Revealing antimicrobial resistance profile of the novel probiotic candidate *Faecalibacterium prausnitzii* DSM 17677

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Abstract

Faecalibacterium prausnitzii, a resident anaerobic bacterium commonly found in healthy gut microbiota, has been proposed as a next generation probiotic with high potential for application in food matrices and pharmaceutical formulations. Despite its recognized health benefits, detailed information regarding its antimicrobial susceptibility profile is still lacking. However, this information is crucial to determine its safety, since the absence of acquired antimicrobial resistance is required to qualify a probiotic candidate as safe for human and animal consumption. Herein, the antimicrobial susceptibility profile of *F. prausnitzii* DSM 17677 strain was evaluated by integrating both phenotypic and *in silico* data. Phenotypic antimicrobial susceptibility was evaluated by determining minimum inhibitory concentrations of 9 antimicrobials using broth microdilution and E-test® methods. Also, the whole genome of *F. prausnitzii* DSM 17677 was analysed, using several databases and bioinformatics tools, to identify possible antibiotic resistance genes (ARG), genomic islands (GI) and mobile genetic elements (MGE). With exception of erythromycin, the same classification (susceptible or resistant) was obtained in both broth microdilution and E-test® methods. Phenotypic resistance to ampicillin, gentamycin, kanamycin and streptomycin were detected, which was supported by the genomic context. Other ARG were also identified but they seem not to be expressed under the tested conditions. *Faecalibacterium prausnitzii* DSM 17677 genome contains 24 annotated genes putatively involved in resistance against the following classes of antimicrobials: aminoglycosides (such as gentamycin, kanamycin and streptomycin), macrolides (such as erythromycin), tetracyclines and lincosamides. The presence of putative ARG conferring resistance to β-lactams could only be detected using a broader homology search. The majority of these genes are not encoded within GI or MGE and no plasmids

were reported for this strain. Despite the fact that most genes are related with general resistance mechanisms, a streptomycin-specific ARG poses the only potential concern identified. This specific ARG is encoded within a GI and a MGE, meaning that it could have been laterally acquired and might be transferred to other bacteria present in the same environment. Thus, our findings provide relevant insights regarding the phenotypic and genotypic antimicrobial resistance profiles of the probiotic candidate *F. prausnitzii* DSM 17677.

Keywords

Antimicrobial resistance genes, *Faecalibacterium prausnitzii*, *in silico* analysis, safety profile, susceptibility testing.

1. Introduction

Globally, antimicrobials remain as one of the main therapeutic approaches indicated for the treatment of several infectious diseases (Aminov, 2017). Nevertheless, overprescription and misuse of such antimicrobial agents has driven the worldwide emergence, evolution and dissemination of antimicrobial resistance (AR) in pathogenic and non-pathogenic bacteria associated with humans, animals and the environment (Hernando-Amado et al., 2019; Sirichoat et al., 2020). Considering the growing emergence of AR, joined with a lack of new antimicrobial agents, AR has been considered a major global public health issue (World Health Organization, 2014). In this context, food microorganism as probiotics are now coming under scrutiny for possible involvement in the spreading of AR (Campedelli et al., 2019; Selvin et al., 2020). The existence of AR in probiotic strains could be advantageous when they are used as adjuvant of antibiotic therapy (Neut et al., 2017). However, if those determinants have an acquired nature, the consumption of probiotics pose a potential risk of dissemination in gut microbiota and in environment (Anisimova and Yarullina, 2019; Selvin et al., 2020).

Currently, several strains of the intestinal commensal bacterium *Faecalibacterium prausnitzii* are proposed as next generation probiotic candidates, mostly due to their specific bioactivities against several intestinal inflammatory conditions, which provoke interest in the application in food matrices and pharmaceutical formulations (Almeida et al., 2020; Andrade et al., 2020). Specifically, the administration of either live *F. prausnitzii* DSM 17677 or its supernatant reduced the severity of induced colitis and counterbalancing the associated dysbiosis, in a mice model (Martín et al., 2014; Sokol et al., 2008). Despite the multiple beneficial effects of these novel probiotic candidates, there is little information regarding their safety profile, namely in terms of antimicrobial

susceptibility pattern (Foditsch et al., 2014; Martín et al., 2017). In European countries, the absence of acquired AR is one of the most important selection criteria for a probiotic candidate in order to be stated as safe for human and animal consumption and, consequently, to be included in the list of qualified presumption of safety (EFSA-FEEDAP et al., 2018). For this purpose, phenotypic testing based on determination of a minimum inhibitory concentration (MIC) for a selected group of antimicrobials, as well as *in silico* detection of AR genes in whole genome sequence, should be performed (EFSA-FEEDAP et al., 2018). Notwithstanding, this methodology has not yet been widely implemented in the scope of probiotics (Rozman et al., 2020). In particular, scarce data on phenotypic and *in silico* analyses of AR in *F. prausnitzii* strains was previously reported (Bag et al., 2017; Foditsch et al., 2014; Martín et al., 2017). In this context, the present study aimed to assess antimicrobial susceptibility profile of novel probiotic candidate *F. prausnitzii* DSM 17677, using phenotypic and *in silico* methods.

2. Material and methods

2.1. Bacterial strain and growth conditions

Faecalibacterium prausnitzii strain DSM 17677 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was used for this study. It was initially cultured according to the recommended conditions proposed by DSMZ (2020). This strain was kept frozen at -80ºC in sBHI [Brain Heart Infusion medium (37g/L; VWR International, Leuven, Belgium) supplemented with yeast extract (5g/L; VWR International), hemin (5mg/L; Alfa Aesar, Kandel, Germany), vitamin K1 (5µL/L; Sigma-Aldrich, St. Louis, Missouri, USA) and L-cysteine (2g/L; Alfa Aesar)], as previously used by Maier et al. (2017), with 20% (v/v) of glycerol (Fisher Scientific, Loughborough, United Kingdom). For each assay, a *F. prausnitzii* DSM 17677 glycerol

