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ORIGINAL ARTICLE

In *vitro* probiotic characterization of high GABA producing strain *Lactobacilluas brevis* DSM 32386 isolated from traditional "wild" Alpine cheese

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Abstract

Purpose γ-Aminobutyric acid (GABA) is recognised as a potential metabolic bioactive food ingredient with increasing evidence of its effects on the gut-brain axis and systemic metabolic health. Different lactic acid bacteria are capable of producing GABA, particularly strains of *Lactobacillus brevis*. In this study, we characterized a *Lb. brevis* isolated from traditional alpine cheese (*Lb. brevis* DSM 32386) for its ability to accumulate high levels of GABA in the culture medium and for other important probiotic phenotypic traits.

Methods In vitro analysis were used to study the *Lb. brevis* DSM 32386 probiotic traits and the gene expression involved in GABA production

Result *Lactobacillus brevis* DSM 32386 converted monosodium glutamate to GABA more efficiently than the type strain *Lb. brevis* DSM 20054, resulting in more than 200% of GABA produced. This ability seemed to be related to the higher transcriptional activation of the gene encoding for the glutamate (gad) decarboxylase antiporter (*gadC*) and regulator (*gadR*). *Lactobacillus brevis* DSM 32386 performed well in vitro under the stress conditions mimicking the gastro-intestinal tract, being resistant to acid pH (pH 2.5) and growing in simulated pancreatic fluid and 0.3% ox-bile.

Conclusion These preliminary studies indicate that *Lb. brevis* DSM 32386 holds promise as a starter for GABA-rich dairy fermented foods and possibly a promising next-generation probiotic microorganism in the context of the gut (microbiota):brain axis.

Keywords Probiotic · *Lactobacillus brevis* · Oxbile · γ-Aminobutyric acid · GABA · GAD genes

Introduction

Formulation of the neurotransmitter γ-aminobutyric acid (GABA)–enriched foods or functional foods capable of delivery GABA is an opening research and development target for the food industry. These foods include dairy products, soybean, kimchi and juice products (Inoue et al. 2003; Hayakawa et al. 2004; Park and Oh 2007; Kim et al. 2009; Chang et al. 2009; Gangaraju et al. 2014). Moreover, the isolation of GABA-producing strains from diverse fermented food and from the human gut is providing considerable natural biotechnological solutions for efficacious functional food design (Komatsuzaki et al. 2005; Siragusa et al. 2007; Hiraga et al. 2008; Li et al. 2008). Those isolates could represent next-generation probiotics with specific mode of action based around their GABA-producing capability and possible modulation of the gut:brain axis (Bravo et al. 2011; Forsythe and Kunze 2013).

As defined by the Food and Agriculture Organization and the World Health Organization, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Other

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definitions advanced through the years have been restrictive by specification of mechanisms, site of action, delivery format (e.g. food) or host (Hill et al. 2014). To accumulate in the intestinal tract, probiotics must first survive the various conditions specific to the digestive system, such as low pH in the stomach and the presence of bile acids in the intestines: these represent important selection criteria for putative probiotics (Kimoto-Nira et al. 2015). Probiotic efficacy has been shown to be species- and even strain-dependent since different bacterial strains can affect host via different modes of action (Maassen and Claassen 2008; Cani and Van Hul 2015). With a growing emphasis on demonstrating probiotic health effects, rational selection of probiotic strains based on their ability to modulate very specific physiological traits e.g. BSH activity, GABA or bacteriocin production, is becoming a prerequisite for probiotic selection.

