Final Screening Assessment for

***Bacillus amyloliquefaciens* 13563-0**
***Bacillus atrophaeus* 18250-7**
***Bacillus licheniformis* ATCC 12713**
***Bacillus subtilis* ATCC 6051A (=ATCC 6051a)**
***Bacillus subtilis* ATCC 55405**
***Bacillus subtilis* subspecies *subtilis* ATCC 6051**
***Bacillus subtilis* subspecies *inaquosorum* ATCC 55406**
***Bacillus* species 16970-5**
***Bacillus* species 2 18118-1**
***Bacillus* species 4 18121-4**
***Bacillus* species 7 18129-3**

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Synopsis

Pursuant to paragraph 74*(b)* of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Minister of the Environment and the Minister of Health have conducted a screening assessment of the following living organism strains that are listed on the DSL:

- *Bacillus amyloliquefaciens* 13563-0
- *Bacillus atrophaeus* 18250-7
- *Bacillus licheniformis* ATCC 12713
- *Bacillus subtilis* ATCC 6051A (also referred to as *Bacillus subtilis* ATCC 6051a)
- *Bacillus subtilis* ATCC 55405
- *Bacillus subtilis* subspecies *subtilis* ATCC 6051 (= type strain)
- *Bacillus subtilis* subspecies *inaquosorum* ATCC 55406
- *Bacillus* species 16970-5
- *Bacillus* species 2 18118-1
- *Bacillus* species 4 18121-4
- *Bacillus* species 7 18129-3

For the purposes of this assessment, the DSL micro-organisms listed above will collectively be referred to as the 'DSL *Bacillus licheniformis/subtilis* group' (*B. licheniformis/subtilis* group). The term '*Bacillus subtilis* complex' will denote information that is not specific to these DSL strains, but relates to the broader group of species that includes the DSL strains.

Under the *Masked Name Regulations* pursuant to section 113 of CEPA 1999, Environment Canada assigned masked names and accession numbers to *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 in place of these organisms' explicit biological names, which are considered confidential and must not be publicly disclosed.

Members of the broader *Bacillus Subtilis* (*B. subtilis*) complex have the ability to adapt to and thrive in many terrestrial and aquatic habitats. They may be contaminants in food and aviation fuel and transient members of the bowel microflora. Some members of the *B. subtilis* complex are used in the fermentation of foods. They form endospores that permit survival in sub-optimal environmental conditions. Numerous physiological variants exist in nature, indicating that members of this complex establish successfully in nearly every environment. Various characteristics of the DSL *B. licheniformis/subtilis* group make them suitable for use as active ingredients in commercial and consumer products.

Certain strains of *Bacillus licheniformis* (*B. licheniformis*) can cause bovine, porcine and ovine abortion as well as mastitis in cattle, but the overall impact of *B. licheniformis* disease in livestock is low. Members of the DSL *B. licheniformis/subtilis* group are susceptible to veterinary antibiotics so that in the case of livestock infection, effective treatment options are available. Negative effects in aquatic and terrestrial invertebrates exposed to strains of *B. subtilis* and *B. licheniformis* have been reported. One report implicated an isolate of *B. licheniformis* as the causative agent of pistachio dieback. *B. subtilis* complex strains also have antimicrobial properties, and can promote growth in both plants and animals.

Certain members of the *B. subtilis* complex are occasionally reported to cause disease in susceptible humans, including those with debilitating disease or compromised immunity, young infants and the elderly, but do so rarely in the general population. Some produce extracellular enzymes and toxins that could cause food poisoning. In laboratory analyses done by scientists at Health Canada, the DSL *B. licheniformis/subtilis* group strains did not produce these food poisoning toxins.

This assessment considers the aforementioned characteristics of these strains with respect to environmental and human health effects associated with product use and industrial processes subject to CEPA 1999, including releases to the environment through waste streams and incidental human exposure through environmental media. To update information about current uses, the Government launched a mandatory information-gathering survey under section 71 of CEPA 1999, as published in the *Canada Gazette*, Part I, on October 3, 2009 (section 71 notice). Information submitted in response to the section 71 notice indicates that the DSL *B. licheniformis/subtilis* group strains were used in biodegradation and bioremediation; products for surface and drain cleaning, degreasing and deodorizing; enzyme and chemical production; waste and wastewater treatment.

Considering all available lines of evidence presented in the Screening Assessment, there is low risk of harm to organisms and the broader integrity of the environment from the DSL *B. licheniformis/subtilis* group strains. It is concluded that *Bacillus amyloliquefaciens* 13563-0, *Bacillus atrophaeus* 18250-7, *Bacillus licheniformis* ATCC 12713, *Bacillus subtilis* ATCC 6051A, *Bacillus subtilis* ATCC 55405, *Bacillus subtilis* subsp. *subtilis* ATCC 6051, *Bacillus subtilis* subsp. *inaquosorum* ATCC 55406, *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 or *Bacillus* species 7 18129-3 do not meet the criteria under paragraphs 64(*a*) or (*b*) of CEPA 1999, as they are not entering

the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity or that constitute or may constitute a danger to the environment on which life depends.

Also, based on the information presented in the Screening Assessment, it is concluded that *Bacillus amyloliquefaciens* 13563-0, *Bacillus atrophaeus* 18250-7, *Bacillus licheniformis* ATCC 12713, *Bacillus subtilis* ATCC 6051A, *Bacillus subtilis* ATCC 55405, *Bacillus subtilis* subsp. *subtilis* ATCC 6051, *Bacillus subtilis* subsp. *inaquosorum* ATCC 55406, *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 or *Bacillus* species 7 18129-3 do not meet the criteria under paragraph 64*(c)* of CEPA 1999, as they are not entering the environment in a quantity or concentration or under conditions that constitute or may constitute a danger in Canada to human life or health.

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Introduction

Pursuant to paragraph 74(b) of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Minister of the Environment and the Minister of Health are required to conduct screening assessments of living organisms listed on the DSL that were in commerce between 1984 and 1986, to determine whether they present or may present a risk to the environment or human health (according to criteria as set out in section 64 of CEPA 1999)[1]. These strains were added to the DSL under section 105(1) of CEPA 1999 because they were manufactured in or imported into Canada between January 1, 1984, and December 31, 1986 and they entered or were released into the environment without being subject to conditions under CEPA 1999 or any other federal or provincial legislation.

This Screening Assessment considers hazard information obtained from the public domain as well as from unpublished research data and comments from researchers in related fields. Exposure information was obtained from the public domain and from a mandatory CEPA 1999 section 71 Notice published in the *Canada Gazette*, Part I, on October 3, 2009. Further details on the risk assessment methodology used are available in the Risk Assessment Framework document "Framework on the Science-Based Risk Assessment of Micro-organisms under the *Canadian Environmental Protection Act, 1999*" (Environment Canada and Health Canada 2011).

In this report, data that are specific to the DSL *Bacillus licheniformis/subtilis* group strains are identified as such and includes information from the Nominators, the American Type Culture Collection (ATCC), and unpublished data generated by Environment Canada[2] and Health Canada[3] research scientists. Where strain-specific data were not available, surrogate information from literature searches was used. When applicable, literature searches conducted on the organism included its synonyms, and common and superseded names. Surrogate organisms are identified in each case to the taxonomic level provided by the source. Literature searches were conducted using scientific literature databases (SCOPUS, CAB Abstracts and Google Scholar), web searches, and key search terms for the identification of human health and environmental hazards of each of the DSL strains assessed in this report. Information identified as of May 2014 was considered for inclusion in this report.

[1] A determination of whether one or more of the criteria of section 64 of CEPA 1999 are met is based upon an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA 1999, on DSL *Bacillus licheniformis/subtilis* group strains, is not relevant to, nor does it preclude, an assessment against the hazard criteria for Workplace Hazardous Materials Information System (WHMIS) that are specified in the Controlled Products Regulations for products intended for workplace use.
[2] Testing conducted by Environment Canada's Biological Methods Division
[3] Testing conducted by Health Canada's Environmental Health Science and Research Bureau

Decisions from Domestic and International Jurisdictions

Domestic

The members of the DSL *B. licheniformis/subtilis* group are recognized as Risk Group 1 micro-organisms by the Public Health Agency of Canada (PHAC) and by the Canadian Food Inspection Agency (CFIA).

Strains of *B. subtilis* have been approved in Canada for the production of enzymes used in food. Fermentation extracts from strains of *B. subtilis* are accepted as a feed ingredient under the Feeds Regulations, as long as they are free from antimicrobial activity and are not a source of viable microbial cells. The DSL *B. licheniformis/subtilis* strains have not been approved for use on the Canadian market under this Act at this time. The CFIA Fertilizer Safety Office conducted a comprehensive safety assessment of *B. subtilis* and exempted all strains from full safety data requirements (CFIA 2014). Strains isolated from the natural environment must be identified and distinguished to the strain level.

The Pest Management Regulatory Agency of Health Canada (PMRA-HC) has approved several other strains of the *B. subtilis* complex for use as biocontrol agents including *B. subtilis* var. *amyloliquefaciens* strain FZB24 (2011) (PMRA-HC 2012), *B. subtilis* strain MBI 600 (2005) (PMRA-HC 2007a; PMRA-HC 2007c), *B. subtilis* strain QST 713 (2006) (PMRA-HC 2007b) and *B. subtilis* strain GB03 (2011) (PMRA-HC 2013). An evaluation for each microbial pest control agent and end-use product determined that they did not present an unacceptable risk to human health or the environment.

International

The United States Environmental Protection Agency (U.S. EPA) assessed several strains of *B. subtilis* and *B. licheniformis* used in the production of enzymes. It was concluded that no unreasonable risks to human health or the environment were associated with the use of these strains for the production of enzymes, antibiotics or other specialty chemicals. The Unites States Food and Drug Administration (U.S. FDA) recognizes enzymes produced by *B. subtilis* to be generally recognized as safe (GRAS) for use in food. The U.S. EPA approved many of the same biocontrol agents registered by PMRA-HC. In addition to the strains approved for use as biofungicides in Canada, the U.S. EPA has also approved B. amyloliquefaciens strain D747 (U.S. EPA 2011) and *B. licheniformis* strain SB3086 (U.S. EPA 2001).

In Australia, modified *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* were approved for use in enzyme production (ANZ 2012a; ANZ 2012b; ANZ 2013). *B. subtilis* PB6 has been applied in poultry to reduce clostridial isolates (*C. difficile* and *C. perfringens*) (ANZ 2011). Other strains are being considered for biocontrol purposes.

1. Hazard Assessment

1.1 Characterisation of the DSL strains under assessment

1.1.1 Taxonomy, identification and strain history

Taxonomic designation:

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: *Bacillaceae*
Genus: *Bacillus*
Species: *Bacillus amyloliquefaciens* 13563-0
Bacillus atrophaeus 18250-7
Bacillus licheniformis ATCC 12713
Bacillus subtilis ATCC 6051A
Bacillus subtilis ATCC 55405
Bacillus subtilis subsp. *subtilis* ATCC 6051 (=type strain)
Bacillus subtilis subsp. *inaquosorum* ATCC 55406
Bacillus species 16970-5
Bacillus species 2 18118-1
Bacillus species 4 18121-4
Bacillus species 7 18129-3

Eleven strains of the '*Bacillus subtilis* species complex' that are listed on the DSL are the subject of this assessment. They will be assessed collectively as the 'DSL *Bacillus licheniformis/subtilis* group' in this report. The term '*Bacillus subtilis* complex' and the grouping of these species are supported in the literature and include the DSL strains (Sorokulova et al. 2008; De Jonghe et al. 2010). This term will be used when surrogate information is discussed. As indicated above, the names of several of the DSL *B. licheniformis/subtilis* group strains have been masked to the genus level at the request of the nominators, pursuant to the Masked Name Regulations of CEPA 1999, and may not be disclosed.

Synonyms for species of the DSL *B. licheniformis/subtilis* group were obtained from the 'List of Prokaryotic Names with Standing in the Nomenclature' (Euzéby 2013), the 'NCBI taxonomy browser' (Benson et al. 2009; Sayers et al. 2009) and the 'Catalogue of Life' (Shimura et al. 2013) unless otherwise indicated (Table 1-1).

Table 1-1: Synonyms of micro-organisms in the *B. subtilis* complex

Current Nomenclature	Synonyms
B. amyloliquefaciens • subspecies *amyloliquefaciens*[a] • subspecies *plantarum*[a]	*Bacillus amyloliquifaciens*[b] *Bacillus subtilis* var. *amyloliquefaciens*[c] *Bacillus velezensis*[d]

Current Nomenclature	Synonyms
B. atrophaeus	*Bacillus subtilis* var. *niger* *Bacillus globigii* *Bacillus niger*
B. licheniformis	*Denitrobacillus licheniformis* *Clostridium licheniforme*
B. subtilis • subsecies *inaquosorum*[e] • subspecies *spizizenii* • subspecies *subtilis* • subspecies *virginiana*[f]	*Vibrio subtilis* *Bacillus globigii* *Bacillus uniflagellatus* *Bacillus natto*

[a] (Borris et al. 2011)
[b] (Priest et al. 1987)
[c] (PMRA-HC, 2012)
[d] Later heterotypic synonym of *B. amyloliquefaciens* (Wang et al. 2008)
[e] Some strains were reclassified from *B. licheniformis* (Rooney et al. 2009)
[f] (Zhao et al. 2011)

1.1.1.1 Phenotypic identification and biochemical profile

Bacillus species are Gram positive but stain variably, with some species staining clearly Gram positive in young cultures only. They have rod-shaped cells with rounded or squared ends ranging from 0.5 × 1.2 to 2.5 × 10 µm in size, occurring singly or in chains, and the stability of these chains determines the form of the colony, which may vary from strain to strain (Logan and De Vos 2009; Rooney et al. 2009). While most species within the genus are aerobic some are facultatively or strictly anaerobic (Logan and De Vos 2009; Murray et al. 1995). *Bacillus* species are capable of forming spores that may be cylindrical, oval, round, or kidney-shaped, placed centrally, terminally or subterminally, none of which swell the sporangium (Murray et al. 1995).

Members of the *B. subtilis* complex can be differentiated from known human and animal pathogens of the *B. cereus* group (*B. anthracis*, *B. cereus* and the insect pathogen *B. thuringiensis*) by both morphological and biochemical means. Members of the *B. subtilis* complex have cell diameters which measure less than 1 µm whereas members of the *B. cereus* group have cell diameters which are greater than 1 µm (Logan and De Vos 2009). Biochemical profiles can be used to differentiate between members of the *B. subtilis* complex and the *B. cereus* group; select distinguishing features are provided in Table 1-2 (Santini et al. 1995).

