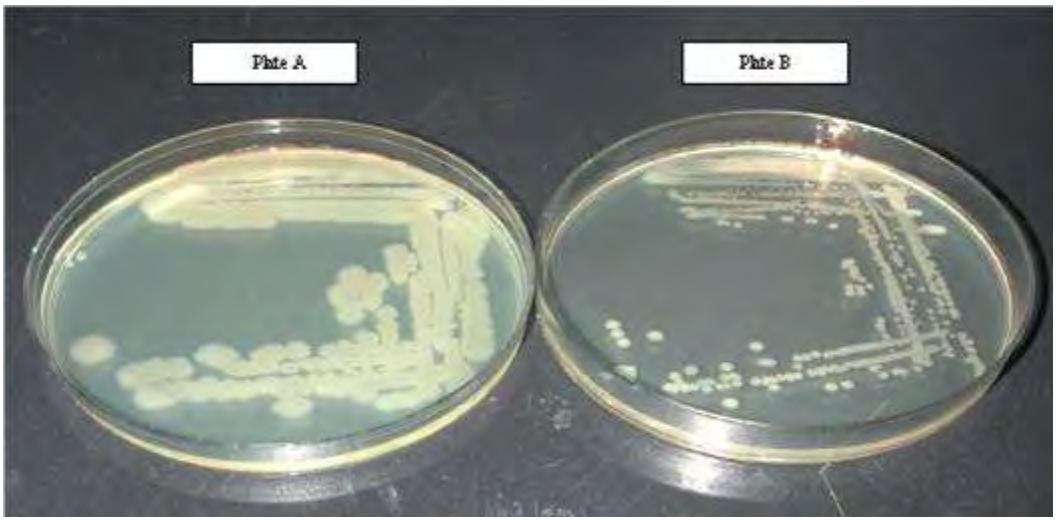

Figure 9: Results of bile salt hydrolase activity: (Plate A) *B. subtilis* streak on TSA as control plate, (Plate B) *B. subtilis* streak on TSA + 0,5% TCA

After this initial *in vitro* test, whole genome sequencing was performed on this strain to more accurately determine if the bacteria had any bile salt hydrolase that was not appearing in these tests. The genome mining determined that there was an open reading frame that coded for choloylglycine hydrolase (EC. 3.5.1.24.). The above tests were performed with taurine coupled bile acids, which the choloylgylcine hydrolase specifically targets glycine coupled bile acids. This perhaps indicates why this strain did not grow in the presence of taurine coupled bile acids.

6.3.3. Presence of Antibiotic Resistance Genes

The antibiotic resistance profile of *Bacillus subtilis* R0179 was established using several complementary methods:

First, the phenotypic resistance was assessed by determining Minimum Inhibitory Concentrations and comparing them to established breakpoints. Then the strain *Bacillus subtilis* R0179 was screened for the presence or absence of antibiotic resistance (ABR) genes by using the following genomic screening tools:

- ResFinder
- ARG-ANNOT
- CARD

6.3.3.1. Phenotypic resistance

The generally recognized method to assess antibiotic susceptibility of microorganisms is by measuring the Minimum Inhibitory Concentration (MIC) and comparing it to standard microbiological breakpoints. Strains with MICs higher than the breakpoints are considered resistant. However, this result does not imply that the resistance can be transferred to other microorganisms. Microbiological breakpoints were

suggested by the FEEDAP Panel (EFSA 2018) for *Bacillus spp* in their "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance", published in June 2018[1].

The microbiological breakpoints were set for 8 antimicrobial agents, which were chosen to maximize the identification of resistance genotypes to the most commonly used antimicrobials. It is mentioned in the guidance that the values should be reviewed on a regular basis and modified when necessary, as new data become available. The MIC of several antimicrobial agents were determined for *Bacillus subtilis* R0179 and compared with FEEDAP 2018 breakpoints. Strains with MICs higher than the breakpoints are considered as resistant.

The Minimal Inhibitory Concentrations of several antimicrobial agents were determined for *B. subtilis* R0179. The minimal inhibitory concentrations (MIC) were determined according to CLSI recommended methods (CLSI M45-A2 2010).

Minimal Inhibitory Concentration (MIC) µg/ml in Tryptic Soy Broth

Antimicrobial Agent	**Minimum Inhibitory Concentration (µg/ml) of *B. subtilis R0179***	**Microbiological breakpoints (µg/ml) FEEDAP 2018, *Bacillus spp***
Ampicillin	<0.031	n.r.
Chloramphenicol	0.5	8
Clindamycin	0.25	4
Erythromycin	<0.031	4
Gentamicin	<0.031	4
Kanamycin	0.25	8
Streptomycin	4	8
Tetracycline	1	8
Vancomycin	<0.031	4
SOURCE: Lallemand Health Solutions, 2020 (unpublished)		

n.r.: not required

According to those breakpoints, *B. subtilis* R0179 is not considered resistant to any of the tested antimicrobial agents.

6.3.3.2. Whole genome screening

The report of the genome analysis by Prof. Cutting described in section 6.3.6. has evaluated the presence of antibiotic resistance genes. Some antibiotic resistance genes have been identified (fosfomycin, streptothricin, β-lactamase, fluoroquninolones, novobiocin, streptomycin and actinomycin D). These

[1] This guidance document replaces the previous EFSA opinion on the updating of the criteria used in the assessment of bacteria of human or veterinary importance, adopted on 18 June 2008, and later revised in 2012.

genes are believed to be intrinsic to the *Bacillus* taxonomic group since they are not flanked by mobile genetic elements and are chromosomally borne, which limits the capacity of transfer.

