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BRIEF ARTICLE

Safety assessment of *Bifidobacterium longum* JDM301 based on complete genome sequences

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Abstract

AIM: To assess the safety of *Bifidobacterium longum* (*B. longum*) JDM301 based on complete genome sequences.

METHODS: The complete genome sequences of JDM301 were determined using the GS 20 system. Putative virulence factors, putative antibiotic resistance genes and genes encoding enzymes responsible for harmful metabolites were identified by blast with virulence factors database, antibiotic resistance genes database and genes associated with harmful metabolites in previous reports. Minimum inhibitory concentration of 16 common antimicrobial agents was evaluated by *E*-test.

RESULTS: JDM301 was shown to contain 36 genes associated with antibiotic resistance, 5 enzymes related to harmful metabolites and 162 nonspecific virulence factors mainly associated with transcriptional regulation, adhesion, sugar and amino acid transport. *B. longum* JDM301 was intrinsically resistant to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptible to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and trimethoprim-sulphamethoxazol. JDM301 was moderately resistant to bacitracin, while an earlier study showed that bifidobacteria were susceptible to this antibiotic. A tetracycline resistance gene with the risk of transfer was found in JDM301, which needs to be experimentally validated.

CONCLUSION: The safety assessment of JDM301 using information derived from complete bacterial genome will contribute to a wider and deeper insight into the safety of probiotic bacteria.

Key words: *Bifidobacterium longum*; Safety assessment; Genome; Antibiotic resistance; Harmful metabolite; Virulence factor

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INTRODUCTION

Bifidobacteria spp are high-GC content, Gram-positive bacteria which belong to the *Actinobacteria* branch and these species naturally colonize the gastrointestinal tract (GIT) of mammals, birds and insects[1]. Scientists have determined the major probiotic properties of *Bifidobacteria* spp isolated from the human intestine and these properties include the strengthening of the intestinal barrier, modulation of the immune response and antagonism of pathogens[2].

Bifidobacterium spp has been reported to possess various glycosyl hydrolases (GH) and these hydrolases metabolize plant- or milk-derived oligosaccharides including nondigestible ones such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS)[3,4]. The capability to utilize nondigestible oligosaccharides confers a competitive advantage to *Bifidobacterium* spp in the human gut.

Bifidobacterium longum (*B. longum*) and various other bifidobacteria strains are often added to probiotic products in combination with other lactic acid bacteria (LAB). Through their long and safe history of application, LAB have acquired the status of "Generally Regarded As Safe" (GRAS), but the safety of *Bifidobacteria* and other LAB strains selected for probiotics still need to be carefully evaluated. The key safety aspects for use of bifidobacteria and other LAB strains in probiotics include antibiotic resistance, production of harmful metabolites and the potential for virulence. Antibiotic resistance in potential probiotic strains is not considered a risk factor unless resistance is transferred to pathogens or it renders the *probiotic untreatable* in very rare cases of infection[5]. Biogenic amines, D-lactic acid, azoreductases and nitroreductases produced by bifidobacteria and other LAB strains are potential health hazards[6,7] and the safety of some of these compounds have been evaluated[8]. Virulence genes may be present in commensal bacteria and absence of virulence in these bacteria needs to be proved on a case by case basis.

Probiotic agents are widely used in the food and drug industry and as more commercial probiotic products are being introduced in the market, it is timely to reassess the safety of these probiotic products using the latest technology. Information from the complete genome sequences of *Bifidobacteria* will provide additional insight into the genetic basis for their safety. We sequenced the complete genome sequences of *B. longum* JDM301 (GenBank accession number CP002010), a commercial strain used widely in China with several probiotic functions, for this purpose[9].

The aim of the present work was to assess the safety of *B. longum* JDM301 based on complete genome sequences. The criteria used were the potential to transfer antibiotic resistance to pathogens, the potential for production of harmful metabolites and the potential for virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions

JDM301 was isolated from commercial probiotic product and identified using a sequence analysis of its 16S rRNA gene. De Man-Rogosa-Sharpe (MRS) broth (Difco) supplemented with 0.05% L-cysteine·HCl (Sigma) was used for cultivating JDM301. Cultures were incubated at 37 ℃ under anaerobic conditions.

Genome sequencing and assembly

We determined the complete genome sequence of JDM301 at the Chinese National Human Genome Center in Shanghai using the GS 20 system (454 Life Science Corporation, Branford, Connecticut). A total of 192 888 reads with an average length of 210 bps were assembled into 112 contigs by the 454 assembly tool. The order of most large contigs, which were larger than 500 bp, was determined through the basic local alignment search tool (BLAST) analysis with the reference strain *B. longum* ATCC15697 (GenBank accession number CP001095) and the others were arranged by multiplex polymerase chain reaction (PCR). Gap closure was carried out by sequencing gap-spanning PCR products or clones using ABI 3730 xl DNA sequencers. Primer design and sequence assembly were performed by the Phred/Phrap/Consed software package[9]. The locations of low-quality sequences in genome were verified by directly resequencing the PCR products spanning the low-quality sequences using the ABI 3730 xl DNA sequencers.

