the ratio of their number of shared protein families and their total number of combined proteins. The probiotic genomes are more similar to each other (small black triangle) and to three other nonpathogenic strains (big black triangle) than they are to the pathogenic strains CFT073 and O157:H7

tent can vary over time and is generally not a good indicator of strain similarity.

Figure 2 presents a Genome Atlas of the chromosome of strain G3/10, around which blast hits are shown for genes that detected a homolog in the other Symbioflor2 genomes. From this graphical representation, it is obvious that two genome islands can be identified that are unique to strain G3/10. This exemplifies the mosaic structure of *E. coli* genomes, where islands of colocated genes are often introduced or deleted by activity of mobile elements. The island named GI-A, around 0.85 MB, contains mainly transposases and other remnants of mobile DNA elements. The island GI-B, around 2.4 MB, mainly contains remnants of a prophage. It is not surprising that the variation in protein gene content between G3/10 and the other probiotic genomes is mostly due to mobile DNA, as this is frequently observed in bacteria. The two outer lanes of the atlas show the BLAST hits detected in the proteome of commensal *E. coli* K12 MG1655 and in a pathogenic *E. coli* O157 genome. The Blast lanes in which G3/10 protein genes are compared to pathogenic O157 genes does not differ much from the other blast lanes *(Fig. 2)*. This can be explained by the relatively small genome of G3/10 compared with the much larger O157 genome: with its relatively small genome, G3/10 mostly contains proteins found in many other *E. coli* genomes. The genes that are typical for O157 are mostly absent in G3/10 and these are missed in this comparison (in total, there are 934 genes, comprising 782 gene families, present in strain O157:H7 EDL933 that are missing from the Symbioflor2-specific pan genome).

Comparison of Symbioflor2 E. coli *with other* E. coli *strains*

We aimed to assess further how unique the individual Symbioflor2 components were and compared their genomes with those of a selection of other sequenced *E. coli* strains. This selection contained both nonpathogenic (environmental) as well as pathogenic strains, of various pathotypes. In view of the large variation in gene content within this species, we applied two analyses that assessed mutually exclusive information: on the one hand, we performed a phylogenetic analysis of concatenated conserved housekeeping genes; and on the other hand, a cluster analysis of the variable gene content was carried out. For the phylogenetic analyses, seven gene fragments were selected that are frequently used for multilocus sequence typing (MLST) [17]. The results of the