Table 1-2: Biochemical characteristics of *B. cereus* group species compared with *B. subtilis* complex species

***Bacillus* species**[a]	**D-xylose**	**Mannose**	**Inositol**	**Mannitol**	**ONPG**[b]
B. anthracis	-[c]	-	-	-	-
B. cereus	-	-	-	-	-
B. thuringiensis	-[d]	-[e]	N/A[f]	-[d]	N/A
B. amyloliquefaciens	N/A[g]	+[h]	N/A	+[g]	N/A
B. atrophaeus	+[h]	+[h]	N/A	+[h]	N/A
B. licheniformis	+[I]	+	+	+	+
B. subtilis	+[J]	+	+	+	+

[a] (Santini et al. 1995)

[b] *o*-nitrophenyl-β-D-galactopyranoside
[c] -, ≤19% positive reactions
[d] (Fakruddin et al. 2012)
[e] (Swiecicka et al. 2002)
[f] N/A, Not Available
[g] (Borriss et al. 2011)
[h] (Nakamura, 1989)
[i] +, ≥ 81% positive reactions
[j] 20-80% positive reactions

1.1.1.2 Molecular identification

The genus *Bacillus* is large, consisting of 11 phylogenetic subclusters and over 140 species (Logan and De Vos 2009). Using 16S rRNA gene sequencing analysis, the *B. subtilis* complex can be differentiated from the *B. cereus* group due to the presence of a *Hinfl* restriction site between the V4 and V5 region in the *B. subtilis* complex (Jeyaram et al. 2011). Figure 1-1 describes the phylogenetic relationships of *Bacillus* species and closely related genera based on the alignment of 16S ribosomal RNA gene sequences generated by Health Canada scientists and publicly available sequences. This figure clearly demonstrates that species of the *B. subtilis* complex cluster together and apart from known pathogens of the *Bacillus* genus, particularly those of the *B. cereus* group.

The identity of the DSL *B. licheniformis/subtilis* group strains was independently verified by Health Canada scientists. Colony morphologies (Appendix 1) were consistent with descriptions in the literature. For example, *B. atrophaeus*, unlike other group members, forms a black pigment when grown on media containing tyrosine or other organic nitrogen sources (Logan and De Vos 2009; Rooney et al. 2009). The ability of *B. atrophaeus* strain 18250-7 to produce dark pigments was confirmed.

At Health Canada laboratories, the identification of most DSL *B. licheniformis*/*subtilis* group strains, including those that are masked at the genus level, was confirmed by 16S ribosomal RNA gene sequence, fatty acid methyl ester (FAME) analyses and total cellular content of select fatty acids to be correctly identified (Appendices 2 to 4). *B. subtilis* is difficult to distinguish from closely related *Bacillus* species, particularly *B. amyloliquefaciens* (Ash et al. 1991; Logan and De Vos 2009); however, *B. amyloliquefaciens* carries distinctive differences in the 16S ribosomal RNA gene sequence: the absence of two *Rsa*I restriction sites in the V3 region that differentiates it from *B. subtilis* (Jeyaram et al. 2011). The lack of *Rsa*I sites is characteristic of *B. amyloliquefaciens* and was observed in the ribosomal RNA gene sequence of *B. subtilis* ATCC 55405. Other methods used also demonstrated that *B. subtilis* ATCC 55405 is more similar to *B. amyloliquefaciens* than *B. subtilis*, suggesting that it may be misidentified. The *Cfol* restriction site, between the V4 and V5 regions, can be used to differentiate between *B. subtilis* and *B. licheniformis* (Jeyaram et al. 2011).

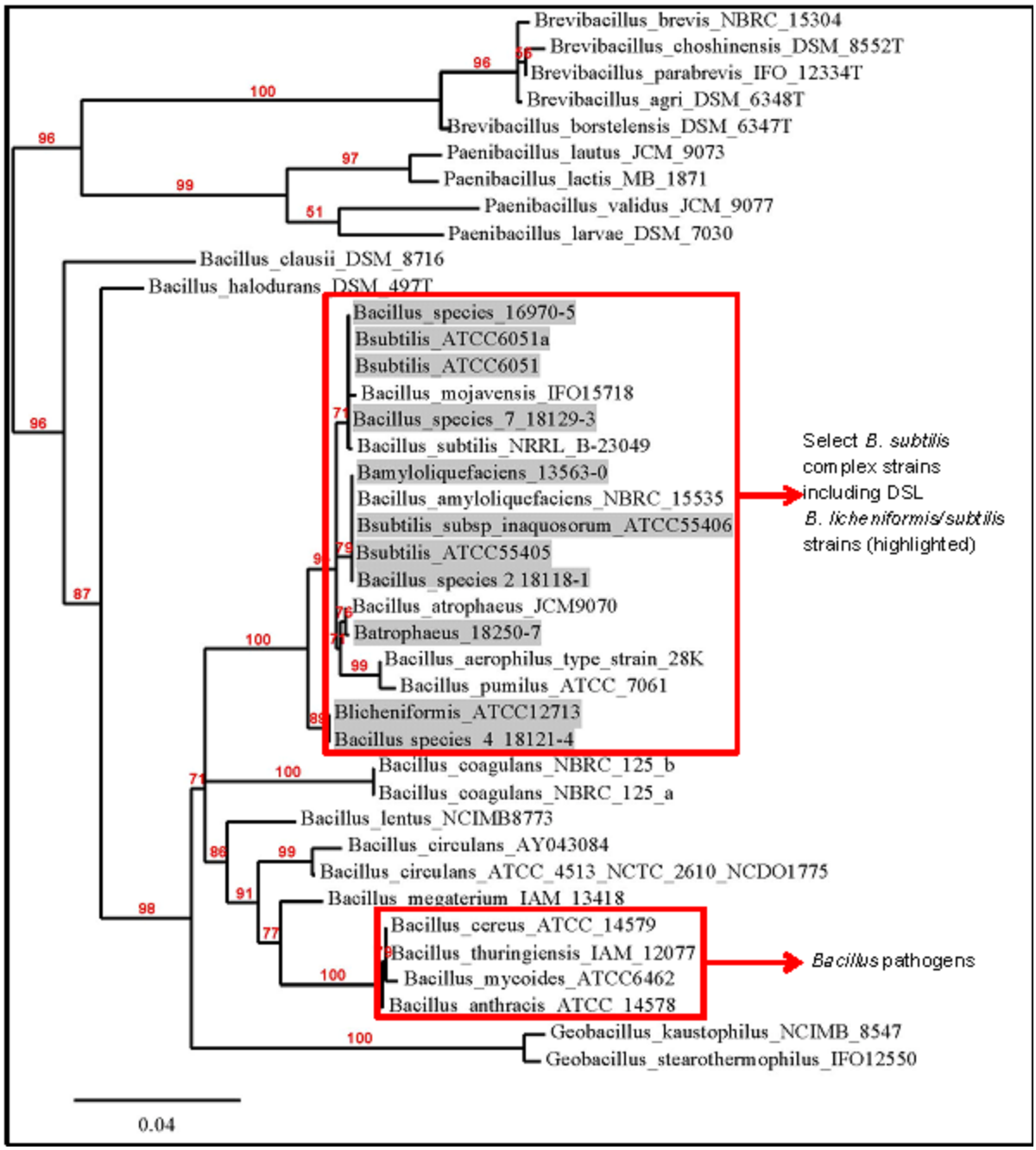

Figure 1-1: Phylogenetic relationships of *Bacillaceae* species based on the alignment of the 16S ribosomal RNA gene sequence coding region

B. subtilis subsp. *inaquosorum* and *B. licheniformis* both have properties that distinguish them from other *B. subtilis* complex members, including a lower salt tolerance, anaerobic growth and the production of toxic compounds in some strains (Salkinoja-Salonen et al. 1999). *B. subtilis* subsp. *inaquosorum* is distinguished from *B. licheniformis*, other subspecies of *B. subtilis* and other members of the *B. subtilis* complex by the production of a novel surfactin-like lipopeptide demonstrated by an additional major ion (mass *m/z* 1120.8) in its matrix-assisted laser desorption/ionization-

time-of-light mass spectrometer profile, as well as differences in the total cellular content of fatty acids (Rooney et al. 2009) (Appendix 4). Recent genomic sequencing of strains of *B. subtilis* subsp. *inaquosorum* supports its taxonomic status as an independent subspecies of *B. subtilis* (Yi et al. 2014). For the purposes of this report, information relating to *B. subtilis* subsp. *inaquosorum* ATCC 55406 will be grouped with information on the DSL *B. subtilis* strains.

1.1.1.3 Strain history

The sites of isolation of most members of the DSL *B. licheniformis*/*subtilis* group are unknown. Certain members were isolated from soil (*B. subtilis* ATCC 55405, *B. subtilis* subsp. *inaquosorum* ATCC 55406 and *Bacillus* species 16970-5) and industrial settings (*Bacillus* species 2 181181-1).Various strains of the DSL *B. licheniformis/subtilis* group that are in the American Type Culture Collection (ATCC) are also recognised under other strain designations in culture collections around the world (Table 1-3). The type strain, *B. subtilis* subsp. *subtilis* ATCC 6051, has been deposited to many culture collections and is known as the Marburg strain (Table 1-4).

Table 1-3: Culture collections holding DSL *B. licheniformis/subtilis* group strains and alternative recognized strain designations

Strain	Culture Collection	Other Strain Designation
B. licheniformis ATCC 12713	Agricultural Research Service Database Culture Collection/NRRL Collection	NRRL B-1001
B. licheniformis ATCC 12713	Prairie Regional Laboratory	PRL B479
B. subtilis ATCC 6051A	Not applicable	P31K6
B. subtilis ATCC 55405	Not applicable	300
B. subtilis subsp. *inaquosorum* ATCC 55406	National Collection of Industrial, Food and Marine Bacteria	NCIMB 14014

Table 1-4: Major culture collections holding the Marburg strain (type strain), *B. subtilis* subsp. *subtilis* ATCC 6051 and alternative strain designations

Culture Collection	Other Strain Designations
Agricultural Research Service Database Culture Collection/NRRL Collection	NRRL B-4219, NRS 1315, NRS 744
American Type Culture Collection	ATCC 6051-U
Collection Française des Bactéries Phytopathogenes and Pasteur Institute Collection (France)	CFBP 4228, CIP 52.65
Deutsche Sammlung von Mikroogansimen und Zellkulturen (Germany)	DSM 10, IMET 10758
Institute for Fermentation, Osaka (collection transferred to NBRC) (Japan)	IFO 12210, IFO 13719, IFO 16412
Japan Collection of Micro-organisms	JCM 1465, IAM 12118
National Collection (United Kingdom)	NCFB 1769, NCIB 3610, NCTC 3610
Netherlands Culture Collection of Bacteria	NCCB 32009, NCCB 53016, NCCB 70064

1.1.2 Biological and ecological properties

1.1.2.1 Natural occurrence

B. subtilis complex members can adapt to and thrive in many environments. In general, *Bacillus* species have been isolated from a diversity of habitats, including terrestrial (soil and vegetation) (Logan and De Vos 2009; Murray et al. 1995; Thatoi et al. 2013) and aquatic environments (Rajarajan et al. 2013; Shakir et al. 2012; Shields et al. 2013; Smitha and Bhat, 2012). *Bacillus* species have also been isolated from animals and as a transient part of the human bowel flora (Kramer and Gilbert, 1989; Turnbull and Kramer 1985); as contaminants of raw and prepared foods (reviewed in Fangio et al. 2010; Hosoi et al. 2000; Inatsu et al. 2006; Kramer and Gilbert 1989; Ray et al. 2000; Turnbull et al. 2001); and aviation fuels (Rauch et al. 2006). The broad range of environments exploited by the genus reflects the wide physiological variation among *Bacillus* species (Murray et al. 1995).

Naturally-occurring cell densities of viable *B. licheniformis*, *B. amyloliquefaciens* and *B. subtilis* in indoor air and settled dust of schools and daycare centres (Table 1-5) and agricultural buildings (cow shed and piggery) (Table 1-6) have been reported (Andersson et al. 1999).

Table 1-5: Naturally-occurring cell densities of viable *B. amyloliquefaciens, B. licheniformis* and *B. subtilis* in schools and daycare centers

Organism	Indoor air (CFU/m^3)	Settled Dust (CFU/g)
B. amyloliquefaciens	No data	10-10^2
B. licheniformis	10^2	10^3
B. subtilis	No data	No data

Table 1-6: Naturally-occurring cell densities of viable *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* in agricultural settings (cow shed and piggery)

Organism	Indoor air (CFU/m^3)	Settled Dust (CFU/g)
B. amyloliquefaciens	No data	No data
B. licheniformis	10^4-10^7	10^4-10^6
B. subtilis	10^4-10^7	10^4-10^6

1.1.2.2 Survival and persistence in the environment

Bacillus species form spores that allow them to survive inhospitable conditions and this gives them a competitive advantage over non-spore forming species in variable environments (Grossman and Losick 1988; Kramer and Gilbert 1989). Spores are more resistant to heat, chemicals, radiation and desiccation than their vegetative counterparts (Brown 2000; Logan 2012). The physiology of *Bacillus thuringiensis* spores is similar to those of the *B. subtilis* complex making it an appropriate surrogate. Spores of *B. thuringiensis* are reported to persist at high levels in soil for at least 13 years (Hendriksen and Hansen 2002; Hendriksen and Carstensen 2013). Nevertheless, in general, introduced microbial populations gradually decline, regardless of the source of

their original isolation, due to the hostility of biotic and abiotic conditions in the soil environment (Van Veen et al. 1997). Biotic factors include predation and antagonism; abiotic factors include adverse soil pH, temperature and moisture, and nutrient scarcity (Van Veen et al. 1997). High numbers of vegetative cells are unlikely to be maintained in water or soil due to competition from other microflora (Leung et al. 1995). Plant colonization and biofilm formation may also increase the resistance of the bacteria to unfavourable conditions (Sella et al. 2012).

Three studies were identified that investigate the persistence of the *B. subtilis* complex in soils. In one study, long-term persistence of *B. subtilis* ATCC 6051 and *B. subtilis* ATCC 13933 in agricultural soil was investigated (Xiang et al. 2010). DNA from *B. subtilis* ATCC 6051 and *B. subtilis* ATCC 13933 could be amplified from laboratory microcosms for 8 and 127 days respectively after inoculation with cell culture suspensions containing 10^8 to 10^{10} CFU/mL of the test strains (Xiang et al. 2010). Using amplified fragment length polymorphisms to develop specific DNA markers for the strains being investigated combined with quantitative real-time PCR the fate of *B. subtilis* ATCC 6051 and *B. subtilis* ATCC 13933 extracted from soil can be quantitatively tracked and can be used to estimate the concentration of cells in the soil (Figure 1-2).