stock was grown in sBHI for 16 h at 37 °C in an anaerobic incubator (Whitley A35 HEPA Anaerobic Workstation, Bingley, United Kingdom) under an atmosphere of 85% N_2, 5% H_2 and 10% CO_2. Afterwards, 0.1 mL of the previously grown culture was transferred to 10 mL of sBHI medium, and this bacterial suspension was incubated anaerobically for 9-10 h at 37ºC for the antimicrobial susceptibility testing.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentration of 9 clinically relevant antimicrobials recommended by EFSA-FEEDAP guidelines (2018) were determined by broth microdilution method in accordance with guidelines from Clinical and Laboratory Standards Institute (2020) and Wiegand et al. (2008), with some minor modifications. The recommended antimicrobials were: ampicillin (0.25-2 µg/mL), vancomycin (1-8 µg/mL), gentamicin (1-8 µg/mL), kanamycin (4-32 µg/mL), streptomycin (2-16 µg/mL), erythromycin (0.25-2 µg/mL), clindamycin (1-8 µg/mL), tetracycline (0.5-4 µg/mL) and chloramphenicol (1-8 µg/mL). All antimicrobials were purchased from Sigma, with the exception of chloramphenicol that was purchased from Merck (Darmstadt, Germany). The stock solutions of antimicrobials were prepared according to the manufacturer's instructions. Briefly, *F. prausnitzii* DSM 17677 grown culture in sBHI broth was used to prepare a bacterial suspension with cell concentration of 10^6 CFU/mL. Afterwards, 50 µL of two-fold serial dilutions of each antimicrobial previously prepared in sBHI broth were added to each well of a 96-well round base microtiter plate (Sarstedt, Nümbrecht, Germany). Then, each well was inoculated with 50 µL of bacterial suspension (10^6 CFU/mL) and the 96-well microtiter plates were incubated at 37 ºC during 48 h under anaerobic conditions. Moreover, a negative control, containing only sBHI medium (100 µL/well), and a positive control that contained 50 µL of bacterial

suspension (10^6 CFU/mL) with 50 µL of sBHI medium per well were included. After incubation, the MIC was evaluated and defined as the lowest antimicrobial concentration that inhibited visible bacterial growth. Additionally, MIC for these 9 antimicrobials were also determined using the E-test® (Biomérieux, Marcy-l'Etoile, France), in accordance with guidelines recommended by the supplier with some minor changes. In brief, the bacterial suspension prepared in sBHI broth, with a cell concentration of $3x10^8$ CFU/mL was spread evenly onto sBHI agar plates using a sterile cotton swab. Then, the E-test® strips were placed onto the surface of the plates that incubated under same conditions as for the broth microdilution method. After 48 h of incubation, MIC values (in µg/mL) were read where the pointed end of the ellipse intersects the strip. Both antimicrobial susceptibility tests were performed at least three independent times with two replicates in each assay. Using microbiological cut-off values established by EFSA-FEEDAP guideline, *F. prausnitzii* DSM 17677 strain was classified as susceptible or resistant to a certain antimicrobial (EFSA-FEEDAP et al., 2018).

2.3. *In silico* analysis of antibiotic resistance genes (ARG)

Genome sequences of *F. prausnitzii* strains were obtained from GenBank (Benson et al., 2018) in August 2020, in a total of 104 sequences. The presence and distribution of ARG in these genomes was investigated using PATRIC – the Pathosystems Resource Integration Center (Davis et al., 2020). PATRIC retrieves genome annotations and other curated data associated with each genome, including laboratory-derived AR phenotypes if available, host organisms, isolation sources and geographical information. For the analysis of the 104 *F. prausnitzii* genome sequences, the annotated "Speciality genes" were filtered to retrieve only the "Antibiotic Resistance" related genes, whose source

was PATRIC. The total number of hits was then subdivided into antibiotic related classes and the percentage of genes falling in each class was assessed.

PATRIC was also used to retrieve the ARG encoded within *F. prausnitzii* DSM 17677 genome (= A2-165; Accession number: NZ_CP022479.1), the strain used in the present study for the phenotypic tests, following the same criteria. Additionally, the same genome was screened using ResFinder 3.1 (Bortolaia et al., 2020) and CARD (version 3.1.0) – The Comprehensive Antibiotic Resistance Database (Alcock et al., 2019). Particularly CARD's RGI (Resistance Gene Identifier) tool (version 5.1.1) (Jia et al., 2017) was used, with homology search criteria defined to show not only "Perfect" and "Strict" matches, but also "Loose" similarities.

Genomic islands (GI), regions of probable horizontal origin, within *F. prausnitzii* DSM 17677 genome were predicted using IslandViewer4 (Bertelli et al., 2017), while possible integrative and conjugative elements (ICE) were detected using ICEberg 2.0 (Liu et al., 2019). Prophage-like sequences, putatively acquired from prophage integration within *F. prausnitzii* DSM 17677 genome, were also identified, using Prophage Hunter (Song et al., 2019) and PHASTER (Arndt et al., 2016; Zhou et al., 2011) web tools. The guanine/cytosine (GC) content was evaluated using the genome browser Artemis (version 16.0.0) (Carver et al., 2012). A representation of the full genome, including all the delimited GI, integrative elements, prophage-like sequences, as well as a GC distribution graphic, was constructed using SnapGene Viewer software (from Insightful Science; available at snapgene.com).

3. Results and discussion

Antimicrobial resistance in stablished and potential probiotic strains does not constitute a safety concern *per se*, when the resistance mechanisms responsible for the resistant

phenotype is intrinsic (EFSA-FEEDAP et al., 2018; Gueimonde et al., 2013). In fact, EFSA-FEEDAP reported that only the presence of acquired AR genes coding for, or contributing to, resistance to clinically relevant antimicrobials in those microorganisms to be incorporated in foods or feeds should be considered a hazard (EFSA-FEEDAP et al., 2018). In the present study, the antimicrobial susceptibility profile of *F. prausnitzii* DSM 17677 strain was evaluated using phenotypic and *in silico* approaches.