Several GABA-producing LAB species isolated from traditional fermented food and beverages have been reported. These include *Lb. paracasei* (Komatsuzaki et al. 2005, 2008; Siragusa et al. 2007), *Lb. buchneri* (Cho et al. 2007; Park and Oh 2007; Zhao et al. 2015), *Lactococcus lactis* (Nomura et al. 1998; Siragusa et al. 2007), *Lb. delbrueckii* subsp. *bulgaricus* (Siragusa et al. 2007), *Lb. plantarum* (Siragusa et al. 2007) and *Lb. brevis* (Ueno et al. 1997; Yokoyama et al. 2002; Park and Oh 2007; Siragusa et al. 2007; Li et al. 2008; Zhang et al. 2012). Cheese represents a rich source of LAB with potential GABA-producing properties (Nomura et al. 1998; Siragusa et al. 2007; Franciosi et al. 2015). The results of these findings offer potential alternatives to take advantage of GABA's health benefits through GABA-enriched cheeses. In a previous work, we isolated 276 strains from a specific raw cow milk "Nostrano-cheese", typical of the Trentino province (north, Alpine area) in Italy. Among those, 71% bacterial strains were able to produce GABA (Franciosi et al. 2015) and, in particular, the *Lb. brevis* DSM 32386 possessed the highest GABA-producing rate (Franciosi et al. 2015; Carafa et al. 2019). In this work, we characterized the genetic basis of DSM 32386 strain's GABA production, as well as we assessed some characteristics considered important for probiotic selection, making it a good candidate as a starter of ingredient for functional GABA-enriched foods.

Material and methods

Reagents

All media constituents were purchased from Oxoid Ltd. (Basingstoke, UK) and Sigma Aldrich (Milan, Italy), chemicals were purchased from Sigma Aldrich.

Bacterial strains, culture medium and growth conditions

The stock culture collection of *Lb. brevis* DSM 32386 (Fondazione Edmund Mach collection and deposited at Leibniz-Institut DSMZ, Germany) and DSM 20054 (Leibniz-Institut DSMZ, Germany), as well as of *Lb. delbrueckii* subs. *lactis* LL199 from the Department of Food and Drug Science—University of Parma collection (Belletti et al. 2009), were maintained at − 80 °C in 20% v/v glycerol. Bacterial cells were propagated twice in MRS broth by incubation at 37 °C for 16 h before each experiments.

γ-Aminobutyric acid (GABA) production and quantification

Glutamate decarboxylase (GAD) activity of *Lb. brevis* DSM 32386 and the production of GABA were measured as reported by Nomura et al. (Nomura et al. 1999). Briefly, *Lb. brevis* DSM 32386 and DSM 20054 were grown in MRS for 24 h at 37 °C temperature. Cell cultures were then centrifuged (8600 rcf for 15 min at 4 °C), washed twice with sterile PBS, and suspended in sterile 0.85% w/v NaCl solution in order to achieve the A620 nm value of 2.5. One hundred microliters of cell suspension was then mixed with 900 μL of 50 mM sodium acetate buffer (pH 4.7) containing 7.0 mM L-glutamate and 0.1 mM pyridoxal phosphate. The reaction mixture was incubated for 24 h at 37 °C and filtered through a 0.22-μm pore size filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany). The sample, diluted 10 times with sodium tetraborate 0.1 M (pH adjusted to 10.5) and added to glycine, as internal standard to a final concentration of 10 mg/L, was stored at − 20 °C before the analysis. L-Glutamic acid, glycine, and GABA were quantified as o-phthalaldehyde (OPA) adducts. The detection limit for GABA was estimated at 0.025 mg/L (three times the standard deviation of the GABA contents measured repeating ten times the analysis of a sample at unquantifiable content).

GAD genes sequencing

DNA was extracted with QIAamp DNA Blood Mini Kit (QIAGEN, Milan, Italy) from Lb. brevis DSM 32386 overnight broth culture following the manufacturer protocol. PCR amplification for the GABA genetic locus (gadR, gadA, gadC and gadB) was performed by using of specific primers (see Supplementary Table S1). PCR reactions were carried out in a 2720 Applied Biosystems Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplified products were subsequently purified using the Promega PCR and Gel Clean Up system kit according to the manufacturer's instructions (Promega Corporation, Milan, Italy). Sequencing was carried out by Sequencing Platform Unit, Fondazione Edmund Mach

(San Michele a/A, Trento, Italy). The identifications were refined by BLAST (1 www.ncbi.nlm.nih.gov/BLAST) and Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) alignment of the GAD DNA sequences to the reference genome.

