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两歧双歧杆菌BGN4和长双歧杆菌BORI的安全性评估

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Safety Evaluations of Bifidobacterium bifidum BGN4 and Bifidobacterium longum BORI

摘要：在过去的十年中，各种乳酸菌已经商业上可供消费者使用并被消费者稳定使用。但是，最近的研究表明，某些乳酸菌会产生有毒物质并显示出毒性。为了建立乳酸菌的安全指导方针，联合国粮食及农业组织（FAO）/世界卫生组织（WHO）建议通过多种实验（例如抗生素抗药性，代谢活性，毒素产生，溶血活性，免疫功能缺陷的动物物种的感染性，人的副作用和不良结果分析 ）对乳酸菌进行表征并证明其对消费者健康是安全的。在乳酸菌中，双歧杆菌和乳杆菌种是益生菌菌株，最常被使用于商业生产和积极研究。两歧双歧杆菌BGN4和长双歧杆菌BORI已在全球功能性食品市场（例如中国，德国，约旦，韩国，立陶宛，新西兰，波兰，新加坡，泰国，土耳其和越南）中用作营养成分数十年，没有发生任何不良事件。但是，鉴于最近对一些新筛选的益生菌物种的安全性的争论，确认每种被用于商业的菌株对消费者的安全性是至关重要的。因此，本文详细介绍了通过评估氨的产生，血细胞的溶血，生物胺的产生，抗菌药的敏感性模式，抗生素抗性基因的可转移性，抗生素抗性基因的PCR数据，粘蛋白降解，基因组稳定性以及是否具有毒力因子来对两歧双歧杆菌BGN4和长双歧杆菌的BORI的安全性进行评估。这些益生菌菌株既不显示溶血活性也不显示粘蛋白降解活性，并且不产生氨或生物胺（即尸胺，组胺或酪胺）。两歧双歧杆菌BGN4和长双歧杆菌BORI产生少量的腐胺，通常在活细胞中被发现，其含量与其他食物（例如菠菜，番茄酱，青豆，酸菜和香肠）相似或更低。两歧双歧杆菌BGN4对庆大霉素的耐药性高于欧洲食品安全局（EFSA）的临界值。但是，本文显示两歧双歧杆菌BGN4的庆大霉素抗性并未通过与嗜酸乳杆菌 ATCC 4356 结合而转移，后者对庆大霉素高度敏感。两歧双歧杆菌BGN4的整个基因组序列已在GenBank中公开（登录号：CP001361.1），记录了缺乏能够转移抗生素抗性基因的质粒的保留。此外， 两歧双歧杆菌BGN4的第1代和第25代之间几乎没有遗传突变。四环素抗性基因在长双歧杆菌菌株中普遍存在。长双歧杆菌BORI在其染色体DNA上具有tet（W）基因，并且还显示出对四环素的抗性。但是，这项研究表明，其四环素抗性并未通过与发酵乳杆菌AGBG1的结合而转移，而后者对四环素高度敏感。这些发现支持了这两种已经被几项临床研究报告为安全的，并且已经用于食品补充剂多年的两歧双歧杆菌BGN4和长双歧杆菌BORI作为益生菌的继续使

用。

关键词：益生菌；安全性；抗生素耐药性；功能性食品；保健品

1.简介

自“益生菌”于1960年代首次出现以来，该术语已被各种学者和团体定义。近年来，益生菌已被多个监管机构明确定义。根据粮农组织/世界卫生组织的定义，益生菌可以定义为“活微生物，如果给予足够的量，可以给宿主带来健康益处”。其他专家也类似地将益生菌定义为“活的微生物，当它们被摄入或局部应用足够数量时，会为消费者提供一种或多种已经证实的健康益处”。被认为是益生菌的可食用微生物是从各种已经对人类健康益处进行了研究的菌株，物种和属中衍生出来的。 包括芽孢杆菌属，乳杆菌属，双歧杆菌属，链球菌属和丙酸杆菌属的各种微生物被认为是益生菌，通过免疫介导参与宿主营养代谢和生理功能的维生素生物合成。在这些益生菌微生物中，乳酸杆菌属和双歧杆菌属在全球范围内已被用于发酵食品和商业生产的食品补充剂。截至2010年7月，已经对11个双歧杆菌和21个乳杆菌的基因组进行了全面分析，其微生物基因组序列为益生菌的属和物种提供了准确的证据。一些专家发现，消费者对含有乳酸菌的食品或食品补充剂的需求已导致全球食品市场健康趋势指数呈增长趋势 。然而，这种现象不能忽略微生物的安全标准，也不能让乳酸菌在没有科学研究和安全验证的情况下随意使用。此外，益生菌安全性评估应考虑益生菌的生理特性，治疗方法（例如口服，皮肤喷雾，凝胶，胶囊等），暴露剂量，消费者健康状况以及有效益生菌性能所需的生理功能。

2002年，联合国粮农组织制定了食品工业使用益生菌的四项基本准则，因为各种各样的商用微生物在没有明确的标签标准的情况下被作为益生菌出售给消费者。Huys等人总结的粮农组织准则。具体如下：（i）“菌株鉴定（即属、种、菌株水平）；（ii）筛选潜在益生菌菌株的体外试验（例如，对胃酸、胆汁酸和消化酶的抗性，对潜在致病菌的抗菌活性等）；（iii）安全性评估：需要证明益生菌菌株是安全的，且其运载形式没有污染；以及（iv）证实目标宿主健康影响的体内研究体内研究，以证实目标宿主的健康影响”。 此外，粮农组织建议使用益生菌进行各种试验（例如，抗生素耐药性、代谢活性、毒素产生、溶血活性、免疫受损动物模型的感染性、人类副作用的分析，以及消费者的不良后果），以证明其对宿主的安全性，并详细说明上述指南的第三节。然而，这些安全评估项目是建议而不是法律要求。不同的研究小组根据益生菌的细胞类型和微生物功能，结合附加的实验方法，对益生菌的安全性进行了评估。2002年，欧盟动物营

养科学委员会发布了《食品和动物饲料中使用的食用微生物安全评估和监管指南》。自2016年起，相应的“合格安全推定（QPS）”指南如下：（1）微生物分类的定义；（2）收集足够的信息，为QPS状态提供依据，包括任何科学文献、使用历史、工业应用、生态数据和人为干预数据；（3）排除致病性；（4）最终用途的定义。根据本指南，如果特定分类单元没有安全问题或安全问题得到缓解，则可以授予欧盟食品市场中益生菌细胞QPS的资格。人们普遍认为，微生物的安全性应证明（i）具有遗传信息的物种特征，（ii）表型证据，（iii）隔离史，（iv）缺乏/存在抗生素抗性特性，（v）潜在毒性和/或致病性因素。

商业化生产的乳酸菌最大的安全问题之一是，以饮食形式供应的一些微生物可能作为对肠道病原体的抗生素抗性质粒的供体。一些报道发现，在抗生素治疗的情况下，由于质粒的转移耐药，一些菌株在人体胃肠道中存活下来。多种微生物基因可以通过质粒转移到肠道内的肠道细菌中，导致耐药性的传播。因此，在大规模生产商业用途的乳酸菌之前，确保益生菌菌株的安全是必要的。

尽管一些双歧杆菌和乳酸杆菌已经在体内和体外研究中显示出希望，但是缺乏明确的临床证据来支持这些微生物对健康的益处。因此，许多团体和研究人员正试图通过临床实验来证明乳酸菌的疗效。从母乳喂养的健康婴儿的粪便中分离出的两歧双歧杆菌BGN4和长双歧杆菌BORI，自2000年以来已作为食品成分被商业化使用。包括两歧双歧杆菌和长双歧杆菌在内的一些双歧杆菌菌株在韩国《保健功能食品法典》中被登记为功能性成分益生菌（II.2.51）。多年来，许多研究揭示了两歧双歧杆菌BGN4的功能，其完整的基因组序列已报告给GenBank 。 长双歧杆菌 BORI，同样从母乳喂养的健康婴儿中分离出来，并存放在KCCM（韩国微生物培养中心，14092）中，在对轮状病毒感染的婴儿进行的临床研究中，证明其在统计学上可缩短腹泻的持续时间。 两种益生菌菌株均已证明可有效形成健康的肠道菌群，而无任何不良影响。 但是，还需要进一步的系统研究来证明它们在学术和商业上的应用是安全的。本研究的目的是通过进行联合国粮食及农业组织（FAO）/世界卫生组织（WHO）推荐的实验和其他公开发表的安全性研究来验证两歧双歧杆菌BGN4和长双歧杆菌BORI的安全性。

2.结果与讨论

2.1.氨生产

肠道细菌可以降解肠道粪便中存在的各种氮源（如蛋白质、肽和氨基酸）。 这些自然产生的微生物群和人工施用的菌群具有在脱氮阶段通过氮衍生物产生各种有毒物质的潜力。 多种潜在的有毒产品（即苯酚、氨和吲哚），可能是蛋白水解过程的产物，特别是在大肠中。 因此，细菌的氨的产生与人体肠道健康密切相关，是证明商业益生菌安全性评价的必要组成部分。 此外，最近的研究还表明，肠道微生物产生的氨会影响肝脏，并在慢性肝损害中起辅助因子的作用。 Vince和Burridge报告说，革兰氏阴性厌氧菌、梭菌

（包括产气荚膜梭菌）、肠杆菌和芽孢杆菌会产生大量的氨。一些链球菌、微球菌和革兰氏阳性非芽孢厌氧菌会产生中等浓度的氨。 相比之下，革兰氏阳性需氧杆菌，特别是乳酸杆菌，产生的氨很少。

为了验证这些益生菌的安全性，对两歧双歧杆菌BGN4和长双歧杆菌BORI的产氨量进行了评估。 在本研究中，两歧双歧杆菌BGN4、长双歧杆菌BORI和其他益生菌菌株不产生氨。 相比之下，在本研究中被用作阳性对照的已知有害细菌为拟杆菌属，产气荚膜梭状芽胞杆菌和肠杆菌属，产生的氨为12.9±1.3至161.0±6.6μg/ mL（表1）。 这个测试包括三个重复，给出的值是平均标准偏差。本研究未发现两歧双歧杆菌BGN4和长双歧杆菌BORI产生氨的迹象。

表1. 两歧双歧杆菌BGN4，长双歧杆菌BORI和其他商业微生物的氨水平变量的平均值和标准偏差（n = 3）。

Strain	Ammonia (μg/mL)
Bifidobacterium bifidum BGN4	negative
Bifidobacterium longum BORI	negative
Bifidobacterium breve ATCC 15701	negative
Lactobacillus plantarum KFRI 708	negative
Bacteroides fragilis ATCC 25285	14.7 ± 1.5
Bacteroides thetaiotaomicron ATCC 29741	23.3 ± 3.0
Clostridium perfringens ATCC 13124	23.5 ± 1.6
Enterobacter cloacae ATCC 13047	161.0 ± 6.6
Enterobacter faecalis ATCC 19433	12.9 ± 1.3

2.2.溶血性试验

2002年 FAO / WHO益生菌安全性考虑因素指南明确指出：“如果所评估的菌株属于已知有溶血潜力的物种，则需要测定溶血活性”。 微生物溶血特性是对致病菌(如肠球菌和链球菌)普遍关注的问题，因为它可能导致宿主贫血和水肿。 虽然双歧杆菌是正常的、自然产生的肠道菌群，已被广泛地应用在功能性食品中，并被营养保健行业所利用，但它们可能表现为类似于常见共生微生物的条件致病性微生物。 因此，应该对潜在的益生菌进行溶血试验。 通过在含有动物或人类血液的培养基上培养微生物来观察因溶血活性引起的物理变化是评估病原体溶血特性的常用工具。在这项研究中，使用血琼脂平板法评估了两歧双歧杆菌BGN4和长双歧杆菌BORI的潜在溶血活性。Listeria ivanovii subsp. ivanovii ATCC 19119（阳性对照）在细胞集落周围显示β-溶血无色区，而两歧双歧杆菌BGN4和长双歧杆菌BORI在集落周围无溶血现象且颜色不变（图1）。

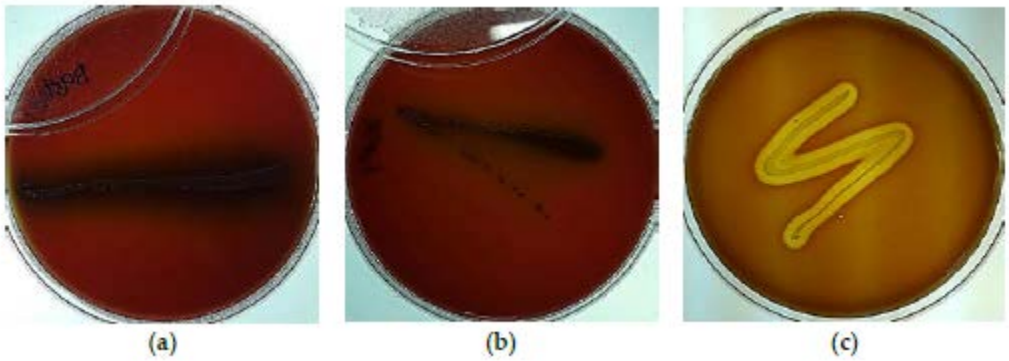

图1. B. bifidum BGN4（（a）;逆光）和B. longum BORI（（b）;逆光）生长而无血细胞溶解。观察到红细胞完全溶解，在Listeria ivanovii subsp.和 ivanovii ATCC 19119菌落周围有清晰的区域（（c）;阳性对照;逆光）。

2.3.生物胺生产评价

生物胺（如尸胺、组胺、酪胺和腐胺）具有疏水性骨架和从动物和人类氨基酸中衍生的天然有机聚阳离子分子。这些分子参与哺乳动物的多种代谢和细胞内活动（如突触传递、血压控制、过敏反应和细胞生长控制）。传统上，各种益生菌因其有益作用和增香特性而被人工添加到发酵食品中。它们的生物胺水平被视为微生物活性和食品新鲜度的指标，因为生物胺是通过微生物代谢活动（即蛋白质分子的脱羧和转氨）产生的。虽然生物胺通常存在于新鲜肉类、蔬菜和奶酪中，但摄入大量生物胺可能会导致人和动物出现类似严重过敏反应的症状。近年来益生菌领域最常见的问题之一是益生菌是否促进生物胺的生产，以及它们如何促进生物胺的生产。复合生物胺（即具有一个以上氨基的多胺）最初被认为是天然存在于各种新鲜食品中的，但最近的研究表明，这些化学物质会因微生物活性而积累。 据报道，一些食用微生物和益生菌菌株能产生生物胺。 因此，本研究的目的是检测作为益生菌安全性评估整体组成部分的两歧双歧杆菌BGN4和长双歧杆菌BORI的生物胺生产。 双歧杆菌的生物胺含量见表2。

表2. 两歧双歧杆菌BGN4和长双歧杆菌BORI的生物胺水平。

Strains	Cadaverine (μg/mL)	Histamine (μg/mL)	Putrescine (μg/mL)	Tyramine (μg/mL)
B. bifidum BGN4	N/D [1]	N/D [1]	24.23	N/D [1]
B. longum BORI	N/D [1]	N/D [1]	16.58	N/D [1]

[1] N/D; not detected.