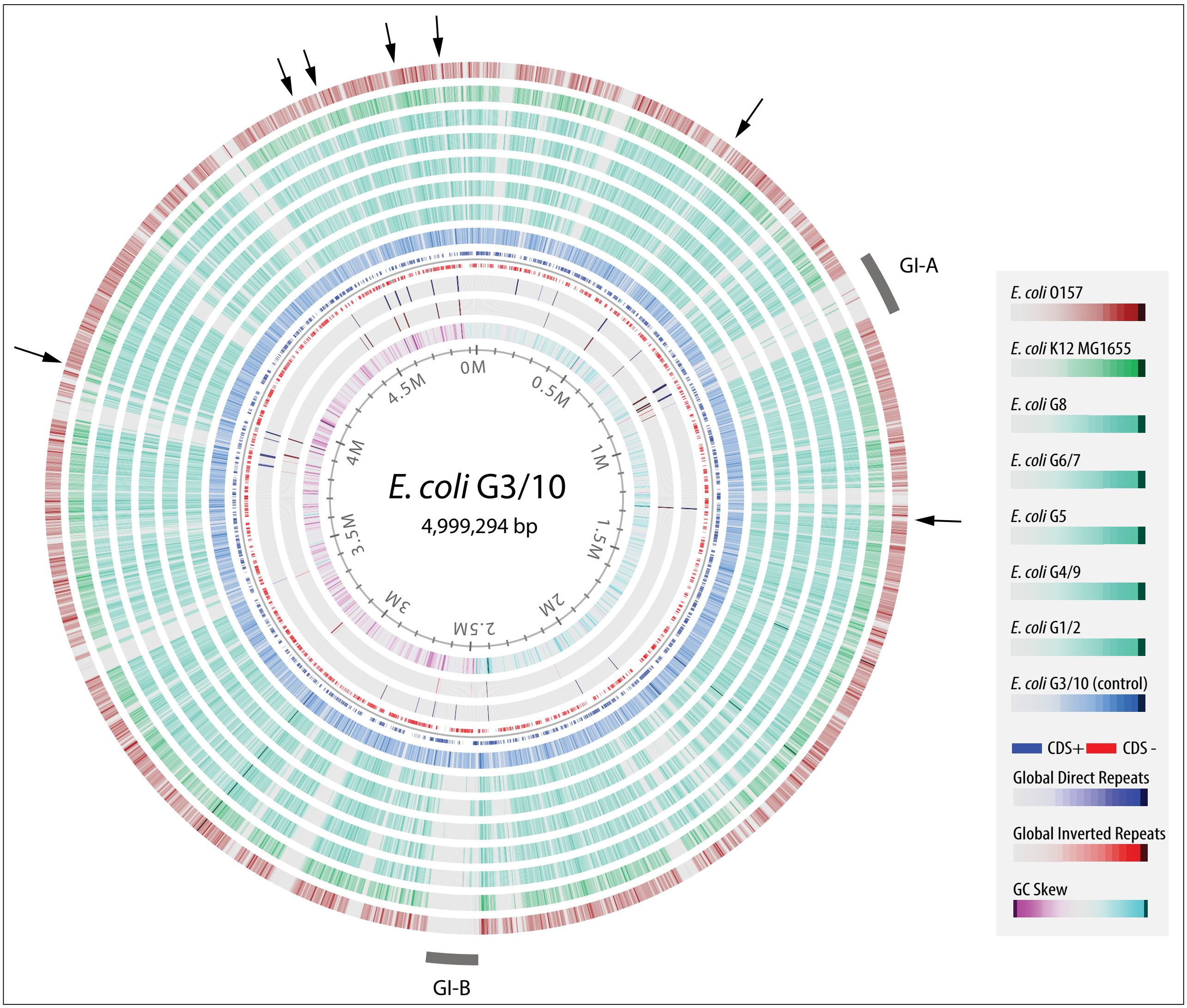


Fig. 2. Blast atlas of Symbioflor2 genomes with strain G3/10 as the reference genome. Protein genes from *E. coli* G3/10 were compared by BlastP to other genomes, including a pathogenic strain (*E. coli* O157, outward lane, in red), a commensal (*E. coli* K12 MG 1655, dark green) and the Symbioflor2 genomes, as indicated in the legend (showing the lanes from outward, at the top, to inward, at the bottom of the legend). The dark blue Blast lane shows the positive control, blasting the protein genes of strain G3/10 against itself. Two areas with genes unique to G3/10 are indicated as GI-A and GI-B. Arrowheads indicate the position of the seven ribosomal gene loci

phylogenetic analysis are shown to the left of *Fig. 3*. Four of the five Symbioflor2 strains have sequence type (ST) 10, belonging to Clonal Complex 10 (http://mlst.ucc.ie/mlst/dbs/Ecoli), while strain G3/10 has different alleles for *fumC*, *gyrB*, *icd*, and *recA*, resulting in ST472. As a result, in the tree to the left of *Fig. 3*, strain G3/10 is separated from the ST10 Symbioflor2 strains, which cluster together with commensal *E. coli* K12. The cluster analysis of nonconserved genes is shown to the right of the figure where, again, G3/10 is separated from the other Symbioflor2 strains. In this analysis, a cluster of three commensal strains is placed within the cluster that contains all Symbioflor2 strains. Although the two analyses are based on different genetic information, and the topology of the trees is substantially different, in both analyses, the Symbioflor2 components are grouped together with commensal or other nonpathogenic *E. coli* strains. Combined with the Blast results summarized in *Figs 1* and *2*, it can be concluded that these probiotic *E. coli* strains are relatively closely related to each other.

Identification of potential virulence-associated genes

The six Symbioflor genomes were screened for presence of genes encoding potential virulence factors, since presence of such genes might be considered undesirable in probiotic organisms. A comparison of their protein-coding genes to the MvirDB database [12] resulted in between 88 and 181 hits for the different Symbioflor2 genomes. In comparison, the genome of *E. coli* K-12 strain MG1655 resulted in 161 hits following the same procedure, while pathogenic strain O157:H7 produced 455, and pathogenic strain CFT073 resulted in 468 hits. The other database used to identify virulence genes, VFDB [13], produced between 34 and 80 hits for Symbioflor2 genomes, 41 hits