GAD genes expression

Lactobacillus brevis DSM 32386 and DSM 20054 were inoculated in MRS in the presence or absence of 30 mg/mL of monosodium glutamate (MSG) for 12 h. Total RNA was extracted from cultures at 0, 3, 7 and 12 h using TriZol® (LifeTechnologies, Monza, Italy), according to the manufacturer's instructions, then samples were reverse transcribed using SensiFAST cDNA Synthesis Kit (BioLine AUROGENE s.r.l., Rome, Italy). The expression of target genes (*gadR*, *gadA*, *gadB* and *gadC*) was quantified by quantitative Real Time PCR (Supplementary Table S2). The assay was performed using 2× qPCRBIO SyGreen mix (PCRBIOSYSTEM, Resnova S.r.l., Rome, Italy) and carried out with a LightCycler 480 (Roche, LifeScience, Italy). The housekeeping gene *tuf1* was used to normalize the expression of target genes. The comparative critical threshold method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative gene expression with respect to time 0. The RT-qPCR was performed in duplicates for each cDNA sample and independent experiments were carried out in triplicates.

Tolerance to pH, oxbile and pancreatic fluid

Effect of low pH was studied by the method of Tsai et al. 2008. Briefly, one millilitre of culture containing about 10^9 CFU/mL of LAB was transferred into 9 ml phosphate-buffered saline (PBS; NaCl, 137 mM/L; KCl, 2.7 mM/L; Na_2HPO_4, 10 mM/L; KH_2PO_4, 1.8 mM/L). The pH was adjusted to 2.0, 2.5 and 3.2 using 0.1 N HCl and cells incubated at 37 °C for 3 h. Control was performed at pH 7.2. After incubation, serial dilution plating on MRS agar was performed to determine viable bacterial counts. Plates were incubated anaerobically at 37 °C for 48 h and acid tolerance was estimated by comparing the viable LAB bacteria counts in MRS agar for surviving cells. Data are presented as log(CFU/mL) ± standard deviation. Five independent experiments were performed.

Tolerance for bile acids was performed on LAB cells exposed to low pH. After the 3-h treatment described above, cells were centrifuged (5000*g*, 5 min), washed with PBS (pH 7.2) and then grown in 9 mL MRS broth with and without 0.3% (w/v) Oxgall bile for 3, 12 and 24 h. Bile tolerance was estimated by comparing the viable LAB bacteria count in MRS with and without bile salt. Data are presented as log(CFU/mL) ± standard deviation. Three independent experiments were performed.

Tolerance for pancreatic fluid was tested by inoculating actively growing bacteria (10% v/v inoculum size) to the test medium [150 mM $NaHCO_3$ and 1.9 mg/mL pancreatin (Sigma, USA); pH 8.0]. The cultures were kept for 3 h in a shaking water bath (Certomat WR, B. Braun, Melsungen, Germany) at 37 °C. Survival of LAB strains was examined by plating on MRS agar after 0, and 3 h of incubation. Data are presented as log(CFU/mL) ± standard deviation. Three independent experiments were performed.

Antibiotic susceptibility test

Lactobacillus brevis DSM 32386 and DSM 20054 phenotypic antibiotic resistance to ampicillin, vancomycin, gentamicin, erythromycin, clindamycin and tetracycline was assessed using the strip test M.I.C. Evaluator (Oxoid Ltd., Basingstoke, UK) following the manufacturer's instruction. The strips consist of a gradient of stabilised antimicrobial covering 15 doubling dilutions. M.I.C.E. strips were used on a pre-inoculated agar plate, with formation of defined concentration gradient in the area around it. Minimum Inhibitory Concentration (MIC) was determined at the border of growth inhibition around the strip. Values were compared to the guidelines for facultative heterofermentative lactobacilli as indicated in the "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance" by the European Food Safety Authority (EFSA) (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) 2012).