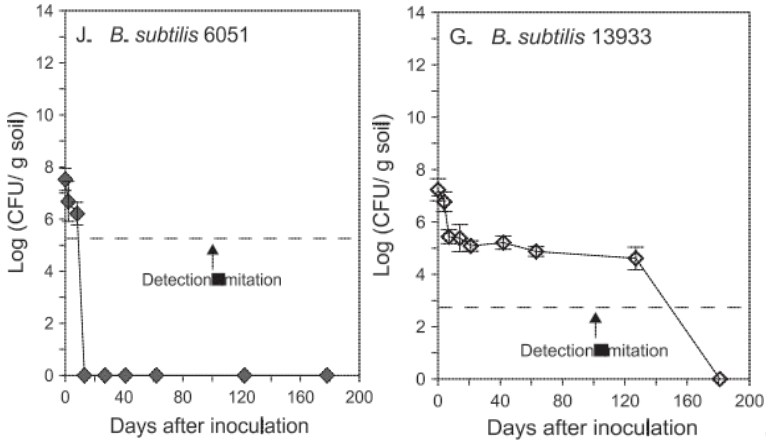

Figure 1-2: Persistence of *Bacillus subtilis* ATCC 6051 and *Bacillus subtilis* 13933 in soil, based on qPCR analyses of extractable soil DNA

The very different detection limits between these two strains make comparison of their persistence difficult. Sporulation of vegetative cells and less efficient recovery of DNA from spores may have played a role in the observed decline. Recovery of DNA from spores depends on the spore type, concentration of spores and the environment.

In another study, a strain of *B. subtilis* was inoculated into field soils and the population was observed to decline rapidly before stabilising (van Elsas et al. 1986). The populations remained low and mainly as spores over the course of 120 days.

In a third study, the persistence of *B. amyloliquefaciens* 13563-0, *B. licheniformis* ATCC 12713, *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 in soil was investigated (Providenti et al. 2009). The authors

suggested that if 1×10^6 CFU/g soil of the vegetative cells were initially released, the detectable concentration of bacteria would likely decrease to 1×10^2 CFU/g soil or less within one to six months.

On the basis of these three studies, concentrations of the *Bacillus* species under assessment applied to soil are expected to decrease several fold over time, but would be likely to persist at some lower concentration as spores.

1.1.2.3 Growth parameters

Growth temperature and pH ranges vary between members of the *B. subtilis* complex and may vary between strains (Table 1-7) (Logan and De Vos 2009; Rooney et al. 2009).

Table 1-7: Growth temperature and pH ranges of members of the *B. subtilis* complex

Species	Growth Temperature Range (°C)	Optimal Growth Temperature (°C)	pH Range[a]
B. amyloliquefaciens	15-50	30-40	5.5-8.5[b]
B. atrophaeus	5-55	28-30	5.3-5.7[c]
B. licheniformis	15-55	30-40	5.7-6.8[b]
B. subtilis subsp. *subtilis*	5-55	28-30	5.5-8.5[b]
B. subtilis subsp. *inaquorsorum*	15-55[d]	28-30[d]	5.5-5.7[d]

[a] pH in Voges-Proskauer broth
[b] (Logan and De Vos 2009)
[c] (Nakamura 1989)
[d] (Rooney et al. 2009)

B. licheniformis and *B. subtilis* subsp. *inaquosorum* are facultative anaerobes and some strains of *B. subtilis* have restricted growth under anaerobic conditions (Logan and De Vos 2009). The ability to grow in both aerobic and anaerobic conditions contributes to the success of these *Bacillus* species in colonizing a variety of niches. In BALB/c mice inoculated orally with high concentrations of *B. subtilis* spores the quantity of *B. subtilis* (spores and vegetative cells) excreted in the feces was higher than the initial inoculation concentration (Hoa et al. 2001). This increase suggests that spores may be able to persist and germinate in the gastrointestinal tract of mice despite the anaerobic environment (Hoa et al. 2001).

1.1.2.4 Biocontrol and growth promotion[4]

Biocontrol

B. subtilis complex strains have characteristics which make them effective biocontrol agents. As an endophytic bacterium, *B. licheniformis*, colonises the same sites as certain plant pathogens and may be better suited than rhizosphere bacteria to

[4] Biocontrol or growth promotions activities are not within the scope of this assessment.

outcompete or antagonise plant pathogens (Mekete et al. 2009). *B. licheniformis* ATCC 14580 has chitinase and chitobiase activity which may be useful against fungal pathogens (ATCC 2012e). *B. subtilis* complex members are able to produce antibiotics and extracellular chitinolytic enzymes that may inhibit plant fungal pathogens (Cordero-Ramírez et al. 2013; reviewed in Hameeda et al. 2006; Jamalizadeh et al. 2008; Pérez-García et al. 2011; Toledo et al. 2011). Bacteriocins are antagonistic peptides that may kill or inhibit the growth of other bacteria. (He et al. 2006; Tagg et al. 1976). Bacteriocins produced by *B. licheniformis* strains exhibit a broad range of antagonistic activity against various Gram positive and fungal pathogens but not Gram negative organisms (He et al. 2006). Antimicrobial compounds, such a bacteriocins, produced by members of the *B. subtilis* complex could affect microbial populations in habitats such as soils, and the microbiomes of plants, animals and humans. Recently, *B. atrophaeus* CAB-1 was demonstrated to have antifungal activity, making it a potential biocontrol agent (Zhang et al. 2013).

Strains of *B. amyloliquefaciens* and *B. subtilis* have been approved for use as biocontrol agents against fungal disease in terrestrial plants in Canada since 2011 and 2005, respectively (PMRA-HC 2007a; PMRA-HC 2007b; PMRA-HC 2007c; PMRA-HC 2012; PMRA-HC 2013; PMRA-HC 2014). Strains of *B. amyloliquefaciens, B. licheniformis* and *B. subtilis* have been approved for use as biocontrol agents of fungal disease in terrestrial plants in the United States since 2000, 2007 and 1992, respectively (Mendelsohn and Vaituzis 1999; U.S. EPA 2001; U.S. EPA 2006; U.S. EPA 2010; U.S. EPA 2011; U.S. EPA 2012; U.S. EPA 2013b).

Growth promotion

Members of the *B. subtilis* complex may promote plant growth by fixing nitrogen, producing biofertilizers and phytohormones, enhancing root nodulation, controlling plant pathogens and through their interactions with other symbiotic bacteria and fungi. These functions may be related to plant colonization and biofilm formation (Beauregard et al. 2013; Chung et al. 2010; Weng et al. 2012). *B. licheniformis, B. amyloliquefaciens* and *B. subtilis* have been isolated from the inner tissues of healthy plants and may have roles in growth promotion and plant protection (Logan, 2012). *B. amyloliquefaciens, B. atrophaeus* and *B. licheniformis* have been described within the rhizosphere of mangrove forests where they solubilize phosphate, increasing nutrient availability to the plants (Thatoi et al. 2013).

B. licheniformis and *B. subtilis* produce a number of enzymes (e.g. protease, lipase and amylase) that can be applied in aiding the digestion of proteins from animal feed (Ahmadnia Motlagh et al. 2012; Link and Kovác 2006). As an alternative to prophylactic antibiotic treatment, *B. licheniformis* has been demonstrated to protect against pathogens in aquaculture (Vinoj et al. 2013) and has been used as a probiotic for weight gain or pathogen resistance in rainbow trout (Merrifield et al. 2010a; Merrifield et al. 2010b), pigs (Link and Kovác 2006) and chickens (Rahimi and Kahsksefidi 2006). The use of some of the *B. subtilis* complex strains as probiotics in animals and the addition of their enzymes to feeds have been reported to result in increased weight gain and improvement of health. Other studies have investigated the immune stimulating

potential of probiotic strains to enhance resistance of animal hosts against pathogens (Huang et al. 2013; reviewed in Vinoj et al. 2013).

1.1.2.5 Gene transfer

B. subtilis is naturally competent for transformation, a phenomenon that is growth-stage specific and nutrient sensitive (Dubnau and Losick 2006; Veening et al. 2008). Genetic exchange by this mechanism seems to be biased towards closely related species since the transformation frequency decreases exponentially with DNA sequence divergence (Majewski and Cohan 1998; Roberts and Cohan 1993; Zawadzki et al. 1995). This is expected to limit the possibility of *B. subtilis* acquiring pathogenic traits from distant species.

B. subtilis has also been implicated in the conjugal transfer of plasmids; however, most *B. subtilis*-like bacteria do not contain endogenous plasmid DNA (Kreft and Hughes 1982; Meijer 1995; Meijer et al. 1998; Tanaka et al. 1977). Transposable elements and prophages were reported in the genome of *B. licheniformis* ATCC 14580 (the type strain) (Lapidus et al. 2002), including nine identical copies of the 1,285 base pair insertion sequence *IS3Bli1* and prophage sequences NZP1 and NZP3 (Rey et al. 2004).The identified prophage sequences have not been characterized. *B. subtilis* can also transfer transposons and integrons (Auchtung et al. 2005; Celli and Trieu-Cuot 1998; Kimura et al. 2011; Koehler and Thorne 1987; Marra and Scott 1999; Meijer et al. 1998), and especially those of the class of integrative and conjugative elements (ICE) such as ICEBs1 (Auchtung et al. 2005), which can be transferred from *B. subtilis* to other *Bacillus* or *Listeria* species under conditions of host cell distress or in the presence of a high concentration of cells lacking ICEBs1. ICEs encode for proteins required for conjugal transfer, resistance to antibiotics and metabolism of alternative carbon sources (Auchtung et al. 2005).

Mobile genetic elements for some strains of the *B. subtilis* complex are reported in Appendix 5. Genes associated with virulence in strains of the *B. subtilis* complex are reported in Appendix 6. It is unknown if the DSL strains carry genes conferring virulence factors or antimicrobial resistance on mobile elements. Given their capacity for horizontal gene transfer, they could theoretically acquire such genes, but this potential is no greater for the DSL strains than for strains that are naturally present in the environment given what has been reported in the current scientific literature.

1.1.2.6 Pathogenic and Toxigenic Characteristics

Spores

The ability to form spores is integral to the etiology of *Bacillus* food poisoning, which has been associated with certain strains of *B. licheniformis* and *B. subtilis*. *Bacillus* spores survive disinfection, irradiation and cooking (Baril et al. 2012; Logan 2012). All of the DSL strains under assessment are capable of forming spores. Spores of the *B. subtilis* complex are highly heat resistant, with temperatures between 94.9^{o}C and 97.7^{o}C for *B. licheniformis* and between 103.2^{o}C and 108.0^{o}C for *B. subtilis* required to inactivate

90% of spores within 10 minutes (André et al. 2013). Under favourable conditions, such as when food is held at temperatures between 10°C and 50°C, the spores can germinate and proliferate (Baril et al. 2012; Brown 2000), and this permits the accumulation of sufficient cell concentrations for foodborne illness to occur.

Determinants of infectivity

In order to be an effective bacterial pathogen, a micro-organism must be able to adhere to host cell surfaces, invade host tissues and evade host defences. In one study, certain *B. licheniformis* and *B. subtilis* isolates had some ability to adhere or invade (Hep-2 and Caco-2 cell lines) while others were completely incapable of adherence or invasion (Rowan et al. 2001).

Strong hemolytic activity (as well as lecithinase activity) may indicate the presence of cytotoxic phospholipases that may facilitate invasion and are associated with virulence (Rowan et al. 2001; Sorokulova et al. 2008). Isolates of *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* exhibit varying levels of hemolysis. In one study, *B. amyloliquefaciens* demonstrated beta-hemolysis; *B. licheniformis* no hemolysis; and *B. subtilis* alpha, beta or no hemolysis, depending on the isolate (Cordero-Ramírez et al. 2013). However, analysis by Health Canada scientists on the DSL *B. licheniformis/subtilis* group strains indicated no strong hemolytic activity in any strain (Appendix 7).

Catalase activity can enable a micro-organism to protect itself from reactive oxygen-induced killing from immune cells potentially making it a more effective pathogen. Catalase activity was assessed for the DSL *B. licheniformis/subtilis* strains by Health Canada scientists; all strains tested positive for catalase activity (Appendix 8).

Secondary Metabolites

Members of the *B. subtilis* complex also produce an array of secondary metabolites. Surfactin (*B. subtilis*) and lichenysin (*B. licheniformis*) are amphiphilic lipopeptides (Li et al. 2010). Both are powerful surfactants and have antimicrobial and hemolytic properties. Although they differ by only two amino acids, the hemolytic activity of lichenysin is much higher than that of surfactin (15 µmol/L vs. 200 µmol/L required to achieve 100% hemolysis, respectively) (Li et al. 2010).

Amylosin was first detected in *B. amyloliquefaciens* (Logan, 2012; Mikkola et al. 2007). It is an ionophore that forms K^+ and Na^+ channels in host cell membranes causing toxic responses including complete cell death, with extensive lysis in exposed cell lines and inhibition of motility in a boar spermatozoa assay (Mikkola et al. 2007).

Toxins

Strains of *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* have been reported to produce both heat-labile and heat-stable toxins (Appendix 9) (Beattie and Williams, 1999; reviewed in De Jonghe et al. 2010; Mikkola et al. 2007; Nieminen et al. 2007).

Toxins produced include some that are similar to the *B. cereus* emetic toxin (cereulide) (Salkinoja-Salonen et al. 1999; Taylor et al. 2005), hemolysin BL (Hbl) enterotoxin (Lindsay et al. 2000; Rowan et al. 2001) and a non-hemolytic enterotoxin (Nhe). A non-emetic heat-stable cytotoxic component has also been reported in certain strains of *B. subtilis* and *B. amyloliquefaciens* (De Jonghe et al. 2010). Isolates of *B.* licheniformis have been reported to produce a non-proteinaceous heat-stable toxin, which damages cell membrane integrity, depletes cellular ATP and has beta-hemolytic activity (Salkinoja-Salonen et al. 1999).

The Hbl toxin complex and BceT diarrheal toxin genes were identified in *B. licheniformis* and *B. subtilis* clinical and food isolates (Rowan et al. 2001). The growth medium was reported to affect toxin production, with more strains producing Hbl if grown in infant milk formula than in brain-heart infusion (BHI) broth. Toxin production is not related to the source of the isolate (clinical or environmental) (Beattie and Williams 1999; Madslien et al. 2012).

In testing conducted in Health Canada laboratories, strains were cultured on BHI. Three different commercial assay kits were used: all strains were tested for the HblC subunit of the Hbl enterotoxin using a commercial RPLA kit (Oxoid) and six strains[5] were tested for the NheA subunit of the Nhe enterotoxin using an ELISA assay (TECRA kit). The Duopath Cereus (Millipore) kit was also used to detect both Nhe and Hbl enterotoxin production in the DSL strains. None of the DSL strains produced these toxins.