Statistical analysis

The genome sequences of *Bifidobacteria* except JDM301 were retrieved from GenBank at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/)[10]. Potential open reading frames (ORF) were identified using Glimmer[11] and ZCURVE[12] 1.0 using default settings. Clusters of orthologous group (COG) functional categories were used for functional classification of all genes in the genome sequences of JDM301 and the COGs. A BLAST analysis of the translations with GenBank's nonredundant database was performed, which was followed by manual curation. The best matches were chosen for preliminary product assignments. Insertion sequences (IS) elements, prophage sequences and clustered regularly interspaced short palindromic repeats (CRISPR) were identified by IS finder (http://www-is.biotoul.fr/is.html), Prophage Finder[13] and CRISPRFinder (http://crispr.u-psud.fr/crispr/)[14] respectively. Putative orthologues were determined by Omics Explorer (http://omics.biosino.org:14000/kweb/about.jsp) using default values. Ribosomal RNA genes were detected on the basis of BLASTN searches and transfer RNA genes were identified using tRNAscan-SE[15]. The atlas of genome was drawn using GenomeViz1.1[16]. Putative virulence factors and putative antibiotic resistance genes were identified by blast with virulence factors database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm)[17] and antibiotic resistance genes database (ARDB) (http://ardb.cbcb.umd.edu/)[18] respectively.

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) of 16 common

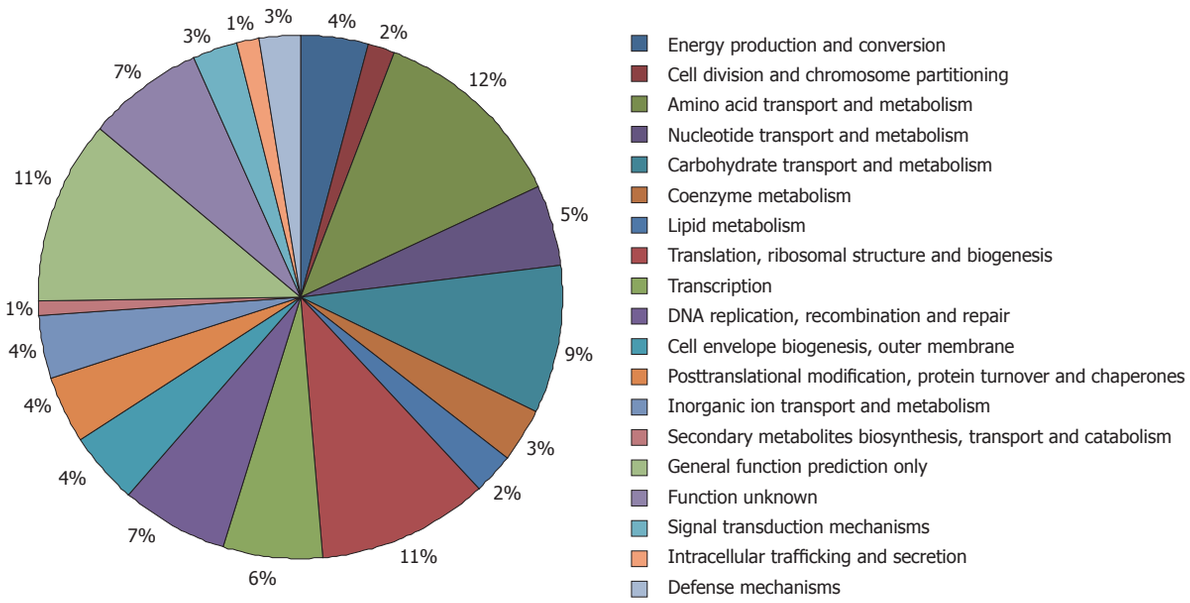

Figure 1 Functional distribution of *Bifidobacterium longum* core proteins. A total of 1265 proteins were conserved in all four *Bifidobacterium longum* (*B. longum*) strains (*B. longum* JDM301, *B. longum* NCC2705, *B. longum* DJO10A and *B. longum* ATCC15697), representing the "core" genome of *B. longum*.

antimicrobial agents was evaluated by *E*-test (AB Biodisk, Solna, Sweden) including amoxicillin (0.016-256 mg/L), amikacin (0.016-256 mg/L), ampicillin (0.016-256 mg/L), bacitracin (0.016-256 mg/L), cephalothin (0.016-256 mg/L), ciprofloxacin (0.002-32 mg/L), cefotaxime (0.016-256 mg/L), chloramphenicol (0.016-256 mg/L), erythromycin (0.016-256 mg/L), gentamicin (0.016-256 mg/L), imipenem (0.002-32 mg/L), rifampicin (0.016-256 mg/L), streptomycin (0.016-256 mg/L), tetracycline (0.016-256 mg/L), trimethoprim-sulphamethoxazol (0.002-32 mg/L), and vancomycin (0.016-256 mg/L). Tests were done with MRS agar supplemented with 0.05% L-cysteine·HCl (Sigma) and were conducted in triplicate for each antibiotics. Cultures sub-inoculated into the MRS agar supplemented with 0.05% L-cysteine·HCl were incubated anaerobically at 37 ℃ for 24 h.