3.1. Phenotypic antimicrobial susceptibility profile

The MIC values of 9 clinically relevant antimicrobials determined using the broth microdilution and E-test® methods are displayed in Table 1. Phenotypic antimicrobial susceptibility profile of *F. prausnitzii* DSM 17677 was stablished according to microbiological cut-off values available in the EFSA-FEEDAP guideline (2018) that covers the characterisation of microorganisms used as feed additives or as production organisms in European Union. Considering the microbiological cut-off values for *Corynebacterium* and other Gram positive bacteria from EFSA-FEEDAP guidelines (2018), *F. prausnitzii* DSM 17677 strain was susceptible to vancomycin, clindamycin, tetracycline and chloramphenicol. In contrast, this strain was resistant to ampicillin, gentamicin, kanamycin and streptomycin. Only erythromycin failed to meet the same classification in both methods, with broth microdilution technique classifying the bacterial strain as resistant (MIC higher than 2 µg/mL) while the E-test® method classified the strain as susceptible (MIC value ranging 0.5-1 µg/mL). Indeed, disagreements in the classification of microbial species using different antimicrobial susceptibility testing methods have been reported in the literature (Kulengowski et al., 2019; Matuschek et al., 2018). However, it is important to note that broth microdilution

method is widely accepted as reference method for antimicrobial susceptibility testing (Haeili et al., 2019; Hakvoort et al., 2020; Humphries et al., 2018).

Regarding ampicillin E-test®, it was possible to observe a phenotypic heterogeneity in which small colonies grew within the broader inhibition zone, throughout a range of ampicillin concentrations. According to E-test® guidelines, the inner inhibition zone, where no colonies were observed, was considered for the determination of the resistance phenotype (Table 1), although considering either of them would result in the same classification (EFSA-FEEDAP cut-off: 1 µg/mL). This occurrence can be related with the phenomenon of heteroresistance, which describes the ability of a pre-existing subpopulation of cells, within an homogeneous culture, to show increased levels of resistance compared with the main population (Andersson et al., 2019; Band and Weiss, 2019). Heteroresistance was already reported for various classes of antimicrobials, including β-lactams. The mechanisms underlying this phenomenon are still unclear, but it is assumed that they are related with differentially expressed, mutated or even duplicated ARG within the same population (El-Halfawy and Valvano, 2015). Although it can sometimes influence the result of an antimicrobial clinical treatment, this is usually a transient characteristic, which is reverted upon removal of the antimicrobial selective pressure (Andersson et al., 2019; Band and Weiss, 2019).

To the best of our knowledge, to date, only two studies had evaluated the antimicrobial susceptibility profile of *F. prausnitzii* strains (Foditsch et al., 2014; Martín et al., 2017). In fact, Foditsch et al. (2014) studied the antimicrobial susceptibility profile of several *F. prausnitzii* strains isolated from fecal samples of healthy calves and piglets. Using the E-test® on VTR2RF agar plates, these researchers verified that all isolates were resistant to ciprofloxacin and sulfamethoxazole-trimethoprim, more than 50% of isolates were resistant to tetracycline, cefepime and cefoxitin; 34.5% were resistant to

ceftriaxone; 27.6% were resistant to ampicillin. Around 55.2% of the isolates grew in the highest concentration of amikacin tested and only 1 isolate was resistant to gentamicin, being all isolates susceptible to chloramphenicol (Foditsch et al., 2014). Later, Martín et al. (2017) tested the antimicrobial susceptibility to tetracycline, kanamycin, chloramphenicol, linezolid, quinupristin/dalfopristin, trimethoprim, gentamicin, erythromycin, cefpirome, clindamycin, streptomycin, vancomycin, and ampicillin for *F. prausnitzii* DSM 17677 strain and other *F. prausnitzii* strains isolated from human healthy volunteers using the E-test® assay on Wilkins-Chalgren agar. Among the 9 antimicrobials recommended by EFSA-FEEDAP to test, these researchers showed that *F. prausnitzii* DSM 17677 strain was only resistant to streptomycin, contrasting with our results. These discrepancies may be explained by the different growth conditions, specifically growth medium composition (sBHI agar in our work *vs* Wilkins-Chalgren agar in Martín et al. work) or in incubation atmosphere (85% N_2, 10% CO_2 and 5% H_2 in our work *vs* 90% N_2, 5% CO_2 and 5% H_2 in Martín et al. work). Indeed, growth conditions, such as media composition or CO_2 content in incubation, have been reported as important factors influencing the antimicrobial susceptibility of several bacterial strains (De Vecchi et al., 2008; Loose et al., 2020). Underlining this rationale, EFSA-FEEDAP alerts to potential interference of medium components, test type (broth microdilution *vs* agar dilution), and culture conditions (pH, temperature, time of incubation) on the antimicrobial susceptibility profile (EFSA-FEEDAP et al., 2018).

3.2. Prevalence of ARG within the genomes of *F. prausnitzii* species

Genotypic approaches have been used as a valuable complement to the phenotypic antimicrobial resistance studies for several years (Courvalin, 1991). Homology-based

genome searches can be used to predict ARG and infer a possible resistance profile (Brinkac et al., 2017). The major advantage of such approach is that it allows the detection of virtually any known ARG and, the identification of new variants of these genetic determinants. However, this also constitutes the major pitfall of any genotypic approach: only known mechanisms can be detected, while resistance caused by new mechanisms or resulting from gene expression modulation might remain unnoticed. Yet, sequence data can be store indefinitely and be re-analysed when new ARG are phenotypically characterized (Anjum et al., 2017).

In this study, the genomic information regarding possible ARG was gathered, starting with a brief overview of the *F. prausnitzii* genomes deposited so far. The 104 *F. prausnitzii* genomes (deposited until August 2020) were analysed using PATRIC, to identify ARG. PATRIC integrates metadata publicly available from genomes deposited in GenBank (Benson et al., 2018) and RefSeq (Pruitt et al., 2012). Each entry is also annotated using RAST (Rapid Annotations using Subsystems Technology) (Aziz et al., 2008), while maintaining the original GenBank or RefSeq annotations. Thus, PATRIC allows an integrated and parallel analysis of genes of interest across the various platforms (Wattam et al., 2014). More recently, and given the increasing importance of studying the widespread phenomena of AR, PATRIC has evolved to facilitate the analysis of ARG (Antonopoulos et al., 2019). A total number of 2324 ARG were identified across all the genomes analysed, conferring resistance to 19 classes of antimicrobial compounds (Supplementary Table A.1). On average, each genome possesses 23 annotated resistance genes, but the effective number of ARG ranges from 2 (*F. prausnitzii* strain ATCC 27766) to 43 (*F. prausnitzii* strain AHMP21) (Supplementary Table A.2). Regarding the antibiotic classes, the most abundant ARG confer resistance to peptide antibiotics (15%), followed by triclosans (13%) and