A summary of the findings of Prof. Cutting and further testing of phenotypic resistance is provided in the table below.

Antimicrobial resistance marker	Similar genes in other *Bacilli*	Assessment & further phenotypic testing
Fosfomycin resistance	yes	**Low Risk** Probably the gene is intrinsic to the *Bacillus* taxonomic group. MIC for R0179 for fosfomycin has been determined (16 µg/µL) and compared to published MICs for *Bacillus subtilis* (Cao et al. 2001). According to that, R0179 is actually not resistant to fosfomycin.
Streptothricin resistance	yes	**Low Risk** Probably the gene is intrinsic to the *Bacillus* taxonomic group. Streptothricins are broad-spectrum antibiotics that are not used in humans due to their nephrotoxicity (Hoffman et al. 1986b, Hoffman et al. 1986a). Accordingly, the identification of this resistance gene is not of immediate concern.
β-lactamase resistance	yes	**Low Risk** Probably the gene is intrinsic to the *Bacillus* taxonomic group. MIC for R0179 for ampicillin has been determined (< 0.031 mg/L, Tompkins et al. 2008). The resistance is low. Additionally, no MIC breakpoints for ampicillin have been required by EFSA for *Bacillus* species (EFSA Journal 2012). Accordingly, this resistance gene is of minor concern.
Fluoroquinolone resistance	yes	**Low Risk** Genes present that could acquire spontaneous mutations generating resistance. No evidence from the sequence that resistance proteins are produced. May be intrinsic to the Bacillus taxonomic group. Additionally, MICs for R0179 have been determined for ciprofloxacin (0.25 µg/mL) and levofloxacin (0.12 µg/mL) and compared to breakpoints of the Clinical and Laboratory Standards Institute (CLSI) for *Bacillus* species (M45-A2, table 3[2]). According to these results, R0179 is sensitive to these two fluoroquinolones. In addition, floroquinones are not used as primary line antibiotics.
Resistance to fusidic acid, novobiocin, streptomycin and actinomycin D	yes	**Low Risk** Genes present that could acquire spontaneous mutations generating resistance. Genes linked to low-level resistance to fusidic acid, novobiocin, streptomycin and actinomycin D (Kim et al. 2009). R0179 has been shown to have low level of resistance to streptomycin, with a MIC of 4 µg/mL (EFSA breakpoint for Bacillus species: 8 µg/mL). The sensitivity of R0179 to novobiocin and actinomycin-D was also tested despite the absence of MIC breakpoints for these antibiotics. The MIC values are sufficiently low (0.25 µg/ml for Novobiocin and 0.125 µg/ml for Actinomycin-D) that it is evident that R0179 is sensitive to both antibiotics. Genes are intrinsic to the *Bacillus* taxonomic group.

[2] Clinical and Laboratory Standards Institute (CLSI) document: M45-A2 Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline – Second Edition. Vol. 30 No. 18. 2010.

Additionally, it has been shown that *Bacillus subtilis* R0179 is sensitive to all antibiotics listed by EFSA (EFSA Journal 2012).

In addition to this, whole genome screening for ABR genes was also performed using multiple databases. The ResFinder v2.1 database is a peer-reviewed database that is used for screening acquired antibiotic resistance (ABR) genes (Kleinheinz et al. 2014). This validated database contains more than 2000 ABR genes and is updated periodically. ResFinder uses a homology search tool such as BLAST to screen the input sequences.

The ARG-ANNOT ABR gene database is downloadable software that can be used to detect existing and putative new antibiotic resistance in bacterial genomes (Gupta et al. 2014). This database also utilizes a BLAST approach for sequence complementary screening of 1689 ABR genes. *B. subtilis* R0179 screening using these two databases revealed the presence of *aadK* and *tet (L)* genes encoding a resistance to aminoglycosides and to tetracycline, respectively.

As shown in the section 6.3.3.1 on MIC, *B. subtilis* R0179 is not considered resistant to any of the tested antimicrobial agents, including:

- streptomycin,
- kanamycin and gentamicin (aminoglycosides), despite the presence of genes *aadK,* which is supposed to confer resistance to aminoglycosides.
- tetracycline, despite the presence of gene tetL, which is supposed to confer resistance to tetracycline.

These results show that the genes *aadK* and *tetL* are either not expressed or they are inactive.

The Comprehensive Antibiotic Resistance Database (CARD) tool is a validated and regularly updated online platform to analyze DNA sequences and compare them to over 2500 ABR genes reference sequences (Jia et al. 2017). Analysis of the *B. subtilis* R0179 sequence with CARD revealed the presence of the following genes: mphK (macrolide), vmlR (macrolide, lincosamide, streptogramin, tetracycline, oxazolidinone, phenicol, pleuromutilin), ykkC and D (aminoglycosides, tetracycline), pgsA-A64V (daptomycin), tmrB (tunicamycin). These genes are believed to be intrinsic to the *Bacillus* taxonomic group since they are not preceded by mobile genetic elements and are chromosomally borne (i.e., not mobile), which prevents the capacity of transfer. These genes do not appear to confer resistance functionally, as *B. subtilis* R0179 was sensitive to all antibiotics listed by EFSA.