Cell-free culture supernatants of some clinical and food isolates of *B. licheniformis* and *B. subtilis* that had been implicated in food poisoning had cytotoxic activity towards both human Caco-2 and HEp-2 epithelial cell lines (Rowan et al. 2001). The growth medium affected the cytotoxic potential, and heat or trypsin treatment of the culture supernatant reduced or eliminated cytotoxic activity, indicating that it was attributable to the proteinaceous fraction (Rowan et al. 2001). In another study, food poisoning isolates of *B. licheniformis* and *B. subtilis* from street vendor food were cytotoxic to McCoy cells (Mosupye et al. 2002). In addition, whereas *B. cereus* isolates lost cytotoxicity following heat-treatment, some *B. licheniformis* and *B. subtilis* isolates retained their cytotoxicity (Mosupye et al. 2002). A *B. licheniformis* strain isolated from raw milk that was associated with food-poisoning was also cytotoxic to McCoy cells (Lindsay et al. 2000).

In testing conducted by Health Canada scientists, the cytotoxicity of the DSL *B. licheniformis/subtilis* strains was assessed in two cell lines, J774A.1 (macrophage cells) and HT29 (human colonic epithelial cells), with and without gentamicin. The strains did not demonstrate strong cytotoxicity towards either cell line (Appendix 10).

[5] *B. licheniformis* ATCC 12713, *B. subtilis* ATCC 6051A, *B. subtilis* subsp. *subtilis* ATCC 6051, *B. subtilis* subsp. *inaquosorum* ATCC 55406, *Bacillus* species 2 18118-1 and *Bacillus* species 7 18129-3.

1.1.2.7 Antibiotic Susceptibility Profile

Information in the scientific literature on antibiotic susceptibility in *B. amyloliquefaciens* and *B. atrophaeus* is scant, presumably because these have not been implicated in cases of infection.

Variable antibiotic susceptibility profiles have been reported as part of case reports of infection with *B. licheniformis* and *B. subtilis* (Table 1-8). *B. licheniformis* susceptibility to the beta-lactam antibiotics ampicillin, piperacillin and ticarcillin depends on the isolate (Banerjee et al. 1988; Castagnola et al. 1997). Some isolates have an inducible beta-lactamase that may be responsible for this variable susceptibility (Filée et al. 2002; Zhu et al. 1992). Similarly, *B. licheniformis* ATCC 12713 is resistant to erythromycin, whereas the type strain ATCC 14580 is susceptible and variations in bacitracin synthase gene sequences are postulated to determine erythromycin resistance (Ishihara et al. 2002). A case of *B. subtilis* endocarditis was successfully treated with cefazolin (Tuazon et al. 1979), but in a later study, isolates were reported to be cefazolin resistant (Banerjee et al. 1988).

Table 1-8: Antibiotic susceptibilities of *B. licheniformis* and *B. subtilis* reported in the scientific literature

Antibiotic	*B. licheniformis*	*B. subtilis*	Reference
Amikacin	S[a]	S	(Banerjee et al. 1988; Sorokulova et al. 2008)
Aminoglycosides	S	N/A[b]	(Ozkocaman et al. 2006)
Amoxicilin	I[c]	I	(Sorokulova et al. 2008)
Amoxicillin clavulanic acid	S	N/A	(Lépine et al. 2009)
Ampicillin	V[d]	S	(Banerjee et al. 1988; Sorokulova et al. 2008)
Azlocillin	S	S	(Banerjee et al. 1988)
Bactrim	S	S	(Sorokulova et al. 2008)
Carapenem	S	N/A	(Ozkocaman et al. 2006)
Carbenicillin	S	S	(Sorokulova et al. 2008)
Ceftazimide	R[e]	R	(Banerjee et al. 1988)
Cefamandol	I	S	(Sorokulova et al. 2008
Cefatolin	S	N/A	(Lépine et al. 2009)
Cefazolin	S	V	(Banerjee et al. 1988; Sorokulova et al. 2008)
Cefepime	S	N/A	(Ozkocaman et al. 2006)
Cefotaxim	R	I	(Sorokulova et al. 2008)
Cefoxitin	R	I	(Sorokulova et al. 2008)
Ceftriaxon	R	I	(Sorokulova et al. 2008)
Cephalotin	S	S	(Sorokulova et al. 2008)
Chloramphenicol	R	V	(Banerjee et al. 1988; Sorokulova et al. 2008)
Ciprofloxacin	S	S	(Castagnola et al. 1997; Sorokulova et al. 2008)
Clindamycin	R	S	(Banerjee et al. 1988; Sorokulova et al. 2008)
Cotrimoxazole	S	N/A	(Castagnola et al. 1997)

Antibiotic	*B. licheniformis*	*B. subtilis*	Reference
Doxycycline	S	N/A	(Lépine et al. 2009)
Enrofloxacin	S	S	(Sorokulova et al. 2008)
Erythromycin	V	S	(Ishihara et al. 2002; Sorokulova et al. 2008)
Gentamicin	S	S	(Banerjee et al. 1988; Sorokulova et al. 2008)
Imipenem	S	S	(Banerjee et al. 1988; Sorokulova et al. 2008)
Kanamycin	S	S	(Sorokulova et al. 2008)
Linezolid	S	S	(Sorokulova et al. 2008)
Meropenem	S	N/A	(Mochiduki et al. 2007)
Methicillin	R	I	(Sorokulova et al. 2008)
Mezlocillin	I	S	(Sorokulova et al. 2008)
Nafcillin	S	N/A	(Blue et al. 1995)
Neomycin	S	S	(Sorokulova et al. 2008)
Netilmicin	S	N/A	(Castagnola et al. 1997)
Nitrofurantoin	S	S	(Sorokulova et al. 2008)
Norfloxacin	S	S	(Banerjee et al. 1988; Sorokulova et al. 2008)
Ofloaxcin	S	N/A	(Lépine et al. 2009)
Oxacillin	R	R	(Castagnola et al. 1997; Sorokulova et al. 2008)
Penicillin	R	R	(Banerjee et al. 1988)
Piperacillin	V	S	(Banerjee et al. 1988)
Quinupristin + dalfopristin	N/A	N/A	(Sorokulova et al. 2008)
Rifampicin	S	S	(Sorokulova et al. 2008)
Streptomycin	S	S	(Sorokulova et al. 2008)
Telcoplanin	S	N/A	(Castagnola et al. 1997)
Tetracycline	S	S	(Sorokulova et al. 2008)
Ticarcillin	V	S	(Banerjee et al. 1988)
Tobramycin	S	S	(Castagnola et al. 1997; Sorokulova et al. 2008)
Trimethoprim	S	S	(Sorokulova et al. 2008)
Vancomycin	S	S	(Banerjee et al. 1988; Sorokulova et al. 2008)

[a] S, susceptible, also includes successful treatments where no other antibiotics were used
[b] N/A, not available
[c] I, intermediate
[d] V, variable (different sources gave different resistance results)
[e] R, resistant

Vegetative cells of the DSL *B. licheniformis/subtilis* group strains were tested for their resistance to antibiotics from a number of families by Health Canada scientists[6] (Table 1-9 to Table 1-13). Interpretive categories (susceptible, intermediate, resistant or nonsusceptible) are classifications based on an in vitro response of an organism to an

[6] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Work conducted using TSB-MTT liquid assay method to determine the MIC values for bacteria based on replicate experiments (Seligy et al. 1997). Values correspond to the minimal inhibitory concentration (µg/mL) for select *Bacillus* species grown in the presence of antibiotic for 24 hours at 37°C.

antimicrobial agent at levels corresponding to blood or tissue levels attainable with usually prescribed doses of that agent (CLSI, 2010). Minimum inhibitory concentration values were interpreted where possible. Interpretive criteria were not identified for some of the tested antibiotics.

Table 1-9: Minimum inhibitory concentrations (MIC) of *B. amyloliquefaciens* 13563-0

Antibiotic	Susceptible[a]	Intermediate[a]	Resistant[a]	MIC µg/mL (interpretation)
Amoxycillin	N/A[b]	N/A	N/A	0.37 ± 0
Cephotaxime	≤8	16-32	≥64	21.3 ± 8 (I[c])
Ciprofloaxcin	≤1	2	≥4	0.37 ± 0 (S[d])
Doxycycline	N/A	N/A	N/A	0.37 ± 0
Erythromycin	≤0.5	1-4	≥8 (≥4[e])	0.37 ± 0 (S)
Gentamicin	≤4	8	≥16 (≥4[e])	0.52 ± 0.21 (S)
Meropenem	N/A	N/A	N/A	0.37 ± 0
Nalidixic acid	N/A	N/A	N/A	10.2 ± 8.4
Trimethoprim	≤2	N/A	≥4	>24 (R[f])
Vancomycin	≤4	N/A	N/A (≥4[e])	0.45 ± 0.17 (S)

[a] Interpretive criteria (MIC µg/mL; CLSI 2010)
[b] N/A, not available
[c] I, intermediate susceptibility
[d] S, susceptible
[e] Interpretive criteria (MIC µg/mL; EFSA 2008)
[f] R, resistant

Table 1-10: Minimum inhibitory concentrations (MIC) of *B. atrophaeus* 18250-7

Antibiotic	Susceptible[a]	Intermediate[a]	Resistant[a]	MIC µg/mL (interpretation)
Amoxycillin	N/A[b]	N/A	N/A	0.75 ± 0
Cephotaxime	≤8	16-32	≥64	1.5 ± 0 (S[c])
Ciprofloaxcin	≤1	2	≥4	0.37± 0 (S)
Doxycycline	N/A	N/A	N/A	0.37± 0
Erythromycin	≤0.5	1-4	≥8 (≥4[d])	0.37 ± 0 (S)
Gentamicin	≤4	8	≥16 (≥4[d])	0.37 ± 0 (S)
Meropenem	N/A	N/A	N/A	0.37 ± 0
Nalidixic acid	N/A	N/A	N/A	3 ± 0
Trimethoprim	≤2	N/A	≥4	>24 ± 0 (R[e])
Vancomycin	≤4	N/A	N/A (≥4[d])	0.75 ± 0 (S)

[a] Interpretive criteria (MIC µg/mL; CLSI 2010)
[b] N/A, not available
[c] S, susceptible
[d] Interpretive criteria (MIC µg/mL; EFSA 2008)
[e] R, resistant

B. licheniformis ATCC 12713 appeared to be resistant to many antibiotics (most for which interpretive criteria were available; Table 1-11). This was unexpected, given that the literature on the species indicates susceptibility to a variety of antibiotic classes (Table 1-8). Resistance to vancomycin was particularly unexpected (CLSI 2010). For this reason the test results were revisited. The MIC had been strictly interpreted as the lowest concentration that completely inhibited growth of the micro-organism (CLSI

2010); however for some antibiotics, the vast majority of bacteria had been eliminated at much lower concentrations, with a small number of residual bacteria persisting through several higher concentration increments. Examination by microscopy revealed that these residual bacteria were in the form of aggregates, which is a unique behavior of this strain growing in liquid cultures. This aggregate formation may protect internal cells from contact with the antibiotic. When tests results were re-interpreted with a 95% bioreduction activity cutoff, the revised MICs were more consistent with values expected of this species. This was confirmed for vancomycin using test-strips, which showed low MIC values (1.1 ± 1.0; n=6). It was concluded that the apparent high resistance observed was an artifact of the liquid culture MIC assay.

Table 1-11: Minimum inhibitory concentrations (MIC) of *B. licheniformis* ATCC 12713

Antibiotic	S[a]	I[b]	R[c]	MIC µg/mL (interpretation)	95% bioreduction activity (interpretation)
Amikacin	≤16	32	≥64	>24 (not S[d])	>24 (not S)
Amoxycillin	N/A[e]	N/A	N/A	>24	>24
Ampicillin	≤0.25	N/A	≥0.5	0.37 ± 0 (I[f])	0.37 ± 0
Ceftazidime	≤8	16	≥32	>24 (R[g])	>24 (R)
Cephotaxime	≤8	16-32	≥64	12 (I)	6.0 ± 0 (S)
Chloramphenicol	≤8	16	≥32 (≥8[h])	12 (I)	12.0 ± 0 (I)
Ciprofloaxcin	≤1	2	≥4	18 ± 9 (R)	0.37 ± 0 (S)
Doxycycline	N/A	N/A	N/A	24	0.56 ± 0.19
Erythromycin	≤0.5	1-4	≥8 (≥4[f])	>24 (R)	>24 (R)
Gentamicin	≤4	8	≥16 (≥4[f])	18 ± 9 (R)	2.53 ± 1.54(S)
Meropenem	N/A	N/A	N/A	24	0.37 ± 0
Nalidixic acid	N/A	N/A	N/A	>24	>24
Penicillin	≤0.12	N/A	≥0.25	0.75 ± 0 (R)	0.75 ± 0 (R)
Rifampin	≤1	2	≥4	0.5 ± 0.2 (S)	0.37 ± 0 (R)
Tetracyclin	≤4	8	≥16 (≥8[f])	3 ± 0 (S)	3.0 ± 0 (S)
Trimethoprim	≤2	N/A	≥4	>24 (R)	0.37 ± 0 (S)
Vancomycin	≤4	N/A	N/A (≥4[f])	18 ± 9 (non-S)	0.61 ± 0.37(S)

[a] Interpretive criteria (MIC µg/mL; CLSI 2010) S, susceptible
[b] Interpretive criteria (MIC µg/mL; CLSI 2010) I, intermediate susceptibility
[c] Interpretive criteria (MIC µg/mL; CLSI 2010) R, resistant
[d] S, susceptible
[e] N/A, not available
[f] I, intermediate susceptibility
[g] R, resistant
[h] Interpretive criteria (MIC µg/mL; EFSA 2008)
[i] Confirmed using test strips (1.1 ± 1.0 µg/mL, n=6)

Table 1-12: Minimum inhibitory concentrations (MIC, µg/mL) of DSL strains of *B. subtilis*

Antibiotic	S[a]	I[b]	R[c]	*B. subtilis* subsp. *subtilis* ATCC 6051	*B. subtilis* ATCC 6051A	*B. subtilis* ATCC 55405	*B. subtilis* subsp. *inaquosorum* ATCC 55406
Amoxycillin	N/A[d]	N/A	N/A	0.4	12.2 ± 13.6	4.3 ± 9.6	0.6 ± 0.5
Ampicillin	≤0.25	N/A	≥0.5	>24 (R[e])	>24 (R)	>24 (R)	No data