这些菌株的生物胺含量是通过减去每个培养基中生物胺的背景含量得到的。 两歧双歧杆菌BGN4和长双歧杆菌BORI不产生尸胺，组胺或酪胺；然而，他们分别产生了24.23和16.58 μg / mL的腐胺。所产生

的水平无关紧要。 腐胺是存在于各种食物中的一种天然物质。 腐胺是鸟氨酸和精氨酸脱羧形成的，在活细胞中也有少量存在。 腐胺、亚精胺、精胺和尸胺是活细胞的重要组成部分，在核酸的形成、蛋白质的合成和细胞膜的稳定性等方面起着重要作用。 在各种水果、果汁和蔬菜中检测到的各种生物胺中，腐胺是最常见的。 Kalaˇc报告说，腐胺常见于冷冻菠菜泥（平均12.9mg/kg）、番茄酱（平均52.5mg/kg）、浓缩番茄酱（平均25.9mg/kg）和冷冻青豆（平均46.3mg/kg）。 发酵食品和饮料的腐胺含量在雪利酒中为9 mg/kg（3-25mg/kg，n=28），在泡菜中为154mg/kg（6-550mg/kg，n=8），在荷兰奶酪中为19mg/kg（1-71 mg/kg，n=8）， 在发酵香肠中为52 mg/kg（1-190 mg/kg，n=14）。

此外，在意大利撒丁岛用母羊全脂乳制成的传统奶酪中发现的腐胺在成熟过程中增加到1658毫克/升。双歧杆菌属（即Bifidobacterium CCDM 94，B. adolescentis CCDM 223，B. animalis ssp. lactis CCDM 239，240，241，374，B. bifidum CCDM 559，B. longum CCDM 569）已知可产生尸胺、腐胺、酪胺和亚精胺。根据Pollark等人研究， 腐胺存在于母乳（0~3804nmol/L）和商品配方奶（0~1057±25 nmol/L）中。 因此，腐胺的自然发生量是很重要的。一些研究人员认为食物中的腐胺对组胺毒性有协同作用。 但是，据我们所知，这种协同作用尚未得到实验数据的证明或报道。此外，欧洲食品安全局（EFSA）也指出，缺乏研究来确定增加组胺副作用所需的腐胺的确切水平。两歧双歧杆菌BGN4和长双歧杆菌BORI在发酵过程中不产生任何尸胺，组胺或酪胺。 两歧双歧杆菌BGN4和长双歧杆菌BORI在两种培养基中都能产生低水平的腐胺（即全乳培养基=24.43μg/mL， 两歧双歧杆菌BGN4培养基（全乳）=48.67μg/mL，MRS培养基=26.60μg/mL，长双歧杆菌BORI培养基（MRS）=43.17μg/mL）。单胺和二胺氧化酶的人体氧化系统包括少量的生物胺，它们通常是代谢的和无害的，因为人和动物有能力在体内分解它们。

2.4.抗菌药物敏感性和耐药转移性

2.4.1.抗生素敏感性

各种乳酸菌研究小组已经发出警告，一些作为食物或饲料使用的乳酸菌可能具有耐抗生素特性。由于这种抗药性可以通过质粒转移给其他病原体，因此对抗生素抗药性的评估是评估食品和饲料中菌株安全性的重要标准。此外，已获得的可转移基因在双歧杆菌和乳酸杆菌中已被鉴定。为了区分对抗生素耐药的微生物与对抗生素敏感的微生物，EFSA已经确定了用作食品和/或饲料添加剂的微生物对微生物的耐药性的微生物临界值。这些微生物临界值是根据所选抗菌药物在属于单个分类单位的细胞群体中的最小抑菌浓度（MICs）的分布确定的。

在本研究中所有双歧杆菌属均对氨苄西林、氯霉素、克林霉素、红霉素、青霉素G、利福平和万古霉素敏感(MIC范围为0.01至4μg/ml） 并且通常对氨基糖苷类抗生素，如庆大霉素、卡那霉素、新霉素和链霉素耐药（MIC范围大于32μg/ml，表3）。除庆大霉素和四环素外，两歧双歧杆菌BGN4和长双歧杆菌BORI的MI

C值均等于或低于EFSA动物饲料中使用的添加剂和产品或物质小组（FEEDAP）建议的既定EFSA临界值。 除了长双歧杆菌BORI中四环素的MIC高,两歧双歧杆菌BGN4和长双歧杆菌BORI的敏感性趋势与其他研究相似。 青霉素G、氨苄西林、万古霉素、庆大霉素、红霉素、甲氧苄啶磺胺甲恶唑和甲硝唑是儿科患者常用的抗生素。两歧双歧杆菌BGN4和长双歧杆菌对甲氧苄啶磺胺甲恶唑耐药，但10株双歧杆菌中有6株在本研究中的MIC值也超过128μg/ml（表3）。

Mättö 等人报告了双歧杆菌菌株对链霉素和庆大霉素的MIC普遍较高，表明它们的耐药性是固有的。Ammor等人从21种食品样品中分离出益生菌，例如酸奶，酸奶型发酵乳和药品，并发现了22株双歧杆菌。在他们的研究中，双歧杆菌对氨基糖苷类药物具有耐药性（MIC_{90}的范围为64-1000g/ml），对卡那霉素具有很强的耐药性（MIC_{90}=1000g/ml）。他们还证明了一些MIC范围没有重叠，这意味着与这些MIC范围相关的抗生素可用作选择性培养基中的成分。他们认为双歧杆菌对庆大霉素的选择性范围为32至64μg/ mL，卡那霉素的选择范围为64至500μg/ mL 。因此，含有庆大霉素培养基和含有培养基莫匹罗星已经被用于双歧杆菌的筛选和计数。因此，这种抵抗可以被认为是内在的。进行了抗生素耐药性转移性研究，以证实这种耐药性的性质。

表3. 两歧双歧杆菌BGN4和长双歧杆菌BORI及其他双歧杆菌的抗药性（MIC值）。

Antibiotics	EFSA Cut-Off of *Bifidobacterium* spp.	*B. longum* ATCC 15707	*B. longum* BB536	*B. longum* KCCM 91563	*B. longum* BORI	*B. infantis* ATCC 15697	*B. lactis* BB-12	*B. bifidum* BGN4	*B. bifidum* KCTC 3440	*B. adolescentis* ATCC 15703	*B. breve* M-16V	*E. faecalis* ATCC 29212
Penicillin G		0.25	0.125	0.5	1	0.125	0.125	0.063	0.063	0.25	0.25	0.5
Carbenicillin disodium salt		2	2	4	8	0.5	2	0.5	0.5	4	4	8
Methicillin		8	4	16	16	1	2	1	0.5	8	8	16
Ampicillin sodium salt	2	0.5	0.25	1	0.5	0.125	0.125	0.063	0.063	0.25	0.25	0.25
Dicloxacillin sodium salt hydrate		4	4	8	8	0.5	4	0.5	1	256	8	4
Gentamicin sulfate	64	32	64	32	32	16	128	128	256	128	128	256
Streptomycin sulfate salt	128	32	128	64	64	>256	128	64	32	128	256	>256
Kanamycin sulfate	N/R[1]	512	1024	1024	512	32	1024	1024	1024	1024	1024	256
Neomycin sulfate		1024	512	512	512	64	512	1024	512	512	1024	1024
Cephalothin sodium salt		8	4	16	32	4	8	4	2	16	16	16
Tetracycline	8	1	1	1	64	2	16	1	1	8	16	32
Polymyxin B sulfate salt		256	32	256	256	128	256	512	512	512	1024	>1024
Erythromycin	1	0.125	0.5	0.5	0.5	0.125	0.125	0.125	0.125	0.125	0.125	8
Metronidazole		8	8	>256	>256	8	4	4	64	>256	8	>256
Vancomycin hydrochloride	2	0.5	<0.25	<0.25	1	0.5	0.5	1	2	0.5	0.5	2
Chloramphenicol	4	2	2	2	4	2	2	2	2	2	2	8
Rifampicin		<0.125	<0.125	<0.125	0.25	<0.125	2	0.5	0.25	0.5	1	0.5
Clindamycin hydrochloride	1	<0.032	0.063	0.063	0.125	0.25	<0.032	0.063	0.063	<0.032	<0.032	>16
Phosphomycin disodium salt		128	256	256	256	16	64	128	256	64	32	32
Mupirocin		>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	64
Trimethoprim-Sulfamethoxazole		128	256	128	256	256	1	128	64	1	2	32

[1] N/R denotes not required.

2.4.2. 抗生素耐药可转移性

由于两歧双歧杆菌BGN4和长双歧BORI在这些抗菌药敏试验中表现出对庆大霉素和/或四环素的高度耐药性，因此使用对四环素高度敏感的受体菌株发酵乳杆菌AGBG1进行了四环素抗性转移性测试。为了测试两歧双歧杆菌 BGN4和长双歧杆菌 BORI 的庆大霉素抗性的转移性，嗜酸乳杆菌 ATCC 4356由于其对

庆大霉素的高敏感性而被用作受体菌株。结合结果示于表4。

表4. 四环素抗性从供体（长双歧杆菌 BORI和两歧双歧杆菌 BGN4）到受体（发酵乳杆菌 AGBG1和嗜酸乳杆菌 ATCC 4356）的转移性（cfu / mL）。

Antibiotics	AGBG1 (Aerobic)	AGBG1 + BORI		BORI (Anaerobic)
		Aerobic	Anaerobic	
None [1]	4.38×10^8	3.38×10^8	2.27×10^8	4.56×10^8
T8 [2]	0	0	4.44×10^6	7.11×10^7
Antibiotics	ATCC 4356 (Aerobic)	ATCC 4356 + BORI Aerobic	Anaerobic	BORI (Anaerobic)
None [1]	3.65×10^8	1.67×10^8	2.34×10^8	3.14×10^8
G64 [3]	0	0	2.78×10^6	1.46×10^8
Antibiotics	ATCC 4356 (Aerobic)	ATCC 4356 + BGN4 Aerobic	Anaerobic	BGN4 (Anaerobic)
None [1]	3.65×10^8	3.29×10^8	2.54×10^8	3.86×10^8
G64 [3]	0	0	4.64×10^6	1.43×10^8

[1] No antibiotics were included in the counting agar medium. [2] Tetracycline (8 µg/mL) was included in the counting agar medium. [3] Gentamicin (64 µg/mL) was included in the counting agar medium.

在含四环素的培养基中单独培养或与长双歧杆菌 BORI 共培养时，发酵乳杆菌 AGBG1 不会生长。本文报道的抗菌药敏试验发现，尽管两歧双歧杆菌BGN4对四环素非常敏感（MIC 1.0µg/ mL），但长双歧杆菌BORI对四环素耐药（MIC 64µg / mL）。但是，在这项研究中，长双歧杆菌 BORI 的四环素抗性并未转移至受体发酵乳杆菌 AGBG1 。对庆大霉素高度敏感的嗜酸乳杆菌 ATCC 4356在正常MRS培养基中生长良好；然而，嗜酸乳杆菌ATCC 4356在含有庆大霉素的MRS培养基或与两歧双歧杆菌BGN4或长双歧杆菌BORI共培养的培养基中未生长。相比之下，两歧双歧杆菌BGN4和长双歧杆菌 BORI在本研究中显示出对64µg/ mL庆大霉素的表现出耐药性。因此，这证明两歧双歧杆菌 BGN4对庆大霉素的抗性和长双歧杆菌 BORI对庆大霉素和四环素的抗性没有转移到受体菌株中。值得注意的是，美国医疗保健研究与质量局（AHRQ）于2011年发布的一份报告广泛审查了关于六个属（即乳酸杆菌、双歧杆菌、酵母菌、链球菌、肠球菌和芽孢杆菌属）的 622项研究。没有发现任何临床证据表明益生菌基因转移到其他微生物的理论可能性。

2.4.3.抗生素耐药基因的pcr检测

尽管两歧双歧杆菌 BGN4（登录号：CP001361.1）和长双歧杆菌 BORI 的整个基因组均显示它们均不包含能够转移抗药性基因的质粒，但对庆大霉素（aaac（6）-aph（2））、卡那霉素（AphA3，aaaD）、链霉素（aadE）、甲氧苄啶（dfrA）和四环素（tet（K）、tet（L）、tet（M）、tet（O）、tet（S））等十种抗生素基因进行PCR分析。在这项研究中所有测试的双歧杆菌属均使用16S rRNA 双歧杆菌属特异性引物进行鉴定（图2）。抗生素基因的PCR结果如图3所示。在两歧双歧杆菌 BGN4，长双歧杆菌 BORI和其他双歧杆菌属中均没有指示抗性基因的扩增子。

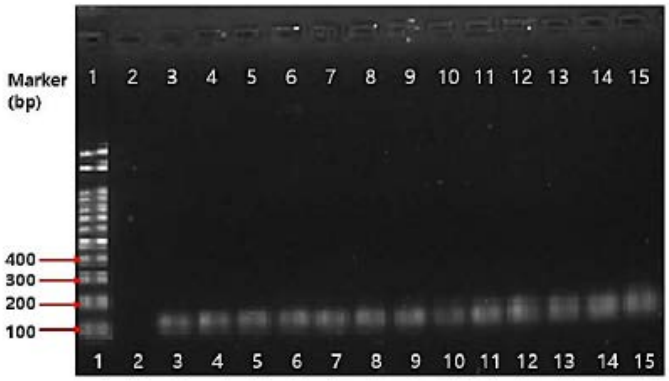

图2. 各种双歧杆菌属的PCR分析结果。泳道1：标记；泳道2：无负载；泳道3：B. lactis AS60；泳道4：B.bifidum KCTC 3440；泳道5:B.longum BORI；泳道6：B.longum KCCM 91563；泳道7：B.lactis BB-12；泳道8：B.longum RD47；泳道9：B.bifidum BGN4；泳道10：B.thermophilum KCCM 12097；泳道11：B.adolescentis ATCC 15703；泳道12：B. lactis AD011；泳道13：B.infantis ATCC 15697；泳道14：B. breve M-16V；泳道15：B.animalis ATCC 25527。

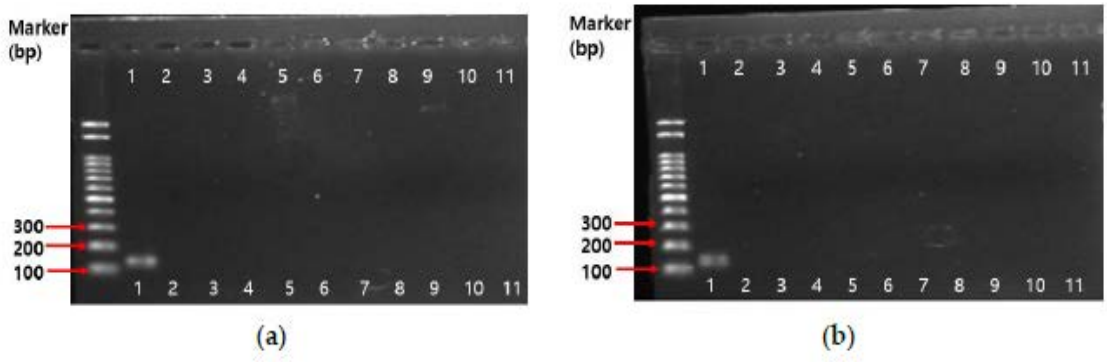

图3. 两歧双歧杆菌BGN4和长双歧杆菌BORI中的抗生素抗性基因的PCR分析结果：（a） B.bifidum BGN4；（b）B. longum BORI；泳道1：双歧杆菌属特异性引物；泳道2：庆大霉素（aaac（6）-aph（2））；泳道3：卡那霉素（AphA3），泳道4：链霉素（aadE），泳道5：甲氧苄啶（dfrA）；泳道6：四环素K（tet（K））；泳道7：四环素L（tet（L））；泳道8：四环素M（tet（M）），泳道9：四环素O（tet（O）），泳道10：四环素S（tet（S））；泳道11：卡那霉素（aaaD）。

最近，双歧杆菌属固有的庆大霉素抗性被认为是由于存在两个基因，即Bbr_0651和Bbr_1586，这两个基因是双歧杆菌染色体DNA中存在的酶，同时它们都编码假定的磷酸转移酶。四环素抗性基因广泛分布在双歧杆菌属中；然而，它被称为核糖体保护蛋白。四环素W（tet（W））基因在长双歧杆菌BORI染色体DNA中被发现。在Mättö等人的研究中，与人和益生菌有关的双歧杆菌物种（203株）对四环素显示出较高