The whole genome sequence of *B. subtilis* R0179 was annotated using the Rapid Annotation using Subsystem Technology (RAST; http://rast.nmpdr.org/rast.cgi). The predicted number of protein coding sequences was 4417 open-reading frames with an additional 62 RNA coding sequences for a total of 4479 features. RAST predicted that another 64 open-reading frames were “possibly missing.” There were 2680 (61%) open-reading frames with assigned functions and 1737 (39%) open-reading frames with putative function or hypothetical proteins with unknown function. These analyses revealed the presence of the *FosA* gene encoding a resistance for fosfomycin, as well as a regulator of this gene called sigmaW. However, using MIC analyses, it was demonstrated that *B. subtilis* R0179 is sensitive to fosfomycin, with

a MIC value of 16 µg/ml, which is well below the resistant strains' MIC value of 800 µg/ml (J. Belvis, Internal Report, Unpublished data).

Overall, the genomic examination of the strain R0179, considered in parallel to *in vitro* and *in vivo* analyses, provides no evidence that it could be harmful to human health when used orally in healthy individuals.

6.3.4. Antibiotic Production

Bacillus subtilis as a species is known to produce more than two dozen antibiotics. A review by Stein (2005) reported their large variety of structures. The antimicrobial active compounds produced predominantly include peptides that are either ribosomally synthesized and post-translationally modified or non-ribosomally generated, as well as non-peptidic compounds such as polyketides, an aminosugar, and a phospholipid.

Bacillus subtilis R0179 possesses genes encoding for production of four antibiotics: surfactin, plipastin, bacilysin, and potentially cephalosporin. Only the two lipopeptide antibiotics surfactin and plipastin are relevant for evaluating safety in humans. Their lipophilic nature makes them capable of binding to cell membranes and may represent a risk of cell toxicity and hemolysis. Both toxins are also found in *Bacillus subtilis* 168, the type-strain, which is part of the QPS list published by EFSA. Moreover, the toxicity studies in animals and clinical studies in humans have reported no adverse effects. Antibiotic production represents no real issues for human consumption and should rather be considered as positive since it is one of the mechanisms of action by which certain strains of bacteria can confer beneficial effects to the host.

6.3.5. Toxigenic activity

6.3.5.1 Enterotoxins

The genomic analysis did not identify any of the known enterotoxins commonly associated with other species of Bacillus (e.g., *B. cereus* & *B. anthracis*). The absence of these genes was confirmed using polymerase chain reaction with specific primers to these genes (*hbl*A, *hbl*C, *hbl*D, *nhe*A, *nhe*B, *nhe*C, *cyt*K and *ces*). These results were published by Tompkins et al. (2008) and are detailed in Table 14.

Table 14: Testing of the presence of toxin genes in *B. subtilis* R0179

Gene	Toxin	Annealing Temper-ature (ºC)	Positive control	Presence of gene in R0179
hblA[1]	Hemolysin, causes diarrhea	65	R0311	Negative
hblC[1]	Hemolysin, causes diarrhea	62	R0311	Negative
hblD[1]	Hemolysin, causes diarrhea	54	R0311	Negative
bceT[2]	Enterotoxin T, causes diarrhea	63	R0311	Negative
nheA[3]	Non-hemolytic enterotoxin, causes diarrhea, responsible for the food-poisoning syndrome	50	R0311	Negative
nheB[3]	Non-hemolytic enterotoxin, causes diarrhea, responsible for the food-poisoning syndrome	50	R0311	Negative
nheC[3]	Non-hemolytic enterotoxin, causes diarrhea, responsible for the food-poisoning syndrome	46.4 to 65.6	R0311	Negative
cytK[4]	Necrotic, hemolytic enterotoxin	44	R0311	Negative
cer[5]	Emetic toxin cereulide	46.4 to 65.6	No positive available	Negative

[1]Rowan NJ, Deans K, Anderson JG, Gemmell CG, Hunter IS, and Chaithong T**.** Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. *Appl Environ Microbiol* 2001; 67**:**3873-81.
[2]Hansen B M, Hoiby PE, Jensen GB, and Hendriksen NB. The Bacillus cereus *bceT* enterotoxin sequence reappraised. *FEMS Microbiol Lett* 2003; 223**:**21-4.
[3]Granum PE, O'Sullivan K, and Lund T. The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiol Lett* 1999; 177**:**225-9.
[4]Lund T, De Buyser ML, and Granum PE. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol Microbiol* 2000; 38**:**254-61.
[5]Horwood PF, Burgess GW, and Oakey HJ**.** Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. *FEMS Microbiol Lett* 2004; 236**:**319-24.

B. subtilis R0179 was negative for all toxin genes tested (Tompkins et al. 2008). Based on these results, *B. subtilis* R0179 has a low pathogenic potential because it does not carry toxigenic genes.

In addition, two different phenotypic tests were performed to confirm the absence of *Bacillus* toxins, using biological and immunological detection systems (tests described by Tompkins et al. 2008). The BCET-RPLA toxin detection kit (TD0950, Oxoid Ltd.), which detects diarrheal-type enterotoxins, was used. Assays were performed on *B. subtilis* R0179 and ATCC 6051T (negative control), and *B. cereus* R0311 (positive control). Strains were streaked on tryptic soy agar prior to subculture into brain heart infusion (BHI) broth and grown with aeration at 37°C overnight, according to manufacturer's instructions. Agglutination was performed in 96-well round-bottom plates using supplied negative and positive controls and culture supernatant of each strain. The absence of diarrheal-type toxin activity in strain R0179 was confirmed.