285 aminoglycosides (11%) (Fig. 1). The antibiotic classes with only a few ARG detected
286 include β-lactams (4 ARG detected), glycopeptides (4), chloramphenicol (3) and
287 streptogramin (1) (Fig. 1 and Supplementary Table A.3).
288 The human microbiome has been considered as a large reservoir of known and new
289 ARG, including some genes that can be genetically distant from known genes (Rolain,
290 2013; Rolain et al., 2016). Antibiotic production and resistance development are natural
291 mechanisms that provide evolutionary advantages to microorganisms in competitive
292 environments, including the human gut (Rolain, 2013). Just like the gut microbiota, the
293 respective resistome is highly variable, depending on the eating habits and environment
294 exposure. Consequently, strains will have a diverse ARG pattern depending on the
295 source where they were isolated from (Baron et al., 2018). The ARG most frequently
296 reported in the human gut microbiome are those related with resistance to tetracycline,
297 β-lactams, aminoglycosides, glycopeptides, chloramphenicol and macrolides (Baron et
298 al., 2018). Consistently, all of these classes could also be found in *F. prausnitzii*
299 genomes: 41 genomes possess one or more ARG putatively conferring resistance to
300 aminoglycosides; 37 possess one or more tetracycline resistance genes and 19 possess
301 macrolide resistance genes (Supplementary Table A.3). Although less represented, β-
302 lactams (4), chloramphenicol (3) and glycopeptides (2) resistance genes could also be
303 found in a small number of genomes (Supplementary Table A.3), which might indicate
304 that they do not belong to the usual resistome of *F. prausnitzii* species. In this context, it
305 is important to distinguish between intrinsic and acquired ARG. The first is usually
306 encoded within the chromosome and includes non-specific mechanisms that might
307 mediate the resistance phenotypes against several classes of antibiotics, such as non-
308 specific efflux pumps and inactivating enzymes (Peterson and Kaur, 2018). Conversely,
309 acquired ARG are usually associated with horizontal gene transfer (HGT) mechanisms

through mobile elements, and involve mechanisms of resistance that specifically target a single antibiotic or class of antibiotics, including enzymes that modify the antibiotic or the antibiotic target (Peterson and Kaur, 2018). Most ARG identified within *F. prausnitzii* genomes are associated with general targets within the species or unspecific mechanisms of resistance (Table 2). Hence, it can be presumed that they consist mainly of intrinsic mechanisms of resistance, associated with the species, rather than resistance genes acquired by HGT.

The high incidence of peptide antibiotics resistance genes can be explained by their wide structural and functional diversity, which is most likely due to their ubiquitous production in all domains of life – including prokaryotes, animals and plants –, and as well as being an immune-modulatory component of the human innate defence system in aiding pathogen clearance (Abdi et al., 2019; Mahlapuu et al., 2016). Thus, it is expectable that commensal bacteria develop their own resistance mechanisms, to adapt and successfully establish within the hosts' organism, which otherwise could be harmful to them (Baron et al., 2018). Notably, the mechanisms involved in the resistance to this kind of antibiotics are general resistance mechanisms, such as non-specific efflux pumps or membrane alterations (Table 2). This means that they are not specifically targeting a given compound, which, as mentioned, constitutes an indication of an intrinsic resistance rather than an acquired trait (Peterson and Kaur, 2018).

The present *in silico* data lacks phenotypic confirmation, since phenotypic data regarding this species is not available, except for the type-strain *F. prausnitzii* A2-165 (= DSM 17677) and a few other isolates (this study and references Foditsch et al. (2014); Martín et al. (2017)).

3.3. ARG and putative mobile elements within *F. prausnitzii* DSM 17677 genome

In silico analysis is becoming a major tool in antibiotic resistance surveillance, allowing the prediction of the potential resistance profile of a given strain. Indeed, EFSA-FEEDAP guidelines encourage the use of this approach to characterize possible resistance profiles of new candidate strains that are to be applied for human consumption (EFSA-FEEDAP et al., 2018). In this way, alongside the phenotypic characterization, *F. prausnitzii* DSM 17677 genome was also analysed in more detail using PATRIC (Antonopoulos et al., 2019). A total of 24 ARG hits were obtained in PATRIC, including genes that might mediate resistance against aminoglycosides, macrolides, tetracycline and lincosamides (Supplementary Table B.1). Thus, there is genomic evidence supporting the phenotypic observation of resistance to gentamycin, kanamycin and streptomycin (aminoglycosides) and erythromycin (belonging to macrolide class) (Table 1). No phenotypic evidence of resistance was observed for tetracycline and clindamycin (from lincosamides class). Notice that the presence of a specific ARG in a bacterial strain does not necessarily translate into the expression of a resistant phenotype, for various reasons: *i)* the gene itself can be truncated; *ii)* it might not be expressed in the growth conditions; *iii)* the product of a specific gene might only provide a low level of resistance to the strain, thus, not reaching the cut-off values defined by EFSA-FEEDAP to classify it as a resistant strain (EFSA-FEEDAP et al., 2018). Additionally, the whole principle of ARG search and annotation relies on homology-based searches, therefore, it might not be possible to ensure that the product of the detected ARG sequence retains the function of the original query sequence (Brinkac et al., 2017). This variability might also explain the inconsistency observed for erythromycin, between broth microdilution and E-test® methods. Erythromycin related ARG are associated with non-specific resistance mechanisms, and thus, they might be differentially expressed under the test conditions for each method.

In addition, ResFinder, which allows the detection of acquired ARG within genomes, using a defined threshold of 98.00% identity, was also used to detect putative ARG. Thus, it detects whole resistance genes, although not providing information regarding their functional integrity and expression (Zankari et al., 2013). From the annotated genes retrieved by PATRIC, only aminoglycoside-encoded resistance was also confirmed by ResFinder (Supplementary Table B.1). This might indicate that resistance to streptomycin could have been acquired via HGT.