Detection of antibiotic resistance genes

Genomic DNA from overnight cultures of *Lb. brevis* DSM 32386, *Lb. brevis* DSM 20054 and *Lb. delbrueckii* subs. *lactis* LL199 was extracted with QIAamp DNA Blood Mini Kit (QIAGEN, Milan, Italy). Polymerase chain reactions was used to determine antibiotic resistance genes by gene-specific primers (Table 4), by using genomic DNA from *Lb. delbrueckii* subs. *lactis* resistant strain LL199 as positive control. After an initial denaturation step of 94 °C for 5 min, the amplification programme consisted of 35 cycles of: 94 °C for 1 min, annealing temperature (Table 4) for 1 min, 72 °C for 2 min. PCR reactions were carried out in a 2720 Applied Biosystems Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and amplicons were analysed on 1% agarose gel to confirm presence and fragment size.

Statistics

Paired *t* test was used to compare differences between the effect of a particular stress condition and the control, or between different gene expression levels. The level of significance was set at $p < 0.05$.

Results

Lb. brevis DSM 32386 GABA production

Our previous study identified several cheese isolates capable of producing GABA (Franciosi et al. 2015). Starting from this preliminary information, we focused on *Lb. brevis* DSM 32386. Firstly, the GABA production rate of *Lb. brevis* DSM 32386 was compared to that of the type strain *Lb. brevis* DSM 20054. After incubation at 37 °C for 24 h, DSM 32386 was able to produce considerable higher quantity of GABA (262.06 ± 15.42 mg/L) compared to the type strain (78.27 ± 18.61 mg/L) ($p < 0.00001$).

Two different GAD encoding genes have been characterized in different *Lb. brevis* stains, namely *gadA* and *gadB* (Nomura et al. 1998; Siragusa et al. 2007; Komatsuzaki et al. 2008; Hiraga et al. 2008). *gadA* is located adjacent to and downstream of the glutamate/GABA antiporter gene (*gadC*), commonly referred as *gad*CA. They form an operon with the operon regulator *gadR*, being immediately upstream of *gad*CA. *gadB* is located separately from the other *gad* genes (Li et al. 2013) (Fig. 1A). By the use of fourteen sets of primers based on the nucleotidic sequence of the reference strain ATCC367 (Supplementary Table S1), the DSM 32386 operon and *gadB* sequences were amplified confirming the presence of each genetic locus involved in the GABA production within DSM 32386 strain. The gene sequences shared high similarity with the ATCC367 strain, revealing the absence of any polymorphisms in the operon system or in the antiporter. We thus asked whether this high ability was related to an increase in gene expression of such genes in the presence of glutamate. The RT-qPCR analysis of *gad* genes showed a different expression profile in *Lb. brevis* DSM 32386 and DSM 20054 genes, both in the presence or absence of the operon inducer glutamate (Fig. 1B). With the exception of *gadA* after 7 h and *gadC* after 12 h, *gad* genes are repressed in the absence of glutamate, while the presence of glutamate induced their expression in both strains. All *gad* genes were up-regulated to a greater degree by DSM 32386 than in the type strain (*gadA*, $p < 0.001$; *gadB*, $p < 0.001$;*gadC*, $p < 0.01$; *gadR*, $p < 0.01$), especially after 7 h of growth (Fig. 1B).

Lb. brevis DSM 32386 tolerance to simulated gastrointestinal conditions

One of the characteristics required for being a probiotic is the ability to survive the gastrointestinal (GI) physiochemical environment. Therefore the *Lb. brevis* DSM 32386 tolerance to acid pH, bile and pancreatic fluid, mimicking the acidic and liptolytic environment present along the GI tract was measured. *Lb. brevis* DSM 32386 was more resistant to acid pH than the type strain DSM 20054, being able to survive to 3 h exposure at pH 2.5 and 3.2, but not to pH 2.0 (Table 1). Moreover, incubation of DSM 32386 strain in growth medium containing pancreatic fluid had no effect on its viability and fitness (Table 1). The type strain DSM 20054 behaved in a similar manner and not statistically significant differences were observed between the survivals of both strains in pancreatic fluid (Table 1).

Subsequently DSM 32386 cells that survived pH 2.5 (3 h) acid treatment were cultured in MRS broth in the presence or absence of 0.3% Oxbile. *Lb. brevis* DSM 32386 cells were able to resist to bile salts, even with a significant cell reduction over time (Table 2). DSM 32386 cells surviving the pH 2.5 acid treatment grew at rate comparable to the not pH-treated cells when transferred into MRS broth (Table 2).