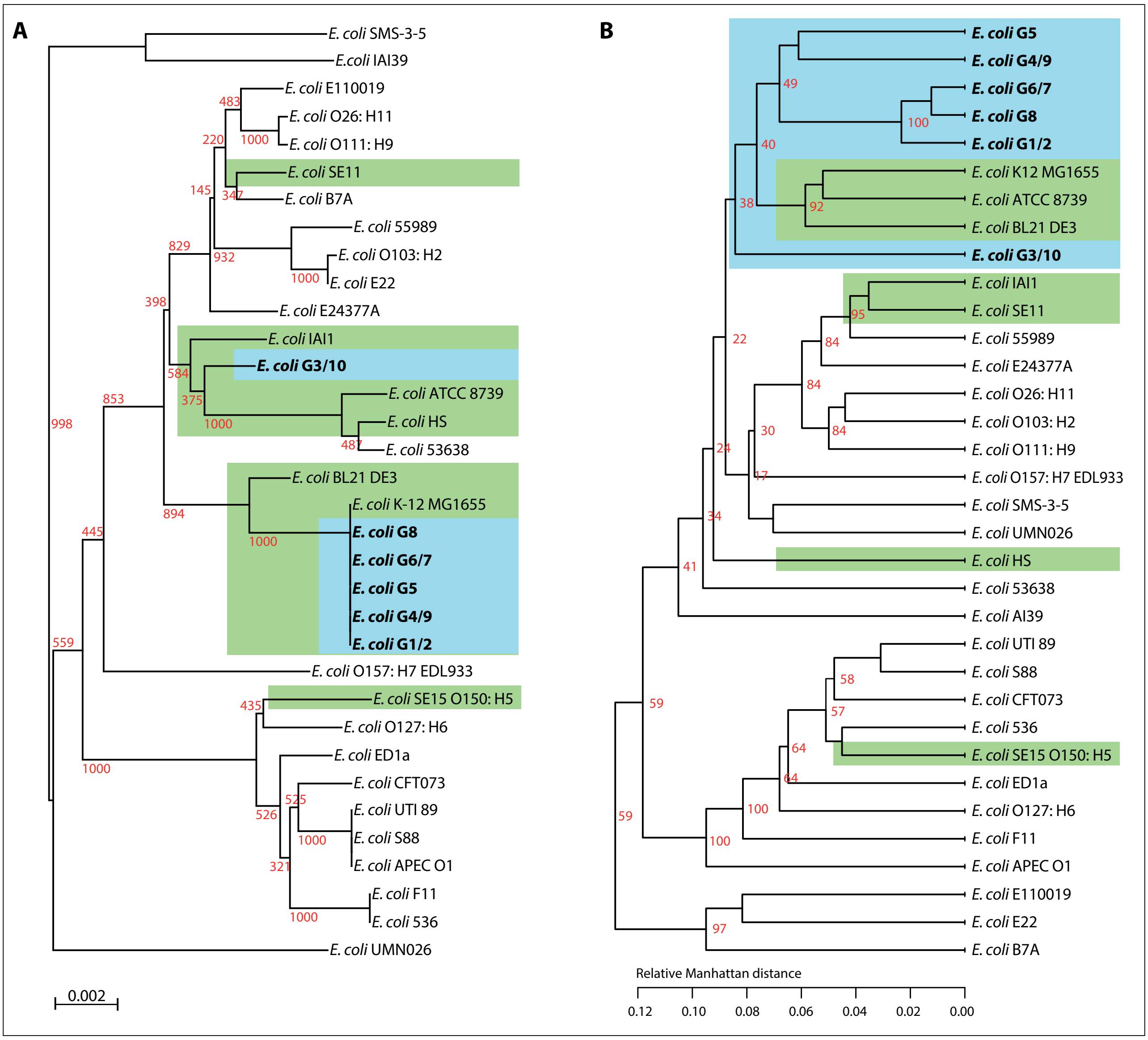


Fig. 3. Comparison of Symbioflor2 *E. coli* with commensal and pathogenic strains. In panel A, a phylogenetic tree (constructed by neighbor joining) is shown of the concatenated MLST genes *adk*, *fumC*, *icd*, *gyrB*, *mdh*, *purA*, and *recA*. In panel B, a cluster analysis of variable gene content is shown. Green shading indicates commensal *E. coli* strains (information extracted from Ref. [8] and from www.genomesonline.org). The Symbioflor2 strains are marked blue