A bioassay examining boar sperm motility was used to screen for the presence of the emetic toxin cereulide. *Bacillus subtilis* R0179 was sent to Dr. M. Salkinoja-Salonen (University of Helsinki, Finland) for analysis. The toxicity test was performed according to the protocol detailed in Andersson et al. (1998), using *B. subtilis* ATCC 6015R and *B. cereus* ATCC 14579 as negative controls and *B. cereus* F4810/72 as a positive control. This test confirmed that strain R0179 does not have emetic toxin activity.

6.3.5.2 Hemolysins

There were six open-reading frames encoding putative hemolysins. These genes (yhdP, yhdT, yrkA, yqhB, yplQ, ytjA) were also found in the genome of *B. subtilis* 168, which contains at least eight putative hemolysins (yhdP, yhdT, yugS, yrkA, yqhB, yqxC, yplQ, ytjA). Gene yplQ is a homolog of hemolysin-III (hlylll). The potential role of these genes in *B. subtilis* 224, which is a human isolate strain used in China, has recently been investigated for ytjA, yhdT, yugS, yplQ. These four genes were shown to impart hemolysis when cloned into non-hemolytic bacteria (Liu et al. 2007, Liu et al. 2009; Liu et al. 2010). However, knock-out of any one of these genes, e.g., yplQ, from *B subtilis* 224, did not prevent hemolysis of sheep blood, suggesting that there were alternate hemolytic genes still functional (Yu et al. 2010).

Several bacilli were evaluated for their hemolytic potential. Alpha-hemolysis (i.e., incomplete hemolysis) was observed when R0179 and natto strain ATCC 15245 were incubated with 5% sheep blood (see Table 15). The type strain of *B. subtilis* ATCC 6051 (equivalent to *B. subtilis* 168) and one of the isolates from natto culture showed beta-hemolysis (i.e., complete hemolysis). See Table 15 for results of testing of the hemolytic potential of *B. subtilis* R0179 and other *Bacillus* species. Furthermore, using PCR and gene specific primers, it was demonstrated that the gene ytjA was present in all *B. subtilis* isolates from natto. Thus, it appears that these genes are ubiquitously distributed in *B. subtilis,* including existing commercial strains, but there appear to be other regulators in the genome which suppress their hemolytic activity.

Table 15: Hemolytic potential of strains of *Bacilli*.

Strain	Type of Hemolysis Observed
B. subtilis R0179	alpha (i.e., incomplete hemolysis)
B. subtilis natto ATCC 15245	alpha
B. subtilis natto isolate #7	alpha
B. megaterium 899	alpha
B. megaterium QMB 1551	alpha
B. pumilus ATCC 7065	alpha
B. cereus R0311	beta (i.e. complete hemolysis)
B. subtilis ATCC 6051 (type strain)	beta
B. subtilis natto isolate #1	beta
B. amyloliquefaeciens ATCC 23350	beta
B. amyloliquefaeciens IT45	beta
B. megaterium ATCC 19213	beta

B. cereus R0310	beta
B. cereus ssp. toyoi R1125	beta
B. subtilis natto JCM 20036	gamma (i.e. no hemolysis)
B. thurigiensis R3025	gamma

Bacillus subtilis R0179 is weakly hemolytic (alpha-hemolysis). However, in his report, Prof. Cutting states that: "For oral use in healthy people there is no reason to consider the capacity to produce hemolysins as a safety issue. On the other hand, entry of large quantities in wound tissue, or by invasion in immunocompromised individuals, may provide an opportunity for proliferation where the secretion of hemolysins confers some virulent effect" (Cutting SM, 2021, personal communication).

Conclusion of genomic analysis

A genetic assessment of safety has been performed on the draft genome sequence by Professor Simon Cutting from the school of Biological Science at the Royal Holloway University in United Kingdom and concludes that *Bacillus subtilis* R0179 represents no major safety risk based on the genomic analysis.

6.3.6. Prophages

The genome of *B. subtilis* R0179 harbors a resident but defective prophage, PBSX, which is present in all *B. subtilis* genomes that have been sequenced to date. The genome is defective, and the prophage, if induced (i.e., excised from the host genome), fails to package its own DNA effectively (Wood et al. 1990). The PBSX genome is well characterized and carries 32 genes, all of which are found in R0179. According to Cutting (2011), although *B. subtilis* carries the prophage, it is unable to excise unless induced by Mitomycin C. *B. subtilis* lysates contain phage particles carrying host chromosomal DNA, but they are unable to transduce, that is, the DNA can not be transferred from cell to cell.

Cutting (2011) cited studies that have shown that during sporulation a unique chromosomal rearrangement occurs at an intermediate stage (about hour 2) of sporulation. A 42-kb-stretch of chromosomal DNA (known as the skin element) is excised, and this brings together two truncated genes, *spoIIIC* and *spoIVCB*. The fusion of these two genes is irreversible, and so does not occur in the germ line cell, and creates a new gene, *sigK*, that encodes a unique developmental sigma factor known as σ^K (Stragier et al. 1989) that directs the final stages of spore formation. This process is well understood, and the 42 kb of excised DNA carries many genes of PBSX origin, indicating that a prophage has at some time been adopted by the host bacterium to excise DNA and create a novel gene. This process enhances the fidelity of spore formation, with the result that the prophage is utilized by *B. subtilis* for its own benefit. In strain R0179 as well as other strains of *B. subtilis*, two clusters of PBSX genes are carried. One cluster (cluster 2 in strain R0179) is the PBSX genome and cluster 1 is the skin element that is excised and was once a resident PBSX genome (Krogh et al. 1996; Kimura et al. 2010).