的MIC值（即≥16mg / mL；患病率为4-18%），这归因于四环素基因的存在（tet），其中检测到tet（W）和tet（O）。tet（W），和tet（M）分别在26，7%的双歧杆菌菌株列内被发现。tet（W）的基因被推测为TRAFAC家族的翻译因子GTPase，其诱导核糖体的非共价修饰，破坏四环素的作用，抑制蛋白质合成。

2.5.粘蛋白降解

肠粘液凝胶层是由糖蛋白家族组成的肠屏障的重要组成部分。多个研究小组报告说，即使肠道粘液起到了抵御微生物的生物学作用，细菌移位仍可能发生在婴儿和免疫功能低下的宿主中。这种细菌易位可能导致败血症，并且是最严重的益生菌安全性问题之一。一些科学家还报道了由于使用益生菌菌株而引起菌血症性心内膜炎的可能性。根据Ruas-Madiedo等人的说法，一些双歧杆菌属具有粘液溶解活性，并具有诱导粘蛋白降解酶的基因。但是，大多数双歧杆菌属物种，例如长双歧杆菌和假双歧杆菌，不表现出粘液溶解活性。

为了确认其微生物安全性，有必要通过每种菌株的粘液溶解能力分析来评估其易位能力。在这项研究中，两歧双歧杆菌BGN4和长双歧杆菌BORI的易位能力是使用体外粘液溶解测定法测量的。在五种改良的MRS培养基中，通过测量其在550 nm时的吸光度来检查培养后的细胞生长速率：基础培养基（无葡萄糖MRS，◇），具有0.5%粘蛋白（×），1.0%粘蛋白（○），0.5%的葡萄糖（Δ）和1.0%的葡萄糖的基础培养基。（□）（图4）。

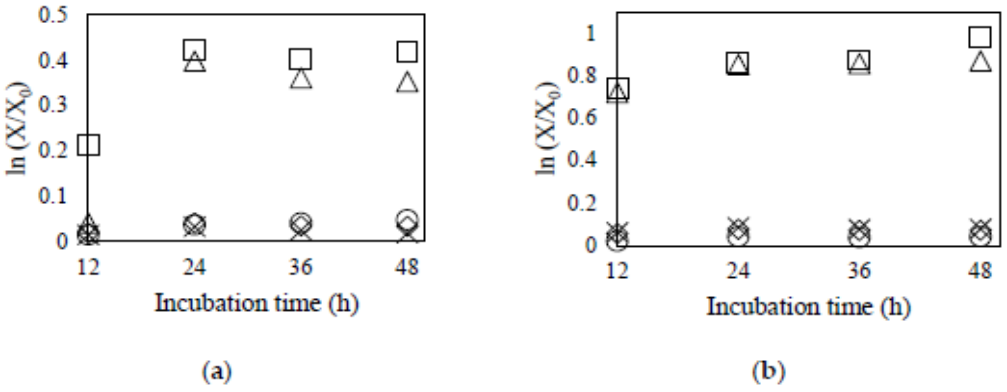

图4. 两歧双歧杆菌BGN4（a）和长双歧杆菌 BORI（b）在具有多种碳源的改良MRS中的 生长曲线：基础培养基（无葡萄糖MRS，◇），具有0.5%粘蛋白（×），1.0%粘蛋白（○），0.5%的葡萄糖（Δ）和1.0%的葡萄糖的基础培养基。（□）

通常，当添加单糖（例如葡萄糖，果糖，麦芽糖和蔗糖）时，由于分解代谢抑制，粘蛋白酶的产生可

以被抑制。尽管微生物有可能产生粘蛋白分解酶，但仍可能得到假阴性结果。因此，为了获得准确的数据，有意从培养实验细胞的培养基中除去了通常在MRS培养基中用作碳源的葡萄糖。如果两歧双歧杆菌BGN4和长双歧杆菌BORI能够产生粘蛋白酶，那么它们将能够通过粘蛋白消化获得碳源并积极生长。如图2所示当添加葡萄糖作为碳源时，两种益生菌菌株的生长均被积极诱导。然而，当添加粘蛋白代替葡萄糖时，在任一菌株中均未观察到生长。这些观察结果清楚地表明，两歧双歧杆菌BGN4和长双歧杆菌BORI并未使用粘蛋白作为生长的碳源。这项研究中，正如其他的研究所建议的那样，无论是两歧双歧杆菌BGN4还是长双歧杆菌BORI均不能降解粘蛋白，说明该菌株不能够破坏肠道表面的能力并且不具有易位能力。

2.6.遗传稳定性

食用微生物的遗传变异可能导致插入缺失（即基因缺失和插入）和突变。益生菌商业化的一个关键考虑因素是是否有可能长期维持遗传安全。然而，商业益生菌菌株的遗传稳定性还没有被报告。从理论上讲，遗传稳定性的评估需要菌株的整个基因组序列。

双歧杆菌BGN4的全基因组序列已经发表， 由2,223,664 bp的环状染色体（62.65%G + C）组成，无质粒。从核苷酸序列中总共编辑了1835个编码序列（CDS），7个假基因，3个rRNA操纵子和52个tRNA。这项研究表明，通过直系同源平均核苷酸同一性（OrthoANI）值比较，第1代和第25代样品在基因组比较中的相似性为99.9996∽99.9998%。（表5）。

表5. OrthANI 值

Strain/Sample	*B. bifidum* BGN4-1/13075.BBGN41.1 [1]	*B. bifidum* BGN4-2/13075.BBGN42.1 [2]	*B. bifidum* BGN4-3/13075.BBGN43.1 [3]
B. bifidum BGN4-1/13075.BBGN41.1 [1]	100	99.9997	99.9996
B. bifidum BGN4-2/13075.BBGN42.1 [2]	99.9997	100	99.9998
B. bifidum BGN4-3/13075.BBGN43.1 [3]	99.9996	99.9998	100

[1] *B. bifidum* BGN4-1/13075.BBGN41.1 denotes the 1st generation; [2] *B. bifidum* BGN4-2/13075.BBGN42.1 denotes the 25th generation; [3] *B. bifidum* BGN4-3/13075.BBGN43 and *B. bifidum* BGN4-2 are the 25th generations.

0.0002%和0.0004%之间的差异相当于整个核苷酸序列的4.4到8.8 bp突变，可以认为是由于测序错误或自发的进化突变。因此，可以得出结论，在培育25世代的过程中，遗传突变很少，遗传信息也没有改变。

2.7.毒力因素

将两歧双歧杆菌BGN4和长双歧杆菌BORI的基因组序列与四种常见致病菌（大肠杆菌，肠球菌，李斯

特菌和金黄色葡萄球菌）的基因组序列进行了比较。毒力因子包括大肠杆菌志贺毒素基因和金黄色葡萄球菌外切酶基因，宿主免疫改变或逃避基因和毒素基因。在两歧双歧杆菌 BGN4和长双歧杆菌 BORI 的基因组序列中未发现毒力因子。因此，该结果表明两歧双歧杆菌BGN4和长双歧杆菌BORI的基因组序列不包括与大肠杆菌，肠球菌，李斯特菌和金黄色葡萄球菌有关的毒性或致病基因。

3.材料与方法

3.1.微生物

表6列出了本研究中使用的细菌菌株，包括来源，培养基和测试方法。

表6：菌株列表和方法

Strains	Origin	Medium	Method
Bifidobacterium bifidum BGN4	BIFIDO Co., Ltd. (Hongcheon, Korea)	BHI [1], Blood agar [2], whole milk [3], LSM-Cys [4], MRS [5–8]	3.2., 3.3., 3.4., 3.5.2., 3.5.3., 3.5.4., 3.6., 3.7
Bifidobacterium longum BORI	BIFIDO Co., Ltd. (Hongcheon, Korea)	BHI [1], Blood agar [2], MRS [3,5–7], LSM-Cys [4]	3.2., 3.3., 3.4., 3.5.2., 3.5.4., 3.6
Bacteroides fragilis ATCC 25285	American Type Culture Collection (Manassas, VA, USA)	BHI [1]	3.2
Bacteroides thetaiotaomicron ATCC 29741	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Bifidobacterium adolescentis ATCC 15703	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium animalis ATCC 25527	American Type Culture Collection (Manassas,VA, USA)	MRS [6]	3.5.4
Bifidobacterium animalis subsp. *lactis* AD011	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4

Bifidobacterium animalis subsp. *lactis* AS60	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4
Bifidobacterium animalis subsp. *lactis* BB-12	Isolated from a pharmaceutical product, USA	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium bifidum KCTC 3440	Korean Collection for Type Cultures, (Jeongeup, Korea)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium breve ATCC 15701	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Bifidobacterium breve M-16V	Isolated from a pharmaceutical product, USA	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium infantis ATCC 15697	American Type Culture Collection (Manassas, VA, USA)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium longum ATCC 15707	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4]	3.5.2
Bifidobacterium longum BB536	Isolated from a pharmaceutical product, USA	LSM-Cys [4]	3.5.2
Bifidobacterium longum KCCM 91563	Korean Culture Center of Microorganisms (Seoul, Korea)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium longum RD47	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4
Bifidobacterium thermophilum KCCM 12097	Korean Culture Center of Microorganisms (Seoul, Korea)	MRS [6]	3.5.4
Clostridium perfringens ATCC 13124	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Enterococcus faecalis ATCC 29212	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4]	3.5.2
Enterobacter cloacae subsp. *cloaca* ATCC 13047	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Enterobacter faecalis ATCC 19433	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Lactobacillus acidophilus ATCC 4356	American Type Culture Collection (Manassas,VA, USA)	MRS [5]	3.5.3
Lactobacillus fermentum AGBG1	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [5]	3.5.3
Lactobacillus plantarum KFRI 708	Korea Food Research Institute (Wanju, Korea)	BHI [1]	3.2
Listeria ivanovii subsp. *ivanovii* ATCC 19119	American Type Culture Collection (Manassas,VA, USA)	Blood Agar [2]	3.3

[1]氨生产测试（3.2）： B. bifidum BGN4, B. longum BORI, B. breve ATCC 15701, L. plantarum KFRI708, B. fragilis ATCC 25285, B. thetaiotaomicron ATCC 29741, C. perfringens ATCC 13124, E. cloacae ATCC 13047,and E. faecalis ATCC 19433 在脑心浸液（BHI）（BD BBL™, Franklin Lakes, NJ, USA）中于37℃厌氧培养5天。[2]溶血试验（3.3）： B. bifidum BGN4和B. longum BORI 在血琼脂（补充有1.5%琼脂和5%羊血的BHI肉汤培养基）中于37°C厌氧培养2天。 Listeria ivanovii subsp ，ivanovii ATCC 19119，溶血的阳性对照，在血液琼脂培养需氧在37℃下2天。[3]生物胺生产测试（3.4）：B. bifidum BGN4和B. longum BORI在全脂牛奶（韩国首尔牛奶）或 de Man–Rogosa–Sharpe (MRS)肉汤（BD Difco™, Franklin Lakes, NJ, USA）中补充0.05%（w / w）L-半胱氨酸盐酸盐（Sigma，St.Louis，MO，USA）中在37°C的条件下厌氧培养15个小时 。[4]药敏试验（3.5.2.）：添加0.03%L-半胱氨酸HCL的LSM-Cys肉汤培养基，由90%的IST和10%的MRS肉汤培养基

组成。[5]抗生素耐药性传递能力测试（3.5.3.）：将双歧杆菌菌株在添加了0.05％（w / v）L-半胱氨酸HCl的MRS肉汤培养基中厌氧培养，并将乳酸杆菌菌株在不添加L-半胱氨酸盐酸盐的条件下37°C 培养18小时。[6]对抗生素抗性基因（3.5.4.）的PCR测定：将双歧杆菌菌株在添加了0.05％的L-半胱氨酸盐酸盐在MRS肉汤培养基中37°C下厌氧培养18 h。[7]粘蛋白降解试验（3.6.）：将B. bifidum BGN4和B. longum BORI在添加了0.05％（w / v）L-半胱氨酸盐酸盐的MRS肉汤培养基中厌氧培养37小时到48小时。[8]遗传稳定性测试（3.7.）： B. bifidum BGN4在补充0.05％（w / v）L-半胱氨酸盐酸盐的MRS肉汤培养基中厌氧培养。

3.2.氨生产测试

两歧双歧杆菌 BGN4，长双歧杆菌 BORI，短双歧杆菌 ATCC 15701，植物乳杆菌 KFRI 708，脆弱拟杆菌 ATCC 25285，多形拟杆菌 ATCC 29741，产气荚膜梭菌 ATCC 13124，阴沟肠杆菌 ATCC 13047和粪肠球菌将ATCC 19433在脑心浸液（BHI）（BD BBL™，Franklin Lakes，NJ，USA）中于37°C厌氧培养5天。根据Chaney和Marbach的方法确定了催化的吲哚酚反应产生的氨。为了评估产生的细胞外氨水平，通过以10,000×g速度在4°C下离心30分钟获得每个菌株的培养基上清液。然后使用1N的NaOH将培养基的pH调节至7。制备如下两种溶液：溶液1由2g苯酚和0.01g脱水亚硝酸铁氰化钠溶于200 mL蒸馏水中组成，溶液2由1g氢氧化钠和0.08g次氯酸钠由溶于200 mL蒸馏水中组成。将溶液1和2的等分试样（10μL）加到96孔板中，每个菌株的培养基上清液为100μL。在每个菌株上进行三个重复。将96孔板在室温下保持1小时，并在625nm处测量吸光度。无细菌的BHI培养基用作阴性对照，并使用标准曲线计算氨浓度。

3.3.溶血试验

将两歧双歧杆菌BGN4和长双歧杆菌BORI在血琼脂（添加有1.5％琼脂和5％羊血的BHI肉汤培养基）中于37°C厌氧培养2天。Listeria ivanovii subsp. ivanovii ATCC 19119 （溶血的阳性对照）在血琼脂中于37°C有氧培养2天。然后通过将平板举到光源处并通过板的两侧进行观察来分析是否存在微生物溶血特性。在菌落周围产生绿色色调区域（α-溶血）或在血琼脂板上不产生任何溶血（γ-溶血）的菌株被认为是非溶血性的。在菌落周围显示出溶血带（白色的区域）的菌株被分类为具有溶血（β-溶血）特性的微生物。

3.4.生物胺生产测试

两歧双歧杆菌 BGN4和长双歧杆菌BORI在添加 0.05％（w/w）L-半光氨酸盐酸盐（Sigma，St. Louis，MO，USA)的全脂牛奶（韩国首尔牛奶）或 de Man-Rogosa-Sharpe（MRS）肉汤（BD Difco™，

Franklin Lakes, NJ, USA）中37°C的条件下厌氧培养15个小时。四种生物胺（尸胺（≥97.0%，Cat. #33211 ），组胺（≥97.0%，Cat. #H7125 ），腐胺（≥98.5%，Cat. #51799 ）和酪胺（99%，Cat. #T90344 ）购自Sigma-Aldrich（St. Louis, MO, USA ）。1,7-二氨基庚烷（(internal standard; ISTD, 98%, Cat. #D174708 ），丹磺酰氯（≥99.0%，Cat.#39220）和L-脯氨酸（≥99.0%，Cat. #P0380 ）也购自Sigma-Aldrich（ St. Louis, MO, USA ）。Whatman No.4滤纸购自Whatman Intl., Ltd. (Maidstone, UK)。碳酸钠（99.0%，Cat.＃433401201），乙醚（99.0%，Cat.＃33475S1280）和丙酮（99.7%，Cat.＃A0108）购自Samchun Pure Chemical Co., Ltd.(Pyeongtaek, Korea)。