Antibiotic	S[a]	I[b]	R[c]	*B. subtilis* subsp. *subtilis* ATCC 6051	*B. subtilis* ATCC 6051A	*B. subtilis* ATCC 55405	*B. subtilis* subsp. *inaquosorum* ATCC 55406
Aztreonam	N/A	N/A	N/A	>24	>24	>24	No data
Cephotaxime	≤8	16-32	≥64	6.1 ± 4.7 (S[f])	5 ± 1.7 (S)	1.3 ± 1.3 (S)	>24
Ciprofloaxcin	≤1	2	≥4	No data	No data	No data	>24 (R)
Doxycycline	N/A	N/A	N/A	0.4	0.4	0.4	8.4 ± 3.3
Erythromycin	≤0.5	1-4	≥8 (≥4[g])	0.4 (S)	0.4 (S)	0.4 (S)	0.4 (S)
Gentamicin	≤4	8	≥16 (≥4[g])	0.6 ± 0.2 (S)	0.6 ± 0.2 (S)	0.4 (S)	0.4 (S)
Meropenem	N/A	N/A	N/A	ND	No data	No data	1.2 ± 1.1
Nalidixic acid	N/A	N/A	N/A	>24	8 ± 3.5	8 ± 3	9.6 ± 3.3
Trimethoprim	≤2	N/A	≥4	>24 (R)	>24 (R)	>24 (R)	>24 (R)
Vancomycin	≤4	N/A	N/A (≥4[g])	0.9 ± 0.7 (S)	0.4 (S)	0.4 (S)	0.37 (S)

[a] Interpretive criteria (MIC µg/mL; CLSI 2010) S, susceptible
[b] Interpretive criteria (MIC µg/mL; CLSI 2010) I, intermediate susceptibility
[c] Interpretive criteria (MIC µg/mL; CLSI 2010) R, resistant
[d] N/A, not available
[e] R, resistant
[f] S, susceptible
[g] Interpretive criteria (MIC µg/mL; EFSA 2008)

Table 1-13: Minimum inhibitory concentrations (MIC, µg/mL) of the masked *Bacillus* species on the DSL

Antibiotic	S[a]	I[b]	R[c]	*Bacillus* species 2 18118-1	*Bacillus* species 4 18121-4	*Bacillus* species 16970-5	*Bacillus* species 7 18129-3
Amoxycillin	N/A[d]	N/A	N/A	Variable	0.37	0.9 ± 0.6	0.4
Ampicillin	≤0.25	N/A	≥0.5	No data	No data	>24 (R[e])	>24 (R)
Aztreonam	N/A	N/A	N/A	>24	No data	>24	>24
Cephotaxime	≤8	16-32	≥64	1.6 ± 0.7 (S[f])	3 (S)	11 ± 2.4 (I[g])	6.1 ± 4.7 (S)
Ciprofloaxcin	≤1	2	≥4	No data	0.37 (S)	No data	No data
Doxycycline	N/A	N/A	N/A	0.8 ± 0.4	0.37	0.4	0.4
Erythromycin	≤0.5	1-4	≥8 (≥4[h])	0.37 (S)	0.37 (S)	0.4 (S)	0.4 (S)
Gentamicin	≤4	8	≥16 (≥4[h])	1.2 ± 0.5 (S)	1.5 (S)	0.5 ± 0.2 (S)	0.6 ± 0.2 (S)
Meropenem	N/A	N/A	N/A	No data	0.37	No data	No data
Nalidixic acid	N/A	N/A	N/A	8 ± 3	12	9.0 ± 3.3	>24
Trimethoprim	≤2	N/A	≥4	>24 (R)	0.37 (S)	24 ± 23 (R)	>24 (R)
Vancomycin	≤4	N/A	N/A (≥4[h])	0.37 (S)	0.75 (S)	0.4 (S)	0.9 ± 0.7 (S)

[a] Interpretive criteria (MIC µg/mL; CLSI 2010) S, susceptible
[b] Interpretive criteria (MIC µg/mL; CLSI 2010) I, intermediate susceptibility
[c] Interpretive criteria (MIC µg/mL; CLSI 2010) R, resistant
[d] N/A, not available

[e] R, resistant
[f] S, susceptible
[g] I, intermediate susceptibility
[h] Interpretive criteria (MIC µg/mL; EFSA 2008)

1.1.3 Effects

1.1.3.1 Environment

B. amyloliquefaciens

B. amyloliquefaciens is widely distributed in nature in a variety of habitats. Certain strains have been released to agricultural ecosystems as biological pesticides for the control of fungal plant pathogens (PMRA-HC 2012; U.S. EPA 2011; U.S. EPA 2012); others have been released to aquatic habitats as a water treatment/conditioner (Advanced Water Technologies 2012). Despite its natural presence in and history of release into, a variety of environments, a comprehensive search of the scientific literature across a number of sources yielded no cases of infection or evidence of adverse effects in aquatic or terrestrial plants, vertebrates or invertebrates.

Studies on the effects of *B. amyloliquefaciens* strains FZB24 and D747 on a variety of environmental species were submitted to support their registration as biofungicides for use on terrestrial plants (Appendix 11, Table A-51 and Table A-52). Briefly, no significant pathogenicity or toxicity was observed in terrestrial vertebrates (CD and Sprague Dawley rats, Northern Bobwhite quail), aquatic vertebrates (rainbow trout), terrestrial invertebrates (honeybee adults and larvae, earthworm) or aquatic invertebrates (*Daphnia magna*) at the tested concentrations. Although studies on aquatic or terrestrial plants were not reported as part of the pesticide registrations, pesticides containing these strains are deliberately applied to terrestrial plants to control fungal and bacterial plant pathogens and no adverse effects on the treated plants have been reported in the scientific literature.

Murine exposure assays were conducted by Health Canada scientists. Female BALB/c mice remained asymptomatic after exposure to 10^6 CFU of *B. amyloliquefaciens* 13563-0 spores or vegetative cells administered in a 25 µL volume via an endotracheal nebulizer. Aside from a transient inflammatory response, no significant changes were observed (Appendix 12, Table A-59 to Table A-63).

B. atrophaeus

B. atrophaeus is widely distributed in nature. It is used as a non-pathogenic surrogate for *B. anthracis* in experiments modelling airborne dispersal of spores (Carrera et al. 2007; Page et al. 2007; U.S. EPA 2013a). In spite of its natural presence in and releases into the environment, a comprehensive search of the scientific literature across a number of sources yielded no cases of infection or evidence of adverse effects in aquatic or terrestrial plants, vertebrates or invertebrates.

Murine exposure assays were conducted by Health Canada scientists. Female BALB/c mice remained asymptomatic after exposure to 10^6 CFU of *B. atrophaeus* 18250-7 spores or vegetative cells administered in a 25 µL volume via an endotracheal nebulizer. Aside from a transient inflammatory response, no significant changes were observed (Appendix 12, Table A-59 to Table A-63).

B. licheniformis

Environmental isolates of *B. licheniformis* have the ability to form biofilms (Dat et al. 2012) which are implicated in the pathogenesis of bovine mastitis (reviewed in Contreras and Rodríguez 2011; Nieminen et al. 2007) and bovine toxemia (Murray et al. 1995). *B. licheniformis* has been reported to cause sporadic abortion or stillbirths in cattle as well as in buffalo, sheep, pigs and camelids (Agerholm et al. 1995; Agerholm et al. 1997; Cabell 2007; Duncanson 2012; Galiero and De Carlo 1998; Gill 1999; reviewed in Kirkbride et al. 1986; Kirkbride 1993; Madslien et al. 2012; Mitchell and Barton 1986). Other adverse effects in terrestrial vertebrates associated with *B. licheniformis* include placentitis, keraconjuntivitis, feather degradation and yolk sac infection in ostriches (Johnson et al. 1994; Gill 1999; Sheldon et al. 2002; Murray 2006; Hare et al. 2008; Rajchard 2010; Goncagul et al. 2012). *B. licheniformis* has been implicated in adverse effects in insects, including bed bugs, root-knot nematodes, *Ecualyptus* snout-beetles and moths (Reinhardt et al. 2005; Mekete et al. 2008; Molina and Santolmazza-Carbone 2010; Bilbech et al. 2012). An isolate of *B. licheniformis* was implicated in effects in plants as the causative agent of pistachio dieback (Baradaran and Ghasemi 2010).

A six month study attempted to determine the cause of 218 naturally-aborted bovine fetuses (Agerholm et al. 1997). The likely cause of 73 abortions was diagnosed; the most common causes were bovine diarrhea virus (13%), *Neospora caninum* (10%), mycosis (5%) and *B. licheniformis* (4%) (Agerholm et al. 1997). In another study, *B. licheniformis* represented 3% of bovine abortions (n=5,662) (Murray 2006). A Canadian bovine abortion update report for years 1998 to 2004 implicated *B. licheniformis* in 1.1 to 3.1% of abortion cases submitted to the Animal Health Laboratory (McEwen and Carman 2005). In comparison, *Neospora* species represented between 8.3 and 19% of cases submitted and other bacterial species represented between 6.1 and 14% for the same period of time. An etiological agent was not identified in up to 60.6% of cases between 2001 and 2002. Despite its presence at high concentrations in agricultural settings (10^4-10^7 CFU/m^3 in indoor air and 10^4-10^6 CFU/g in settled dust (Andersson et al. 1999), abortion from exposure to naturally-occurring *B. licheniformis* populations is not common. Pathogenesis of abortion is not clear but ingestion of poor-quality/mouldy feed during gestation and subsequent hematogenous spread to the reproductive tract as well as introduction during general animal husbandry activities have been implicated (Cabell 2007; Scott 2011; Goncagul 2012). Gentamicin and ciprofloxacin were the most effective antibiotics tested against *B. licheniformis* isolated from the cervicovaginal mucus of repeat-breeding cows (Yadav and Kashyap 2003).

Experimental infection with *B. licheniformis* strain DVL 9315323 in pregnant dairy cows demonstrated placentome tropism after IV challenge doses ranging from 10^9 to 10^{12} CFU per animal (Agerholm et al. 1999). *B. licheniformis* bacteria were closely associated with placentome and fetal lesions, and were hypothesised to have caused abortion or premature delivery (Agerholm et al. 1999). In another mammalian study, immune depressed BALB/c mice were exposed intravenously to environmental and food isolates of *B. licheniformis,* including the type strain *B. licheniformis* ATCC 14580 at doses of $<1 \times 10^6$ to 6×10^{10} CFU per animal (Agerholm et al. 1997; Appendix 11, Table A-54). Mice were able to eliminate high numbers of the bacteria within one week however, some of the tested isolates caused pulmonary and brain lesions. Male albino Wistar rats exposed to a strain of *B. licheniformis* had an oral NOAEL reported to be greater than 1.1×10^{11} CFU/kg body weight (Nithya et al. 2012; Appendix 11, Table A-54).

Studies on the effects of *B. licheniformis* strain SB3086 on a variety of environmental species were submitted to support its registration as a fungicide for use on terrestrial plants (Appendix 11, Table A-53). No pathogenicity or toxicity was observed in terrestrial vertebrates (rats, mallard ducks), aquatic vertebrates (rainbow trout) or terrestrial invertebrates (honeybee larvae) at the tested concentrations (U.S. EPA, 2001). Aquatic invertebrates (*Daphnia magna*) were exposed to the technical grade active ingredient (TGAI). The survival of daphnids exposed to 1×10^7 CFU/mL of the TGAI (1000 times the expected environmental concentration for pesticidal use) was 90% (two died) (PMRA-HC, personal communication). The TGAI was considered to be not toxic in terms of survival, reproduction, length and weight relative to the control. Although pathogenicity and toxicity studies on aquatic or terrestrial plants were not reported as part of the pesticide registration, the pesticide containing this strain is deliberately applied to terrestrial plants to control fungal plant pathogens. No adverse effects on the treated plants have been reported in the scientific literature or in testing performed for efficacy evaluation.

No negative effects were reported in brine shrimp, rainbow trout, pigs and chickens exposed to probiotics containing strains of *B. licheniformis* (Link and Kovác 2006; Merrifield et al. 2010a; Merrifield et al. 2010b; Rahimi and Kahsksefidi 2006; Vinoj et al. 2013). Increased weight gain and/or pathogen resistance were noted.

Murine exposure assays were conducted by Health Canada scientists. Female BALB/c mice remained asymptomatic after exposure to 10^6 CFU of *B. licheniformis* ATCC 12713 spores or vegetative cells administered in a 25 µL volume via an endotracheal nebulizer. Aside from a transient inflammatory response, no significant changes were observed (Appendix 12, Table A-59 to Table A-63).

B. subtilis

B. subtilis occurs naturally in indoor air and settled dust of agricultural settings at elevated cell-densities (Andersson et al. 1999). Certain strains have been released to agricultural ecosystems as fungicides for use on terrestrial plants (Mendelsohn and

Vaituzis 1999; U.S. EPA 2006; PMRA-HC 2007a; PMRA-HC 2007b; PMRA-HC 2007c; U.S. EPA 2010; PMRA-HC 2013); others have been released to aquatic habitats as a water treatment/conditioner (Advanced Water Technologies 2012). Despite its natural presence in, and history of release into, a variety of environments, a comprehensive search of the scientific literature across a number of sources yielded no cases of infection or evidence of adverse effects in aquatic plants or vertebrates.

Studies on the effects of strains of *B. subtilis* on a variety of environmental species were submitted to support the registration of certain strains as biofungicides for use on terrestrial plants (Appendix 11, Table A-56 and Table A-57). No significant adverse effects were reported in birds, mammals, terrestrial insects, earthworms or soil micro-organisms as a result of exposure to *B. subtilis* strain MBI 600 (PMRA-HC 2007a). No significant adverse effects in birds, freshwater and marine fish, mammals or algae were reported as a result of exposure to *B. subtilis* strain QST 713 (PMRA-HC 2007b). There is some evidence of effects in aquatic and terrestrial invertebrates, but results are inconsistent. In studies reviewed by the U.S. EPA, mortalities were reported in *Daphnia magna* and parasitic *Hymenoptera* after exposure to *B. subtilis* QST 713 at varying concentrations (Mendelsohn and Vaituzis 1999). The cause of death and involvement of *B. subtilis* QST 713 in toxicity or pathogenicity could not be determined in these studies.

Murine exposure assays were conducted by Health Canada scientists. Female BALB/c mice remained asymptomatic after exposure to 10^6 CFU of *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 spores or vegetative cells administered in a 25 µL volume via an endotracheal nebulizer. Aside from a transient inflammatory response, no significant changes were observed (Appendix 12, Table A-59 to Table A-63).

Pathogenicity and toxicity studies were performed by Environment Canada scientists[7] using *Festuca rubra* (red rescue), *Folsomia candida* (collembolan or springtail) and *Eisenia andrei* (earthworm) exposed to either *B. subtilis* ATCC 6051A or *B. subtilis* ATCC 55405 in either field-collected sandy clay loam or a formulated artificial sandy loam soil (Appendix 11, Table A-58). For the red fescue, field-collected or artificial soils were inoculated with 10^5 CFU/g soil dry weight of either *B. subtilis* ATCC 6051A or *B. subtilis* ATCC 55405. At the end of the study (day 21), a significant reduction (approximately 18%) in the mean shoot length was detected in plants exposed to *B. subtilis* ATCC 55405 in the field-collected soil, relative to the field-collected soil negative control.