Cutting (2011) concluded that PBSX, although defective and present on the genome, has long lost its ability to mobilize and is being retained by the host bacterium. The skin element has evolved to form a mobile element for a specific purpose while the PBSX genome, although still largely intact, has no function (Westers et al. 2003). Thus, the presence of the PBSX genome in *B. subtilis* strains including R0179 should be considered no more than a curiosity since it is unable to mobilize unless exposed to mitomycin C and, even if so, it cannot transduce.

6.3.7. Bioavailability of *Bacillus subtilis* R0179

6.3.7.1. Acid Stability

Bacillus subtilis R0179 is resistant in acidic conditions. Strain R0179 comprising 20% vegetative cells and 80% spores was resistant to pH as low as 2, as shown in Table 16. The survival at pH 3 is reduced because the strain contains an acid protease which is more active on the spores at this pH. One potential advantage with this strain is that the spores can survive transit through the stomach and reach the gut intact, as suggested in the literature (Spinosa et al. 2000).

Table 16: Percentage of survival of *Bacillus subtilis* R0179 strain at acidic pH for 30 minutes.

Rosell Strain	pH 4	pH 3	pH 2
Bacillus subtilis R0179	99%	26%	78%

6.3.7.2. Bile Stability

The bile exerts a moderate inhibitory effect on *Bacillus subtilis* R0179. This was tested by inoculating a tube of 0.3% bacto-oxgall dehydrated fresh bile (Difco Laboratories), incubating at 37°C, and comparing the growth with a negative control containing no bile. There was 30% less growth of R0179 vegetative cells observed in the sample inoculated with bile vs. the negative control. Here again, however, spores can survive transit through the stomach and reach the gut unaltered as reported in the literature (Casula and Cutting 2002).

6.4. Human Studies

6.4.1. Studies in Adults

6.4.1.1 Studies with *Bacillus Subtilis* R0179

B. subtilis R0179 has been marketed in multiple countries, mainly in Asia (China, Korea), as part of a two-strain product, Medilac. This product has many clinical trials showing safety at levels up to $3.0x10^8$ cfu/day. In addition to these trials on the two-strain supplement containing *B. subtilis* R1079, a safety trial was performed in 2012 and published in 2015 where the single strain was evaluated.

A randomised, double-blind, placebo-controlled study, Hanifi et al. (2015), was conducted to determine the safety status of the strain in higher concentrations. Previous studies, described below, were conducted at levels not higher than $3.0 x10^8$ cfu/day. This study used dosages of 0.1, 1.0, and $10x10^9$ cfu/day of *B. subtilis* R0179 and one placebo group split evenly between 81 healthy subjects, 18-50 years old.

The study included a 4-week intervention period after an initial baseline measurement, with a measurement at the end of the intervention period, and the subjects underwent a 1-week washout period with a final measurement visit at the end of the washout period. Measurements included a daily questionnaire, a weekly Gastrointestinal Symptom Rating Scale (GSRS) questionnaire at baseline, week 4, and post washout, *B. subtilis* quantification in stool samples at baseline, week 4 of intervention, and post washout. The daily questionnaire included GI, cephalic, ear-nose-throat, behavioral, emetic, and epidermal criteria, asking the participants to grade themselves on a 7-point scale, with additional questions to determine compliance. The GI transit of the bacteria was assessed via 16S rRNA amplification and sequencing from the stool samples provided at various points.

81 total participants completed the 6-week study. One participant voluntarily withdrew, and 1 participant was removed from the $10x10^9$ cfu/day group due to non-compliance. One participant in the $0.1x10^9$ cfu/day group was hospitalised, but the reason (hypertension) was determined by the investigators to be unrelated to the study. The participant continued to participate in the intervention and completed the study. The placebo group contained 20 participants, the $0.1x10^9$ cfu/day group had 21 participants, and the $1x10^9$ cfu/day and $10x10^9$ cfu/day groups each had 20 participants.

All groups had similar results in relation to the GSRS and the daily questionnaire. However, the fecal count levels of *B. subtilis* R0179 were significantly different among the groups, showing a dose-dependent result consistent with the differences in the concentrations between different intervention groups. The measurements were presented on a logarithmic scale. The placebo group's results were 1.1 ± 0.1 $\log_{10}$ cfu/g of feces, which were significantly different when compared to 4.6 ± 0.1 $\log_{10}$ cfu/g for the $0.1x10^9$ cfu group, 5.6 ± 0.1 $\log_{10}$ cfu/g for the $1x\ 10^9$ cfu group, and 6.4 ± 0.1 $\log_{10}$ cfu/g for the $10\ x10^9$ cfu group.

After a week of washout, there was significant diminution in the *B. subtilis* R0179 levels detected in fecal samples, with results from the $1x10^9$ cfu group at 0.8 ± 0.1 $\log_{10}$ cfu/g and the $10x10^9$ cfu group at 2.1 ± 0.1 $\log_{10}$ cfu/g. Hanifi et al. (2015) reported that this demonstrates that the bacteria do not persist in the

gastrointestinal tract for a significant time and concluded that the daily consumption of *B. subtilis* R0179 is safe and well tolerated in healthy individuals up to $10x10^9$ cfu/day (= $1.0x10^{10}$ cfu/day).