RESULTS

Comparative genomic analysis of Bifidobacteria

The predicted proteins of *B. longum* JDM301 were functionally categorized. The functional distribution of genes assigned to clusters of orthologous groups of proteins was relatively similar to the other *Bifidobacteria*, e.g., *B. longum* and *B. adolescentis* in the GIT and *B. dentium* in the oral cavity[3,4,19]. The top four functional categories in *B. longum* JDM301, namely, carbohydrate transport and metabolism, amino acid transport and metabolism, were identical with other *Bifidobacteria*[20].

Putative orthologues among *B. longum* strains were determined in a comparative study (Figure 1). Overall, 1265 proteins were conserved in all four *B. longum* strains (*B. longum* JDM301, *B. longum* NCC2705, *B. longum* DJO10A and *B. longum* ATCC15697). These proteins represent the "core" genome of *B. longum*, whereas 219 proteins are unique to *B. longum* JDM301. The most common functional distributions of the core proteins were these involved in housekeeping functions including amino acid transport and metabolism, translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism and DNA replication, recombination and repair. Twenty-one percent of the core proteins were dedicated to carbohydrate and amino acid transport and metabolism, indicating the important roles of these proteins in *Bifidobacteria*.

Stability of the genome of B. longum JDM301

Horizontal gene transfer (HGT) events are responsible for introduction of alien genes, which may reinforce the adaptation of bacteria in their specific niches. Genes on plasmids, bacteriophages, genomic islands and IS are sensitive to HGT[21]. Twelve phage-related fragments were identified in the genome of *B. longum* JDM301[9], but no complete prophages were found. The JDM301 chromosome also possesses 15 complete or disrupted IS elements[9]. The number of IS element in JDM301 is relatively smaller than the other sequenced *B. longum* spp[3,4]. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system. No CRISPR was discovered in the genome.

One complete type Ⅱ restriction-modification (R-M) system and one type Ⅲ R-M system were present in the genome of JDM301. A complete and incomplete type Ⅰ R-M system was also identified in this genome. Two complete type Ⅱ R-M systems and one type Ⅰ R-M system were present in the genome of *B. longum* NCC2705, while one complete type Ⅱ R-M system and type Ⅰ R-M system were found in *B. longum* DJO10A.

Antibiotic resistance determinants

The antibiotic resistance genes in JDM301 were identified

Table 1 Putative antibiotic resistance genes identified in the genome of *Bifidobacterium longum* JDM301

Antibiotics	Antibiotic resistance genes	Product name
Bacitracin	*BLJ_1636*	ABC transporter-related protein
	BLJ_0984	ABC transporter-related protein
	BLJ_0923	ABC transporter-related protein
	BLJ_1055	Undecaprenyl pyrophosphate phosphatase
	BLJ_1119	Bacitracin transport ATP-binding protein bcrA
Vancomycin	*BLJ_0853*	VanU
	BLJ_1764	Dehydrogenase VanH
	BLJ_1084	Sensor protein vanSB
	BLJ_0707	VanSD5
	BLJ_0343	Histidine kinase VanSc3
	BLJ_0287	D-Ala: D-Lac ligase VanD
Multiple drugs	*BLJ_1090*	ATP-binding protein
	BLJ_1650	Lsa
	BLJ_1437	LmrB
	BLJ_0618	Multidrug export protein MepA
	BLJ_0769	Efflux transporter, RND family, MFP subunit
	BLJ_0181	Multidrug efflux protein QacB
	BLJ_1062	Multidrug export protein MepA
Chloramphenicol	*BLJ_1672*	Chloramphenicol resistance protein
	BLJ_1322	Chloramphenicol resistance protein
Thiostrepton	*BLJ_0885*	Thiostrepton-resistance methylase
Penicillin	*BLJ_1301*	Penicillin binding protein
Kasugamycin	*BLJ_2030*	S-adenosylmethionine-6-N', N'-adenosyl (rRNA) dimethyltransferase
Tetracycline	*BLJ_0814*	Tetracycline-resistance determinant tetV
	BLJ_1245	TetW
	BLJ_0594	Tetracycline resistance protein
	BLJ_1401	TetQ
Carbomycin	*BLJ_1625*	Carbomycin resistance protein
Sulfonamide	*BLJ_1629*	Dihydropteroate synthase
Tetracenomycin C	*BLJ_1624*	Tetracenomycin C efflux protein
Trimethoprim	*BLJ_1657*	dihydrofolate reductase
Macrolide	*BLJ_0925*	Macrolide-efflux protein
	BLJ_1936	Macrolide-efflux protein
	BLJ_0819	Macrolide-efflux protein
	BLJ_0042	Macrolide-efflux protein
	BLJ_1154	Macrolide-efflux protein variant

ABC: ATP-binding cassette; RND: Resistance-nodulation-cell division.

using ARDB (E < 1e-2, coverage > 70%)[18]. Homologs of the antibiotic resistance determinants for vancomycin, methicillin, tetracycline, chloramphenicol and trimethoprim were found in the genome of JDM301 (Table 1) and 6 putative resistance genes for vancomycin. *B. longum* JDM301 also possessed 5 putative bacitracin efflux pumps, 5 homologs of macrolide efflux proteins. Additionally, 7 putative multidrug resistance efflux pumps belonging to an ATP-binding cassette (ABC)-type transport system, a major facilitator superfamily transporter and resistance-nodulation-cell division (RND) family were found in the genome. The genome of *B. longum* JDM301 also contains 4 tetracycline resistance genes encoding for TetV, TetW, TetPB and TetQ. The gene for TetW shows a strong difference in G + C content (53.0%) compared to the average value of *B. longum* JDM301 (59.8%) genome and it is flanked by genes encoding for integrases, indicating that this region may have been acquired by HGT.