In the springtail trials, the arthropods were exposed for 28 days to field-collected or artificial soils inoculated with either 10^4 CFU of *B. subtilis* ATCC 6051A or 10^3 CFU of *B. subtilis* ATCC 55405 per gram of dry soil. When compared with the negative control in both soils, a significant reduction (approximately 50%) in juvenile production was observed after exposure to *B. subtilis* ATCC 55405, while no juveniles were produced

[7] Tests done according to Environment Canada's "*Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms* (EPS 1/RM/44, March 2004)".

after exposure to *B. subtilis* ATCC 6051A. Adult survival was not affected by either of these strains.

In the earthworm trials, the invertebrate was exposed for 35 days in field-collected or artificial soils inoculated with either 10^4 CFU of *B. subtilis* ATCC 6051A or 10^5 CFU of *B. subtilis* ATCC 55405 per gram of dry soil. There were no adverse effects on reproduction upon exposure to either strain, regardless of soil type. A significant increase in juvenile production was observed in the field-collected soil, relative to the field-collected soil negative control, after exposure to *B. subtilis* ATCC 55405.

Masked DSL *Bacillus* Strains

Murine exposure assays were conducted by Health Canada scientists. Female BALB/c mice remained asymptomatic after exposure to 10^6 CFU of *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 spores or vegetative cells administered in a 25 µL volume via an endotracheal nebulizer. Aside from a transient inflammatory response, no significant changes were observed (Appendix 12, Table A-59 to Table A-63).

1.1.3.2 Human Health

With the exception of *B. cereus*, *Bacillus* infections in humans are rare. They are diverse and tend to occur in immune compromised people (Pennington et al. 1976), or in association with implanted medical devices (Banerjee et al. 1988) or recent trauma (Logan 2012). Cases of non-*B. cereus* food poisoning caused by *Bacillus* species have been reported (Kramer and Gilbert 1989; Murray et al. 1995). In recent years however, there have been no reports of food poisoning incidents or outbreaks attributed to non-*B. cereus Bacillus* species (Sorokulova, personal communication). As potential contaminants of tobacco products, *Bacillus* species have been implicated in infections, pulmonary inflammation and allergic sensitivities and plasma exudation and tissue dysfunction in the mouth (Rooney 2005; Rubinstein and Pedersen 2002).

B. amyloliquefaciens

B. amyloliquefaciens is globally distributed in a variety of ecological niches and has a history of use in industrial fermentation and pest control. A comprehensive search of the scientific literature across all major sources yielded no reports of human infection linked to the species or of other adverse effects in humans from exposure to the organism, its metabolites or structural components.

Studies submitted to support pesticide registrations for *B. amyloliquefaciens* strains FZB24 and D747 included a variety of exposures in mammalian models used to predict adverse effects in humans (Appendix 11, Table A-51 and Table A-52). Oral, pulmonary and intravenous exposure studies using *B. amyloliquefaciens* strains FZB24 or D747 demonstrated low toxicity and no pathogenicity in CD and Sprague-Dawley rats at maximum challenge doses.

In studies conducted at Health Canada, female BALB/c mice were exposed to 10^6 CFU of *B. amyloliquefaciens* 13563-0 vegetative cells or spores administered in a 25 µL volume via an endotracheal nebulizer, as a model for human pulmonary exposure. The mice appeared normal and remained asymptomatic after exposures to vegetative cells and spores. All treated mice were necropsied 24 hours after exposure to vegetative cells or 1 week after exposure to spores to assess bacterial clearance, pulmonary cytokine expression and acute phase response (Appendix 12, Table A-59 to Table A-63). A statistically significant pro-inflammatory response was observed and some pulmonary cytokines were elevated 24 hours following exposure to vegetative cells. No significant changes were observed after one week following exposure to spores. Mice dosed with spores were not assessed for inflammation or cytokine expression at 24 hours, so the occurrence of transient inflammation would not have been detected. The serum amyloid A levels were slightly elevated in the acute phase response for both vegetative cells at 24 hours and spores at one week post-exposure.

No cases of hypersensitivity from glucanases or amylases produced by *B. amyloliquefaciens* have been reported (Caballero et al. 2007). No hypersensitivity incidents were reported during testing, production, use or handling of *B. amyloliquefaciens* biocontrol strains FZB24 and D747 in controlled laboratory settings during research and development (U.S. EPA 2011; U.S. EPA 2012).

B. atrophaeus

B. atrophaeus has a widespread distribution in nature and a history of environmental release as a surrogate organism for modelling airborne dispersal of pathogenic *Bacillus* species (Carrera et al. 2007; Page et al. 2007; U.S. EPA 2013a). A comprehensive search of the scientific literature across all major sources yielded no reports of human infection with *B. atrophaeus* or of other adverse effects in humans from exposure to the organism, its metabolites or structural components.

In studies conducted at Health Canada, female BALB/c mice were exposed to 10^6 CFU of *B. atrophaeus* 18250-7 vegetative cells or spores administered in a 25 µL volume via an endotracheal nebulizer, as a model for human pulmonary exposure. The mice appeared normal and remained asymptomatic after exposures to both spores and vegetative cells. All treated mice were necropsied 24 hours after exposure to vegetative cells or 1 week after exposure to spores to assess bacterial clearance, pulmonary cytokines expression and acute phase response (Appendix 12, Table A-59 to Table A-63). A statistically significant pro-inflammatory response was observed and some pulmonary cytokines were elevated 24 hours following exposure to vegetative cells. No significant changes were observed after one week following exposure to spores. Mice dosed with spores were not assessed for inflammation or cytokine expression at 24 hours, so the occurrence of transient inflammation would not have been detected. The serum amyloid A levels were slightly elevated in the acute phase response for both vegetative cells and spores.

No cases of hypersensitivity or allergenicity as a result of *B. atrophaeus*, its metabolites or structural components have been reported.

B. licheniformis

Although *B. licheniformis* is naturally present in high concentrations in a variety of environments to which humans are exposed, only 35 case reports of infection have been published in the English literature since 1966. Several were cases of bacteremia or septicemia, but a range of other infections were also reported (Blue et al. 1995; Castagnola et al. 1997; Cotton et al. 1987; Maucour et al. 1999; Murray et al. 1995; Tabbara and Tarabay 1979; Thurn and Goodman 1988). Almost all cases involved predisposing factors: immune deficiency, debilitating disease or significant breaches in natural barriers to infection.

B. licheniformis bacteremia was reported in patients with cancer (Banerjee et al. 1988; Ozkocaman et al. 2006), peritonitis (Sugar and McCloskey 1977), central venous catheters (Blue et al. 1995; Castagnola et al. 1997) and after a bronchoscopic procedure (Hong et al. 2004). It was also seen in association with foot lesions (Gayet et al. 2005) and in a pregnant woman (Peloux et al. 1976). Co-bacteremia of *B. licheniformis* and *B. subtilis* in an elderly patient with predisposing factors was also reported (La Jeon et al. 2012). In three cases of *B. licheniformis* septicemia, one was due to contaminated intravenous lines (Matsumoto et al. 2000), another followed arteriography (Hardy et al. 1986) and the third was in a pre-term infant (Lépine et al. 2009; Thomson et al. 1990). In two accounts, individuals deliberately injected themselves with products containing *B. licheniformis* spores (alone or in combination with spores of other *Bacillus* species), resulting in bacteremia (Galanos et al. 2009; Hannah and Ende, 1999). Bacteremia was recurrent in one case, possibly because spores, which were resistant to antibiotic treatment, remained in the tissues and germinated periodically (Hannah and Ender 1999). This kind of recurrent sepsis caused by *B. licheniformis* was also observed more recently, in an immune competent individual with no apparent underlying conditions (Haydushka et al. 2012).

B. licheniformis ophthalmitis or endophthalmitis (Maucour et al. 1999; Tabbara and Tarabay 1979; Thurn and Goodman 1988) and brain abscess (Jones et al. 1992) each resulting from penetrating eye trauma have been reported. A brain abscess caused by *B. licheniformis* was also described in a patient with acute myeloid leukemia (Mochiduki et al. 2007) and in a healthy patient which later progressed to a malignant brain tumour (Flores et al. 2001). In the last case, subsequent to being the causal organism in the formation of a brain abscess, *B. licheniformis* was postulated to be the oncogenic agent. Although conclusive evidence of such a causal relationship is lacking, *B. licheniformis* has been hypothesized to be an oncogenic bacterium along with others such as *Heliobacter pylori* (Wainwright and Al Talih 2003). Other infections with *B. licheniformis* include parotid gland abscess (Longo et al. 2003), a cutaneous infection as the result of injury (Ameur et al. 2005), prosthetic valve endocarditis (Santini et al. 1995), a pacemaker wire infection with bacteremia (Quan et al. 2000), post-operative ventriculitis

where *B. licheniformis* was isolated from cerebrospinal fluid (Young et al. 1982) and spondylitis in association with bacteremia in a lung cancer patient (Kim et al. 2012).

The safety of *B. licheniformis* strain MeI (isolated from milk) was assessed for use in the food industry (Appendix 11, Table A-54). The oral no observed adverse effect level (NOAEL) was greater than 1.1×10^{11} CFU/kg body weight in male albino Wistar rats (Nithya et al. 2012). Studies submitted to support pesticide registrations for *B. licheniformis* strain SB3086 included a variety of exposures in standard mammalian models used to predict adverse effects in humans (Appendix 11, Table A-53). Oral, pulmonary and intravenous exposure studies using *B. licheniformis* strain SB3086 demonstrated low toxicity and no pathogenicity in rats at maximum challenge doses.

Artificially immune depressed mice (BALB/c mice treated intraperitoneally with cyclophosphamide at 0.2 mg/g body weight), were dosed, intravenously with $<1 \times 10^{6}$ to 6×10^{10} CFU per animal of clinical, environmental and food isolates of *B. licheniformis,* including the type strain ATCC 14580 (Agerholm et al. 1997; Appendix 11, Table A-54). Despite the immune-depressed state of the mice, they were able to eliminate high numbers of the bacteria within one week, but *B. licheniformis* was recovered from the liver and spleen of most mice and from the kidneys of some mice one week after exposure. Some of the tested isolates caused pulmonary and brain lesions. Signs were only observed in two mice and no deaths attributed to treatment were reported. Given the high doses, zero treatment-related mortality and the clearance of most bacteria from tissues, all tested strains of *B. licheniformis* were considered to be of low pathogenicity in immune depressed mice.

In studies conducted at Health Canada, female BALB/c mice were exposed to 10^{6} CFU/25 µL of *B. licheniformis* ATCC 12713 vegetative cells or spores administered in a 25 µL volume via an endotracheal nebulizer, as a model for human pulmonary exposure. The mice appeared normal and remained asymptomatic after exposures to vegetative cells and spores. All treated mice were necropsied 24 hours after exposure to vegetative cells or 1 week after exposure to spores to assess bacterial clearance, pulmonary cytokine expression and acute phase response (Appendix 12, Table A-59 to Table A-63). A statistically significant pro-inflammatory response was observed and some pulmonary cytokines were elevated 24 hours following exposure to vegetative cells. An increase in serum amyloid A level in the acute phase response relative to the control was observed for vegetative cells of *B. licheniformis* ATCC 12713. No data regarding pulmonary cytokines or serum amyloid A level were available for exposure to spores of *B. licheniformis* ATCC 12713.

B. licheniformis has been reported in the literature as being implicated in outbreaks of food poisoning (Appendix 13). Endospore-forming bacteria, like *B. licheniformis*, along with heat-resistant toxic substances they produce, may survive pasteurization and other dairy processes as well as cooking temperatures (Biesta-Peters et al. 2010; Nieminen et al. 2007). For a toxic dose of enterotoxin to be produced in contaminated milk or other foods, cell counts of 10^{5} to 10^{9} CFU/g are estimated to be required (reviewed in Cosentino et al. 1997; Griffiths 1990; Logan, 2012; Lund, 1990;Rosenkvist and Hansen

1995; Salkinoja-Salonen et al. 1999). Food poisoning symptoms resulting from ingestion of *B. licheniformis*-contaminated food occur 5 to 12 hours after consumption (8 hour median). *B. licheniformis* food poisoning is similar to the diarrheal syndromes caused by *Clostridium perfringens* and *B. cereus* (reviewed in Drobniewski 1993; Kramer and Gilbert 1989). Death as a result of *B. licheniformis* food poisoning was reported in an infant that had consumed contaminated formula (Mikkola et al. 2000; Salkinoja-Salonen et al. 1999). Two *B. licheniformis* isolates obtained from the formula were reported to be toxigenic (Salkinoja-Salonen et al. 1999). *B. licheniformis* ATCC 14580 (the type strain) has been reported to be non-toxigenic (Pedersen et al. 2002). The DSL strain, *B. licheniformis* ATCC 12713, was tested at Health Canada for Hbl and Nhe toxin production and was not observed to produce these diarrheal toxins. Germination of spores and growth of *Bacillus* spp. in heat-treated raw milk and other foods produce "off-flavours" and poor appearance which may deter consumption and thereby prevent exposure (reviewed in Abo-Elnaga et al. 2002; Davies and Wilkinson 1973).

Glyphosate acetyltransferase from *B. licheniformis* used in an herbicide was evaluated for potential allergenicity and toxicity (Delaney et al. 2008). The authors concluded that at least in the context of agricultural biotechnology there are no expected adverse effects to humans and the potential for human exposure to the protein is low if expressed in transgenic plants (Delaney et al. 2008). *B. licheniformis* strain SB3086 has been screened for delayed contact sensitivity in guinea pigs and was determined to not be a dermal sensitizer. No reports of hypersensitivity or allergenicity implicating the DSL strain *B. licheniformis* ATCC 12713 have been described.

B. subtilis

B. subtilis bacteremia, septicemia and other infections have been reported (De Boer et al. 1991; reviewed in Drobniewski 1993; Ihde and Armstrong 1973; Logan 1988; Murray et al. 1995; Olszewski et al. 1999; Pennington et al. 1976; reviewed in Tuazon et al. 1979; Turnbull et al. 1979); however, *B. subtilis* infections are rare, and involve predisposing conditions including immune deficiency, debilitating disease and significant breaches in normal barriers to infection. Few cases of infection and no fatalities caused by *B. subtilis* have been reported since 1980.

B. subtilis bacteremia has been reported in cancer patients (Banerjee et al. 1988). Nosocomial bacteremia caused by *B. subtilis* was reported in four of eight patients with underlying conditions (cancer, head trauma and recent surgery) who had been given a probiotic containing *B. subtilis* spores (10^9 spores per tablet) (Richard et al. 1988). Septicemia caused by *B. subtilis* was reported in a young child (Cox et al. 1959) and in hospitalized patients who had intravenous lines (Matsumoto et al. 2000).