6.4.1.2 Studies of other formulations containing *Bacillus subtilis* R0179

A product containing *B. subtilis* R0179 has been on the market in Korea since 1985 and in China since 1994. This strain and *E. faecium* R0026 are found in a capsule format at a ratio of 1:9. Two preparations are available, Medilac-S, containing $5x10^8$ cfu per capsule and Medilac-DS (i.e., double-strength) containing $1x10^{10}$ cfu/capsule, thus they contain $5x10^7$ and $1x10^8$ cfu of *B. subtilis* R0179 per capsule, respectively. Both the safety and efficacy of these preparations have been extensively studied in patients suffering from a variety of ailments, and a post-market review of 27 published studies in adults with these products has been published (Tompkins et al. 2010). These studies and 11 new studies published since this review are summarized in Table 17.

Of the 38 published studies summarized in the table, the administered dose of bacteria was $3.0x10^9$ cfu/day while that of *B. subtilis* R0179 was $3.0x10^8$ cfu/day in 29 trials and half those levels in the other 9 trials. All trial participants suffered from medical conditions, including acute or chronic diarrhea, acute pancreatitis, irritable bowel syndrome, constipation, ulcerative colitis, peptic ulcers and *H. pylori* infections, cirrhosis, and antibiotic-associated diarrhea. The most frequent duration was 2 weeks (13 studies), followed by 4 weeks (10 studies) and 12 weeks (5 studies). In all, 3347 patients completed these trials, including 1948 patients who received the treatment.

Overall, the studies show that the product containing *B. subtilis* R1079 and *E. faecium* R0026 is safe, with no reports of serious adverse events (Tompkins et al. 2010). All minor adverse events such as nausea, vomiting, bloating, abdominal cramps, headaches, and dizziness were also common in the control groups. There was no significant difference in the occurrence of adverse events except for nausea and bloating, which occurred significantly less frequently in the experimental group than the control group (Lee et al. 2009). No changes in blood parameters were reported in any of the studies. There were two cases of urinary tract infections which were not attributed to the bacteria (Tompkins et al. 2010). The absence of any reports of adverse events attributable to the administration of $3.0x10^8$ cfu *B. subtilis* R0179/day to these severely compromised and vulnerable individuals provides strong evidence of the safety of this strain for its intended use.

Table 17. Clinical Studies in Adults (Tompkins *et al.* 2010)

Study ID	Start and end dates	Design Control type	Microorganism and control; route	Dose, regime, and duration.	Total enrolment	Control gender M/F Median age (Range)	Treated gender M/F Median age (Range)	Primary objective(s)	Averse events
Wang et al. 2004	Not stated	Randomized, parallel, blind, with control. Multi-center. Intention to treat analysis	Medilac-S vs. Pei Fei Kang; orally	2 capsules t.i.d. ($3.0x10^9$ cfu bacteria/day; $3.0x10^8$ cfu R0179/day); acute: 5 days Chronic: 2 weeks	95 patients with acute diarrhea (48 treated + 47 control) and 48 patients with chronic diarrhea (24 treated + 24 control)	All groups combined: 99 M / 44 F; 39.5 yrs (18-65 yrs)		Compare safety and efficacy of bacteria in the treatment of acute and chronic diarrhea	1 case of nausea, 1 of headache, 1 of dizziness. 1 of palpitation and 3 cases of urinary infection (determined as not linked with the use of Medilac S)
Zhao et al. 2004 and 2005	Not stated	Randomized, with healthy control but not double-blind	Medilac-S or Bifico; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; $3.0x10^8$ cfu R0179/day); 2 weeks	50 liver cirrhosis patients (25 treated with Medilac-S and 25 treated with Bifico) and 20 healthy volunteers	10 M / 10 F; 49.8 yrs (25-70 yrs)	32 M / 18 F; 50.5 yrs (14-75 yrs)	Normalization of intestinal microflora and blood parameters in patients with liver cirrhosis	Not stated
Kim et al. 2006	Mar. 2005 to Nov. 2005	Randomized, double-blind, placebo-controlled	Medilac-DS, or placebo, orally	1 capsule, t.i.d. (3.0×10^9 cfu bacteria/day; $3.0x10^8$ cfu R0179/day); 4 weeks	40 IBS patients but 6 lost in follow-up (17 Medilac DS + 17 placebo)	14 M / 3 F; 39.6±13.2 yrs (18-70 yrs)	11 M / 6 F; 39.1±10.9 yrs (18-70 yrs)	Improvement in clinical symptoms and change in intestinal gas volume in IBS patients	No intervention-related adverse reactions were reported
Li et al. 2006	Jan. 2004 to Nov. 2004	Randomized with active control but not double-blind	Medilac-S, and mesalazine vs. mesalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; $3.0x10^8$ cfu R0179/day); 12 weeks	50 patients with mild to moderate ulcerative colitis (25 with Medilac-S and mesalazine; 25 mesalazine only)	18 M / 7 F; 38±6 yrs)	20 M / 5 F; 40±9 yrs	Improvement of mesalazine treatment of ulcerative colitis	Not stated