Table 2 Minimum inhibitory concentration values of 16 antibiotics for *Bifidobacterium longum* JDM301

Antibiotics	Minimum inhibitory concentration (mg/L)
Ciprofloxacin	> 32
Amikacin	> 256
Gentamicin	> 256
Bacitracin	26.67
Streptomycin	170.67
Vancomycin	0.9
Amoxicillin	0.064
Cephalothin	1.33
Chloramphenicol	0.25
Erythromycin	0.04
Ampicillin	0.058
Cefotaxime	0.19
Rifampicin	0.074
Tetracycline	8
Imipenem	0.19
Trimethoprim-sulphamethoxazol	1.83

The antibiotic susceptibility of *B. longum* JDM301 to 16 antibiotics was determined by an *E*-test to probe the *in silico* analyses of the complete genome sequence. The results of the *E*-test are summarized in Table 2. The breakpoints for determining susceptibility were determined using accepted protocols[22-25]. *B. longum* JDM301 showed a high resistance to ciprofloxacin, amikacin and gentamicin, moderate resistance to streptomycin and bacitracin and were sensitive to tetracycline, vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol.

Putative enzymes for harmful metabolites

Genes encoding enzymes responsible for harmful metabolites, including beta-glucosidase (GS), arylsulphatase (AS), beta-glucuronidase (GN), nitroreductase (NR), azoreductase (AR), D-lactate dehydrogenase (DLD), amino acid decarboxylase (AD) and conjugated bile salt hydrolase (CBSH) were searched for in the genome of *B. longum* JDM301. Two GS genes (BLJ_1280, BLJ_1540) and one CBSH gene (BLJ_0948) were found in the chromosome of *B. longum* JDM301. Homologs of DLD (BLJ_1306, BLJ_1436) and NR (BLJ_1980) were also discovered in the genome. Enzymes involved in putatively harmful metabolites, AR, GN, AD and AS were not found in JDM301 genome.

Putative virulence factors

Published reports of rare infections involving *Lactobacilli* or *Bifidobacteria* are available and the potential virulence of *Lactobacilli* or *Bifidobacteria* used as probiotics should be assessed[5]. Putative virulence genes of *B. longum* JDM301 were determined by BLAST analysis of the VFDB[17]. A total of 141 homologs of virulence factors were identified in the genome of JDM301, including 28 sugar-binding transcriptional regulators, 20 genes associated

Table 3 Putative virulence factors identified in the genome of *Bifidobacterium longum* JDM301