Interestingly, ARG putatively conferring resistance to β-lactams, which could explain ampicillin-resistance phenotype, were not identified within *F. prausnitzii* DSM 17677 genome, using neither PATRIC nor ResFinder (Supplementary Table B.1). To understand if other resistance genes are present within this genome, an homology-based search was performed in CARD database, using Resistance Gene Identifier (RGI) tool (Alcock et al., 2019). CARD contains a large, continuously curated, database of molecular sequence reference data for prediction of AR genotype from genomic data. This includes intrinsic resistance, dedicated resistance genes, and acquisition of resistance via mutation of antimicrobial targets and associated elements (Jia et al., 2017). The newly added RGI software predicts ARG from genomes, including non-annotated sequences, using CARD-deposited ARG as query sequences (Alcock et al., 2019). The RGI software analyses genome sequences under three paradigms: “Perfect”, “Strict”, and “Loose”. The “Perfect” algorithm detects ARG with an exact (100%) match to a CARD reference sequence and it is most often applied to clinical surveillance. The “Strict” algorithm is more flexible, allowing some variation from the CARD reference sequence, as long as the sequence falls within the curated BLAST cut-off, to ensure that the detected variant is likely a functional ARG. This algorithm is useful to detect previously unknown variants of ARG or antibiotic targets altered via

mutation. Finally, the "Loose" algorithm works outside the detection model cut-off, thus allowing the detection of new, emergent threats and more distant homologs of ARG, as well as homologous partial sequences that may not have a role in AR. "Loose" algorithm, also known as "Discovery", when combined with phenotypic screening allows the discovery of novel ARG (Alcock et al., 2019; Jia et al., 2017). Although no "Perfect" or "Strict" matches could be identified, a total of 263 "Loose" hits were retrieved by RGI software upon homology-based search within *F. prausnitzii* DSM 17677 genome (Supplementary Table B.2). The absence of "Perfect" or "Strict" hits is not surprising, considering the novelty of the antibiotic resistance profiling in this species. As postulated, the "Loose" algorithm allowed the detection of otherwise unnoticed genes, which might represent new variants of previously known resistance genes. The "Loose" matches comprise the ARG previously identified in PATRIC and other new hits, which include 25 ARG related with β-lactams resistance (Supplementary Table B.2, highlighted). The majority of these β-lactam-related ARG are involved in general resistance mechanisms, such as unspecific efflux pumps or antibiotic target alteration, while only 9 encode putative β-lactamases, responsible for inactivating the antibiotic itself (Supplementary Table B.2) (Docquier and Mangani, 2018). Together with the phenotypic results, it is possible to infer that one, or more, of these mechanisms are active and thus, confer resistance to ampicillin. The presence of more than one mechanism and the possibility of their differential expression, might also explain the heterogeneous resistance phenotype observed in the E-test® method for ampicillin, with the occurrence of isolated colonies within the defined inhibition zone. As β-lactam resistance does not seem to be a characteristic spread among the *F. prausnitzii* species, the emergence of this phenotype has to be carefully considered and further analysed, as it might constitute an acquired trait.

Although ARG *per se* might be worrisome under the lights of the worldwide spread antibiotic resistance problematic, the HGT of such ARG constitutes a major issue when trying to control the spreading across bacterial species (McInnes et al., 2020; Sun et al., 2019). In fact, HGT occurrence was already documented between commensal and opportunistic bacteria in human gut. The gut's high bacterial cell density provides an environment favourable to conjugation events, which, in turn, promotes the spreading of plasmids and integrative and conjugative elements (ICE) carrying ARG (McInnes et al., 2020). Indeed, EFSA-FEEDAP guidelines consider acquired resistance of major concern when evaluating the potential application of a novel probiotic for human consumption (EFSA-FEEDAP et al., 2018). No plasmids were reported so far for this strain, thus, excluding that vector as a source if mobilizable ARG. To understand the ARG transferability potential in *F. prausnitzii* DSM 176777, ICEfinder tool from IceBerg 2.0 software, was used to predict putative ICE regions within its genome. ICE regions usually contain recombination and conjugation modules, and might even mobilize other genetic elements encoded nearby such as chromosome-borne integrative and mobilizable elements (IME) (Liu et al., 2019). Seven putative integrative regions were identified within *F. prausnitzii* DSM 17677 genome (Supplementary Table B.3; Fig. 2). Of particular interest is the presence of a β-lactamase-encoding gene embedded in region 2 (IME) and the annotated ANT(6)-Ib, putatively conferring resistance to the aminoglycoside streptomycin, within region 6 (ICE) (Supplementary Table B.3; Fig. 2). While the first might require additional elements to be mobilized, the second might pose some risk of genetic transferability and should be further analysed. This also confirms what was observed using ResFinder, regarding the possible streptomycin acquired resistance.

In parallel, the presence of putative GI was also assessed using IslandViewer (Bertelli et al., 2017). Genomic islands are defined as clusters of consecutive genes, differing between closely related strains, likely acquired by HGT in bacterial genomes. This designation refers to genome regions that might be mobile, non-mobile or might have lost their mobility through their evolution. In the first case, several types of mobile genetic elements might be present, which include ICE, among others (Juhas et al., 2009). Genomic Islands usually provide an advantage in a selective environment in terms of bacterial adaptation and fitness. Depending on their origin and evolution, GI usually contain a variation on the GC content when compared with the average GC content of the chromosome where they are included. Also, GI usually include mobility genes and insertion sequences that promote GI integration in the "host's" core chromosome (Bertelli et al., 2019, 2017; da Silva Filho et al., 2018). Thus, IslandViewer 4.0 webserver integrates complementary GI prediction tools, which are based in the nucleotide bias, the presence of mobility genes and comparative genomics, to provide accurate and integrated GI predictions (Bertelli et al., 2017). IslandViewer predicted a total of 69 GI, some of them overlapping each other (Fig. 2A; Supplementary Table B.4). Notice the number of GI that is embedded into the previously predicted ICE regions, thus, confirming a certain past and/or present mobility of these sequences: they were putatively acquired from other genome(s) and have the potential to be laterally transferred to other bacteria, if ideal conditions are provided. Another evidence of the lateral acquisition of the GI is presented in Fig. 2B, where the GI (orange bars on the in the bottom) coincide with regions of lower GC content, compared with the average GC content of the chromosome. Putative acquired/mobilizable genomic regions can also be transferred due to the action of bacteriophages. Also known as phages, they play a major role in HGT mediation, since