Antibiotic susceptibility testing

Regarding the safety assurance of probiotic organisms in food, FAO/WHO guidelines (2002) suggest testing probiotic strains for antibiotic resistance. Primary testing of the antibiotic resistance patterns of *Lb. brevis* DSM 32386 and DSM 20054 strains was carried out by the E-strip method, following manufacturer's instruction. According to the sensitivity guidelines provided by EFSA (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) 2012), DSM 32386 was interpreted to be sensitive to vancomycin, clindamycin, tetracycline, ampicillin and erythromycin, while resistant to gentamycin with MIC being two times more the indicated cut off value (Table 3). Instead, DSM 20054 was interpreted to be sensitive to vancomycin, tetracycline, ampicillin and erythromycin and resistant to gentamycin and clindamycin with MICs being four and sixteen times more the indicated cut-off values (Table 3).

We therefore analysed the presence of the antibiotic resistance cassettes at the genomic level by PCR (Table 4) using *Lb. delbrueckii* subs. *lactis* LL199 as positive control, as already reported resistant to clindamycin, tetracycline, erythromycin and gentamycin (Belletti et al. 2009). Only the erythromycin resistance gene *erm(A)* and tetracycline cassette *tet(W)* were detected in both *Lb. brevis* strains, while clindamycin, ampicillin and gentamycin resistance genes were not detectable.

Discussion and conclusion

The identification of strains with specific mode of action or biochemical traits capable of mediating specific host physiological responses represents the basis of a rational scientific selection of the next generation probiotic strains designed to mediate specific health effects in the host.

High γ-aminobutyric acid (GABA)–producing LAB strains isolated from fermented food, including cheese (Siragusa et al. 2007; Franciosi et al. 2015) and fresh