Query	Identity	Subject	Predicted functions
BLJ_1089	24.9	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_1835	26.36	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_0323	29.3	VFG0934	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
BLJ_1476	22.11	VFG2378	6 kDa early secretory antigenic target esxA
BLJ_0992	32.98	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_1080	34.81	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_1968	37.3	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_0770	37.43	VFG0869	AatC ATB binding protein of ABC transporter
BLJ_0026	35.71	VFG1404	ahpC
BLJ_0136	28.73	VFG2218	ATPase VirB11 homolog
BLJ_0880	24.18	VFG1042	ATP-binding protein FecE
BLJ_0787	47.92	VFG0077	ATP-dependent Clp protease proteolytic subunit
BLJ_0786	53.8	VFG0077	ATP-dependent Clp protease proteolytic subunit
BLJ_0948	37.66	VFG2162	Bile salt hydrolase
BLJ_1243	22.97	VFG2242	Conjugal transfer protein trag
BLJ_0551	26.54	VFG1108	Conserved hypothetical protein
BLJ_1951	29.85	VFG1269	Cyclolysin secretion ATP-binding protein
BLJ_1925	32.31	VFG1269	Cyclolysin secretion ATP-binding protein
BLJ_1863	45.5	VFG0079	Endopeptidase Clp ATP-binding chain C
BLJ_1465	56.77	VFG0079	Endopeptidase Clp ATP-binding chain C
BLJ_0713	30.12	VFG0925	Ferric enterobactin transport ATP-binding protein fepC
BLJ_1872	25.51	VFG2225	GDP-mannose 4,6-dehydratase
BLJ_1324	32.49	VFG1399	glnA1
BLJ_0624	62.11	VFG1399	glnA1
BLJ_1834	29.47	VFG0313	Glucose/galactose transporter
BLJ_1926	30.02	VFG1557	HlyB protein
BLJ_1477	56.12	VFG1855	Hsp60, 60K heat shock protein HtpB
BLJ_0064	26.21	VFG1397	hspX
BLJ_1444	40.85	VFG1563	Hypothetical protein
BLJ_1606	27.78	VFG1593	Hypothetical protein
BLJ_1640	30.81	VFG1593	Hypothetical protein
BLJ_0011	22.16	VFG1604	Hypothetical protein
BLJ_1513	26.3	VFG1604	Hypothetical protein
BLJ_1846	27.67	VFG1604	Hypothetical protein
BLJ_0337	44.25	VFG1630	Hypothetical protein
BLJ_0336	44.38	VFG1630	Hypothetical protein
BLJ_1500	23.53	VFG1963	Hypothetical protein Cj1435c
BLJ_1169	24.64	VFG1390	Hypothetical protein Rv0981
BLJ_0708	36.8	VFG1390	Hypothetical protein Rv0981
BLJ_0802	28.83	VFG1824	Hypothetical protein Rv3133c
BLJ_1357	30.46	VFG1824	Hypothetical protein Rv3133c
BLJ_1113	32.41	VFG1824	Hypothetical protein Rv3133c
BLJ_0835	32.42	VFG1824	Hypothetical protein Rv3133c
BLJ_0859	27.93	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_0348	28.13	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_0530	29.29	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_2016	35.81	VFG1206	Iron(Ⅲ) ABC transporter, ATP-binding protein
BLJ_1875	36.19	VFG1627	IS100 transposase; transposase ORFA
BLJ_1249	37.55	VFG1627	IS100 transposase; transposase ORFA
BLJ_1252	39.22	VFG1627	IS100 transposase; transposase ORFA
BLJ_0930	42.29	VFG1627	IS100 transposase; transposase ORFA
BLJ_1966	30.68	VFG1485	L7045
BLJ_1850	59.7	VFG1411	leuD
BLJ_0379	39.24	VFG0320	Lipopolysaccharide core biosynthesis protein (kdtB)
BLJ_1549	22.02	VFG1817	mbtA
BLJ_1204	25.8	VFG0574	Mg<up>2+</up> transport protein
BLJ_2010	30.62	VFG0574	Mg<up>2+</up> transport protein
BLJ_1270	28.62	VFG1116	N-acetylglucosamine-6-phosphate deacetylase
BLJ_1832	21.89	VFG1109	N-acetylneuraminate lyase, putative
BLJ_0490	25.95	VFG1109	N-acetylneuraminate lyase, putative
BLJ_0021	26.83	VFG0307	Neutrophil activating protein (bacterioferritin)
BLJ_1889	24.14	VFG2227	O-antigen export system permease protein
BLJ_1251	26.05	VFG1461	ORF A protein
BLJ_0214	30.5	VFG0594	Pathogenicity island encoded protein: SPI3
BLJ_0159	33.25	VFG0594	Pathogenicity island encoded protein: SPI3
BLJ_1474	57.32	VFG1386	phoP
BLJ_1703	25.65	VFG2220	Phosphoglucomutase
BLJ_0497	28.35	VFG2362	Phosphomannomutase
BLJ_1137	25.1	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_0508	25.93	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_1453	29.27	VFG1983	ABC-type amino-acid transporter periplasmic solute-binding protein
BLJ_0408	38.22	VFG2059	ATP-binding component of ABC transporter
BLJ_0480	27.04	VFG2061	Phosphoprotein phosphatase
BLJ_0805	28.09	VFG1384	proC
BLJ_1396	31.06	VFG1384	proC
BLJ_0584	26.09	VFG1387	purC
BLJ_1772	22.28	VFG0480	Putative amino acid permease
BLJ_0538	25.17	VFG0480	Putative amino acid permease
BLJ_1329	24.42	VFG1965	Putative aminotransferase
BLJ_0025	30.45	VFG2301	Putative carbonic anhydrase
BLJ_0922	23.51	VFG0031	Putative glycosyl transferase
BLJ_1670	38.88	VFG1668	Putative lysil-tRNA synthetase LysU
BLJ_0563	25	VFG1498	Putative periplasmic solute binding protein
BLJ_1171	28.48	VFG0483	Putative regulatory protein, deoR family
BLJ_1517	29.25	VFG0483	Putative regulatory protein, deoR family
BLJ_0344	37.02	VFG1702	Putative response regulator
BLJ_0040	27.91	VFG1746	Putative two-component response regulator
BLJ_0740	29.13	VFG1746	Putative two-component response regulator
BLJ_1105	24.49	VFG0168	Pyochelin biosynthesis protein PchD
BLJ_0409	25.56	VFG0168	Pyochelin biosynthesis protein PchD
BLJ_0720	41.04	VFG0479	Pyruvate kinase I (formerly F), fructose stimulated
BLJ_1163	55.32	VFG1826	relA
BLJ_0995	25.84	VFG1889	Response regulator GacA
BLJ_1679	28.89	VFG1889	Response regulator GacA
BLJ_1083	40.89	VFG0473	Response regulator in two-component regulatory system with BasS
BLJ_1273	26.57	VFG1115	ROK family protein
BLJ_1620	26.62	VFG1115	ROK family protein
BLJ_1622	31.35	VFG1115	ROK family protein
BLJ_1796	27.31	VFG0526	Salmonella iron transporter: fur regulated
BLJ_0662	29.06	VFG0526	Salmonella iron transporter: fur regulated