如Kim和Ji所述进行生物胺分析提取程序。每5g样品称重，并用25 mL的0.1 N HCl涡旋振荡5分钟。将得到的匀浆在4°C 以10,000× g离心15分钟（2236R高速离心机; Labogene Aps, Lillerød, 丹麦）中处理15分钟，收集水层，并如上所述再次萃取残留物。通过Whatman No.4滤纸过滤收集的提取物。将每种提取物一毫升转移到玻璃试管中，并添加以下物质：0.1 mL内标物（1,7-二氨基庚烷，100 mg / L），0.5 mL饱和碳酸钠，添加 1%丹磺酰氯的1 mL丙酮 。充分混合后，将试管在深色水浴（WBC 1510A; Jeio Tech.Co., Ltd., 韩国首尔）中于45°C孵育60分钟。随后，将0.5mL的10%脯氨酸和5 mL的乙醚加入到每个样品中，静置5分钟以除去残留的丹磺酰氯。在20°C下将上清液悬浮并蒸发直至干燥（Scanvac Speed Vacuum Concentrator; Labogene Aps, Lillerød, 丹麦）。将干燥的残余物用1mL乙腈（ Sigma-Aldrich, St. Louis, MO, USA ）稀释。重构的样品和标准品通过0.2 μm注射器过滤器进行过滤，以进行HPLC分析。在首尔国立大学（韩国首尔）的国家环境管理仪器中心（NICEM）进行了生物胺的HPLC分析。 HPLC测定方法如表7。

表7.HPLC条件

Parameters	**Conditions**		
HPLC	Thermo Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, St Peters, MO, USA)		
Column	VDSpher C-18 column (4.6 × 250 mm, 5 μm) (VDS optilab Chromatographietechnik GmbH, Berlin, Germany)		
Mobile solvent	Time (min)	Distilled Water (%)	Acetonitrile (%)
	0	40	60
	1	40	60
	20	0	100
	25	0	100
	26	40	60
	30	40	60
Flow rate	0.8 mL		
Column temperature	30 °C		
Injection volume	20 μL		
Detector	UV 250 nm		

3.5.抗菌药敏性和抗生素耐药性转移性测试

3.5.1.抗菌剂

使用了20种抗菌剂：氨苄西林钠（Sigma，Lot# BCBW1243）、卡宾西林二钠盐（Sigma，Lot #116M4834V）、头孢氨苄钠（Sigma ，Lot# 056M4858V）、氯霉素（Sigma，Lot#SLBR8869V）、盐酸克林霉素（Sigma，Lot#021M1533）、水合双氯西林钠（Sigma，Lot#SZBD263XV），红霉素（Sigma，Lot＃WXBC4044V），硫酸庆大霉素（Sigma，Lot＃SLBP3082V），硫酸卡那霉素（Sigma，Lot＃066M4019V），甲硝唑（Sigma，Lot＃MKBZ3056V），莫匹罗星（Sigma，Lot＃106M4733V），硫酸新霉素（ Sigma，Lot＃LRAB3300），青霉素G（Sigma，Lot＃087M4834V），磷酸霉素二钠盐（Sigma，Lot＃096M4031V）， 多粘菌素B硫酸盐（Sigma，Lot＃027M4002V），利福平（Sigma，Lot＃MKCC2435），链霉素硫酸盐（Sigma，Lot＃SLBT8451），四环素（Sigma，Lot＃126M4769V），甲氧苄啶-磺胺甲恶唑（三甲氧苄啶（Sigma，Lot ＃097M4017V），磺胺甲恶唑（Sigma，Lot ＃BCBT3855）），盐酸万古霉素（USP，Lot ＃R07250）。盐酸万古霉素购自USP（ Rockville，MD，USA ），其余19种抗生素购自Sigma（St. Louis，MO，USA ）。 每种抗生素粉末在加入LSM-Cys肉汤培养基之前，在适当的稀释剂中溶解和稀释，并过滤消毒，该培养基由90%的IST和10%的MRS肉汤培养基组成。IST肉汤是从Kisanbio有限公司（ Mbcell Iso-Sensitest Broth，韩国首尔）购买的，MRS是从 Becton，Dickinson and Company（BD Difco™ MRS Lactobacilli broth，Franklin Lakes，NJ，USA）购买的。制备了1024～0.0032μg/ml的抗菌剂系列稀释液。

3.5.2.抗菌药敏试验

通过ISO 10932：2010肉汤微量稀释程序确定所有细菌分离株的最小抑菌浓度（MIC）值。添加含有不同浓度抗生素的0.03%（w / v）L-半胱氨酸盐酸盐的LSM-Cys肉汤培养基用于制备微孔板的每个孔。用Hitachi分光光度计(Hitachi High-Technologies Co.，Tokyo，Japan））测得，在625nm处将接种物的浊度调节至0.16-0.2。该溶液对应于大约3×10^{8}cfu / mL。将每种接种物以0.2%的比率添加到双重强度的LSM-Cys肉汤培养基中。将50μL稀释的细菌悬浮液添加到每个孔中；没有使用阴性对照孔。用一系列两倍稀释的抗生素制备微稀释板。微量稀释板在37℃下厌氧培养48小时（5%CO_2，10%H_2和85%N_2）。MIC被定义为与无抗生素对照孔相比完全抑制可见生长的抗生素最低浓度。实验重复三次。

3.5.3.抗生素耐药性转移试验

通过Tannock的方法评估了抗生素耐药性的共生转移。将供体和受体菌株的相等细菌细胞体积（1 mL）混合，并以7000xg的速度离心10分钟(2236R高速离心机；Labogene Aps，Lillerød，Denmark)（见

表8)。弃去上清液后，将细菌细胞沉淀物重悬于MRS肉汤培养基中，并在37°C的厌氧室内培养12小时。收集的细菌细胞通过0.45μm的微滤膜（Whatman Intl., Ltd.., Maidstone, UK）过滤，并将该膜置于MRS琼脂表面，并在37°C下厌氧培养24 h。用4 mL 0.9%无菌盐水洗涤细菌细胞，分别稀释至10^{-3}，10^{-4}和10^{-5}，然后涂布在含有庆大霉素或四环素的MRS琼脂上。将琼脂平板在37℃下需氧或需氧厌氧培养36小时。所有实验进行三个重复。

表8. 测试方案

Donor Strains Recipient Strains	*B. bifidum* BGN4	*B. longum* BORI
L. fermentum AGBG1	N/A [1]	BORI + AGBG1
L. acidophilus ATCC 4356	BGN4 + ATCC 4356	BORI + ATCC 4356

[1]N/A表示不适用，因为双歧杆菌BGN4对四环素高度敏感，这导致在含有四环素的培养基上没有生长

3.5.4.抗生素耐药基因的PCR检测

Guo等人的实验条件用于这些测试。使用MG™细胞基因组DNA提取SV miniprep（MGmed，韩国首尔）提取纯培养细菌的基因组DNA。根据制造商的说明进行提取，并用200μL无菌水洗脱总细菌DNA。为了确保260 nm处的吸光度与280 nm处的吸光度之比为1.8-2.0。，将DNA提取物等分并保存在-20°C下。聚合酶链反应（PCR）用于通过基因特异性引物检测抗生素抗性基因（表9）。将以下反应混合物添加到每个样品中：1.5μLDNA（50 ng），2μL引物（100 pmol），dNTP混合物8μL，2XGC缓冲液I，并用灭菌蒸馏水调节至50μL体积。扩增程序为94°C的初始变性步骤5分钟，然后进行以下30个循环：94°C 30 s，退火温度（表9）30 s，72°C 1 min和72°C 7分钟。在1.5%琼脂糖凝胶上分析扩增子以确认DNA片段大小。

表9. PCR检测的引物和条件[1]。

No.	Primer Name		Oligo Sequence	TM (°C)	Product Size	Reference
1	*Bifidobacterium* genus-specific primers	-	F: 5′-TCGCGTCYGGTGTGAAAG-3′ R: 5′-GGTGTTCTTCCCGATATCTACA-3′	55	128 bp	[72]
2	Gentamicin	*aaac(6)-aph(2)*	F: 5′-CCAAGAGCAATAAGGGCATA-3′ R: 5′-CACTATCATAACCACTACCG-3′	60	220 bp	[73]
3	Kanamycin	*AphA3*	F: 5′-GCCGATGTGGATTGCGAAAA-3′ R: 5′-GCTTGATCCCCAGTAAGTCA-3′	52	292 bp	[74]
4	Streptomycin	*aadE*	F: 5′-ATGGAATTATTCCCACCTGA-3′ R: 5′-TCAAAACCCCTATTAAAGCC-3′	50	565 bp	[74]
5	Trimethoprim	*dfrA*	F: 5′-AAAAGGGGCAGAGCATG-3′ R: 5′-AGAAAATGGCGTAATCGGTA-3′	50	474 bp	[75]
6	Tetracycline(K)	*tet*(K)	F: 5′-TTAGGTGAAGGGTTAGGTCC-3′ R: 5′-GCAAACTCATTCCAGAAGCA-3′	55	169 bp	[76]
7	Tetracycline(L)	*tet*(L)	F: 5′-GTTGCGCGCTATATTCCAAA-3′ R: 5′-TTAAGCAAACTCATTCCAGC-3′	55		
8	Tetracycline(M)	*tet*(M)	F: 5′-GTTAAATAGTGTTCTTGGAG-3′ R: 5′-CTAAGATATGGCTCTAACAA-3′	55	401 bp	[77]
9	Tetracycline(O)	*tet*(O)	F: 5′-GATGGCATACAGGCACAGAC-3′ R: 5′-CAATATCACCAGAGCAGGCT-3′	55		
10	Tetracycline(S)	*tet*(S)	F: 5′-TGGAACGCCAGAGAGGTATT-3′ R: 5′-ACATAGACAAGCCGTTGACC-3′	55	1923 bp	[78]
11	Kanamycin	*aaaD*	F: 5′-TGCGTTTTGACACATCCAC-3′ R: 5′-GGTGTTTATGGCTCTCTTGG-3′	55		

[1]实验条件引自Guo等人。

3.6.粘蛋白降解试验

来自猪胃的部分纯化的粘蛋白（III型）购自Sigma（St. Louis, MO, USA）。没有碳源的MRS肉汤培养基（即，含有酵母基础培养基提取物0.75%（w / v），大豆蛋白胨0.25%（w / v），鱼肉提取物0.25%（w / v），乙酸钠0.25%（w / v），柠檬酸铵0.1%（w / v），磷酸二氢钠0.05%（w / v），磷酸氢二钠0.025%（w / v），吐温80 0.05%（w / v），L-半胱氨酸盐酸盐0.05%（w / v），马来酸0.005%（w / v），牛磺酸0.00625%（w / v），硫酸镁0.005%（w / v），硫酸锰0.0025%（w / v），蒸馏水98.2%（v / v））作为阴性对照。在四种MRS肉汤培养基中每一种分别添加0.5%（w / v）粘蛋白，1.0%（w / v）粘蛋白，0.5%（w / v）葡萄糖和1%（w / v）葡萄糖。在每种MRS培养基中接种微生物后，将样品于37°C的厌氧条件下培养48小时。培养后，分别在12、24、36和48小时通过在550 nm处测量吸光度来评估细菌的生长。从每个测试样品的最终值中减去介质的初始光密度值。

3.7.遗传稳定性测试

将两歧双歧杆菌 BGN4通过从-80°C下储存的原液划线接种到MRS琼脂平板上，并在37°C下厌氧培养24h，以获得单个菌落。将单个菌落接种到补充有0.05%（w / v）L-半胱氨酸盐酸盐的10 mL的MRS肉汤中，并将其视为两歧双歧杆菌BGN4的2^0（第1代）（约10^6 CFU / mL）。两歧双歧杆菌BGN4在厌氧条件下于37°C培养约12 h，达到约10^9 cfu / mL，获得210世代。在第二次传代培养中，0.1 mL

（1％接种，约10^6cfu / mL）的原代培养物中接种至10mL的MRS肉汤，并在相同条件下培养以获得220代两歧双歧杆菌BGN4。对于第三次传代培养，将0.1 mL（1％接种量，约10^6 CFU / mL）的二次培养物接种至10mLMRS肉汤并培养至10^7或10^8 CFU / mL，以获得2^{25}代两歧双歧杆菌BGN4。测量培养期间的存活数以确认世代数。根据制造商的说明，使用MG™细胞基因组DNA提取SV Miniprep（MGmed，韩国首尔）提取纯培养细菌的基因组DNA。使用Illumina MiSeq测序仪和Nextera XT文库制备试剂盒(Illumina，San Diego，CA，USA）完成了全基因组测序和分析。Nextera XT测序索引用于多路复用，参与者可以自由选择任何样本索引组合。运行验收标准是5.6 Gb的测序输出（对于20个基因组大小为2.8 Mb的样品，其平均测序覆盖率为100倍），Q30读取质量分数为75％。使用Miseq原始数据进行生物信息学分析，并使用ChunLab Co.，Ltd.（韩国首尔）的三个Miseq原始数据集完成比较基因组学分析。

3.8.毒力因素研究

使用VirulenceFinder1.5服务器完成了两歧双歧杆菌BGN4和长双歧杆菌BORI中毒力因子的搜索，该服务器是由基因组流行病学中心（CGE）主办的全基因组测序（WGS）分析的公共网络工具的一个组成部分。（http://www.genomicepidemiology.org/）。该数据库系统旨在检测WGS数据中与大肠杆菌，肠球菌，李斯特菌和金黄色葡萄球菌相关的毒力基因的同源序列。结果包括从所选数据库的blast分析中获得的与提交的两歧双歧杆菌BGN4或长双歧杆菌BORI基因组最佳匹配的基因。选定的%id阈值设置为90.00%，选定的最小长度设置为60% 选定的%ID阈值设置为90.00％，选定的最小长度设置为60％。如果存在匹配结果，输出将显示有关预测的毒力基因，％ID，查询和数据库基因的长度，命中在重叠群中的位置以及命中的登录号的信息。

4.结论

尽管益生菌的健康益处已在世界各地的食品市场中被广泛应用，但与它们对消费者健康的临床影响相比，包括益生菌副作用在内的安全性问题应更加仔细地考虑。在这项研究中，表明两歧双歧杆菌BGN4和长双歧杆菌BORI不会产生氨或生物胺，例如组胺，酪胺或尸胺。在两个菌株中都发现了痕量的腐胺。但是，该数量与经常食用的各种食品中检测到的数量相似或更少。益生菌均未显示出溶血活性或粘蛋白降解活性。但是，他们对抗生素的耐药性在本研究中是不可转移的。这些发现表明两歧双歧杆菌BGN4和长双歧杆菌BORI适用于对消费者有害影响风险很小的食品。

作者贡献

M.J.K和S.K.在M.S.P.，G.E.J.和S.Y.K.的监督下设计了该实验，H.H.L.，H.J.，S.K.和R.L.在M.S.P.和G.E.J.，M.J.K.，S.K.的指导下进行了微生物实验，SK， M.S.P.和G.E.J.一起进行了文献研究。 M.J.K.，

S.K.，M.S.P.和G.E.J.设计了研究模板。S.K.和T.V.J.根据田纳西州中部大学和Bifido有限公司之间的保密研究协议编辑和修订了手稿。所有作者讨论了草稿并批准了最终手稿以供出版。

致谢

这项工作得到了韩国卫生和福利局（03-PJI-PG11-VN01-SV04-0018）的支持，以及促进区域专业化产业（R0004140），贸易，工业和能源部（MOTIE）和韩国技术进步研究所（KIAT），由韩国政府（MSIP）资助的韩国国家研究基金会（NRF）赠款（No. 2017R1A2B2012390），由科技部、ICT与未来规划部(NRF-2017M3A9F3041747）资助的国家研究基金生物与医疗技术发展项目(NRF)和中田纳西州立大学(MTSU)资助的教职工研究与创新活动委员会(FRCAC)资助(No. 221745)。作者要感谢普渡大学的Lauren B. Mallet，MTSU的Fred Marino和Karen M. Lauer的评论和反馈。

International Journal of
Molecular Sciences

MDPI

Article

Safety Evaluations of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI

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Received: 6 April 2018; Accepted: 2 May 2018; Published: 9 May 2018