459 they can be found in almost every microbial community (Casjens, 2003; Chiang et al.,
460 2019). Thus, bacteriophages can affect the bacterial gene content, contributing to the
461 genome plasticity through the acquisition and selection of genetic information that
462 might confer some evolutionary advantage, including virulence factors or ARG
463 (Ramisetty and Sudhakari, 2019). A prophage is defined as the latent form of a
464 bacteriophage, in which the viral genes are integrated in the bacterial cell – either in the
465 chromosome or an extrachromosomal plasmid – without causing the disruption of the
466 cell (Fortier and Sekulovic, 2013). If the prophage remains intact, it can be induced, due
467 to environmental stressors or randomly, to excise and replicate, thus undergoing the
468 lytic cycle (Chiang et al., 2019; Ramisetty and Sudhakari, 2019). During this process,
469 the newly formed viral particle might also include genes from the host, thus
470 contributing to HGT of between bacterial genomes (Casjens, 2003; Loh et al., 2020).
471 The occurrence of possible prophage sequences was also assessed in the present work,
472 using several web tools, starting with Prophage Hunter (Song et al., 2019). This online
473 platform uses a machine learning approach that allows the identification of sequences
474 putatively encoding prophages. It also provides a scoring method for categorizing each
475 prophage as “active”, “ambiguous” or “inactive”. Prophages identified as “active”
476 exhibit the complete genomic sequence and thus can be induced to produce phage
477 particles, while “ambiguous” prophages designate sequences that are somehow
478 incomplete, including mutated or truncated genes (Loh et al., 2020; Song et al., 2019).
479 As such, from a total of 81 prophage sequences candidate that were identified, 7 of them
480 were classified as “active” prophages (Supplementary Table B.5). A similar analysis
481 using PHASTER web tool, which is an integrated search and annotation tool to identify
482 and annotate putative prophage gene clusters, as well as to evaluate the completeness of
483 these putative prophages (Zhou et al., 2011). Six putative prophage-like regions were

identified, that were already identified using Prophage Hunter (Supplementary Table B.6). Although 3 of them are classified as "active" by Prophage Hunter, only one is expected to be complete according with PHASTER (Fig. 1 and Supplementary Table B.6). A closer analysis revealed the absence of putative ARG embedded in this prophage region. It is important to stress, that a prophage that is classified as "active" or "intact" might not be prone to produce phage particles, as they can be false positives – homology search does not ensure functionality – or they might lack the proper induction factor (Loh et al., 2020). However, even if this event does occur, the absence of embedded ARG reduces the possibility of antimicrobial resistance spreading to other species within the environment.

Altogether, it is possible to conclude that ARG conferring resistance to β-lactams are most likely acquired (Supplementary Table B.7). Also, some of the identified putative β-lactamases have the potential to be transferred to other bacteria, as they are encoded within putative ICE (Supplementary Table B.7) and should be regarded with caution in further applications. This is consistent with the low number of *F. prausnitzii* strains that possess β-lactams-related ARG (see previous section). The multiple ARG that can be involved in this resistance phenotype, might also explain the existence of a subpopulation with heterogeneous resistance phenotype in the E-test® method.

However, the highest concern is related with the resistance to streptomycin, mediated by a specific mechanism of antibiotic inactivation. In addition, there is a low prevalence of this specific ARG in other *F. prausnitzii* strains, as shown in the previous section. This was also confirmed by a BLAST search against all deposited *F. prausnitzii* genomes (taxid: 853), using either the DNA or the protein sequences, obtained from CARD as query, which retrieved only two hits (data not shown), one of them corresponding to *F. prausnitzii* A2-165 (= DSM 17677), as expected. In addition, this streptomycin-related

ARG belongs to an ICE and a GI. Associated with the phenotypic outcome of resistance to streptomycin, this poses the only considerable risk to a potential application of *F. prausnitzii* DSM 17677 as a probiotic strain, according to EFSA guidelines. However, it is worth mentioning that resistance to streptomycin has already been reported for probiotic candidates *Lactobacillus paragasseri* K7 and *Bifidobacterium animalis* subsp. *lactis* Bl-04 (Rozman et al., 2020), as well as in other gut-derived *Bifidobacterium* strains, which belong to species already classified as safe by the qualified presumption of safety list (Duranti et al., 2017).

Besides the presence of MGE, the transferability of chromosomal regions containing ARG also depends on physical conditions, such as the occurrence of high bacterial cell density or selective pressure, due to the presence of a specific antibiotic. In fact, it is very unlikely that, without antibiotic selective pressure, ARG will be acquired and, specially, fixed, in a given population, since these processes come with a fitness cost for the acquiring-bacteria (Martínez, 2012). Although the human gut can provide the required proximity between commensal/probiotic strains, possessing putative ARG, and pathogenic bacteria, the transferability of these ARG highly depends on the antibiotic pressure selectivity. Regarding streptomycin, this antibiotic is poorly absorbed by the gastrointestinal tract and usually administered by intramuscular injections (except for the treatment gastrointestinal infections). Also, due to its secondary effects, streptomycin is being gradually replaced for other aminoglycosides with less toxic effects for the patients (Grosset and Singer, 2013). Thus, it seems reasonable to expect that the presence of streptomycin resistance gene encoded within MGE does not pose a significant risk of ARG transferability, given the low probability of this antibiotic to induce this phenomenon.