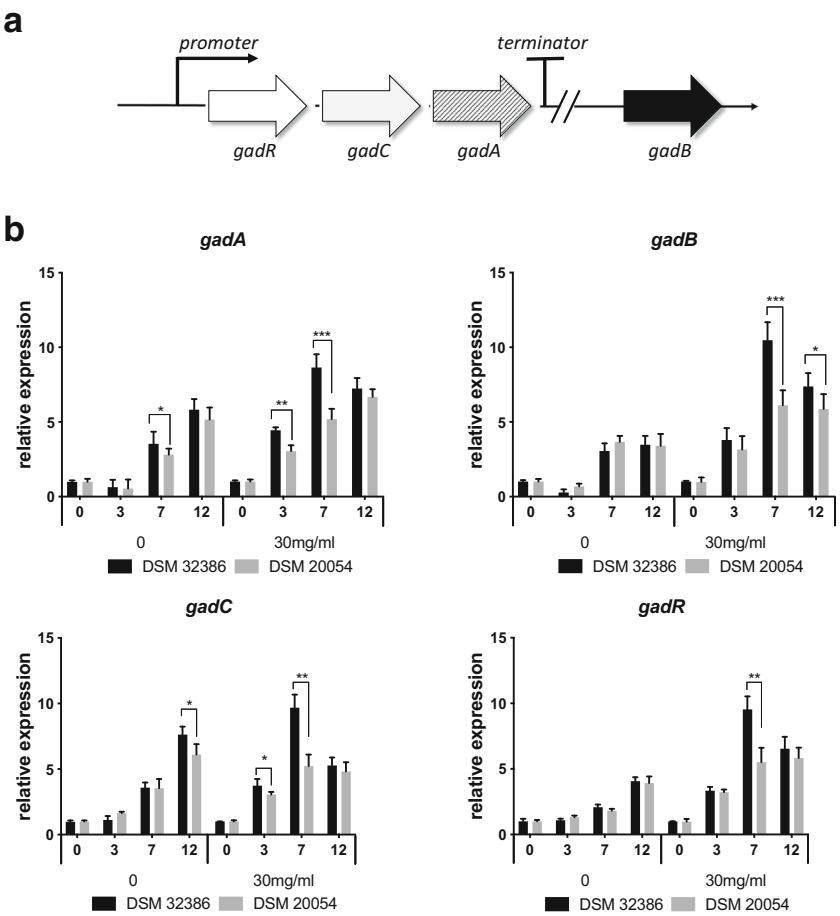

Fig. 1 GAD system in *Lb. brevis* and genes expression in response to 30 mg/ml of monosodium glutamate. (**A**) The *gad* operon genes and *gadB* gene in *Lb. brevis*; (**B**) RT-qPCR has been used to assess the transcription level of the gad in DSM 32386 and DSM 20054 as described in 'Material and methods' section. Comparative critical threshold method ($2^{-}\Delta\Delta^{Ct}$) was used to calculate the relative gene expression with respect to time 0. Data are presented as mean ± sd, $N = 3$. *$p < 0.05$, **$p < 0.01$, paired t test, DSM 32386 relative expression *vs* DSM 20054 relative expression

unpasteurized milk (Fan et al. 2011) or by strains isolated from the human intestinal tract represent a rich and natural bioresource for identification of putative probiotic (Monte et al. 2009; Settanni and Moschetti 2010; Montel et al. 2014). GABA is produced primarily from the irreversible α-decarboxylation of L-glutamate by the enzyme glutamate decarboxylase (GAD) (Cotter and Hill 2003). In the intracellular glutamate decarboxylase (GAD) system, glutamate is imported into cells by the GABA antiporter, decarboxylated by intracellular GAD to produce GABA and subsequently GABA is exported from the cells via the antiporter (Sanders et al. 1998; Small and Waterman 1998; Li et al. 2013). Two GAD-encoding genes, named *gadA* and *gadB* are present in *Lb. brevis* (Li et al. 2013; Wu and Shah 2016; Yunes et al. 2016; Wu et al. 2017; Wu and Shah 2018). The high GABA production by *Lb. brevis* DSM 32386 appears not to be due to mutation in these genes as we identified the presence of the intact *gad* operon at the genomic level. In general, the bacterial GAD system includes (i) a glutamate uptake by a specific transporter followed by (ii) the removal of an intracellular proton during glutamate decarboxylation and (iii) GABA export from the cell via an antiporter. This leads to an increase in the cytoplasmic pH (by the removal of hydrogen ions) and also slightly increasing the extracellular pH (by the exchange of extracellular glutamate for GABA) (Cotter and Hill 2003; Wu et al. 2017). Interestingly, compared to the type strain DSM 20054, *Lb. brevis* DSM 32386 induces a higher expression of both *gadA* and *gadB* genes over time, accompanied by

Table 1 Analysis of acid and pancreatic fluid tolerance *Lb. brevis* DSM 32386 and *Lb. brevis* DSM 20054

Strain	Acid tolerance (pH 2.0, 2.5 and 3.2)					Resistance to pancreatic fluid	
	0 h	pH 7.2, 3 h	pH 3.2, 3 h	pH 2.5, 3 h	pH 2, 3 h	0 h	3 h
DSM 32386	9.64 ± 0.40[a]	9.09 ± 0.21	8.85 ± 0.64	7.75 ± 0.74	nd	7.84 ± 0.56[a]	7.92 ± 0.45
DSM 20054	9.87 ± 0.70	8.61 ± 0.22	8.43 ± 0.37	n.d	n.d	8.11 ± 1.13	7.90 ± 0.51

[a] Bacterial counts are converted to log CFU/ml

Table 2 Effect of bile salts on *Lb. brevis* DSM 32386 after low pH treatment

Time (h)	0	3	12	24
MRS	5.38 ± 0.79[b]	4.16 ± 0.45	6.29 ± 0.51	8.55 ± 0.37
MRS + oxbile[a]	5.78 ± 1.13	2.03 ± 1.3*	2.3 ± 1.41**	3.12 ± 1.55***

[a] MRS + oxbile means MRS broth with 0.3% Oxgall

[b] Bacteria counts are converted to log CFU/ml

*$p < 0.05$

**$p < 0.01$

***$p < 0.001$, paired *t* test, oxbile vs none

an increased level of *gadC*, the gene encoding for the antiporter and *gadR*, encoding for the positive operon regulator. Indeed, the higher activation of the GAD system observed could account for the high GABA production.