for *E. coli* K12 strain MG1655, and 172 and 196 hits for the pathogenic O150:H7 and CFT073 strains, respectively. Thus, although pathogens contain more genes represented in these databases than nonpathogens, the latter nevertheless reports considerable hits. These findings suggest that the used databases not only contain true virulence genes, but also genes related to colonization potential or bacterial fitness; as pointed out before, there is no clear division between these [1], and such genes can be found in pathogenic and nonpathogenic strains alike. The most important findings for the Symbioflor2 genomes are summarized in *Table 4*. Toxin genes were not determined, with the exception of hemolysin: *hlyABCD* is present in strains G1/2, G6/7, and G8, as has been described before, and these strains indeed display weak hemolytic activity *in vitro*, suggesting that the operon is weakly expressed [4]. Fimbriae and their adhesins can play a role in both virulence and colonization, but their biosynthesis requires multiple genes. The presence of fimbrial genes in an incomplete genetic context is frequently observed. For instance, variable *csg* genes, responsible for production of curli fimbriae expression, are found in Symbioflor2 genomes, and when expressed, curli fimbriae are important for biofilm formation and biosynthesis of cellulose [18]. However, none of these genomes contain the complete set (notably, *csgA* is missing in all). Likewise, the regulator gene *mrkE*, required for production of type 3 fimbriae, is missing in G3/10, though the rest of the *mrk* locus is present on its large plasmid (G4/9 contains an even less complete *mrk* locus, not shown in *Table 4*). Functional flagella are often associated with virulence but commensal bacteria can also be motile, for which a complete fla-

Table 4. Genes encoding proteins responsible for colonization behavior and bacterial fitness

Gene	Description	*E. coli* G1/2	*E. coli* G6/7	*E. coli* G8	*E. coli* G5	*E. coli* G4/9	*E. coli* G3/10
hly locus	Hemolysin A (*hlyABCD*)	+	+	+	–	–	–
fim locus	Type 1 fimbriae (*fimABCDEFGHI**)	–	–	–	+	+	+
mrk locus	Type 3 fimbriae (incomplete, *mrkABCDF* but not *mrkE*)	–	–	–	–	–	+†
iuc locus	Aerobactin (*iucABCD*, *iutA*)	+	+	+	–	–	–
ent locus	Enterobactin (*entABCDEFS*)	+	+	+	+	+	+
fec locus	Iron dicitrate system (*fecABCDEIR**)	+	+	–	+	+	–
sigA	Serine protease autotransporter	+	+	+	–	–	–
upaG	Autotransporter, adhesine	–	–	–	+	+	+
iha	Adhesin and siderophore receptor	+	+	+	–	–	–
cib	Colicin IB	+	+	+	–	–	–
cka	Colicin IK	+†	+†	+†	–	–	–
cs	Colicin S4	+†	+†	+†	–	–	–
gad	Glutamate decarboxylase	+	+	+	+	+	+
eib	Immune globulin binding, increased serum survival (*eibCDEFG*)	+	+	–‡	–	+	–
iha	Enterobactin receptor/adhesin	+	+	–	–	–	–

*Only when all genes of the locus are present, this is recorded as +

†Plasmid-encoded

‡Incomplete locus is present

gellar gene set is required. Although most flagellar genes are present in the Symbioflor2 genomes, *fliC* is missing except for G3/10 and G4/9, but these two genomes lack other crucial genes to produce functional flagella (data not shown), which explains why all Symbioflor2 strains are of the H^{-} serotype.

Another group of adhesins is represented by the autotransporter subgroup of proteins. In combination with type IV-secretion mechanisms, these typically form trimeric β-barrel structures that allow transport of a passenger domain across the inner and outer bacterial membrane to reach the extracellular space, where it can bind to extracellular matrix proteins and host cells [19]. Various types of autotransporters are found in the six Symbioflor2 *E. coli* components *(Table 4)*. Autotransporter serin protease SigA is reported to be involved in bacterial pathogenicity of *Shigella flexneri*; in this organism, it encodes an exported cytopathic protease that is involved in intestinal fluid accumulation following infection [20]. Genes encoding colicins can provide a selective advantage to compete with other bacteria; three colicin genes were found present in three of the six Symbioflor2 genomes *(Table 4)*. Such genes are only related to virulence when they promote the colonization of pathogenic strains.

Of note is the observation that genes encoding resistance to antimicrobial drugs were absent from all chromosomes and plasmids.