Su et al. 2006	Jan. 2005 to Jun. 2005	Randomized with active control but not double-blind	Medilac-S and glutamine; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 2 weeks	80 patients with diarrhea-predominant IBS (40 glutamine + 40 bacteria)	I 25 M / 15 F; 38.5±10.3 yrs	22 M / 18 F; 41.6±13.5 yrs	Evaluate efficacy of glutamine in IBS-D	No intervention-related adverse reactions were reported
Wang and Jin 2006	Jun. 2004 to Jun. 2006	Uncontrolled and not double-blind	Medilac-S; orally	1 capsule t.i.d. (1.5×10^9 cfu bacteria/day; 1.5×10^8 cfu R0179/day); 2 weeks	34 patients with diarrhea (21 due to antibiotic therapy and 13 due to chemotherapy)	All groups combined: 25 M / 9 F; 43±28 yrs (26-68 yrs)		Efficacy of Medilac-S in the treatment of diarrhea caused by an intestinal flora imbalance	No intervention-related adverse reactions were reported
Xiang and Feng 2006	2003	Randomized with active control but not double-blind.	Medilac-S, and sulfasalazine vs. sulfasalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 4 weeks	46 patients with mild to moderate ulcerative colitis (24 with Medilac-S and sulfasalazine; 22 sulfasalazine only)	All groups combined: 21 M / 25 F; 46.5±10.5 yrs; no statistical difference between groups at start		Efficacy of sulfasalazine treatment of ulcerative colitis	1 case of nausea
Zhang 2006	Not stated	Randomized with active control but not double-blind	Oral Medilac-S with herbal enema vs. herbal enema only	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 2 weeks	60 patients with ulcerative colitis (30 per arm)	17 M / 13 F; (43-75 yrs)	19 M / 11 F; (41-72 yrs)	Potential of Medilac-S combined with coloclysis in the treatment of ulcerative colitis	No intervention-related adverse reactions were reported
Chen 2007	2004	Randomized with active control but not double-blind	Medilac-S, and sulfasalazine vs. sulfasalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 12 weeks	47 patients with mild to moderate ulcerative colitis (24 with Medilac-S and sulfasalazine; 23 sulfasalazine only)	All groups combined: 19 M / 28 F; 46 yrs (17-61 yrs); no statistical difference between groups at start		Efficacy of sulfasalazine treatment of ulcerative colitis	2 cases of nausea and fatigue
Chen and Zhu 2007	Ended Jan. 2006	Randomized but not double-blind	Medilac-S vs. two other bacterial compounds; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 5 days	123 patients with acute diarrhea (51 with Medilac-S, 51 with Pei Fei Kang, and 51 with *Bifidobacterium*)	Comparison group I: 24 M / 27 F; 40±9 yrs Comparison group II: 26 M / 25 F; 39±11 yrs (20-55 yrs)	25 M /26 F; 39±10 yrs (20-55 yrs)	Compare pharmaco-economics of three products in the treatment of acute diarrhea	Not stated

Feng et al. 2007	Oct. 2006 to Jan. 2007	Randomized but not double-blind	Medilac-S and Tegaserod; orally	Medilac-S: 2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day) and Tegaserod: 6 mg, b.i.d.; 8 weeks	40 patients with IBS-C (20 receiving medication *on-demand*, and 20 *systematic)*	8 M/ 12 F; 39±13 yrs (18-65 yrs)	7 M/ 13 F; 34±15 yrs (20-60 yrs)	Efficacy of co-administrating Tegaserod and Medilac-S on IBS-C	Not stated
Guo and Sun 2007	Jan. 2005 to Dec. 2006	Uncontrolled and not double-blind	Oral Medilac-S with medicated retentive enema	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 2 weeks	66 patients with ulcerative colitis	42 M / 24 F; (20-62 yrs)		Potential of Medilac-S combined with retentive enema in the treatment of ulcerative colitis	Not stated
Gong and Xu 2007	May 2005 to Aug. 2006	Randomized with active control but not double-blind	Medilac-S, Pei Fei Kang and mesalazine vs. mesalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 4 weeks	56 patients with ulcerative colitis (29 in bacteria with mesalazine; 27 in mesalazine control group)	All groups combined: 26 M / 30 F; (25-52 yrs); no statistical difference between groups at start		Improvement of mesalazine treatment of ulcerative colitis	Not stated
Li et al. 2007	Not stated	Randomized but not double-blind	Medilac-S, Pei Fei Kang and smectite; orally vs. rectally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 5 days	68 patients with antibiotic-associated diarrhea (34 orally, 34 by retentive enema)	All groups combined: 41 M / 27 F; 49 yrs (22-88 yrs)		Compare the efficacy of bacteria given by retentive enema to *per os* treatment	Not stated
Lin 2007	Feb. 2006 to Jun. 2007	Uncontrolled and not double-blind	Medilac-S; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 4 weeks	47 patients with chronic diarrhea (42 with chronic colitis, 5 with ulcerative colitis)	No control	16 M / 31 F; 65.2 yrs (46-81 yrs)	Potential use of Medilac-S for treatment of chronic diarrhea	Not stated
Lu and Dong 2007	Jan. 2005 to Oct. 2006	Randomized, single blind with active control	Medilac-S with Smectite vs. Medilac-S only vs. Smectite only, orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 1 week	60 IBS-D patients (21 treated with Smecta and Medilac-S + 18 treated with Smecta only + 21 treated with Medilac-S only)	All groups combined: 24 M / 36 F; 27±6.3 yrs (18-63 yrs)		Evaluate the efficacy of smectite and bacterial co-therapy in IBS-D	Not stated