4. Conclusions

Our study provides novel and important data regarding the phenotypic and genotypic AR profiles of probiotic candidate *F. prausnitzii* DSM 17677. Such information is a paramount requirement for acceptance of this bacterial strain as a probiotic, allowing its incorporation in nutraceutical and pharmaceutical products. Despite minor inconsistencies in the results obtained by broth microdilution and E-test® methods, a phenotypic AR profile was established, which could also be directly linked to the genomic context. Furthermore, other ARG were detected that did not result in a resistance phenotype. Nevertheless, most of detected ARG are related to general resistance mechanisms and are not included within GI or MGE that allied with the absence of plasmids, reduce the AR transferability risk. The only potential concern identified relates with the streptomycin-specific ARG, which is encoded within an MGE and belongs to a GI, which increases the likelihoods of acquired resistance. To meet the criteria of the qualified presumption of safety status that requires the absence of acquired ARG, future research on the transferability and the related mechanisms is urgently needed to clarify the effective risk of the presence of streptomycin ARG in *F. prausnitzii* DSM 17677. Furthermore, the establishment of a standardized protocol for phenotypic AR profiling would be relevant to enable comparative studies among researchers in food microbiology. Also, further studies involving other antimicrobials clinically relevant for humans mentioned in other important guidelines such as CLSI and EUCAST, together with the employment of quality control strains during phenotypic antimicrobial susceptibility testing, would help deeper safety assessment of *F. prausnitzii* DSM 17677 prior its use in food/feed products.

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Declarations of interest

None.

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Figure Captions

Fig. 1. Percentage of antibiotic resistance genes detected in 104 *Faecalibacterium prausnitzii* genomes, divided by antibiotic classes.

Fig. 2. *Faecalibacterium prausnitzii* DSM 17677 genome map. (A) represents the whole chromosome, with ICE (in blue), GI (in red) and Prophage-like region (in green), highlighted in the external circles; possible ARG, identified by CARD are represented inside the chromosome circle: in blue, ARG confirmed with PATRIC/ResFinder; in orange, CARD ARG encoded with an ICE and an GI; in purple, ARG encoded only within the ICE. (B) GI (orange bars) and Prophage-like region (green bar) representation and GC content across the chromosome, with colour coded scheme, to ease the correlation. Images produced in SnapGene Viewer.

849 Table 1. Antimicrobial susceptibility profile of *Faecalibacterium prausnitzii* DSM 17677

Antimicrobial	MIC (µg/mL) in broth microdilution	MIC (µg/mL) in E-test®	EFSA-FEEDAP cut-off values (µg/mL)
Ampicillin	**> 2**	**> 3**	1
Vancomycin	≤ 1	0.25 - 0.5	4
Gentamicin	**> 8**	**12**	4
Kanamycin	**> 32**	**16 - 32**	16
Streptomycin	**> 16**	**≥1024**	8
Erythromycin	**> 2**	0.5-1	1
Clindamycin	≤ 1	≤ 0.016	4
Tetracycline	≤ 0.5	0.023-0.047	2
Chloramphenicol	2-4	1-1.5	4

850 Note: Resistances are indicated in bold i.e., MIC exceeding EFSA-FEEDAP cut-off
851 values.
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855

856 Table 2. Putative ARG detected within 104 *F. prausnitzii* genomes, divided by their mode of action and antibiotic classes. In **bold** are represented

857 mechanisms that are specific for a certain antibiotic.

Mode of Action	**CARD definition**	**# Hits**	**Antibiotics**	**# Hits**
Antibiotic target in susceptible species	Antibiotic-sensitive wild-type bacterial components; might suffer mutations that render them not susceptible	1597	Quinolones	212
			Cycloserine	208
			Fosfomycin	207
			Fusidic acid	128
			Mupirocin	111
			Rifamycin/peptide antibiotics	110
			Fosmidomycin	108
			Peptide antibiotics	107
			Triclosan	105
			Tetracyclines/glycylcyclines	101
			Elfamycins	99

Mode of Action	CARD definition	# Hits	Antibiotics	# Hits
			Aminoglycosides	97
			Sulfonamides	1
			Other	3
Protein altering cell wall charge conferring antibiotic resistance	Cell wall alteration	245	Peptide antibiotics	245
Gene conferring resistance via absence	Deletion of gene or gene product results in resistance. For example, deletion of a porin gene blocks drug from entering the cell.	107	Aminoglycosides	107
Antibiotic inactivation enzyme	Enzyme that catalyses the inactivation of an antibiotic resulting in resistance. Inactivation includes chemical modification, destruction, etc.	80	Aminoglycosides	51
			Nitroimidazoles	19
			Lincosamides	2
			Penams/cephalosporins	2
			Chloramphenicol	3

Mode of Action	**CARD definition**	**# Hits**	**Antibiotics**	**# Hits**
			β-lactams	2
			Streptogramins	1
Efflux pump conferring antibiotic resistance	Subunits of efflux proteins that pump antibiotic out of a cell to confer resistance.	17	Tetracyclines	16
			Macrolides	1
Protein involved in antibiotic sequestration	**A gene whose product inactivates an antibiotic by formation of a complex, preventing interaction of the antibiotic with its target.**	101	Triclosan	101
Antibiotic target replacement protein	**Alternate proteins that have the same functions as other antibiotic target proteins but are structurally different and thus resistant to antibiotics.**	99	Triclosan	99

Mode of Action	CARD definition	# Hits	Antibiotics	# Hits
Antibiotic target protection protein	**These proteins confer antibiotic resistance by bind the antibiotic target to prevent antibiotic binding.**	47	Tetracyclines	47
Antibiotic target modifying enzyme	**Enzymes that confer resistance by modifying (mutational alteration or enzymatic modification) antibiotic targets.**	26	Lincosamides/streptogramins/macrolides	26
Regulator modulating expression of antibiotic resistance genes	**Mechanism activated by the presence of a specific antibiotic.**	5	Glycopeptides	5

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Supplementary Information

File: SupInfo A contains:

Table A.1. List of ARG detected in all deposited *F. prausnitzii* genomes, using PATRIC. Includes the gene designation (when available), function (when available), gene product and the mechanism of resistance mediated by that product. Notice that the availability of these data depends on the annotation data provided at the time of the genome deposit in the database.

Table A.2. Number of ARG in each *F. prausnitzii* strain, calculated from Supplementary Table A.1. The average number of ARG/strain is calculated at the bottom of the list; the maximum and minimum ARG-containing genomes are highlighted.

Table A.3. Number of ARG group by gene product and antibiotic classes to which they might confer resistance. See also Fig. 1.