Colonization potential of Symbioflor2 E. coli *following a single dose*

In order to identify the safety of intake of Symbioflor2 under experimental conditions, a volunteer study was performed; this would also shed light on the colonization potential of the product, since we were able to identify sequences that were specific for Symbioflor2 *E. coli*, and these sequences were used as probes to detect the bacteria from stool. Five human volunteers, who did not have Symbioflor2 strains in their stool prior to the experiment, took a single, high oral dose of Symbioflor2 on day 1. Despite a two to ten times higher intake dose compared to the daily recommended dose of $1 \cdot 10^{8}$ CFU ($2 \cdot 10^{9}$ CFU for volunteers B and E, $1 \cdot 10^{9}$ CFU for A and C, and $2 \cdot 10^{8}$ CFU for D), none of the volunteers reported any side effects; changes in stool consistency or frequency were also not reported. The stools of these volunteers were sampled from day zero onwards, to detect the total *E. coli* counts as well as the presence of Symbioflor2 *E. coli*. The results of the total *E. coli* counts are shown in *Fig. 4*. For four out of five individuals, an increase of total *E. coli* counts during the first week of monitoring was not apparent, with the exception of person E, who had no detectable *E. coli* in the stool prior to the experiment (in contrast to the other volunteers, this person had taken an antibiotic course during the 6 months prior to the experiment, though not in the 4 weeks prior to day zero). For the other volunteers

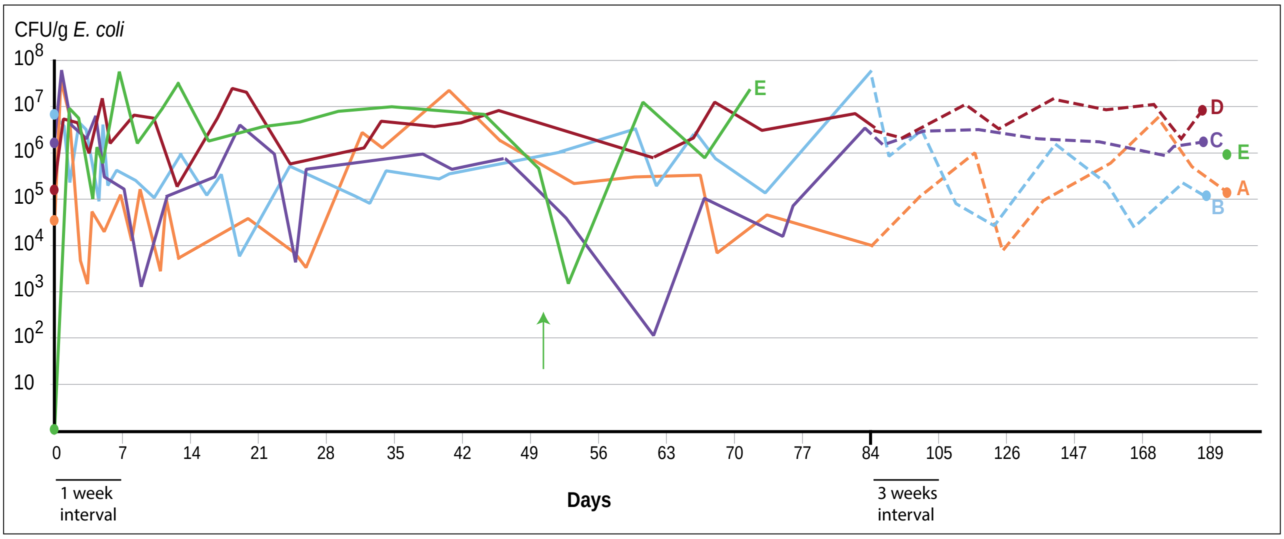


Fig. 4. Total *E. coli* counts in the stools of five volunteers who took a high single dose of Symbioflor2. The counts at day zero, before intake of the probiotic and around day 190, at the end of the experiment, are shown as dots. The time scale beyond 84 days is changed to 3 weeks intervals, indicated by the dotted curves. The arrow indicates the time-point when volunteer E (shown in green) had to take an antibiotic. The detection level was 50 CFU *E. coli* per gram stool

between $3 \cdot 10^4$ (for A) and $6 \cdot 10^6$ (B) CFU/g, *E. coli* was detected in their stools prior to the experiment (geometric mean $3.57 \cdot 10^4$ CFU/g of all five and $4.91 \cdot 10^5$ CFU/g excluding E). During the examined period, the numbers of detected *E. coli* fluctuated considerably over time for each individual, although there was a trend towards more stable counts after 28 days following ingestion of Symbioflor2. Volunteer E had to take an antibiotic course to treat sinusitis during days 47–52 of the experiment, which resulted in a dramatic drop of *E. coli* counts that reversed within 8 days. Unfortunately, for a period of 17 weeks, no samples from person E were available for analysis. The sudden drop in person C, detected at day 62, could not be explained. At the end of the experiment, around day 190, the total *E. coli* counts varied from $1.1 \cdot 10^5$ to $8.8 \cdot 10^6$ CFU/g (geometric mean $7.5 \cdot 10^5$ CFU/g).