Park et al. 2007	Jun. 2002 to Dec. 2004	Randomized (described), single blind with active control	Conventional triple therapy vs. same with Medilac-S, orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 8 weeks	352 patients infected with *H. pylori* (176 triple therapy only + 176 triple therapy and Medilac)	95 M / 81 F; 47.6±18.5 yrs (20-64 yrs)	96 M /80 F; 45.2±19.8 yrs (21-67 yrs)	Improve *H. pylori* eradication and decrease side effects of conventional triple therapy	No intervention-related adverse reactions were reported
Wang and Lui 2007	Oct. 2004 to Jul. 2006	Randomized with active control but not double-blind	Medilac-S, and sulfasalazine vs. sulfasalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 4 weeks	36 patients with mild to moderate colitis (20 with Medilac-S and sulfasalazine; 16 sulfasalazine only)	All groups combined: 23 M / 13 F; 38.9±7.8 yrs (21-56 yrs); no statistical difference between groups at start		Efficacy of sulfasalazine treatment of UC	No intervention-related adverse reactions were reported
Chen 2008	Not stated	Randomized with active control	Medilac-S or Deanxit, or Medilac-S and Deanxit; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 4 weeks	144 patients diagnosed with IBS (48 with Medilac-S, 46 with Deanxit and 50 with combined treatment)	All groups combined: 68 M / 76 F; 22-73 yrs		Efficacy of Medilac-S in combination with Deanxit in the treatment of IBS	No intervention-related adverse reactions were reported
He and He 2008	Nov. 2005 to Oct. 2006	Randomized with non-active control but not double-blind	Medilac-S; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 2 weeks	198 patients diagnosed with chronic diarrhea (112 with Medilac-S, 86 as control)	86 patients; not detailed	38 M / 74 F; 35.6 yrs (16-82 yrs)	Efficacy of Medilac-S treatment of chronic diarrhea	No intervention-related adverse reactions were reported
Huang et al. 2008	Not stated	Uncontrolled	Medilac-S; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 10 days	45 constipated patients	No control	10 M / 35 F; 33.26 yrs ± 2.3 (23-70 yrs)	Efficacy of Medilac-S treatment of constipation during colon hydrotherapy treatment	Not stated
Li et al. 2008	Not stated	Case study, no control	Medilac-S; orally	1 capsule twice a day (1.0×10^9 cfu bacteria/day; 10^8 cfu R0179/day); 2 weeks	65 aged patients with chronic diarrhea		39 M / 26 F; 60-78 yrs	Observe therapeutic effects of the combination of Yunnan Baiyao applied on navel with Medilac-S in treatment of aged patients with chronic diarrhea	No intervention-related adverse reactions were reported

Wang 2008	Not stated	Randomized with active control but not double-blind	Medilac-S and smectite vs. smectite; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 2 weeks	100 patients diagnosed with chronic diarrhea (50 Medilac-S + smectite, 50 smectite control)	Not well defined; (16-60 yrs)		Efficacy of Medilac-s combined with Smecta in the treatment of chronic diarrhea	No intervention-related adverse reactions were reported
Wang and Wang 2008	Not stated	Randomized with active control	Classical treatment or classical treatment + Medilac-S; orally	1 capsule t.i.d. (1.5×10^9 cfu bacteria/day; 1.5×10^8 cfu R0179/day); 4 weeks	60 patients with cirrhosis and spontaneous peritoneal inflammation	All groups combined: 48 M / 12 F; 51.4 ± 5.8 yrs (26-78 yrs)		Efficacy of Medilac-s the treatment of liver cirrhosis	Not stated
Yang et al. 2008	Not stated Not stated	Randomized with active control but not double-blind	Sulfasalazine alone; orally; Medilac-S + SASP; orally; or SASP + Medilac S; retention enema	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 4 weeks	78 patients with mild to moderate ulcerative colitis (26 with Medilac S and Sulfasalazine, orally; 26 with Medilac S and Sulfasalazine, rectally; 26 with Sulfasalazine only)	All groups combined: 41 M / 37 F; (19-57 yrs)		Efficacy comparison of 3 treatments in the treatment of ulcerative colitis	Not stated
Yang et al. 2008	Not stated Not stated	Randomized with active control	Medilac-S or Norfloxacin	1 capsule t.i.d. (1.5×10^9 cfu bacteria/day; 1.5×10^8 cfu R0179/day); 1 week	60 patients with acute diarrhea divided in 2 groups of 30	7 M / 23 F; Age not detailed	12 M / 18 F; Age not detailed	Efficacy of Medilac-S treatment of acute diarrhea	Not stated
Yuan et al. 2008	Jun. 2005 to Dec. 2007	Randomized with active control but not double-blind	Medilac-S, and sulfasalazine vs. sulfasalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 12 weeks	40 patients with mild to moderate ulcerative colitis (20 with Medilac-S and sulfasalazine; 20 sulfasalazine only)	9 M / 11 F; 43±11 yrs	12 M / 8 F; 40±9 yrs	Efficacy of sulfasalazine treatment of ulcerative colitis	No intervention-related adverse reactions were reported
Zeng 2008	Jan. 2006 to Mar. 2007	Randomized with active control but not double-blind	Medilac-S, and sulfasalazine vs. sulfasalazine only; orally	2 capsules t.i.d. (3.0×10^9 cfu bacteria/day; 3.0×10^8 cfu R0179/day); 12 weeks	49 patients with mild to moderate ulcerative colitis (25 with Medilac-S and sulfasalazine; 24 sulfasalazine only)	16 M / 8 F; 39±6 yrs	15 M / 10 F; 40±9 yrs	Efficacy of sulfasalazine treatment of ulcerative colitis	Not stated