Abstract: Over the past decade, a variety of lactic acid bacteria have been commercially available to and steadily used by consumers. However, recent studies have shown that some lactic acid bacteria produce toxic substances and display properties of virulence. To establish safety guidelines for lactic acid bacteria, the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) has suggested that lactic acid bacteria be characterized and proven safe for consumers' health via multiple experiments (e.g., antibiotic resistance, metabolic activity, toxin production, hemolytic activity, infectivity in immune-compromised animal species, human side effects, and adverse-outcome analyses). Among the lactic acid bacteria, *Bifidobacterium* and *Lactobacillus* species are probiotic strains that are most commonly commercially produced and actively studied. *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI have been used in global functional food markets (e.g., China, Germany, Jordan, Korea, Lithuania, New Zealand, Poland, Singapore, Thailand, Turkey, and Vietnam) as nutraceutical ingredients for decades, without any adverse events. However, given that the safety of some newly screened probiotic species has recently been debated, it is crucial that the consumer safety of each commercially utilized strain be confirmed. Accordingly, this paper details a safety assessment of *B. bifidum* BGN4 and *B. longum* BORI via the assessment of ammonia production, hemolysis of blood cells, biogenic amine production, antimicrobial susceptibility pattern, antibiotic resistance gene transferability, PCR data on antibiotic resistance genes, mucin degradation, genome stability, and possession of virulence factors. These probiotic strains showed neither hemolytic activity nor mucin degradation activity, and they did not produce ammonia or biogenic amines (i.e., cadaverine, histamine or tyramine). *B. bifidum* BGN4 and *B. longum* BORI produced a small amount of putrescine, commonly found in living cells, at levels similar to or lower than that found in other foods (e.g., spinach, ketchup, green pea, sauerkraut, and sausage). *B. bifidum* BGN4 showed higher resistance to gentamicin than the European Food Safety Authority (EFSA) cut-off. However, this paper shows the gentamicin resistance of *B. bifidum* BGN4 was not transferred via conjugation with *L. acidophilus* ATCC 4356, the latter of which is highly susceptible to gentamicin. The entire genomic sequence of *B. bifidum* BGN4 has been published in GenBank (accession no.: CP001361.1), documenting the lack of retention of plasmids capable of transferring an antibiotic-resistant gene. Moreover, there was little genetic mutation between the first and 25th generations of *B. bifidum* BGN4. Tetracycline-resistant genes are prevalent among *B. longum* strains; *B. longum* BORI has a *tet*(W) gene on its chromosome DNA and

has also shown resistance to tetracycline. However, this research shows that its tetracycline resistance was not transferred via conjugation with *L. fermentum* AGBG1, the latter of which is highly sensitive to tetracycline. These findings support the continuous use of *B. bifidum* BGN4 and *B. longum* BORI as probiotics, both of which have been reported as safe by several clinical studies, and have been used in food supplements for many years.

Keywords: probiotics; safety; antibiotics resistance; functional foods; nutraceuticals

1. Introduction

Since "probiotics" first emerged in the 1960s [1], the term has been defined by various scholars and groups. In recent years, probiotics have been clearly defined by several regulatory organizations [2]. According to the FAO/WHO, probiotics can be defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" [3]. Other experts similarly define probiotics as "live microorganisms which, when ingested or locally applied in sufficient numbers, provide the consumer with one or more proven health benefits" [4]. Edible microorganisms regarded as probiotic bacteria are derived from various strains, species, and genera, which have been studied with regard to various human health benefits [5]. A variety of microorganisms, including *Bacillus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., and *Propionibacterium* spp., are regarded as probiotics, and are known to be involved in the vitamin biosynthesis of the host's nutrition metabolism and physiological function via immune-mediated effects [6,7]. Of these probiotic microorganisms, *Lactobacillus* spp. and *Bifidobacterium* spp. have been utilized globally in fermented food products and commercially-produced food supplements [8]. As of July 2010, the genomic sequences of approximately 11 *Bifidobacterium* and 21 *Lactobacillus* species have been completely analyzed, whose microbial genomic sequences offer exact evidence of the probiotics' genera and species [9]. Some experts have found that consumer demand for food or food supplements containing lactic acid bacteria have led to the exponential growth of healthy trends in the global food market [10]. However, this phenomenon cannot disregard microbial safety standards or allow lactic acid bacteria to be used indiscriminately without scientific research or safety verification [3,11]. Also, a probiotic safety assessment should consider the probiotic's physiological characteristics, treatment method (e.g., oral administration, skin spray, gel, capsule, etc.), exposure dosage, consumers' health, and the physiological functions required for effective probiotic performance [12].

In 2002, the FAO created four basic guidelines for food industry probiotic application, because a variety of commercially-available microorganisms had been sold to consumers as probiotics without clear labeling standards. The FAO guidelines summarized by Huys et al. [13] are as follows: (i) "the assessment of strain identity (i.e., genus, species, and strain level); (ii) in vitro tests to screen potential probiotic strains (e.g., resistance to gastric acidity, bile acid, and digestive enzymes, antimicrobial activity against potentially pathogenic bacteria, etc.); (iii) assessment of safety: requirement of proof that a probiotic strain is safe and without contamination in its delivery form; and (iv) in vivo studies for the substantiation of the health effects in the target host". In addition, the FAO recommended that various tests (e.g., analysis of antibiotic resistance, metabolic activity, toxin production, hemolytic activity, infectivity in immune-compromised animal models, human side effects, and adverse outcomes in consumers) be conducted with the probiotic microorganisms to demonstrate their safety to hosts and elaborate on section three of the aforementioned guidelines [3]. However, these safety assessment items are recommendations rather than legal requirements. Various research groups have evaluated the safety of probiotic bacteria according to their cell types and microbial functionalities by incorporating additional experimental methods [14–16]. In 2002, the European Union Scientific Committee on Animal Nutrition issued guidelines for the safety assessment and regulation of edible microorganisms utilized in food and animal feeds. The corresponding "qualified presumption of safety (QPS)" guidelines

from 2016 are as follows [11]: (1) definition of the taxonomy of the microbe; (2) collection of sufficient information providing the basis for QPS status, including any scientific literature, history of use, industrial applications, ecological data, and human intervention data; (3) exclusion of pathogenicity; and (4) definition of the end use. Based on this guideline, QPS status may be granted to probiotic cells in European Union food markets if there are no safety problems with a particular taxon or if the safety problem is alleviated. It is commonly agreed that microbial safety should demonstrate the (i) species characteristics with genetic information, (ii) phenotypic evidence, (iii) isolation history, (iv) absence/presence of antibiotic-resistant properties, and (v) potential virulence and/or pathogenic factors [17].

One of the greatest safety concerns for commercially-produced lactic acid bacteria is that some of the microorganisms supplied in the form of diets may act as the donor of antibiotic-resistant plasmids to intestinal pathogens [18,19]. Several reports have found that in the presence of antibiotic treatment, some strains survive in the human gastrointestinal tract due to the transferred resistance of plasmids [20–22]. A variety of microbial genes can be transferred to enteric bacteria in the intestine via plasmids, resulting in the spread of antibiotic-resistance [23]. Therefore, ensuring the safety of a probiotic strain is necessary prior to the mass production of lactic acid bacteria for commercial purposes.

Although some *Bifidobacterium* and *Lactobacillus* spp. have shown promise in in vivo and in vitro studies, there is a lack of clear clinical evidence to support the health benefits of these microorganisms [24]. Therefore, many groups and researchers are trying to prove the efficacy of lactic acid bacteria through clinical experimentation. *B. bifidum* BGN4 and *B. longum* BORI were isolated from the feces of healthy breast-feeding infants, and have been commercially used as food ingredients since 2000 [25–29]. Some bifidobacteria strains, including *B. bifidum* and *B. longum*, are registered as functional ingredient, Probiotics (II.2.51) in Health Functional Food Code of Korea [30]. Over the years, many studies have revealed the functionalities of *B. bifidum* BGN4 [28], and its complete genomic sequence was reported to GenBank [31]. *B. longum* BORI, also isolated from a healthy breast-fed infant and deposited in KCCM (Korean Culture Center of Microorganisms, 14092), was proven to statistically shorten the duration of diarrhea in a clinical study of infants infected with rotavirus [25]. Both probiotic strains have been proven to effectively form healthy intestinal microflora without any adverse effects. However, further systematic research should be conducted to prove their safety for academic and commercial applications. The aim of this study was to validate the safety of *B. bifidum* BGN4 and *B. longum* BORI by conducting FAO/WHO recommended experiments and other published safety research.

2. Results and Discussion

2.1. Ammonia Production

Intestinal bacteria can degrade various nitrogen sources (e.g., proteins, peptides, and amino acids) present in the feces of the intestinal track [32]. These naturally-occurring microbiota and artificially-administered flora have the potential to produce various toxic substances during the deamination stage via nitrogen derivatives. Multiple potentially toxic products (i.e., phenol, ammonia, and indole [33], are possible products of the proteolytic process, especially in the large intestine. Thus, bacterial ammonia production is highly relevant to human intestinal health, and a necessary component of the evaluation to demonstrate the safety of commercial probiotics. Moreover, recent studies have also shown that ammonia produced by gut microorganisms can affect the liver and act as a cofactor in chronic liver damage. Vince and Burridge [34] reported that considerable amounts of ammonia were generated by the Gram-negative anaerobes, *Clostridia* (including *Clostridium perfringens*), *Enterobacter*, and *Bacillus* spp. Some strains of streptococci, micrococci, and Gram-positive non-spore forming anaerobes produced moderate concentrations of ammonia. By contrast, Gram-positive aerobic rods, in particular *Lactobacilli*, produced very little ammonia.

The ammonia production of *B. bifidum* BGN4 and *B. longum* BORI were assessed to verify the safety of these probiotics. In this study, *B. bifidum* BGN4, *B. longum* BORI, and other probiotic strains did not produce ammonia. By contrast, *Bacteroides* spp., *Clostridium perfringens,* and *Enterobacter* spp., which are known harmful bacteria and used as positive controls in this study, produced 12.9 ± 1.3 to 161.0 ± 6.6 μg/mL of ammonia (Table 1). This test included three replications, and the values presented are the means ± the standard deviations. This study found no indication of the production of ammonia by *B. bifidum* BGN4 and *B. longum* BORI.

Table 1. Mean value and standard deviation of ammonia level variables of *B. bifidum* BGN4, *B. longum* BORI, and other commercial microorganisms (*n* = 3).

Strain	Ammonia (μg/mL)
Bifidobacterium bifidum BGN4	negative
Bifidobacterium longum BORI	negative
Bifidobacterium breve ATCC 15701	negative
Lactobacillus plantarum KFRI 708	negative
Bacteroides fragilis ATCC 25285	14.7 ± 1.5
Bacteroides thetaiotaomicron ATCC 29741	23.3 ± 3.0
Clostridium perfringens ATCC 13124	23.5 ± 1.6
Enterobacter cloacae ATCC 13047	161.0 ± 6.6
Enterobacter faecalis ATCC 19433	12.9 ± 1.3

2.2. Hemolytic Property Test

The 2002 FAO/WHO Guidelines on Probiotics Safety Considerations clearly states that "if the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required" [3]. Microbial hemolysis properties are a common concern for pathogenic bacteria (e.g., enterococci, and streptococci) because of the potential for anemia and edema in the host. Although *Bifidobacterium* spp. are normal, naturally-occurring intestinal microbiota that have been widely included in functional foods and utilized by nutraceutical industries, they can potentially behave as opportunistic pathogenic microorganisms similar to common commensal microorganisms. Therefore, hemolysis assay tests should be conducted on potential probiotic bacteria. Visualizing the physical changes caused by hemolytic activity by culturing the microorganisms on a medium containing animal or human blood is a commonly used tool to evaluate the hemolytic properties of pathogens. In this study, the potential hemolytic activity of *B. bifidum* BGN4 and *B. longum* BORI were assessed using the blood agar plating method. *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 (positive control) showed β-hemolysis colorless zones around the cell colonies, whereas *B. bifidum* BGN4 and *B. longum* BORI showed no hemolysis and no change of color in the periphery of the colonies (Figure 1).

(**a**) (**b**) (**c**)

Figure 1. *B. bifidum* BGN4 ((**a**); back light) and *B. longum* BORI ((**b**); back light) growth with no blood cell lysis. Complete lysis of red blood cells was observed, with clear zones around the *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 colonies ((**c**); positive control, back light).

2.3. Evaluation of Biogenic Amine Production

Biogenic amines (e.g., cadaverine, histamine, tyramine, and putrescine) have hydrophobic skeletons and naturally-occurring organic polycation molecules derived from the amino acids in animals and humans [35]. These molecules are involved in multiple metabolic and intracellular activities of mammals (e.g., synaptic transmission, blood pressure control, allergic response, and cellular growth control). Traditionally, a variety of probiotic bacteria have been artificially integrated into fermented foods, due to their beneficial effects and flavor-enhancing properties [36]. Their biogenic amine levels have been regarded as an indicator of microbial activity and food freshness due to the fact that biogenic amines are generated via microbial metabolic activities (i.e., decarboxylation and the transamination of protein molecules) [37]. While biogenic amines are commonly found in fresh meat, vegetables, and cheese, ingestion of large amounts of biogenic amines may cause symptoms in humans and animals that are similar to severe allergic reactions [38]. One of the most common issues in the probiotics field in recent years has been whether probiotics contribute to the production of biogenic amines, and how they contribute to the production of biogenic amines [15]. Complex biogenic amines (i.e., polyamines having more than one amino group) were initially thought to be naturally present in a variety of fresh foods, but recent studies have shown that these chemicals can accumulate as a result of microbial activity. Some edible microorganisms and probiotic strains were reported to produce biogenic amines [39–41]. Therefore, the aim of this study was to examine the biogenic amine production of *B. bifidum* BGN4 and *B. longum* BORI as a component of an overall probiotic safety evaluation. The biogenic amine content of the bifidobacteria is featured in Table 2.

Table 2. Biogenic amine levels of *B. bifidum* BGN4 and *B. longum* BORI.

Strains	Cadaverine (μg/mL)	Histamine (μg/mL)	Putrescine (μg/mL)	Tyramine (μg/mL)
B. bifidum BGN4	N/D [1]	N/D [1]	24.23	N/D [1]
B. longum BORI	N/D [1]	N/D [1]	16.58	N/D [1]

[1] N/D; not detected.

The biogenic amine content of these strains was derived by subtracting the background content of the biogenic amines in each medium. *B. bifidum* BGN4 and *B. longum* BORI did not produce cadaverine, histamine, or tyramine; however, they produced 24.23 and 16.58 μg/mL of putrescine, respectively. The levels produced were not of concern. Putrescine is a natural substance present in various foods [42–44]. Putrescine, also naturally found in small amounts in living cells, is formed by the decarboxylation of ornithine and arginine. It is also a metabolite produced by various edible probiotic cells. Putrescine is also a precursor of spermidine and spermine. The polyamines putrescine, spermidine, spermine, and cadaverine are essential components of living cells, and play an important role in the formation of nucleic acid, protein synthesis, and membrane stability. Of the various biogenic amines detected in a variety of fruits, juices, and vegetables, putrescine was the most common. Kalač [42] reported that putrescine was commonly found in frozen spinach puree (average 12.9 mg/kg), ketchup (average 52.5 mg/kg), concentrated tomato paste (average 25.9 mg/kg), and frozen green pea (average 46.3 mg/kg). The putrescine content of fermented foods and beverages [43] was found to be 9 mg/kg (3–25 mg/kg, n = 28) in sherry, 154 mg/kg (6–550 mg/kg, n = 8) in sauerkraut, 19 mg/kg (1–71 mg/kg, n = 8) in Dutch cheese, and 52 mg/kg (1–190 mg/kg, n = 14) in fermented sausage. Furthermore, the putrescine found in the traditional cheeses made from ewe's whole milk in Sardinia, Italy, increased to 1658 mg/L during ripening [44]. *Bifidobacterium* spps. (i.e., *Bifidobacterium* CCDM 94, *B. adolescentis* CCDM 223, *B. animalis* ssp. *lactis* CCDM 239, 240, 241, and 374, *B. bifidum* CCDM 559, and *B. longum* CCDM 569) are known to produce cadaverine, putrescine, tyramine, and spermidine [41]. According to Pollark et al. [45], putrescine is contained in human breast milk (0~3804 nmol/L) and commercial formula milk (0~1057 ± 25 nmol/L).