BLJ_0712	25.4	VFG0528	Salmonella iron transporter: fur regulated
BLJ_1174	51.39	VFG1405	sigA
BLJ_1258	41.15	VFG1412	sigH
BLJ_1342	33.11	VFG2161	Signal peptidase Ⅱ
BLJ_0906	21.73	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1923	22.38	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1360	22.88	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1421	23.24	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0611	23.31	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1836	23.32	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0459	23.33	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1278	23.43	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1998	23.51	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1522	23.6	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0109	23.63	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0418	23.69	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0118	23.7	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0520	23.85	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1976	24.27	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0099	24.31	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1605	24.34	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0132	24.53	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1912	24.58	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0912	24.69	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0318	24.71	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1515	24.93	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1933	25	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1718	25	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1997	25.36	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0400	25.37	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_0515	27.08	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1321	28.21	VFG2197	Sugar-binding transcriptional regulator, LacI family
BLJ_1232	29.83	VFG1028	Tn21 integrase IntI1
BLJ_1160	43.1	VFG2168	Transcriptional regulator, Cro/CI family
BLJ_0747	28.98	VFG1122	Transposase ORFAB, subunit B
BLJ_1180	43.64	VFG1398	trpD
BLJ_1871	39.62	VFG1967	UDP-galactopyranose mutase
BLJ_1644	39	VFG2361	UDP-glucose 4-epimerase
BLJ_1680	54.49	VFG2361	UDP-glucose 4-epimerase
BLJ_1891	52.63	VFG0963	UDP-glucose 6-dehydrogenase
BLJ_0697	46.15	VFG1414	whiB3

MIC: Minimum inhibitory concentration; ABC: ATP-binding cassette.

Table 4 Putative genes associated with adhesion identified in the genome of *Bifidobacterium longum* JDM301

Locus_tag	Pfam number	Product name
BLJ_1932	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0112	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1284	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1420	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0131	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1604	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1686	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1964	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1994	pfam01547	Family 1 extracellular solute-binding protein
BLJ_1996	pfam01547	Family 1 extracellular solute-binding protein
BLJ_2001	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0288	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0321	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0345	pfam01547	phosphate ABC transporter periplasmic phosphate-binding protein
BLJ_0414	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0522	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0523	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0524	pfam01547	Family 1 extracellular solute-binding protein
BLJ_0012	pfam07174	Hypothetical protein BLJ_0012
BLJ_1801	pfam05738	LPXTG-motif protein cell wall anchor domain-containing protein
BLJ_0140	pfam07811	TadE family protein

with iron, amino acid and sugar transport, 5 transposases, and 2 glutamine synthetase related to plasminogen (Plg)-binding (Table 3).

Although the ability to adhere to the intestinal wall has been one of the selection criteria for probiotics and also a characteristic of commensal bacteria in the intestine, adhesion is also considered to be a significant step in the initial pathogen infections[26]. Thus, predicted proteins for adhesion of JDM301 were also included in the analysis of virulence. A total of 21 predicted proteins for adhesion were identified in JDM301 (Table 4). A large number of predicted surface and extracellular proteins were identified in JDM301, which may be involved in the bacterium-host interaction as in other LAB[27]. A total of 217 proteins with probable Sec-type signal peptides were identified by the tool, Signal P[28]. The genome of JDM301 also harbors 18 copies of extracellular solute-binding protein (SBP, pfam01547) which is predicted to bind oligosaccharides (SBP family 1) as a component of the ABC transporter complex.

DISCUSSION

As more probiotic strains are used in the food and drug industry, more attentions should be paid to the safety of strains used as probiotics. Thus, the safety of LAB used as probiotics need to be reassessed using the latest technology. *B. longum* JDM301, is a commercial probiotic strain used in many probiotic products sold in China. Analysis of the genome of JDM301 reveals several potential risk factors needing further experimental validation, including a tetracycline resistance gene (*tetW*) with the risk of transfer, and the genes associated with harmful metabolites.

Bifidobacteria were considered free of phage infection until prophage-like elements were identified in the genomes of *B. longum* NCC2705, *B. longum* DJO10A and *B. breve* UCC2003[29]. Absence of complete prophages is important for the stability of genomes and for industrial applications of probiotic bacteria[21,30]. Absence of complete prophages and scarcity of IS element may play important roles in promoting genome stability of JDM301[31]. Another set of genes disseminated by HGT in *Bifidobacteria* is the CRISPR-related system (CASS), which is involved in defense against phages and plasmids[32]. No CRISPR was discovered in the genome. R-M systems are diverse and widespread in nature and they are considered as barriers to HGT, e.g., in transformation and phage infection[33]. The diversity of R-M systems in *B. longum* JDM301 may be significant to the stability of genome and its use in industry compared with the other two *B. longum* strains.

B. longum JDM301 was not resistant to tetracycline as the minimum inhibitory concentration (8.0 mg/L) was not higher than the breakpoint value (8.0 mg/L)[34]. However, the MIC for *B. longum* strains ranges from 0.5 to 2 mg/L in a report[35]. Thus, further experiments may be needed to determine the microbiological breakpoint. The *tetW* (BLJ_1245) gene encodes for a ribosomal protection protein and *tetW* genes were responsible for acquired tetracycline resistance in human *B. longum* strains[36]. The rest of the tetracycline resistance genes found in *B. longum* JDM301 were *tetV* (BLJ_0814), *tetQ* (BLJ_1401) and *tetPB* (BLJ_0594). The gene *tetV* encodes for a tetracycline efflux pump and the genes *tetQ* and *tetPB* encode for ribosomal protection proteins. Further experiments are needed to confirm whether the *tetW* gene in the chromosome of *B. longum* JDM301 is a transferable antibiotic resistance determinant and responsible for resistance to tetracycline in human *B. longum* strains.