The physiological activity of GABA makes it an interesting bioactive molecule which has already been used as a food supplement in pure form (Andrighetto et al. 2002). In recent years, researchers have reported a number of placebo controlled studies in which GABA was administered as a food or oral supplement to healthy participants (Am et al. 2005; Nakamura et al. 2009; Kanehira et al. 2011; Yoto et al. 2012; Li et al. 2015; Steenbergen et al. 2015). A pioneering study in patients with mild hypertension reported that daily intake of fermented milk containing 10–12 mg/100 mL of GABA could significantly lower blood pressure within 2 weeks (Inoue et al. 2003). In these terms, fermented milk enriched in GABA produced by *Lb. brevis* DSM 32386 may have commercial potential as a health-oriented dairy product as well as any direct probiotic effect of the high GABA-producing strain.

To be considered a possible probiotic, the bacterial strain, in addition to being a GRAS organism, should be able to survive within the human GI tract and therein mediate a specific health-related activity in the right environment (Lee and Salminen 1995; Salminen et al. 1998). Testing for tolerance of low pH, bile acids and pancreatic fluids have often been considered as good indicators for survival through the GI tract. In this study, *Lb. brevis* DSM 32386 strain performed well in the in vitro tests, and survival through the stomach is likely. In addition, previous studies have shown that food matrix plays an important role in probiotic survival of gastric pH (Charalampopoulos et al. 2003; Stadler and Viernstein 2003; Mancini Andrea 2017) and cheeses in particular appears to effectively protect probiotics from low pH encountered in the stomach (Boylston et al. 2004). However, survival under in vivo conditions in human subjects should be tested.

Lactic acid bacteria are intrinsically resistant to many antibiotics (Delgado et al. 2005; Fukao et al. 2009; Devirgiliis et al. 2013; Guo et al. 2017; Campedelli et al. 2019). In many cases, resistances are not, however, transmissible, and the species are also sensitive to many clinically used antibiotics even in the case of a lactic acid bacteria–associated opportunistic infection. Among 187 isolates, from 55 European probiotic products showed that 79% of the isolates were resistant to kanamycin and 65% of the isolates were vancomycin resistant. Remaining resistances were in the order of tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%). Overall, 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistance (Temmerman et al. 2003). The antimicrobial susceptibility tests indicated that *Lb. brevis* DSM 32386 was resistant to gentamycin, and the type strain DSM 20054 as resistant to gentamycin and clindamycin. In general, *Lactobacillus* species are intrinsically resistant to aminoglycosides such as gentamicin, with resistance reported in strains isolated from different sources, as in the case of *Lb. brevis* isolated from fermented vegetables (Abriouel et al. 2015). EFSA requires the assessment of antibiotic resistance in strain introduced in the food chain, with particular attention in distinguishing between bacterial species with "intrinsic resistance" or "acquired resistance". Since intrinsic resistance is specific for a bacterial species or genus, when all strains within a given taxonomic group show phenotypic resistance to an antimicrobial, this resistance can be considered intrinsic to the taxonomic group (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) 2012). In our study, gentamicin resistance was detected in both *Lb. brevis* DSM 32386 and

Table 3 Resisance of *Lb. brevis* DSM 32386 and *Lb. brevis* DSM 20054 to various antimicrobial agents

	CA	VA	DA	TE	AM	ER
Cut-off value (μg/ml)[a]	16	2	1	8	4	1
DSM 32386 MIC (μg/ml)	32	–	0.03	4	2	0.25
DSM 20054 MIC (μg/ml)	64	–	16	4	2	0.5

[a] Microbiological cut-off values as indicated by EFSA; CA, Gentamycin; VA, Vancomycin; DA, Clindamycin; TE, Tetracycline; AM, Ampicillin; ER, Erythromycin