Therefore, it matters how much putrescine occurs naturally. Some researchers theorize that putrescine in food is likely to show synergistic effects on histamine toxicity. However, such synergy has not been proven or reported with experimental data, as far as we know. Moreover, the European Food Safety Authority (EFSA) [46] also identified a lack of research to identify the exact levels of putrescine required to increase the side effects of histamine. *B. bifidum* BGN4 and *B. longum* BORI did not produce any cadaverine, histamine, or tyramine during the fermentation process. *B. bifidum* BGN4 and *B. longum* BORI produced low levels of putrescine, which was also found in both media (i.e., whole milk medium = 24.43 μg/mL, *B. bifidum* BGN4 culturing medium (whole milk) = 48.67 μg/mL, MRS medium = 26.60 μg/mL, *B. longum* BORI culturing medium (MRS) = 43.17 μg/mL). The human oxidation system of mono-amine and diamine oxidase includes small amounts of biogenic amines that are usually metabolized and harmless, because humans and animals have the ability to decompose them in vivo.

2.4. Antimicrobial Susceptibility and Antibiotic Resistance Transferability

2.4.1. Antibiotic Susceptibility

Various lactic acid bacteria research groups have warned that some lactic acid bacteria consumed as food or feed may have antibiotic-resistant properties. Since this resistance capability could be transferred to other pathogens via plasmids, the assessment of antibiotic resistance is an important criterion for evaluating the safety of strains used in food and feed [47]. Moreover, the acquired transferable genes have been characterized in bifidobacteria and lactobacilli [48]. In order to distinguish antibiotic-resistant from antibiotic-susceptible microorganisms, the EFSA has established microbiological cut-off values for the antibiotic-resistance of microorganisms used as food and/or feed additives. These microbiological cut-off values were determined based on the distribution of the chosen antimicrobials' minimum inhibitory concentrations (MICs) in cell populations belonging to a single taxonomical unit [49].

All *Bifidobacterium* spp. in this study were susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, penicillin G, rifampicin, and vancomycin (MIC ranging from 0.01 to 4 μg/mL) and generally resistant to aminoglycoside antibiotics, such as gentamicin, kanamycin, neomycin, and streptomycin (MIC ranging from >32 μg/mL, Table 3). The MIC values of *B. bifidum* BGN4 and *B. longum* BORI, with the exception of gentamicin and tetracycline, were equal to or lower than the established EFSA cut-off values suggested by the EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) [49]. The susceptibility tendencies of *B. bifidum* BGN4 and *B. longum* BORI were similar to other studies [50–52], with the exception of high MIC to tetracycline in *B. longum* BORI. Penicillin G, ampicillin, vancomycin, gentamicin, erythromycin, trimethoprim–sulfamethoxazole, and metronidazole are known as frequently used antibiotics in pediatric patients [53]. *B. bifidum* BGN4 and *B. longum* BORI are resistant to trimethoprim–sulfamethoxazole but six of ten *Bifidobacterium* spp. strains also showed MIC values over 128 μg/mL in this research (Table 3).

Mättö et al. [54] reported *Bifidobacterium* strains displayed generally high MICs for streptomycin and gentamicin, and suggested their resistances were intrinsic. Ammor et al. [48] isolated probiotic bacteria from 21 food samples, such as yogurt, yogurt-type fermented milk, and pharmaceutical products, and found 22 strains of *Bifidobacterium* spp. In their study, Bifidobacteria were resistant to aminoglycoside (MIC_{90} ranges from 64 to 1000 μg/mL) and strongly resistant to kanamycin (MIC_{90} = 1000 μg/mL). They also demonstrated that some MIC ranges did not overlap, implying that the antibiotics related to these MIC ranges are usable as ingredients in selective media. They suggested the selective range of gentamicin was from 32 to 64 μg/mL and kanamycin was 64 to 500 μg/mL for *Bifidobacterium*. Therefore, gentamicin containing medium [55] and mupirocin containing medium [56,57] have been used for the selection and enumeration of *Bifidobacterium*. Accordingly, this resistance could be considered as intrinsic. Antibiotic resistance transferability studies were conducted to confirm the nature of this resistance.

Table 3. Antimicrobial susceptibility (MIC values) of *B. bifidum* BGN4 and *B. longum* BORI and other *Bifidobacterium* spp.

Antibiotics	EFSA Cut-Off of *Bifidobacterium* spp.	*B. longum* ATCC 15707	*B. longum* BB536	*B. longum* KCCM 91563	*B. longum* BORI	*B. infantis* ATCC 15697	*B. lactis* BB-12	*B. bifidum* BGN4	*B. bifidum* KCTC 3440	*B. adolescentis* ATCC 15703	*B. breve* M-16V	*E. faecalis* ATCC 29212
Penicillin G		0.25	0.125	0.5	1	0.125	0.125	0.063	0.063	0.25	0.25	0.5
Carbenicillin disodium salt		2	2	4	8	0.5	2	0.5	0.5	4	4	8
Methicillin		8	4	16	16	1	2	1	0.5	8	8	16
Ampicillin sodium salt	2	0.5	0.25	1	0.5	0.125	0.125	0.063	0.063	0.25	0.25	0.25
Dicloxacillin sodium salt hydrate		4	4	8	8	0.5	4	0.5	1	256	8	4
Gentamicin sulfate	64	32	64	32	32	16	128	128	256	128	128	256
Streptomycin sulfate salt	128	32	128	64	64	>256	128	64	32	128	256	>256
Kanamycin sulfate	N/R [1]	512	1024	1024	512	32	1024	1024	1024	1024	1024	256
Neomycin sulfate		1024	512	512	512	64	512	1024	512	512	1024	1024
Cephalothin sodium salt		8	4	16	32	4	8	4	2	16	16	16
Tetracycline	8	1	1	1	64	2	16	1	1	8	16	32
Polymyxin B sulfate salt		256	32	256	256	128	256	512	512	512	1024	>1024
Erythromycin	1	0.125	0.5	0.5	0.5	0.125	0.125	0.125	0.125	0.125	0.125	8
Metronidazole		8	8	>256	>256	8	4	4	64	>256	8	>256
Vancomycin hydrochloride	2	0.5	<0.25	<0.25	1	0.5	0.5	1	2	0.5	0.5	2
Chloramphenicol	4	2	2	2	4	2	2	2	2	2	2	8
Rifampicin		<0.125	<0.125	<0.125	0.25	<0.125	2	0.5	0.25	0.5	1	0.5
Clindamycin hydrochloride	1	<0.032	0.063	0.063	0.125	0.25	<0.032	0.063	0.063	<0.032	<0.032	>16
Phosphomycin disodium salt		128	256	256	256	16	64	128	256	64	32	32
Mupirocin		>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	64
Trimethoprim–Sulfamethoxazole		128	256	128	256	256	1	128	64	1	2	32

[1] N/R denotes not required.

2.4.2. Antibiotic Resistance Transferability

Since *B. bifidum* BGN4 and *B. longum* BORI showed high antibiotic resistance to gentamicin and/or tetracycline in these antimicrobial susceptibility tests, tetracycline resistance transferability tests were conducted using *L. fermentum* AGBG1, a recipient strain that is highly susceptible to tetracycline. In order to test the transferability of gentamicin resistance of *B. bifidum* BGN4 and *B. longum* BORI, *L. acidophilus* ATCC 4356 was used as a recipient strain, due to its high gentamicin sensitivity. The conjugation results are shown in Table 4.

Table 4. Transferability of tetracycline resistance from donors (*B. longum* BORI and *B. bifidum* BGN4) to recipients (*L. fermentum* AGBG1 and *L. acidophilus* ATCC 4356) (cfu/mL).

Antibiotics	**AGBG1 (Aerobic)**	**AGBG1 + BORI**		**BORI (Anaerobic)**
		Aerobic	**Anaerobic**	
None [1]	4.38×10^8	3.38×10^8	2.27×10^8	4.56×10^8
T8 [2]	0	0	4.44×10^6	7.11×10^7
Antibiotics	ATCC 4356 (Aerobic)	ATCC 4356 + BORI		BORI (Anaerobic)
		Aerobic	Anaerobic	
None [1]	3.65×10^8	1.67×10^8	2.34×10^8	3.14×10^8
G64 [3]	0	0	2.78×10^6	1.46×10^8
Antibiotics	ATCC 4356 (Aerobic)	ATCC 4356 + BGN4		BGN4 (Anaerobic)
		Aerobic	Anaerobic	
None [1]	3.65×10^8	3.29×10^8	2.54×10^8	3.86×10^8
G64 [3]	0	0	4.64×10^6	1.43×10^8

[1] No antibiotics were included in the counting agar medium. [2] Tetracycline (8 μg/mL) was included in the counting agar medium. [3] Gentamicin (64 μg/mL) was included in the counting agar medium.

L. fermentum AGBG1 did not grow when cultured alone or co-cultured with *B. longum* BORI in the media containing tetracycline. The antimicrobial susceptibility test reported herein found that while *B. bifidum* BGN4 was very susceptible to tetracycline (MIC 1.0 μg/mL), *B. longum* BORI was resistant to tetracycline (MIC 64 μg/mL). However, the tetracycline resistance of *B. longum* BORI was not transferred to the recipient, *L. fermentum* AGBG1, in this study. *L. acidophilus* ATCC 4356, which is highly susceptible to gentamicin, grew well in normal MRS medium; however, *L. acidophilus* ATCC 4356 did not grow in the MRS medium containing gentamicin or the media that was co-cultured with *B. bifidum* BGN4 or *B. longum* BORI. By contrast, *B. bifidum* BGN4 and *B. longum* BORI showed resistance to 64 μg/mL gentamicin in this study. Therefore, this proves *B. bifidum* BGN4's resistance to gentamicin and *B. longum* BORI's resistance to gentamicin and tetracycline were not transferred to the recipient strains. It is worth noting that a 2011 report published by the Agency for Healthcare Research and Quality (AHRQ) [58] extensively reviewed 622 studies on six genera (i.e., *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Enterococcus*, and *Bacillus* spp.), and found no clinical evidence of the theoretical possibility of gene transfer from probiotics to other microorganisms.

2.4.3. PCR Results on Antibiotic Resistance Genes

Even though the whole genome of *B. bifidum* BGN4 (Accession no.: CP001361.1) and *B. longum* BORI show that neither contain a plasmid capable of transferring the antibiotic-resistance gene, PCR analysis on ten antibiotic genes such as gentamicin(*aaac(6)–aph(2)*), kanamycin(*AphA3, aaaD*), streptomycin(*aadE*), trimethroprim(*dfrA*), and tetracycline(*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S)) were conducted. All the tested *Bifidobacterium* spp. in this study were identified using 16S rRNA *Bifidobacterium* genus specific primers (Figure 2). The PCR results on antibiotics genes are shown in Figure 3. There were no amplicons that indicate resistance genes in *B. bifidum* BGN4, *B. longum* BORI, and other *Bifidobacterium* spp. in this study.

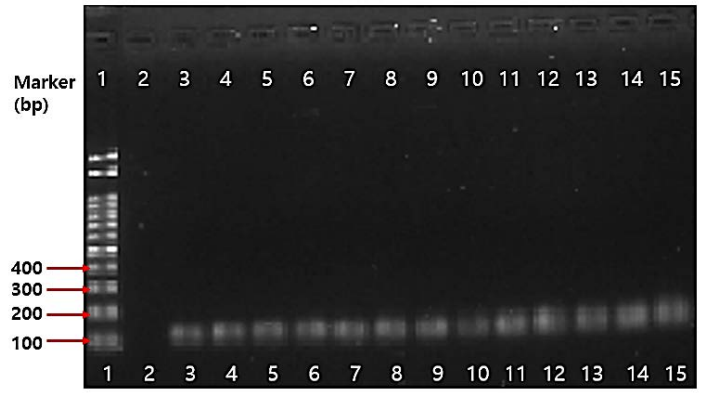

Figure 2. PCR analysis results of various *Bifidobacterium* spp.: Lane 1: marker; Lane 2: without loading; Lane 3: *B. lactis* AS60; Lane 4: *B. bifidum* KCTC 3440; Lane 5: *B. longum* BORI; Lane 6: *B. longum* KCCM 91563; Lane 7: *B. lactis* BB-12; Lane 8: *B. longum* RD47; Lane 9: *B. bifidum* BGN4; Lane 10: *B. thermophilum* KCCM 12097; Lane 11: *B. adolescentis* ATCC 15703; Lane 12: *B. lactis* AD011; Lane 13: *B. infantis* ATCC 15697; Lane 14: *B. breve* M-16V; Lane 15: *B. animalis* ATCC 25527.

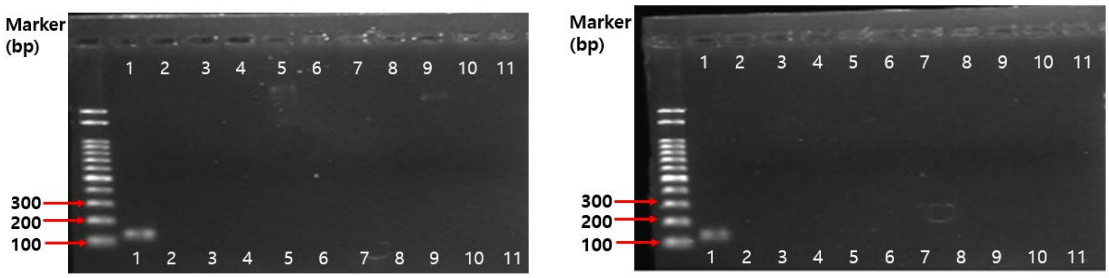

Figure 3. PCR analysis results of the antibiotic resistance gene in *B. bifidum* BGN4 and *B. longum* BORI: (**a**) *B. bifidum* BGN4; (**b**) *B. longum* BORI; Lane 1: *Bifidobacterium* genus-specific primers; Lane 2: gentamicin(*aaac(6)-aph(2)*), Lane 3: kanamycin(*AphA3*), Lane 4: streptomycin(*aadE*), Lane 5: trimethoprim(*dfrA*); Lane 6: tetracycline K(*tet*(K)); Lane 7: tetracycline L(*tet*(L)); Line 8: tetracycline M(*tet*(M)), Lane 9: tetracycline O(*tet*(O)), Lane 10: tetracycline S(*tet*(S)); Lane 11: kanamycin(*aaaD*).

Recently, the intrinsic gentamicin-resistance of *Bifidobacterium* spp. was putatively attributed to the presence of two genes, namely *Bbr_0651* and *Bbr_1586*, which are enzymes present in the *Bifidobacterium* chromosome DNA, with both coding for putative phosphotransferase enzymes [59]. Tetracycline resistance genes (*tet*) are widely distributed in the *Bifidobacterium* genus; however, it is known as a ribosomal protection protein [48,60]. The tetracycline W (*tet*(W)) gene was found in *B. longum* BORI chromosome DNA. In the study of Mättö et al. [54], human- and probiotic-associated *Bifidobacterium* species (203 strains) showed high MIC values for tetracycline (i.e., ≥16 mg/mL; prevalence of 4–18%) that were attributed to the presence of tetracycline genes (*tet*), where *tet*(W), and *tet*(O) were detected. The *tet*(W), and *tet*(M) were found in 26, and 7%, respectively, of the *Bifidobacterium* isolates. The role of the *tet*(W) gene is presumed to be the translation factor GTPase of the TRAFAC family, which induces a noncovalent modification to the ribosome that destroys the effect of tetracycline, inhibiting protein synthesis [61].