B. subtilis was implicated in a case of cellulitis that progressed to necrotizing fasciitis in a cancer patient (Tuazon et al. 1979). Infections where *B. subtilis* was implicated as the causative agent or a concomitant as the result of indwelling medical devices have been reported (Ihde and Armstrong 1973; Schoenbaum et al. 1975). Some reported *B. subtilis* infections were fatal (Ihde and Armstrong 1973; Pennington et al. 1976;

reviewed in Tuazon et al. 1979). In these cases, patients had serious co-morbidities and in some cases *B. subtilis* was thought to be a contaminant and its role as the causative agent was initially overlooked.

Studies submitted to support pesticide registrations for *B. subtilis* strains QST 713 and MBI 600 included a variety of exposures in standard mammalian models used to predict adverse effects in humans (Appendix 11, Table A-56 and Table A-57). Oral, pulmonary and intravenous exposure studies using *B. subtilis* strains QST 713 and MBI 600 demonstrated low toxicity and no pathogenicity in CD rats at maximum challenge doses.

In studies conducted at Health Canada, female BALB/c mice were exposed to 10^6 CFU of *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 vegetative cells and spores administered in a 25 µL volume via an endotracheal nebulizer, as a model for human pulmonary exposure. The mice appeared normal and remained asymptomatic after exposures to vegetative cells and spores. All treated mice were necropsied 24 hours after exposure to vegetative cells or 1 week after exposure to spores to assess bacterial clearance, pulmonary cytokine expression and acute phase response (Appendix 12, Table A-59 to Table A-63). Vegetative cells and spores were enumerated in the lungs, trachea and esophagus. Changes in cytokine level and serum amyloid A in the acute phase response were only reported for vegetative cells of *B. subtilis* subsp. *inaquosorum* ATCC 55406.

Endospore-forming bacteria such as *Bacillus* species, along with the heat-resistant toxic substances they produce, may survive pasteurization and other dairy processes (Nieminen et al. 2007). The proliferation of these micro-organisms in foods represents a potential food poisoning hazard (Beattie and Williams 1999). After consumption of food with high bacterial loads (10^5-10^9 CFU/g) *B. subtilis* food poisoning symptoms may begin 10 minutes to 14 hours (2.5 hour median) with acute onset of vomiting (Rosenkvist and Hansen 1995; Logan 2012). Foods often implicated are meat, seafood, pastry products and rice dishes. *B. subtilis* food poisoning has also been associated with spoiled (ropy) bread where the concentration of *B. subtilis* has been reported to be approximately 10^8 CFU/g. Foodborne illness due to ropy bread is unlikely given the unattractive appearance (discoloured, sticky and soft crumb) of the affected bread as a result of the high number of cells present which breakdown starch and proteins (Rosenkvist and Hansen 1995; Logan 2012; Lund 1990). The DSL strains *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406, were tested at Health Canada for Hbl and Nhe toxin production and were not observed to produce these diarrheal toxins.

In a recent article, liver damage was reported in patients who had consumed nutritional supplements which contained *B. subtilis* (Logan 2012). The strain was later demonstrated to be hepatotoxic in a Hep2G cell culture assay. Several strains of *B. subtilis* were tested in rats and other vertebrates and no negative effects were observed.

No hypersensitivity incidents were reported during testing, production or use of *B. subtilis* strains QST 713 or MBI 600 (PMRA-HC 2007b; PMRA-HC 2007c). *B. subtilis* MBI 600 was a moderate skin sensitizer 24 to 72 hours post challenge (PMRA-HC 2007c; U.S. EPA 2012).*B. subtilis* produces exoenzymes that facilitate the decay of organic matter (Tjalsma et al. 2004). Subtilisins are proteolytic enzymes produced by *B. subtilis* that are known to elicit allergic reactions including dermatitis and respiratory allergies in humans following repeated exposure (Juniper et al. 1977; Norris et al. 1981; Schweigert et al. 2000; Thorne et al. 1986; Tripathi and Grammer 2001; Weissman and Lewis 2002). *B. subtilis* has been reported to produce enzymes that cause symptoms associated with allergenicity including asthma and irritation (Flindt and Hendrick 2002).

Masked DSL *Bacillus* Strains

In studies conducted at Health Canada, female BALB/c mice were exposed to 10^6 CFU of *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 vegetative cells or spores administered in a 25 µL volume via an endotracheal nebulizer, as a model for human pulmonary exposure. The mice appeared normal and remained asymptomatic after exposures to vegetative cells and spores. All treated mice were necropsied 24 hours after exposure to vegetative cells or 1 week after exposure to spores to assess bacterial clearance, pulmonary cytokine expression and acute phase response (Appendix 12, Table A-59 to Table A-63). Vegetative cells and spores were enumerated in the lungs, trachea and esophagus. Changes in the cytokine levels following exposure to vegetative cells and spores of *Bacillus* species 16970-5, *Bacillus* species 2 18118-1 and *Bacillus* species 4 18121-4 were observed. *Bacillus* species 7 18129-3 was not tested. Changes in cytokine level and serum amyloid A in the acute phase response were only reported for vegetative cells of *Bacillus* species 16970-5, *Bacillus* species 2 18118-1 and *Bacillus* species 4 18121-4 and spores of *Bacillus* species 16970-5 and *Bacillus* species 2 18118-1.

1.2 Hazard Severity

Regular exposure to members of the *B. subtilis* complex occurs due to their widespread distribution in the environment (Murray et al. 1995). Strains can be found on dust particles which can be inhaled (Andersson et al. 1999). Dermal contact may occur as strains are commonly found in soils and on most surfaces (Logan and De Vos 2009; Murray et al. 1995; Thatoi et al. 2013). Despite the high natural exposure to these micro-organisms there is a low rate of reported infections (Rooney, personal communication). Furthermore, *B. subtilis* complex members have a history of use in biocontrol, growth promotion and as probiotics, all resulting in direct exposure to humans and environmental species, and without reported adverse effects. Finally, the DSL strains are widely used in a variety of sectors in Canada (see 2.1 Sources of Exposure) and no adverse effects have been reported in association with these uses.

1.2.1 Environmental Hazard

1.2.1.1 *B. amyloliquefaciens*

The environmental hazard severity for *B. amyloliquefaciens* 13563-0 is estimated to be low because no cases of infection or adverse effects in terrestrial and aquatic vertebrates, invertebrates and plants were found in the scientific literature. Testing of *B. amyloliquefaciens* pesticidal strains in terrestrial and aquatic vertebrates and invertebrates indicates low pathogenic or toxic potential. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. amyloliquefaciens* 13563-0 has low pathogenic potential. There is a history of safe use of *B. amyloliquefaciens* 13563-0 and of *B. amyloliquefaciens* pesticidal strains.

1.2.1.2 *B. atrophaeus*

The environmental hazard severity for *B. atrophaeus* 18250-7 is estimated to be low because information from the scientific literature indicates that *B. atrophaeus* has low toxic and pathogenic potential in terrestrial and aquatic vertebrates, invertebrates and plants and no adverse effects were reported. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. atrophaeus* 18250-7 has low pathogenic potential.

1.2.1.3 *B. licheniformis*

The environmental hazard severity for *B. licheniformis* ATCC 12713 is estimated to be low because information from the scientific literature indicates that *B. licheniformis* has low pathogenic potential to terrestrial or aquatic invertebrates or plants. Though *B. licheniformis* abortion occurs naturally in agricultural settings it is rare and under experimental conditions, doses required to establish infection in the bovine placenta were high and resulted in higher blood concentrations of bacteria than would be expected during infection under natural conditions. In the unlikely case of infection, relevant veterinary antibiotics against *B. licheniformis* ATCC 12713 are available. In addition, it has been used as a probiotic in brine shrimp, rainbow trout, pigs and chickens without negative effects reported. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. licheniformis* ATCC 12713 has low pathogenic potential (consistent with the *Bacillus* species assessed in this report).

1.2.1.4 *B. subtilis*

The environmental hazard severity for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 is estimated to be low because information from the scientific literature regarding *B. subtilis* indicates that it has a low toxic and pathogenic potential in terrestrial and aquatic vertebrates, invertebrates and plants. However, some adverse effects were reported following exposure to high concentrations of other strains of *B. subtilis.* Testing

of *B. subtilis* pesticidal strains in terrestrial and aquatic vertebrates and invertebrates generally indicates low pathogenic or toxic potential but some effects were observed in terrestrial and aquatic invertebrates. In testing conducted by Environment Canada scientists, significant reductions in mean shoot length in terrestrial plants and in juvenile production in terrestrial arthropods were observed after exposure to *B. subtilis* ATCC 6051A and *B. subtilis* ATCC 55405. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 have low pathogenic potential. There is a history of safe use for all the DSL *B. subtilis* strains.

1.2.1.5 Masked DSL *Bacillus* Strains

The environmental hazard severity for *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 is estimated to be low because testing conducted by Health Canada scientists in murine models and cell lines indicates that these strains have low pathogenic potential. There is a history of safe use of the masked DSL *Bacillus* strains.

1.2.2 Human Health Hazard

1.2.2.1 *B. amyloliquefaciens*

The human hazard severity for *B. amyloliquefaciens* 13563-0 is estimated to be low because information from the scientific literature indicates a low pathogenic potential and no cases of infection were reported. Testing of pesticidal strains of *B. amyloliquefaciens* in models of human infection indicates a low pathogenic or toxic potential. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. amyloliquefaciens* 13563-0 has low pathogenic potential. Antibiotic susceptibility testing performed by Health Canada scientists demonstrated that clinically relevant antibiotics are effective against this strain. There is a history of safe use of *B. amyloliquefaciens* 13563-0.

1.2.2.2 *B. atrophaeus*

The human hazard severity for *B. atrophaeus* 18250-7 is estimated to be low because information from the scientific literature indicates a low pathogenic potential and no cases of infection were reported. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. atrophaeus* 18250-7 has low pathogenic potential.Antibiotic susceptibility testing performed by Health Canada scientists demonstrated that clinically relevant antibiotics are effective against this strain. There is a history of safe use of *B. atrophaeus* 18250-7.

1.2.2.3 *B. licheniformis*

The human hazard severity for *B. licheniformis* ATCC 12713 is estimated to be low because information from the scientific literature indicates that there is some pathogenic potential, however, case reports are rare, and occur mostly in individuals with compromised immunity, debilitating disease or whose normal barriers to infection are breached by implanted medical devices or wounds. In one instance, recurrent sepsis was reported in an individual with no known predisposition who made full recovery. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. licheniformis* ATCC 12713 has low pathogenic potential.(consistent with the other *Bacilus* species assessed in this report) and no toxicity or pathogenicity was observed. *B. licheniformis*-associated food poisoning has been reported, however the DSL strain did not produce *B. cereus*-like toxins as demonstrated in testing done by Health Canada scientists. Mitigating factors such as off-flavours and appearance would likely discourage consumption of contaminated food. There is a history of safe use of *B. licheniformis* ATCC 12713.

Antibiotic susceptibility testing performed by Health Canada scientists first indicated that *B. licheniformis* ATCC 12713 is resistant to many of the antibiotics it was tested against (most for which interpretive criteria were available, excepting tetracycline and rifampicin); however, after further investigation it was concluded that the apparent high resistance observed was an artefact of the liquid culture MIC assay. Reinterpreted using a 95% bioreduction activity cut-off, the susceptibility profile was consistent with values in the literature on the species, and for vancomycin, this was confirmed using a commercial test-strip method.

1.2.2.4 *B. subtilis*

The human hazard severity for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 is estimated to be low because information from the scientific literature indicates that there is some pathogenic potential in individuals with compromised immunity or whose normal barriers to infection are breached. However, the number of reports is limited, most reports pre-date 1980 and no fatalities have since been reported. Testing conducted by Health Canada scientists in murine models and cell lines indicates that *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 have low pathogenic potential. Although *B. subtilis*-associated food poisoning has been reported, the DSL strains do not produce *B. cereus*-like toxins as demonstrated in testing done by Health Canada scientists. Mitigating factors such as off-flavours and appearance would likely discourage consumption of contaminated food. Testing of pesticidal strains of *B. subtilis* in models of human infection indicates a low pathogenic or toxic potential. There is a history of safe use of the DSL strains.

1.2.2.5 Masked DSL *Bacillus* strains

The human hazard severity for *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 is estimated to be low because Testing conducted by Health Canada scientists in murine models and cell lines indicates that these strains have low pathogenic potential. There is a history of safe use of the masked DSL *Bacillus* strains.

2. Exposure Assessment

2.1 Sources of Exposure

This assessment considers exposure to the DSL *B. licheniformis/subtilis* group strains resulting from their addition to consumer or commercial products and their use in industrial processes in Canada.

The DSL *B. licheniformis/subtilis* group were nominated to the DSL for use in consumer and commercial productsincluding products for cleaning and deodorizing, drain cleaning and degreasing, RV/septic tank treatment and in bioremediation and biodegradation, waste and wastewater treatment and water conditioning.

Responses to a voluntary questionnaire sent in 2007 to a subset of key biotechnology companies, combined with information obtained from other federal government regulatory and non-regulatory programs, indicate that DSL *B. licheniformis/subtilis* group strains were in commercial use in 2006. No information on uses of *B. atrophaeus* was collected at this time, as it was nominated to the DSL after the survey took place.

The Government conducted a mandatory information-gathering survey under section 71 of CEPA 1999, as published in the *Canada Gazette*, Part I, on October 3, 2009 (section 71 Notice). The section 71 Notice applied to any persons who, during the 2008 calendar year, manufactured or imported strains of the DSL *B. licheniformis/subtilis* groupwhether alone, in a mixture, or in a product. Commercial or consumer activity was reported for these micro-organisms in a variety of different sectors (for quantities and concentrations see Table 2-1). Uses reported for members of the DSL *B. licheniformis/subtilis* group include biodegradation; biological waste treatment; bioremediation; custodial cleaning and other related products; drain cleaning and degreasing; fragrance, perfume or deodorizer; enzyme and chemical production; research and development; septic tank or recreational vehicle tank additive; and waste and wastewater treatment. No information on uses of *B. atrophaeus* was collected through the section 71 Notice, as it was nominated to the DSL after the survey took place.