It was investigated which proportion of the detected *E. coli* could be attributed to the Symbioflor2 intake. Three of the individual *E. coli* components of Symbioflor2 could be detected in stool with the use of specific oligonucleotide probes, while the three others (G1/2, G6/7, and G8) were detected in combination. The fractions of Symbioflor2 components of *E. coli* detected in the stool samples are shown in *Fig. 5*. As can be seen, during the first week most or all components of the probiotic mixture were detected, albeit in different amounts per individual; whereas Symbioflor2 strains represented a minority of all *E. coli* detected in person B during the first week, for the other four individuals, these comprised 80% or more (this did not correlate to the different doses). In all five persons, strains G1/2, G6/7, and G8 combined colonized persistently; in three, colonization of these three combined components was detectable even after 12 weeks. These three strains reached a colonization maximum around 3 weeks postinoculation. For person E, who had no detectable *E. coli* in her stool at day zero, following intake of an antibiotic course and a period for which data are not available, host *E. coli* but not Symbioflor2 components could be detected in weeks 28, 34, and 36.

Safety Reports collected from over six years of Symbioflor2 sales

To evaluate the safety of the product further, the Periodic Safety Update Reports that had been collected in Europe according to BfArM and MedDRA pharmacovigilance regulations were reviewed. All safety reports, compiled in accordance to European safety regulations and covering a period of 6.6 years were compiled. The recorded symptoms reported in relation to adverse drug reactions (ADR) are summarized in *Table 5*.

During the reported period over 2,125,000 treatments had been sold in Germany, Switzerland, Austria, and Hungary combined. Seventeen cases of ADR were reported, two of which were serious, according to the definitions in the International Conference on Harmonization (ICH) E2A guidelines. Temporal or spatial clustering of these 17 reported cases was not observed. The two serious cases involved: a) a 29-year old female with chronic sinusitis and allergy developed loss of smell and loss of taste after intake of Symbioflor2 for 5 months; a pharmacological relationship between the observed reactions and the administered product is unknown, and the patient's underlying chronic sinusitis and allergy provided a possible explanation for the reduced sense of smell and taste. b) A female patient of unknown age reported Quincke's oedema, sleep disorder, and agitation; information on the patient's underlying disease history was not available. The woman still suffered from sleep disorder and agitation 4 weeks after discontinuation of Symbioflor2, but in lack of a mechanistic explanation, the probiotic was an unlikely cause of