2.5. Mucin Degradation

The intestinal mucus gel layer is an important constituent of the intestinal barrier that consists of a glycoprotein family. Multiple groups have reported that bacterial translocation can occur in infants and immunocompromised hosts, even if the intestinal mucus acts as a biological shield from

microbes. This bacterial translocation has the potential to cause sepsis, and is one of the most serious probiotic safety concerns. Some scientists have also reported the possibility of bacteremia—endocarditis due to the administration of probiotic strains [62,63]. According to Ruas-Madiedo et al. [64], some *Bifidobacterium* spp. demonstrate mucolytic activities and have genes that induce mucin degrading enzymes. However, the majority of *Bifidobacterium* spp., such as *B. longum* and *B. pseudocatenulatum*, did not display mucolytic activity.

In order to confirm their microbial safety, it is necessary to evaluate translocation ability via mucolytic capacity analysis of each strain. In this study, the translocation capabilities of *B. bifidum* BGN4 and *B. longum* BORI were measured using in vitro mucolytic assays. The cell growth rates after incubation were examined in five kinds of modified MRS media by measuring their absorbances at 550 nm: basal medium (glucose-free MRS, ◇), basal medium with 0.5% mucin (×), 1.0% mucin (◯), 0.5% glucose (Δ), and 1.0% glucose (□) (Figure 4).

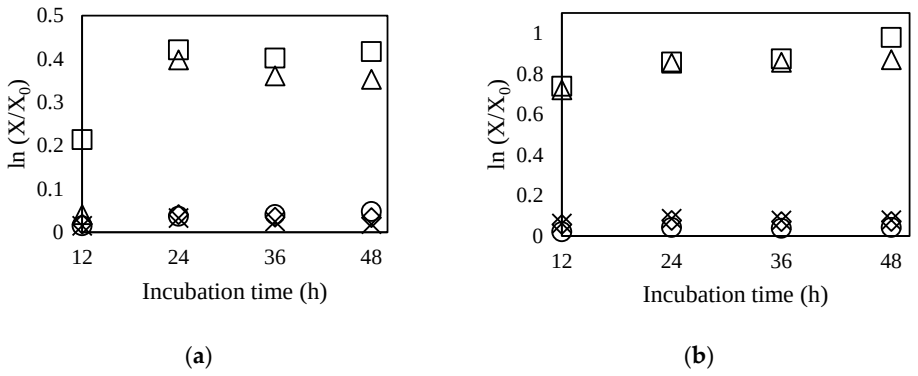

Figure 4. Growth curves of *B. bifidum* BGN4 (**a**) and *B. longum* BORI (**b**) in modified MRS with various carbon sources: basal medium (glucose-free MRS, ◇), basal medium with 0.5% mucin (×), 1.0% mucin (◯), 0.5% glucose (Δ), and 1.0% glucose (□).

In general, when simple sugars (e.g., glucose, fructose, maltose, and sucrose) are added, mucinase production can be inhibited due to catabolic repression. A false negative result can be obtained despite the microorganisms' potential to produce mucinolytic enzymes. Therefore, to obtain accurate data, glucose, which is generally used as a carbon source in the MRS medium, was intentionally removed from the medium in which the experimental cells were cultivated. If *B. bifidum* BGN4 and *B. longum* BORI were able to produce mucinase, they would be able to source carbon and grow actively through mucin digestion. As shown in Figure 2, the growth of both probiotic strains was actively induced when glucose was added as a carbon source. However, when mucin was added instead of glucose, no growth was observed in either strain. These observations clearly indicate that *B. bifidum* BGN4 and *B. longum* BORI did not use mucin as a carbon source for their growth. This study, as suggested by other studies [65,66], shows that neither *B. bifidum* BGN4 nor *B. longum* BORI degrade mucin, indicating that the strains are not capable of damaging intestinal surfaces and do not have translocational abilities.

2.6. Genetic Stability

The genetic variation of edible microorganisms possibly results in indels (i.e., gene deletion and insertion) and mutations. A critical consideration of commercializing probiotics is whether it is possible to maintain genetic safety over the long term. However, the genetic stability of commercial probiotic strains has not yet been reported. Theoretically, an evaluation of genetic stability requires the entire genome sequence of the strain.

The entire genome sequence of *B. bifidum* BGN4 has been published [31], and consists of a 2,223,664 bp circular chromosome (62.65% G+C) with no plasmids. A total of 1835 coding sequences (CDSs), 7 pseudogenes, 3 rRNA operons, and 52 tRNAs were compiled from the nucleotide

sequence. This study shows that the similarity in the genomic comparison between 1st generation and 25th generation samples were 99.9996~99.9998% via the Orthologous Average Nucleotide Identity (OrthoANI) value. (Table 5).

Table 5. OrthANI value

Strain/Sample	***B. bifidum*** **BGN4-1/13075.BBGN41.1** [1]	***B. bifidum*** **BGN4-2/13075.BBGN42.1** [2]	***B. bifidum*** **BGN4-3/13075.BBGN43.1** [3]
B. bifidum BGN4-1/13075.BBGN41.1 [1]	100	99.9997	99.9996
B. bifidum BGN4-2/13075.BBGN42.1 [2]	99.9997	100	99.9998
B. bifidum BGN4-3/13075.BBGN43.1 [3]	99.9996	99.9998	100

[1] *B. bifidum* BGN4-1/13075.BBGN41.1 denotes the 1st generation; [2] *B. bifidum* BGN4-2/13075.BBGN42.1 denotes the 25th generation; [3] *B. bifidum* BGN4-3/13075.BBGN43 and *B. bifidum* BGN4-2 are the 25th generations.

The difference between 0.0002% and 0.0004% is equivalent to 4.4 to 8.8 bp mutation of the entire nucleotide sequence, which can be assumed to be due to sequencing errors or spontaneous evolutionary mutations. Therefore, it is concluded that there was little genetic mutation, and the genetic information did not change in the process of cultivating 25 generations.

2.7. Virulence Factors

The genome sequences of *B. bifidum* BGN4 and *B. longum* BORI were compared with the genome sequences of four well-known pathogens (*E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus*). The virulence factors included *E. coli* Shiga toxin gene and *S. aureus* exoenzyme genes, host immune alteration or evasion genes and toxin genes. No virulence factors were found in the genomic sequences of *B. bifidum* BGN4 and *B. longum* BORI. Thus, this result shows that the genomic sequences of *B. bifidum* BGN4 and *B. longum* BORI do not include toxic or pathogenic genes related to *E. coli*, *Enterococcus*, *Listeria*, and *S. aureus*.

3. Materials and Methods

3.1. Microorganisms

The bacterial strains, including origin, culture medium, and test methods used in this study are presented in Table 6.

Table 6. Strain list and methods.

Strains	Origin	Medium	Method
Bifidobacterium bifidum BGN4	BIFIDO Co., Ltd. (Hongcheon, Korea)	BHI [1], Blood agar [2], whole milk [3], LSM-Cys [4], MRS [5–8]	3.2., 3.3., 3.4., 3.5.2., 3.5.3., 3.5.4., 3.6., 3.7
Bifidobacterium longum BORI	BIFIDO Co., Ltd. (Hongcheon, Korea)	BHI [1], Blood agar [2], MRS [3,5–7], LSM-Cys [4]	3.2., 3.3., 3.4., 3.5.2., 3.5.4., 3.6
Bacteroides fragilis ATCC 25285	American Type Culture Collection (Manassas, VA, USA)	BHI [1]	3.2
Bacteroides thetaiotaomicron ATCC 29741	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Bifidobacterium adolescentis ATCC 15703	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium animalis ATCC 25527	American Type Culture Collection (Manassas,VA, USA)	MRS [6]	3.5.4
Bifidobacterium animalis subsp. *lactis* AD011	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4

Table 6. *Cont.*

Strains	Origin	Medium	Method
Bifidobacterium animalis subsp. *lactis* AS60	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4
Bifidobacterium animalis subsp. *lactis* BB-12	Isolated from a pharmaceutical product, USA	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium bifidum KCTC 3440	Korean Collection for Type Cultures, (Jeongeup, Korea)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium breve ATCC 15701	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Bifidobacterium breve M-16V	Isolated from a pharmaceutical product, USA	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium infantis ATCC 15697	American Type Culture Collection (Manassas, VA, USA)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium longum ATCC 15707	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4]	3.5.2
Bifidobacterium longum BB536	Isolated from a pharmaceutical product, USA	LSM-Cys [4]	3.5.2
Bifidobacterium longum KCCM 91563	Korean Culture Center of Microorganisms (Seoul, Korea)	LSM-Cys [4], MRS [6]	3.5.2., 3.5.4
Bifidobacterium longum RD47	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [6]	3.5.4
Bifidobacterium thermophilum KCCM 12097	Korean Culture Center of Microorganisms (Seoul, Korea)	MRS [6]	3.5.4
Clostridium perfringens ATCC 13124	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Enterococcus faecalis ATCC 29212	American Type Culture Collection (Manassas,VA, USA)	LSM-Cys [4]	3.5.2
Enterobacter cloacae subsp. *cloaca* ATCC 13047	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Enterobacter faecalis ATCC 19433	American Type Culture Collection (Manassas,VA, USA)	BHI [1]	3.2
Lactobacillus acidophilus ATCC 4356	American Type Culture Collection (Manassas,VA, USA)	MRS [5]	3.5.3
Lactobacillus fermentum AGBG1	BIFIDO Co., Ltd. (Hongcheon, Korea)	MRS [5]	3.5.3
Lactobacillus plantarum KFRI 708	Korea Food Research Institute (Wanju, Korea)	BHI [1]	3.2
Listeria ivanovii subsp. *ivanovii* ATCC 19119	American Type Culture Collection (Manassas,VA, USA)	Blood Agar [2]	3.3

[1] Ammonia production test (3.2.): *B. bifidum* BGN4, *B. longum* BORI, *B. breve* ATCC 15701, *L. plantarum* KFRI 708, *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. perfringens* ATCC 13124, *E. cloacae* ATCC 13047, and *E. faecalis* ATCC 19433 were anaerobically cultured in brain heart infusion (BHI) (BD BBL™, Franklin Lakes, NJ, USA) medium at 37 °C for 5 days. [2] Hemolytic test (3.3): *B. bifidum* BGN4 and *B. longum* BORI were anaerobically cultured in Blood agar (BHI broth medium supplemented with 1.5% agar and 5% sheep blood) at 37 °C for 2 days. *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, a positive control for hemolysis, was aerobically cultivated in Blood agar at 37 °C for 2 days. [3] Biogenic amine production test (3.4): *B. bifidum* BGN4 and *B. longum* BORI, were anaerobically cultured in whole milk (Seoul Milk, Seoul, Korea) or de Man–Rogosa–Sharpe (MRS) broth (BD Difco™, Franklin Lakes, NJ, USA) with supplementation of 0.05% (*w*/*w*) L-cysteine-HCl (Sigma, St. Louis, MO, USA) at 37 °C for 15 h. [4] Antimicrobial susceptibility test (3.5.2.): LSM-Cys broth medium supplemented with 0.03% L-cysteine-HCl, which is composed with 90% of IST and 10% of MRS broth medium. [5] Antibiotic resistance transferability test (3.5.3.): *Bifidobacterium* strains were anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl and *Lactobacillus* strains were cultured without L-cysteine-HCl at 37 °C for 18 h. [6] PCR assay on antibiotic resistance gene (3.5.4.): *Bifidobacterium* strains were anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl at 37 °C for 18 h. [7] Mucin degradation test (3.6.): *B. bifidum* BGN4 and *B. longum* BORI were anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl at 37 °C for 48 h. [8] Genetic stability test (3.7.): *B. bifidum* BGN4 was anaerobically cultured in MRS broth medium with supplementation of 0.05% (*w*/*v*) L-cysteine-HCl.

3.2. Ammonia Production Test

B. bifidum BGN4, *B. longum* BORI, *B. breve* ATCC 15701, *L. plantarum* KFRI 708, *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. perfringens* ATCC 13124, *E. cloacae* ATCC 13047, and *E. faecalis*

ATCC 19433 were anaerobically cultured in brain heart infusion (BHI) (BD BBL™, Franklin Lakes, NJ, USA) media at 37 °C for 5 days. The production of ammonia by catalyzed indophenol reaction was determined according to the method of Chaney and Marbach [67]. To evaluate the generated extracellular ammonia levels, the media supernatants of each strain were obtained by centrifuging at 10,000× *g* at 4 °C for 30 min. The media was then adjusted to pH 7 using 1 N NaOH. Two solutions were prepared as follows: Solution 1 consisted of 2 g phenol and 0.01 g sodium nitroferricyanide dehydrate dissolved in 200 mL distilled water and Solution 2 consisted of 1 g sodium hydroxide and 0.08 g sodium hypochlorite dissolved in 200 mL distilled water. Aliquots (10 µL) of Solutions 1 and 2 were added to 96 well plates with 100 µL of the media supernatants of each strain. Three replications of this test were conducted on each strain. The 96 well plates were maintained at room temperature for one hour, and the absorbance was measured at 625 nm. Bacteria-free BHI medium was used as a negative control and the ammonia concentration was calculated using a standard curve.

3.3. Hemolytic Test

B. bifidum BGN4 and *B. longum* BORI were anaerobically cultured in blood agar (BHI broth medium supplemented with 1.5% agar and 5% sheep blood) at 37 °C for 2 days. *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, a positive control for hemolysis, was aerobically cultivated in blood agar at 37 °C for 2 days. The plates were then analyzed for the presence or absence of microbial hemolysis properties by holding the plate up to a light source and viewing through both sides of the plate. Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any hemolysis on the blood plates (γ-hemolysis) were considered non-hemolytic. Strains that displayed blood lyses zones (white-hued zones) around the colonies were classified as microorganisms with hemolytic (β-hemolysis) properties.

3.4. Biogenic Amine Production Test

B. bifidum BGN4 and *B. longum* BORI were anaerobically cultured in whole milk (Seoul Milk, Korea) or de Man–Rogosa–Sharpe (MRS) broth (BD Difco™, Franklin Lakes, NJ, USA)) with supplementation of 0.05% (*w*/*w*) L-cysteine-HCl (Sigma, St. Louis, MO, USA) at 37 °C for 15 h. Four biogenic amines (cadaverine (≥97.0%, Cat. #33211), histamine (≥97.0%, Cat. #H7125), putrescine (≥98.5%, Cat. #51799), and tyramine (99%, Cat. #T90344)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,7-Diaminoheptane (internal standard; ISTD, 98%, Cat. #D174708), dansyl chloride (≥99.0%, Cat. #39220), and L-proline (≥99.0%, Cat. #P0380) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Whatman No. 4 filter paper was obtained from Whatman Intl., Ltd. (Maidstone, UK). Sodium carbonate (99.0%, Cat. #433401201), ether (99.0%, Cat. #33475S1280), and acetone (99.7%, Cat. #A0108) were obtained from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea).