Table 4 Gene specific primers and condition for PCR reactions

Antibiotic	Antibiotic resistance cassette	Primers 5′-3′	Annealing temperatures (°C)	Size (bp)	References
Ampicillin	*mecA*	CGTSTTTAACTAAGTATSGY GGGATCATAGCGTCATTATTC	58	1429	Guo et al. (2017)
	bla	TAGGTTCAGATTGGCCCTTAG CATARTTCCGATAATASMGCC	51	297	Guo et al. (2017)
	claZ	GCATGRTAACCATCACAWAC ACTTCAACACCTGCTGCTTTC	58	240	Guo et al. (2017)
Erythromycin	*erm(A)*	AAGCGGTAAACCCCTCTGA TTCGCAAATCCCTTCTCAAC	55	190	Nawaz et al. (2011)
	erm(B)	GAAAAGRTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	52	642	Nawaz et al. (2011)
Gentamycin	*aac*	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	60	220	Nawaz et al. (2011)
Clindamycin	*lun(A)*	GCTAATATTGTTTAAATCGTCAAT GGTGGCTGGGGGGTAGATGTATTAACTGG	55	323	Guo et al. (2017)
	lun(B)	GCTTCTTTTGAAATACATGGTATTTTTCGATC CCTACCTATTGTTTGTGGAA	54	925	Guo et al. (2017)
Tetracycline	*tetM1*	GCTTGATCCCCAGTAAGTCA GGTGAACATCATAGACACGC	55	401	Guo et al. (2017)
	tetL1	GTMGTTGCGCGCTATATTCC GTGAAMGRWAGCCCACCTAA	55	696	Fukao et al. (2009)
	tet(W)	GAGAGCCTGCTATATGCCAGC GGGCGTAT CCACAATGTTAAC	64	168	Fukao et al. (2009)
	tet(S)	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	56	573	Nawaz et al. (2011)
	tet(L)	CATTTGGTCTTATTGGATCG ATTACACTTCCGATTTCGG	50	456	Nawaz et al. (2011)
	tet(K)	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	55	697	Nawaz et al. (2011)
	tet(O)	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	52	515	Nawaz et al. (2011)
	tet(Q)	AGAATCTGCTGTTTGCCAGTG CGGAGTGTCAATGATATTGCA	56	169	Nawaz et al. (2011)
	tet(M)	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	55	576	Nawaz et al. (2011)

20054 strains, being more evident in the type strains, thus allowing us to consider it as natural and not acquired antibiotic resistance, therefore no particular safety concern is associated with this intrinsic type of resistance.

From a molecular point of view, any gentamicin amplicons was evidenced by PCR, while *Lb. brevis* strains harboured the *erm(A)* and *tet(W)* cassette, respectively, for erythromycin and tetracycline resistance, that were phenotypically confirmed but they were below the EFSA cut-off values. In *Lactobacillus* ssp., the most common *erm* cassette is *erm(B)* also detected in *Lb. brevis*, while *erm(A)* is rarely found (Nawaz et al. 2011). Genes that confer tetracycline resistance in lactobacilli can be *tet*(*S*), (*W*), (*K*), (*L*) and (*O*), widely distributed between species and mainly represented in *Lb. brevis* by *tet*(*M*) and *tet*(*S*) (Fukao et al. 2009; Nawaz et al. 2011; Devirgiliis et al. 2013; Campedelli et al. 2019). A more comprehensive overview and information on the genetic basis of *Lb. brevis* DSM 32386 antibiotic resistance will be derived by genome sequencing that represent the further step in the study of this strain.

Even though in vivo investigations are needed, altogether these preliminary results showed that the *Lb. brevis* GABA producing DSM 32386 strain represents a promising starter for manufacturing GABA-rich cultured dairy foods to be used as functional food as well as a promising next generation probiotic in the context of the gut(microbiota):brain axis. Tests on GABA level availability in DSM 32386 dairy products are ongoing. Overall, our data indicate the importance of studying and preserving the traditional raw milk cheese microbiome. Traditional cheeses represent an important source of microbial biodiversity where new LAB strains with potential health-promoting properties can be isolated.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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