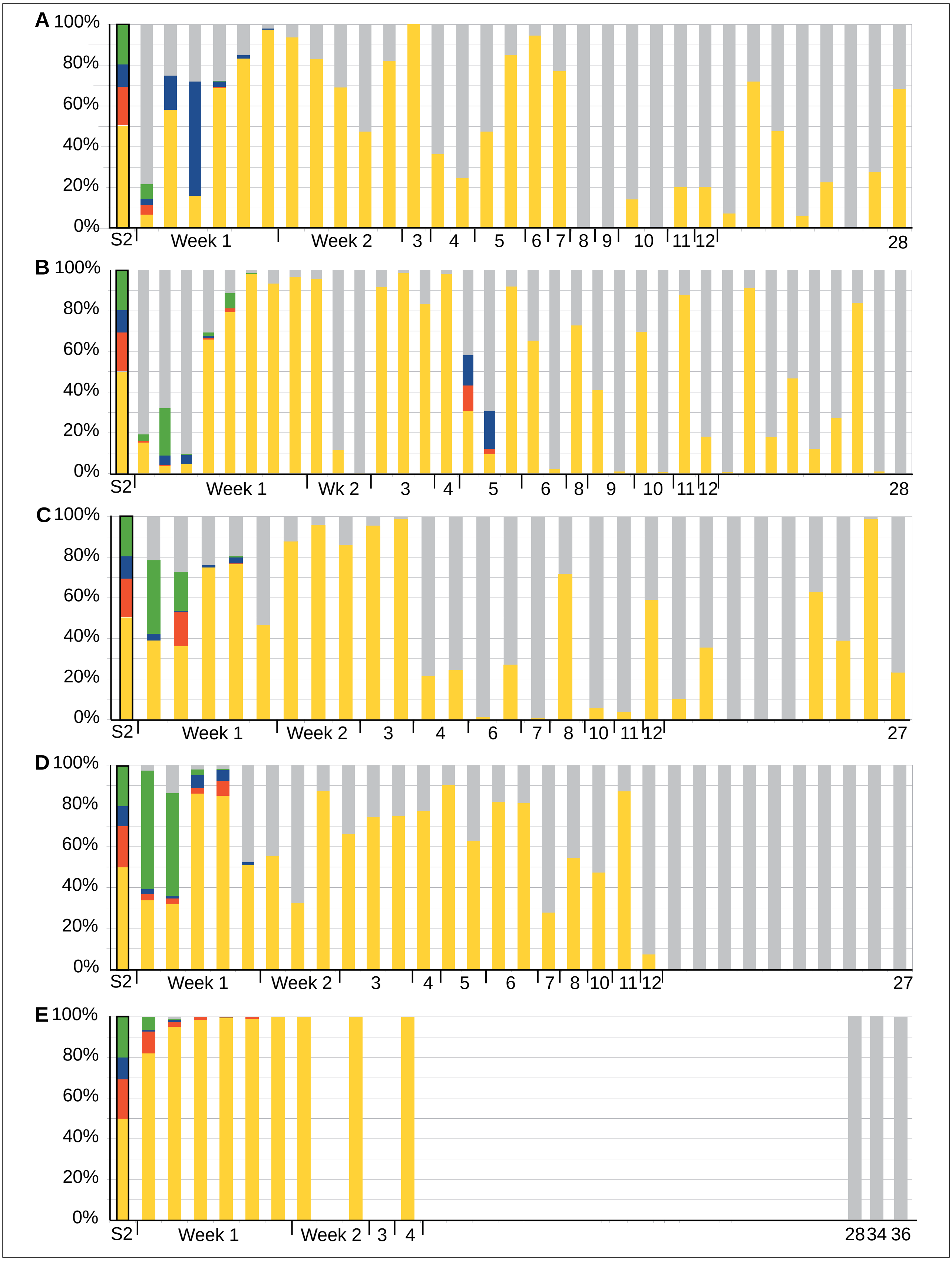


Fig. 5. Distribution of Symbioflor2 strains in stool samples after a single dose. Distribution of the components of Symbioflor2 given as percentage of total *E. coli* determined per time point, for persons A to E (top to bottom). The distribution of G1/2, G6/7, and G8 combined (yellow), G4/9 (red), G5 (blue), and G3/10 (green) that was present in the inoculum is shown to the left of each plot, marked "S2." Host *E. coli* is given in grey. For volunteer E, samples were not available for analysis between week 4 and week 28, for which reason, this person was sampled longer than the others

Table 5. Summary of all adverse drug reactions for the period June 2005 to December 2011, sorted by MedDRA system organ classes

MedDRA system organ class	MedDRA preferred term	No. of symptoms	Main events	Secondary events
Gastrointestinal disorders		**16**[*]	**8**	**8**
	Abdominal discomfort	1	0	1
	Abdominal pain	2	1	1
	Abdominal pain upper	1	0	1
	Diarrhea	3	1	2
	Flatulence	1	1	0
	Gingival bleeding	1	1	0
	Glossodynia	1	0	1
	Lip swelling	1	0	1
	Nausea	4	4	0
	Swollen tongue	1	0	1
General disorders and administration site conditions		**2**	**1**	**1**
	Drug intolerance	1	1	0
	Pyrexia	1	0	1
Immune system disorders		**1**	**1**	**0**
	Hypersensitivity	1	1	0
Nervous system disorders		**4**	**2**	**2**
	Ageusia	1	0	1
	Anosmia	1	1	0
	Burning sensation mucosal	1	0	1
	Headache	1	1	0
Psychiatric disorders		**2**	**0**	**2**
	Agitation	1	0	1
	Sleep disorder	1	0	1
Respiratory, thoracic and mediastinal disorders		**1**	**1**	**0**
	Dyspnoea	1	1	0
Skin and subcutaneous tissue disorders		**4**	**4**	**0**
	Acne	1	1	0
	Angioedema	1	1	0
	Rash	1	1	0
	Rosacea	1	1	0
Total		**30**	**17**	**13**

[*] The numbers of reported adverse reactions for each MedDRA system organ class (shown in bold) are further subdivided into the MedDRA preferred terms

these symptoms. A total of 30 symptoms were reported from the 17 case reports; most frequently recorded were nausea (four times) and diarrhea (three times). Abdominal pain was recorded twice, and 21 symptoms were recorded once each. Taken together, in consideration of 2,125,000 sold treatments, the low number of 30 adverse symptoms emphasizes the favorable safety profile of Symbioflor2.