The MIC of *B. longum* JDM301 to bacitracin was 26.7 mg/L, which indicated a moderate resistance. A previous report[25] indicated that *B. longum* strains were susceptible to bacitracin. A total of 7 putative bacitracin resistance genes were identified, including 6 genes encoding for ABC transporters and 1 for an uncharacterized bacitracin resistance protein. These genes may be responsible for the resistance to bacitracin.

The resistances to ciprofloxacin, amikacin, gentamicin and streptomycin and susceptibility of JDM301 to vancomycin, amoxicillin, cephalothin, chloramphenicol, erythromycin, ampicillin, cefotaxime, rifampicin, imipenem and an antimicrobial compound, trimethoprim-sulphamethoxazol were consistent with reported findings[22-25,36]. However, there are discrepancies between the phenotype and the genotype. *B. longum* JDM301 was sensitive to vancomycin and chloramphenicol but the genome contained vancomycin and chloramphenicol resistance genes. Further analysis will be needed to determine this discrepancy.

Several cases of D-lactic acidosis associated with consumption of LAB in patients with short bowel syndrome were reported[37,38], implying that bacteria used as probiotics should be screened for the ability to generate D-lactate. In this study, two homologs of DLD genes were identified in the genome of JDM301. Since there were no reported cases of D-lactic acidosis caused by bifidobacteria[37-39], the activities of these homologous DLDs in bifidobacteria may be low so that the amount of lactate produced is insufficient to cause D-lactic acidosis.

Although biogenic amines (BA) play an important physiological role in mammals, a high amount of BA in the diet may have a variety of toxic effects[40]. The main BA contained in food and beverage includes histamine, tyramine, putrescine, and cadaverine, some of which are associated with toxicological characteristics of food poisoning[41]. The decarboxylase activities of histidine, tyrosine and ornithine were reported in lactobacilli and the capabilities might be strain-dependent rather than species-dependent[42]. Therefore, BA production, especially thylamine and tyramine, must be carefully evaluated for individual strains.

Bacterial enzymes, such as GN, GS, NR, AR and AS, play important roles in the metabolism of carcinogens and other toxicants in the intestine. Homologs of GS are common in sequenced *Bifidobacteria* genomes where GS and GN facilitate the absorption of a variety of toxicants and may contribute to the development of colon cancer. The link between *Bifidobacteria* and the genotoxic enzyme activities of intestinal microflora has been reported[43,44], with *Bifidobacteria* inhibiting the activity of some genotoxic enzymes[45]. NR activity is common in oral bacteria and it plays an important role in bacterial nitrate reduction. Although NR activities have been reported in *Bifidobacteria*, the activity of this enzyme is lower than the NR activity of other gut bacteria[6].

CBSH mediates microbial bile tolerance and enhances microbial survival in the intestine[46]. Metagenomic analyses demonstrated that CBSH activity is enriched in the human gut microbiome, and has the potential to greatly influence host physiology[46]. In *Bifidobacterium* spp. and *Lactobacillus* spp., CBSH activity is also common and nearly all *Bifidobacteria* species and strains have bile salt hydrolase activities[47]. However, bile salt hydrolase activity releases free bile acids which are harmful to the human body and may act as mutagens[48,49]. Recommendations have been made for absence of bile salt transformation capacity in bacteria added to food[50]. However, it is noteworthy that the evidence for harmful effects is inconclusive so far and bile salt deconjugation activity may play a role in reducing human serum cholesterol[51]. Given the huge CBSH pool in intestinal microflora, the CBSH activities of the small number of additional bacteria consumed as probiotics can be ignored[48].

Putative genes for Plg-binding proteins, DnaK (BLJ_0123) and glutamine synthetase (BLJ_0624 and BLJ_1324) were found in the JDM301 genome, where these proteins play a role in the interaction with human epithelial cells. The protein DnaK has been shown to be present on the surface of pathogens, such as *Neisseria meningitides*[52]. The glutamine synthetases BLJ_0624 and BLJ_1324 had a 62.11% and 32.49% similarity to the glutamine synthetases in *Mycobacterium tuberculosis* H37 Rv. In

the presence of Plg activators, Plg binding to the bacterial surface is converted to plasmin, which is a broad-spectrum serine protease involved in degradation of fibrin and noncollagenous proteins of extracellular matrices and activates latent procollagenases[53]. It is believed that the capability to intervene with the Plg/plasmin system of a host is a strategy for host colonization and bacterial metastasis shared by several pathogens and commensals of the human intestinal tract[53,54]. The plasminogen-dependent proteolytic activity of *B. lactis* BI07 and *B. longum* was shown to be dose-dependent[55,56].

A homolog (BLJ_0880, 24.18% identity) of a gene encoding a component in ferric dicitrate uptake system (Fec) of *Shigella flexneri* serotype 2a, FecE, was identified in the genome of JDM301. As an iron uptake system, Fec is critical for bacterial survival and plays an important role in bacterial virulence[57]. In addition, BLJ_1105 and BLJ_0409 proteins associated with iron acquisition in JDM301 were 24.49% and 25.56% similar to pyochelin biosynthesis protein in *Pseudomonas aeruginosa,* and BLJ_0712, BLJ_1796 and BLJ_0662 proteins were 25.4%, 27.31 and 29.06% similar to iron transporters of *Salmonella enterica*.