File: SupInfo B contains:

Table B.1. *Faecalibacterium prausnitzii* DSM 17677 ARG retrieved by PATRIC. This table includes the position of each ARG within the genome, the gene designation and its product, the type mechanism possibly involved in conferring resistance, and the antibiotic class and antibiotics to which they might confer resistance. The identification of the same gene by CARD and ResFinder is also indicated. Genes encoded within putative integrative regions are highlighted.

Table B.2. *Faecalibacterium prausnitzii* DSM 17677 ARG identified by CARD RGI tool. This table includes the cut-off used for the identification, the position of each ARG within the genome, the gene family, the type mechanism possibly involved in conferring resistance, and the antibiotic class to which they might confer resistance. The identification of the same gene by PATRIC and ResFinder is also indicated. Genes encoding β-lactams resistance-mediating products are highlighted.

Table B.3. List of putative integrative regions detected by ICEfinder. This table includes the position of each region and the CARD RGI hits encoded within each region with the respective information, from Supplementary Table B.2. The GC percentage for each region is also indicated. See also Fig. 1.

Table B.4. List of putative genomic islands predicted by IslandViewer. The position of each island within the chromosome is indicated as well as genes that were considered acquired; highlighted in yellow, the genes that were identified by CARD as ARG; the integrative regions are also provided, to facilitate an integrated observation (Supplementary Table B.3). See also Fig. 2.

Table B.5. List of putative prophage-like regions predicted by Prophage Hunter. The position of each region within the chromosome is indicated as well as the closest homologous phage; highlighted in yellow, prophages that are considered active by this tool; hits that were also retrieved by PHASTER are indicated. See also Fig. 2.

Table B.6. List of putative prophage-like regions predicted by PHASTER. The position of each region within the chromosome is indicated as well as the corresponding

Prophage Hunter hits; highlighted in yellow and orange, prophages that are considered active and ambiguous by Prophage Hunter, respectively; the integration integrative regions and genomic islands are also provided, to facilitate an integrated observation (Supplementary Table B.3 and Table B.4). Predicted CARD ARG that are embedded within these prophage-like regions are also shown (Supplementary Table B.2). See also Fig. 2.

Table B.7. Summary table integrating the *in silico* data from Supplementary Table B.1 toTable B.4, with special emphasis on the most concerning ARG identified in this study.

Figure 1

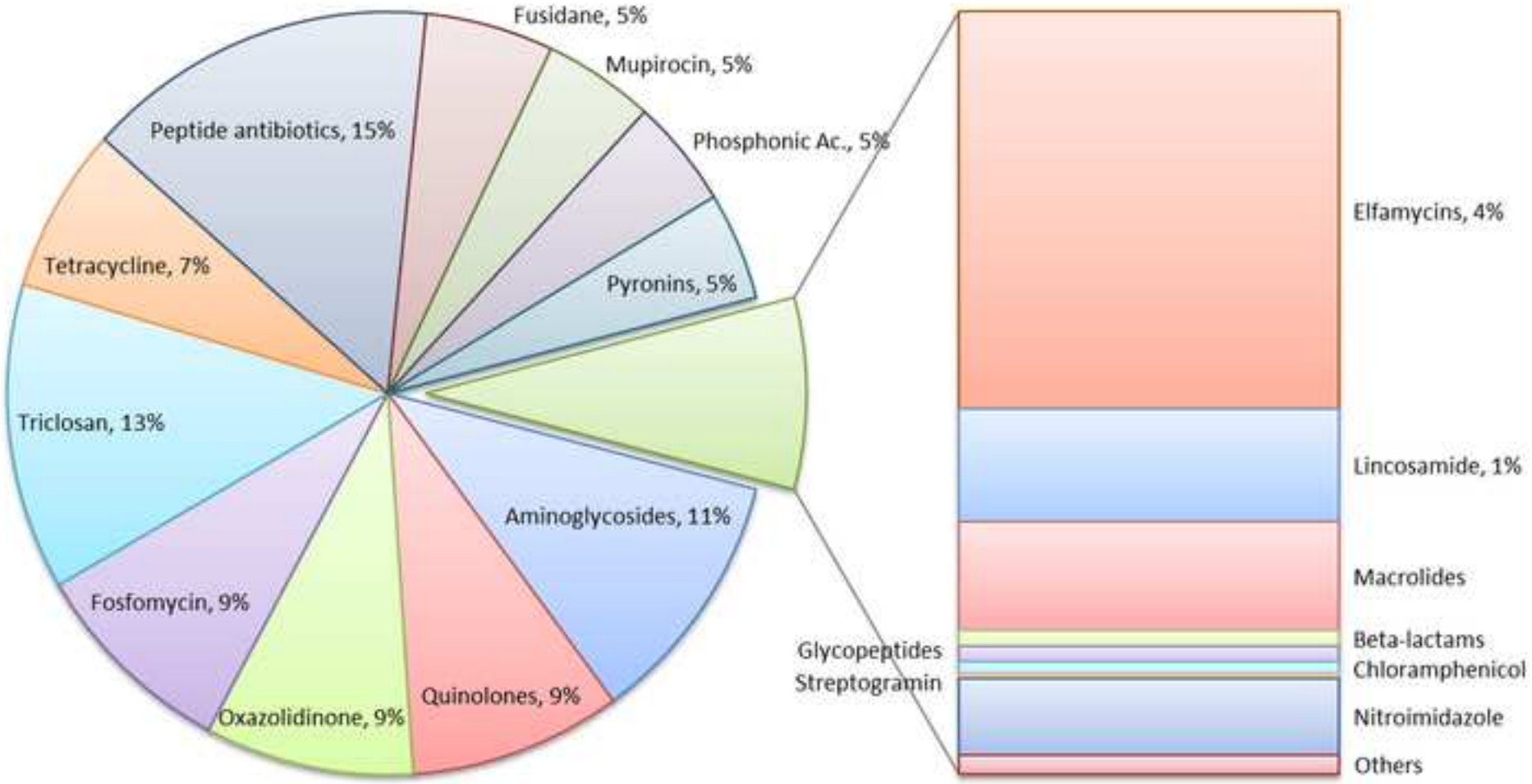

Figure 2

Click here to access/download;Figure;Figure 2.tif