Discussion

This study emphasizes the importance of generating a genome sequence of bacterial probiotics, although some of the observations obtained from a complete sequence blueprint may at first sight appear to be disadvantageous. The advantage of having the sequences of Symbioflor2 strains available was demonstrated, as these enabled the design of strain-specific probes, which could then be used to assess the colonization capacity of the product components. Identification of strain-specific sequences would have been impossible without the genome sequence. That the Symbioflor2 components G1/2, G6/7, and G8 could not be distinguished probably reflects the close resemblance of these three strains; they may have originated from one clone, either in the host from which all Symbioflor2 components were isolated, or during continuous subculturing in the past – this could no longer be assessed.

The genome sequences further confirmed absence of antibiotic resistance genes, an absence that is desired for a probiotic strain. However, the genome sequence also revealed the presence of a number of genes that have been linked to virulence in specific pathogens. Although some of those findings were not novel (the presence of hemolysin in three of the strains had been observed before, e.g., Ref. [4]), the discovery of other genes, such as those coding for the autotransporter SigA *(Table 4)*, was unexpected. Of course, gene presence does not guarantee gene expression. Possibly, a number of the virulence genes are not, or only weakly expressed. Moreover, the identification of virulence-associated genes that can only function in combination with other genes should be interpreted in the context of presence of those other genes; e.g., incomplete gene sets for biosynthesis of fimbriae suggest that these structures cannot be built. An automated analysis tool that could identify such incomplete gene sets would be a welcomed addition to the virulence gene database websites currently available.

The identification of putative virulence genes in the genomes of these probiotic strains is in apparent conflict to the long term clinical experience gained with Symbioflor2. Moreover, the volunteers in our study who took ten or twenty times the recommended dose did not report any symptoms or discomfort.

The finding of a complete operon for hemolysin A production in G1/2, G6/7, and G8 is particularly puzzling, although its expression *in vitro* is much lower than that observed in hemolytic pathogenic *E. coli* strains [L. Beutin (BfR, Berlin, Germany), personal communication]. Expression levels of these genes during colonization have not yet been determined. Irrespective of its *in vivo* expression levels, the presence of this pore-forming toxin can be considered problematic, in particular, since the product is recommended to patients suffering from irritable bowel disease. Recently, it was shown that α-hemolysin producing *E. coli* was frequently present in individuals suffering from ulcerative colitis (UC) or Crohn's disease, and in a mouse model, this toxin was responsible for microlesions resulting in a "leaky gut" that triggered intestinal inflammation [21]. This is in sharp contrast with the low number of reported adverse drug reactions, collected during over 6 years of commercial use of Symbioflor2. It can be expected that many UC or Crohn's patients were among these users, as the product is specifically recommended to treat these conditions. Lack of colonization potential of the hemolysin-producing components of Symbioflor2 cannot explain the absence of adverse effects, since precisely those components that produce hemolysin formed the highest fraction of colonizing Symbioflor2 and remained persistent for longest, following a single dose only. Thus, there is a sharp contrast between virulence prediction based on gene presence and *in vivo* data obtained from mouse models on the one hand and, on the other hand, the lack of adverse effects from long-term commercial use in the general population and during exposure under experimental conditions.

We conclude that the safety of the product is not questioned despite the reported findings of virulence-associated genes of *Table 4*. Applying the precautionary principle, the presence of such genes would deem these strains unsuitable for probiotic use, but that would be a mistake, in view of the reported ADR. In addition to a genome sequence, a volunteer exposure study that measures strain-dependent colonization levels and records possible side effects can aid to correctly evaluate absence of virulence.

An alternative explanation for the apparent conflict of presence of virulence genes and simultaneous safe use could be that the factors for which these genes encode may be involved in colonization potential or fitness and not in virulence per se. For pathogens, it is well known that colonization fitness contributes to virulence and *vice versa* [1, 22]. It is quite possible that similar or even identical factors that increase fitness in pathogens may improve colonization fitness in bacteria with commensal or probiotic properties. The function of "virulence" genes could be a two-sided sword. Their products may have evolved to enhance colonization in a commensal host–microbe relationship, and this is also employed by pathogens, but the evolutionary pressure may not have necessarily applied to a pathogenic relationship.

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Possible conflicts of interest

K.Z. is employed at the research department of SymbioPharm and was involved in conception of the work and writing of the article; SymbioPharm had no influence on the analyses and interpretation or presentation of data. T.M.W. works as a consultant for SymbioPharm. The other authors have no conflict of interest to declare.

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