The biogenic amine analysis extraction procedure was conducted as described by Kim and Ji [68]. Each 5 g sample was weighed and vortexed with 25 mL of 0.1 N HCl for 5 min. After the resulting homogenate was centrifuged at 10,000× *g* for 15 min at 4 °C (2236R high-speed centrifuge; Labogene Aps, Lillerød, Denmark), the aqueous layer was collected, and the residue was re-extracted as described above. The collected extracts were filtered through Whatman No. 4 filter paper. One milliliter of each extract was transferred to a glass test tube, and the following was added: 0.1 mL of internal standard (1,7-diaminoheptane, 100 mg/L), 0.5 mL of saturated sodium carbonate, and 1 mL of 1% dansyl chloride in acetone. After thoroughly mixing, the test tubes were incubated in a dark water bath (WBC 1510A; Jeio Tech. Co., Ltd., Seoul, Korea) at 45 °C for 60 min. Subsequently, 0.5 mL of 10% proline and 5 mL ether were added to each sample and allowed to rest for 5 min to remove the residual dansyl chloride. The supernatants were suspended and evaporated (Scanvac Speed Vacuum Concentrator; Labogene Aps, Lillerød, Denmark) at 20 °C until dry. The dry residue was diluted with 1 mL of acetonitrile (Sigma-Aldrich, St. Louis, MO, USA). The reconstituted sample and standard were filtered through a 0.2 µm syringe filter for HPLC analysis. The HPLC analysis of the biogenic amines was performed at the National Instrumentation Center for Environmental Management (NICEM) at

Seoul National University (Seoul, Korea). The HPLC determinations were performed as described in Table 7.

Table 7. HPLC conditions.

Parameters	Conditions		
HPLC	Thermo Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, St Peters, MO, USA)		
Column	VDSpher C-18 column (4.6 × 250 mm, 5 µm) (VDS optilab Chromatographietechnik GmbH, Berlin, Germany)		
Mobile solvent	Time (min)	Distilled Water (%)	Acetonitrile (%)
	0	40	60
	1	40	60
	20	0	100
	25	0	100
	26	40	60
	30	40	60
Flow rate	0.8 mL		
Column temperature	30 °C		
Injection volume	20 µL		
Detector	UV 250 nm		

3.5. Antimicrobial Susceptibility and Antibiotic Resistance Transferability Test

3.5.1. Antimicrobial Agents

Twenty antimicrobial agents were used: ampicillin sodium salt (Sigma, Lot#BCBW1243), carbenicillin disodium salt (Sigma, Lot#116M4834V), cephalothin sodium salt (Sigma Lot#056M4858V), chloramphenicol (Sigma, Lot#SLBR8869V), clindamycin hydrochloride (Sigma, Lot#021M1533), dicloxacillin sodium salt hydrate (Sigma, Lot#SZBD263XV), erythromycin (Sigma, Lot#WXBC4044V), gentamicin sulfate (Sigma, Lot#SLBP3082V), kanamycin sulfate (Sigma, Lot#066M4019V), metronidazole (Sigma, Lot#MKBZ3056V), mupirocin (Sigma, Lot#106M4733V), neomycin sulfate (Sigma, Lot#LRAB3300), penicillin G (Sigma, Lot#087M4834V), phosphomycin disodium salt (Sigma, Lot#096M4031V), polymyxin B sulfate salt (Sigma, Lot#027M4002V), rifampicin (Sigma, Lot#MKCC2435), streptomycin sulfate salt (Sigma, Lot#SLBT8451), tetracycline (Sigma, Lot#126M4769V), trimethoprim–sulfamethoxazole (trimethoprim (Sigma, Lot#097M4017V), sulfamethoxazole (Sigma, Lot#BCBT3855)), vancomycin hydrochloride (USP, Lot#R07250). vancomycin hydrochloride was purchased from USP (Rockville, MD, USA), and the remaining 19 antimicrobiotics were purchased from Sigma (St. Louis, MO, USA). Each of the antibiotic powders was dissolved and diluted in appropriate diluents and filter sterilized prior to addition to LSM-Cys broth medium, composed of 90% of IST and 10% of MRS broth medium. IST broth was purchased from KisanBio Co., Ltd. (Mbcell Iso-Sensitest Broth, Seoul, Korea) and MRS was purchased from Becton, Dickinson and Company (BD Difco™ MRS Lactobacilli broth, Franklin Lakes, NJ, USA). Serial dilutions of antimicrobial agents ranging from 1024 to 0.0032 µg/mL were prepared.

3.5.2. Antimicrobial Susceptibility Test

Minimal inhibitory concentration (MIC) values for all bacterial isolates were determined by the ISO 10932:2010 broth microdilution procedure [69]. The LSM-Cys broth medium supplemented with 0.03% (*w*/*v*) L-cysteine HCl containing antibiotics at different concentrations was used to prepare each well of a microwell plate. The inoculum was adjusted to a turbidity equivalent to 0.16 to 0.2 at 625 nm as measured by a Hitachi Spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). The solution corresponded to approximately 3×10^8 cfu/mL. Each inoculum was added to a double strength LSM-Cys broth medium at a rate of 0.2%. A 50 µL diluted bacterial suspension was added to each well; no negative control well was employed. The microdilution plates were prepared with a series of twofold dilutions of antibiotics. The microdilution plates were incubated at 37 °C for 48 h in an anaerobic (5% CO_2, 10% H_2 and 85% N_2) chamber. The MIC was defined as the lowest concentration

of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. The experiments were replicated three times.

3.5.3. Antibiotic Resistance Transferability Test

Conjugal transfer of antibiotic resistance was assessed via the methods of Tannock [70]. Equal bacterial cell volumes (1 mL) of the donor and recipient strains were mixed and centrifuged at 7000× *g* for 10 min (2236R high-speed centrifuge; Labogene Aps, Lillerød, Denmark) (see Table 8). After disposing of the supernatant, the bacterial cell pellet was resuspended in the MRS broth medium and cultivated at 37 °C for 12 h in an anaerobic chamber. The collected bacterial cells were filtered through a 0.45 μm microfilter membrane (Whatman Intl., Ltd., Maidstone, UK) and the membrane was placed on the surface of MRS agar and incubated anaerobically at 37 °C for 24 h. The bacterial cells were washed with 4 mL of 0.9% sterile saline, diluted to 10^{-3}, 10^{-4}, and 10^{-5}, respectively, and then plated on MRS agar containing gentamicin or tetracycline. The plates were incubated aerobically or anaerobically at 37 °C for 36 h. Three replicates of all experiments were conducted.

Table 8. Test scheme.

Donor Strains Recipient Strains	*B. bifidum* BGN4	*B. longum* BORI
L. fermentum AGBG1	N/A [1]	BORI + AGBG1
L. acidophilus ATCC 4356	BGN4 + ATCC 4356	BORI + ATCC 4356

[1] N/A denotes not applicable because *B. bifidum* BGN4 was highly susceptible to tetracycline, which resulted in no growth on the media containing tetracycline.

3.5.4. PCR Assay on Antibiotic Resistance Genes

The experimental conditions of Guo et al. [71] were used for these tests. The genomic DNA of the pure culture bacteria was extracted using MG™ Cell Genomic DNA Extraction SV miniprep (MGmed, Seoul, Korea). The extraction was performed according to the manufacturers' instructions, and the total bacterial DNA was eluted with 200 μL of sterile water. To ensure that the ratio of absorbance at 260 nm to absorbance at 280 nm was 1.8–2.0., DNA extracts were aliquoted and stored at −20 °C. Polymerase chain reactions (PCR) were used to detect antibiotic resistance genes by gene-specific primers (Table 9). The following reaction mixture was added to each sample: 1.5 μL DNA (50 ng), 2 μL primer (100 pmol), dNTP mixture 8 μL, 2XGC buffer I, and adjusted to 50 μL volume by sterilized distilled water. The amplification program was an initial denaturation step of 94 °C for 5 min, and then 30 cycles of: 94 °C for 30 s, annealing temperature (Table 9) for 30 s, 72 °C for 1 min, and 72 °C for 7 min. The amplicons were analyzed on 1.5% agarose gel to confirm the DNA fragment size.

Table 9. Primers and conditions for PCR detection [1].

No.	Primer Name		Oligo Sequence	TM (°C)	Product Size	Reference
1	*Bifidobacterium* genus-specific primers	-	F: 5′-TCGCGTCYGGTGTGAAAG-3′ R: 5′-GGTGTTCTTCCCGATATCTACA-3′	55	128 bp	[72]
2	Gentamicin	*aaac(6)-aph(2)*	F: 5′-CCAAGAGCAATAAGGGCATA-3′ R: 5′-CACTATCATAACCACTACCG-3′	60	220 bp	[73]
3	Kanamycin	*AphA3*	F: 5′-GCCGATGTGGATTGCGAAAA-3′ R: 5′-GCTTGATCCCCAGTAAGTCA-3′	52	292 bp	[74]
4	Streptomycin	*aadE*	F: 5′-ATGGAATTATTCCCACCTGA-3′ R: 5′-TCAAAACCCCTATTAAAGCC-3′	50	565 bp	[74]
5	Trimethoprim	*dfrA*	F: 5′-AAAAGGGGCAGAGCATG-3′ R: 5′-AGAAAATGGCGTAATCGGTA-3′	50	474 bp	[75]
6	Tetracycline(K)	*tet*(K)	F: 5′-TTAGGTGAAGGGTTAGGTCC-3′ R: 5′-GCAAACTCATTCCAGAAGCA-3′	55	169 bp	[76]
7	Tetracycline(L)	*tet*(L)	F: 5′-GTTGCGCGCTATATTCCAAA-3′ R: 5′-TTAAGCAAACTCATTCCAGC-3′	55		
8	Tetracycline(M)	*tet*(M)	F: 5′-GTTAAATAGTGTTCTTGGAG-3′ R: 5′-CTAAGATATGGCTCTAACAA-3′	55	401 bp	[77]
9	Tetracycline(O)	*tet*(O)	F: 5′-GATGGCATACAGGCACAGAC-3′ R: 5′-CAATATCACCAGAGCAGGCT-3′	55		
10	Tetracycline(S)	*tet*(S)	F: 5′-TGGAACGCCAGAGAGGTATT-3′ R: 5′-ACATAGACAAGCCGTTGACC-3′	55	1923 bp	[78]
11	Kanamycin	*aaaD*	F: 5′-TGCGTTTTGACACATCCAC-3′ R: 5′-GGTGTTTATGGCTCTCTTGG-3′	55		

[1] The experiment conditions are secondary quoted from Guo et al. [71].

3.6. Mucin Degradation Test

Partially purified Mucin from porcine stomach—Type III, was purchased from Sigma (St. Louis, MO, USA). An MRS broth medium without a carbon source (i.e., basal medium containing yeast extract 0.75% (*w*/*v*), soy peptone 0.25% (*w*/*v*), fish extract 0.25% (*w*/*v*), sodium acetate 0.25% (*w*/*v*), ammonium citrate 0.1% (*w*/*v*), sodium phosphate monobasic 0.05% (*w*/*v*), sodium phosphate dibasic 0.025% (*w*/*v*), Tween 80 0.05% (*w*/*v*), L-cysteine HCl 0.05% (*w*/*v*), maleic acid 0.005% (*w*/*v*), taurine 0.00625% (*w*/*v*), magnesium sulfate 0.005% (*w*/*v*), manganese sulfate 0.0025% (*w*/*v*), and distilled water 98.2% (*v*/*v*)) was used as a negative control. To each of the four MRS broth media, 0.5% (*w*/*v*) mucin, 1.0% (*w*/*v*) mucin, 0.5% (*w*/*v*) glucose, and 1% (*w*/*v*) glucose were added. After the inoculation of the microorganisms in each MRS medium, the samples were cultured at 37 °C for 48 h under anaerobic conditions. After incubation, the bacterial growth was assessed by measuring absorbance at 550 nm at 12, 24, 36, and 48 h. The initial optical density value of the media was subtracted from the final value for each test sample.

3.7. Genetic Stability Test

B. bifidum BGN4 was plated on a MRS agar plate via streaking from a stock stored at −80 °C and incubated anaerobically at 37 °C for 24 h to obtain a single colony. A single colony was inoculated into 10 mL of MRS broth supplemented with 0.05% (*w*/*v*) L-cysteine HCl and regarded as the 2^0 (1st) generation (about 10^6 CFU/mL) of *B. bifidum* BGN4. *B. bifidum* BGN4 was incubated at 37 °C for about 12 h under anaerobic conditions to reach about 10^9 cfu/mL and obtain 210 generations. In the second subculture, 0.1 mL (1% inoculation, about 10^6 cfu/mL) of the primary culture was inoculated with 10 mL of MRS broth and cultured under the same conditions to obtain 220 generations of *B. bifidum* BGN4. For the third subculture, 0.1 mL (1% inoculation, approximately 10^6 CFU/mL) of the secondary culture was inoculated with 10 mL of MRS broth and incubated to 10^7 or 10^8 CFU/mL to obtain 2^{25} generations of *B. bifidum* BGN4. The viable count during cultivation was measured to confirm the generation number. The genomic DNA of the pure culture bacteria was extracted using MG™ Cell Genomic DNA Extraction SV Miniprep (MGmed, Seoul, Korea), according to the manufacturer's instructions. Whole genome sequencing and analysis were completed using an Illumina MiSeq sequencer and a Nextera XT Library Preparation kit (Illumina, San Diego, CA, USA). Nextera XT sequencing indices were used for multiplexing, and the participants were free to choose any sample index combination. The run acceptance criteria were a sequencing output of 5.6 Gb (to achieve an average sequencing coverage of 100-fold for the 20 samples with genome sizes of 2.8 Mb) and a Q30 read quality score of 75% [79]. The bioinformatics analysis was performed using Miseq raw data, and the comparative genomics analysis was completed with three Miseq raw data sets in ChunLab Co., Ltd. (Seoul, Korea).

3.8. Virulence Factors Researching

The search for virulence factors in *B. bifidum* BGN4 and *B. longum* BORI was completed using the VirulenceFinder1.5 Server, which is a component of the publicly available web-based tool for whole-genome sequencing(WGS) analysis hosted by the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/). The database system is designed to detect homologous sequences for the virulence genes related to *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* in WGS data [80]. The output consists of best-matching genes from BLAST analysis of the selected database against the submitted genome of *B. bifidum* BGN4 or *B. longum* BORI. The selected %ID threshold was set at 90.00%, and the selected minimum length was set at 60%. If there is a matching result, the output shows information on the predicted virulence gene, the % ID, the length of query and database gene, the position of the hit in the contig, and the accession number of the hit.

4. Conclusions

Although probiotics have been widely used for their health benefits in food markets around the world, safety issues, including the side effects of probiotics, should be considered even more carefully than their clinical effects on consumers' health. In this study, it is shown that *B. bifidum* BGN4 and *B. longum* BORI did not produce ammonia or biogenic amines such as histamine, tyramine, or cadaverine. A trace amount of putrescine was found in both strains; however, the quantities were similar to or less than the amount detected in various foods regularly consumed. Neither probiotic demonstrated hemolysis activity nor mucin degrading activity. Their resistance to antibiotics, however, was not transferable in this study. These finding suggest that *B. bifidum* BGN4 and *B. longum* BORI are suitable for use in foods with little risk of harmful effects on the consumer.

Author Contributions: M.J.K. and S.K. designed the experiment under the supervision of M.S.P., G.E.J. and S.Y.K., H.H.L., H.J., S.K. and R.L. performed the microbiology experiments under the mentorship of M.S.P. and G.E.J. M.J.K., S.K., M.S.P. and G.E.J. performed the literature research together. M.J.K., S.K., M.S.P. and G.E.J. designed the research template. S.K. and T.V.J. edited and revised the manuscript based on a non-disclosure research agreement between Middle Tennessee State University and BIFIDO Co., Ltd. All authors discussed drafts and approved the final manuscript for publication.

Acknowledgments: This work was supported by Health and Welfare (03-PJI-PG11-VN01-SV04-0018), Republic of Korea, and the Promoting Regional specialized Industry (R0004140), the Ministry of Trade, Industry and Energy (MOTIE) and Korea Institute for Advancement of Technology (KIAT), the National Research Foundation of Korea (NRF) grant (No. 2017R1A2B2012390) funded by the Korea government (MSIP), the Bio &Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2017M3A9F3041747) and the Faculty Research and Creative Activity Committee (FRCAC) grant (No. 221745) funded by Middle Tennessee State University (MTSU). The authors wish to thank Lauren B. Mallet at Purdue University, Fred Marino and Karen M. Lauer at MTSU for their review and feedback.

Conflicts of Interest: The authors declare no conflict of interest.

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