The human pathogen, *Helicobacter pylori*, produces a neutrophil activating protein (NAP) which activate human leukocytes and induces an inflammation, which facilitates the growth of the pathogen[58]. A homolog (BLJ_0021; 26.83% identity) of the gene encoding a NAP was identified in the genome of JDM301.

In JDM301, BLJ_0012 encodes a protein harboring fibronectin-binding motif (Pfam number 07174) that allows mycobacteria to bind to fibronectin in the extracellular matrix and may mediate the adhesion of JDM301 to its host[59]. A potential protein for *Bifidobacteria* adhesion to intestinal cells is the putative LPXTG-motif protein with collagen binding motifs (Cna_B, pfam05738) encoded by BLJ_1801, which shows a 34% identity to a predicted fimbrial subunit in the genome of *B. dentium* Bd1. This protein may be involved in the recognition of and adhesion to mucosal epithelial cell surfaces[19]. Its homologous proteins were also identified in the genome sequences of both *B. longum* NCC2705 and *B. longum* DJO10A genomes[3,60]. *B. longum* subsp. *infantis* 15697, *B. longum* NCC2705 and *B. adolescentis* contains 21, 10 and 11 copies of extracellular solute-binding protein, respectively[3,4]. Comparably, the SBP family 1 proteins are more abundant in JDM301 than the three other *Bifidobacteria* strains due to the genome size.

Finally, JDM301 encodes a number of proteases and peptidase that may contribute to virulence owing their ability to degrade host proteins for bacterial nutrition sources[61]. However, not all the genes associated with virulence have been known until now. Thus, despite the evaluation based on the whole genome sequences, it is recommended that the rat endocarditis and the immunocompromised mouse model should be used for *in vivo* assessment of safety for the low pathogenicity of LAB[48].

Recently, there has been more interest in using probiotic products to promote health and treat diseases. Probiotics have been investigated in clinical trials, such as treatment for diarrhea, D-lactic acidosis, necrotizing enterocolitis, inflammatory bowel disease and so on[39,62-64]. The mechanisms by which probiotics exert their effects are still obscure, which may include modification of gut pH, antagonism of pathogens, modulation of immunity as well as supplements of some nutrients[65]. However, safety issues of probiotics have been discussed in many reports[5,48]. There are reported cases of infections associated with probiotic strains[5]. Although the strain is safe based on phenotype, the information derived from complete bacterial genome sequences reveals some putative unfavorable genes, such as genes encoding for Plg-binding proteins, proteases and genes associated with production of D-lactate. In addition, patients are generally more susceptive to infection and harmful metabolites, such as D-lactate than healthy persons. Thus, the biosafety of probiotics, especially strains used in therapy, must be assessed more carefully and comprehensively.

In conclusion, this study compared the genome of JDM301 with other *Bifidobacteria* and assessed the genomic stability, the potential for antibiotic resistance, the potential for virulence and the potential production of harmful metabolites of this strain. The core genome of *B. longum* is composed of 1265 genes, and 219 genes are unique in JDM301. Our data showed putative virulence genes in the genomes of JDM301 as well as putative genes associated with production of harmful metabolites. In addition, a potentially transferable antibiotic resistance gene was detected in the chromosome of JDM301, which needs to be experimentally validated. This assessment provides information on potential risk factors, which should be further evaluated experimentally, e.g., *in vivo* assessment using animal models.

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COMMENTS

Background

Bifidobacterium longum JDM301 is a commercial strain used widely in China with several probiotic functions. Recently, there has been more interest in using probiotic products to promote health and treat diseases. As model probiotic bacteria, *Bifidobacteria* are often added to probiotic products in combination with other lactic acid bacteria. The biosafety of probiotic bacteria is attracting more attentions with its enlarged applications. As more commercial probiotic products are being introduced in the market, it is necessary to reassess the safety of these probiotic products using the latest technology.

Research frontiers

With a long and safe history of application, lactic acid bacteria have acquired the status of "Generally Regarded As Safe". However, published reports of rare infections involving *Lactobacilli* or *Bifidobacteria* are available. The strains selected as probiotics are needed to be assessed carefully and comprehensively. This study may contribute to a better biosafety assessment of probiotic bacteria.

Innovations and breakthroughs

This is the first study to assess the biosafety of probiotic bacteria based on

complete genome sequences. Through bioinformatics analysis of the genome sequences, the authors found that although the strain was safe based on phenotype, the information derived from complete bacterial genome sequences revealed some putative unfavourable genes that should be paid attention to.

Applications

The study provides a comprehensive assessment on potential risk factors of a probiotic strain based on complete genome sequences. The information related to biosafety derived from the genome of JDM301 will contribute to a wider and deeper insight into the safety of probiotic bacteria.

Peer review

This is a very nice and comprehensive study assessing the genomic stability, potential of antibiotic resistance, virulence and production of harmful metabolites. This adds valuable information to current knowledge about probiotics.

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