Table 2-1: Quantities of DSL *B. licheniformis/subtilis* group strains reported to be imported or manufactured in Canada in 2009[a]

Species[b]	Total Amount Range[c] (kg)	Concentration range[d](CFU/mL)
Bacillus amyloliquefaciens	10,000-100,000	2.0×10^{8} to 1.0×10^{11}

Bacillus licheniformis	100,000-1,000,000	4.0×10^6 to 1.0×10^{11}
Bacillus subtilis[e]	100,000-1,000,000	1.0×10^5 to 1.0×10^{11}

[a] No information on uses of *B. atrophaeus* was collected through the Notice as it was nominated to the DSL after the survey took place
[b] Includes all DSL strains of the species
[c] Combined amount of all products containing the micro-organisms manufactured in or imported to Canada
[d] Concentration range of micro-organisms reported to be imported or manufactured in Canada
[e] Including *B. subtilis* subsp. *inaquosorum* ATCC 55406

A search of the public domain (internet, patent databases, MSDS, etc.) suggests multiple potential uses of the *B. subtilis* complex including the DSL *B. licheniformis/subtilis* group strains.

B. amyloliquefaciens

- As a production micro-organism of enzymes (e.g. amylase, isoprene, protease, non-structural protein 3, ribonuclease, and phytases), biosurfactants, antibiotics and detergents which have industrial and commercial applications (Madslien et al. 2012) including cleaning, degreasing, antibacterial applications (ATCC 2012c; James et al. 1995; Madslien et al. 2012; Moons et al. 2009; Pérez-García et al. 2011; Rendueles and Ghigo, 2012; Rivardo et al. 2009).
- Application to surfaces to favour the formation of a *B. amyloliquefaciens* biofilm to displace undesirable or unknown micro-organisms (James et al. 1995; Moons et al. 2009; reviewed in Rendueles and Ghigo 2012; Rivardo et al. 2009).
- Application in a mixture with other bacterial species for water and wastewater treatment to treat algal blooms, odours and sludge build-up (Advanced Water Technologies 2012; RoeTech 2014).

B. atrophaeus

- Use of spores as a surrogate for weaponized *B. anthracis* in fine-tuning of defense monitoring equipment and as a challenge agent (Blecka et al. 2012; Carrera et al. 2007; Grinshpun et al. 2012; Page et al. 2007; U.S. EPA 2013a).
- Use of spores to test the efficacy of sterilization by dry heat, ethylene oxide and steam sterilization as part of quality assurance and control in the production of pharmaceutical and personal care products (ATCC 2012d).
- Pathogen transmission modelling (Gerhardts et al. 2012).

B. licheniformis

- As a production organism of enzymes and biosurfactants including alpha-amylase, lichenysin, pentosanases, deoxyribonuclease (NucB), nitroreductase and levansucrase (ATCC 2013; reviewed in Komolprasert and Ofoli 1991; Moons et al. 2009; Nerurkar, 2010; reviewed in Rendueles and Ghigo 2012; Rey et al. 2004; Rivardo et al. 2009; Thatoi et al. 2013; Yakimov and Golyshin 1997).
- Biosynthesis of silver nanocrystals (Kalimuthu et al. 2008) and gold nanocubes (Kalishwaralal et al. 2009).

- Degradation of feather waste generated by poultry farms and processing plants (Ichida et al. 2001).
- Bioremediation of heavy metals (e.g. zinc, cadmium and aluminum) (Kamika and Momba 2013).
- Water and wastewater treatment to reduce algal blooms, odours and sludge build-up (Advanced Water Technologies 2012).
- Bioindicator of the toxicity of sediment elutriates (Campbell et al. 1993).
- Beneficial biofilm formation (James et al. 1995; Moons et al. 2009; reviewed in Rendueles and Ghigo 2012; Rivardo et al. 2009).
- In probiotic products for humans and animals (Cutting 2011; Nithya et al. 2012).

B. subtilis

- As a production organism of lipopeptides (biosurfactants), enzymes (e.g. amylase, protease and antibiotic compounds (e.g. aterrimin) and isoprene (ATCC 2012b; ATCC, 2012f; Moons et al. 2009; Rendueles and Ghigo 2012; Rivardo et al. 2009; reviewed in Thatoi et al. 2013).
- Water and wastewater treatment to reduce algal blooms, odours, sludge build-up, septic tanks and agricultural waste pits (Advanced Water Technologies 2012; RoeTech 2014).
- Fermentation of traditional foods (Inatsu et al. 2006; Leejeerajumnean 2003).
- Beneficial biofilm formation (James et al. 1995; Moons et al. 2009; reviewed in Rendueles and Ghigo 2012; Rivardo et al. 2009).
- Use of spores to test sterility assurance and in bacterial resistance of latex paint (ATCC 2012f).
- Applications in research as a bacteriophage host (ATCC 2012f).
- Diagnostic applications in blood screening for phenylketonuria (ATCC 2012f).
- Application in the production of feed supplements (ATCC 2012a).
- In probiotic products for humans and animals (Cutting 2011).

2.2 Exposure Characterisation

2.2.1 Environment

2.2.1.1 *B. atrophaeus*

Environmental exposure to *B. atrophaeus* 18250-7 is possible for terrestrial species, and to a lesser extent aquatic species, during its environmental release as a surrogate organism for *B. anthracis* in dispersal modelling and fine tuning of defense monitoring equipment. The extent of exposure will depend on the method of release, release volume, weather conditions and wind velocity. In general, exposure is expected to be low for these applications as it is a specialized activity occurring at a single, remote site in Canada. Inhalation would be the main route of exposure. Exposure as the result of dermal contact with contaminated surfaces and inadvertent ingestion through secondary contamination of food resources is expected to be low. The overall environmental exposure estimation for *B. atrophaeus* 18250-7 is low.

2.2.1.2 *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis* and masked DSL *Bacillus* strains

Environmental exposure to the other DSL *B. licheniformis/subtilis* strains will be considered together, as the known and potential uses are similar.

Members of the *B. subtilis* complex have the ability to adapt to and thrive in many terrestrial and aquatic habitats. Numerous physiological variants exist in nature, making the complex highly successful in nearly every environment. Despite the widespread distribution of the species complex, there is evidence to demonstrate a decline in introduced populations artificially inoculated into soil microcosms and marine environments (Medina et al. 2003; Nybroe et al. 1992). High numbers of vegetative cells are unlikely to be maintained in water or soil due to competition for nutrients (Leung *et al.* 1995) and microbiostasis, which is an inhibitory effect of soil, resulting in the rapid decline of populations of introduced bacteria (Van Veen et al. 1997).

To estimate expected environmental concentrations from expected applications, case studies in bioremediation and wastewater treatment were explored. A mixture of *Bacillus* species including *B. amyloliquefaciens* and *B. subtilis* (up to 10^{11} CFU/g) was added to treat municipal wastewater at a rate of 7.5 ppm of flow (RoeTech 2014), resulting in a concentration up to 7.5×10^5 CFU/mL in the treated wastewater. In a bench scale proof of concept study, 1.5×10^9 cells of a strain of *B. subtilis* were added to 60 g of petroleum hydrocarbon contaminated soil for a final concentration of 2.5×10^7 cells/g (Wu et al. 2013). Such concentrations are unlikely to be maintained in wastewater effluent or soils as vegetative cells of the DSL *B. licheniformis/subtilis* strains do not have any competitive advantage over naturally-occurring populations of similar micro-organisms and would be subject to competition for nutrients with indigenous flora. Populations of vegetative cells of DSL *B. licheniformis/subtilis* strains introduced to soil and water will likely decrease to background levels over time. Under sub-optimal conditions, spores of the DSL *B. licheniformis/subtilis* strains are likely to persist and accumulate in the environment.

Exposure to the DSL strains is expected to be greatest for organisms in and around the vicinity of direct application to aquatic ecosystems for water treatment (e.g. aquaria and ponds) or to soils for bioremediation of contaminants.

Indirect exposure of environmental species resulting from the use and disposal of cleaning products is expected to be low relative to direct applications to aquatic ecosystems or soils. Growth in the market for “greener” microbial-based products may, however, increase such exposures (Spök and Klade 2009).

No relevant reports concerning the persistence of toxins produced by strains of the *B. subtilis* complex in the environment were found in a comprehensive search of the scientific literature over a number of sources.

The environmental exposure to the other DSL *B. licheniformis/subtilis* strains is expected to be medium based on the wide range of uses reported in response to the Notice.

2.2.2 Humans

2.2.2.1 *B. atrophaeus*

Human exposure to *B. atrophaeus* 18250-7 is possible for bystanders during its environmental release as a surrogate organism for *B. anthracis* in dispersal modelling and fine tuning of defense monitoring equipment. The extent of exposure will depend on the method of release, release volume, weather conditions, wind velocity and the proximity of bystanders to the site of application. In general, exposure is expected to be low for these applications as it is a specialized activity occurring at a single, remote site in Canada. Inhalation would be the main route of exposure. Exposure as the result of dermal contact with contaminated surfaces and inadvertent ingestion through secondary contamination of foodstuffs is expected to be low. The overall human exposure estimation for *B. atrophaeus* 18250-7 is low.

2.2.2.2 *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis* and masked DSL *Bacillus* strains

Human exposure to the other DSL *B. licheniformis/subtilis* strains will be considered together, as the known and potential uses are similar.

Human exposure is expected to be greatest through the direct use of consumer products containing spores or viable cells used for cleaning or water treatment. Handling and application of such products would be expected to result in direct exposure of the skin and inhalation of aerosolized droplets or lofted spores. Inadvertent ingestion following use on or near food preparation surfaces and contact with the eyes, are possible secondary routes of exposure.

Humans may also be exposed as bystanders during commercial application of cleaning, water treatment, agricultural or biodegradation products. The extent of bystander exposure will depend on the mode of application, the volume applied and the proximity of bystanders to the site of application. In general, exposure is expected to be low for these applications.

Indirect human exposure to the DSL *B. licheniformis/subtilis* strains released into the environment subsequent to their use in water treatment, agricultural applications or biodegradation is also expected to occur in the vicinity of treated sites, but is expected to be less than direct exposure from the use of these organisms in consumer products. Human exposure to bodies of water and soils treated with the DSL *B. licheniformis/subtilis* strains (e.g., through recreational activities), could result in exposure of the skin and eyes, as well as inadvertent ingestion; however, dilution of these products is expected to significantly reduce exposure relative to household

application scenarios. Human activity on soils recently treated with the DSL *B. licheniformis/subtilis* strains could loft spores, which could then be inhaled and could expose the skin and eyes, but this exposure is also expected to be low relative to direct use of consumer products.

Release of the DSL *B. subtilis/licheniformis* strains from facilities manufacturing enzymes or biochemicals could occur, but is expected to be limited by the application of good manufacturing practices, in which measures should be taken to minimise the probability of releases of production micro-organisms.

For uses of pre- or probiotics containing spores of *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* strains, direct exposure would be principally by oral ingestion. Indirect exposure could occur following disposal of probiotics or through shedding in feces into the wastewater system. In the case of feces or disposal into the sewage system, municipal wastewater treatment would be expected to reduce the microbial burden prior to the release of effluent into the environment. Human exposure to the strains through the environment is expected to be low. Disposal of unused probiotics to municipal landfills is not expected to result in significant human exposure.

In the event that spores of the DSL *B. subtilis/licheniformis* group enter the source waters of municipal drinking water treatment systems through release from intended and potential uses, drinking water treatment processes (e.g. coagulation, flocculation, ozonation, filtration and chlorination) are expected to effectively eliminate these micro-organisms and so limit their ingestion.

Exposure to the other DSL B. subtilis/licheniformis strains is expected to be medium from the use of consumer products and low for indirect exposures subsequent to environmental release for biodegradation, bioremediation and water and wastewater treatment or release of effluents from facilities manufacturing enzymes and biochemicals.

Growth in the market for “greener” microbial-based products may increase direct human exposure to the DSL *B. subtilis/licheniformis* group which have potential applications in these products (Spök and Klade 2009).

3. Risk Characterisation

In this assessment, risk is characterized according to a paradigm embedded in section 64 of CEPA 1999 that a hazard and exposure to that hazard are both required for there to be a risk. The risk assessment conclusion is based on the hazard, and on what is known about exposure from current uses.

The determination of risk from current uses is followed by consideration of the estimated hazard in relation to foreseeable future exposures (from new uses).

B. amyloliquefaciens

Hazard has been estimated for *B. amyloliquefaciens* 13563-0 to be low for both the environment and human health. Environmental exposure to *B. amyloliquefaciens* 13563-0 is expected to be medium based on the wide range of uses reported in response to the section 71 Notice. Human exposure is expected to be medium for direct use of consumer products and low for indirect exposures subsequent to environmental release based on the wide range of uses reported in response to the section 71 Notice. The risk associated with current uses is estimated to be low for both the environment and human health.

Growth in the market for "greener" microbial-based products may increase human exposure to the DSL *B. subtilis/licheniformis* group which have potential applications in these products (Spök and Klade 2009), however the risk from foreseeable future uses is also expected to be low, given the low hazard associated with *B. amyloliquefaciens* 13563-0.

B. atrophaeus

Hazard has been estimated for *B. atrophaeus* 18250-7 to be low for both the environment and human health. Environmental exposure to *B. atrophaeus* 18250-7 is expected to be medium and human exposure is expected to be low based on the known uses. The risk associated with current uses is estimated to be low for both the environment and human health.

The risk from foreseeable future uses is also expected to be low, given the low hazard associated with *B. atrophaeus* 18250-7.

B. licheniformis

Hazard has been estimated for *B. licheniformis* ATCC 12713 to be low for both the environment and human health because the scientific literature and laboratory results specific to the DSL strain indicate a low pathogenic potential (consistent with the other strains under assessment), and there is a history of safe use of the DSL strain. *B. licheniformis* has been associated with livestock abortion. Routes of exposure leading to *B. licheniformis* abortion in livestock are thought to includeingestion of poor-quality, mouldy feed during gestation and subsequent hematogenous spread to the reproductive tract as well as introduction during general animal husbandry activities (e.g. natural breeding, artificial insemination, parturition and during examination) (Cabell, 2007; Scott, 2011; Goncagul, 2012). Current applications of the DSL strain are not expected to significantly increase exposure of livestock by these routes. Environmental exposure to *B. licheniformis* ATCC 12713 is expected to be medium based on the wide range of uses reported in response to the section 71 Notice. Human exposure is expected to be medium for direct use of consumer products and low for indirect exposures subsequent to environmental releases based on the wide range of uses reported in response to the section 71 Notice. The risk associated with current uses is estimated to be low for both the environment and human health.

Growth in the market for "greener" microbial-based products may increase human exposure to the DSL *B. subtilis/licheniformis* group which have potential applications in these products (Spök and Klade, 2009), however, the risk from foreseeable future uses

is expected remain low for both humans and the environment given the low hazard associated with *B. licheniformis* ATCC 12713.

B. subtilis

Hazard has been estimated for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 to be low for both the environment and human health. Environmental exposure to *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 is expected to be medium based on the wide range of uses reported in response to the section 71 Notice. Human exposure is expected to be medium for direct use of consumer products and low for indirect exposures subsequent to environmental release based on the wide range of uses reported in response to the section 71 Notice. The risk associated with current uses is estimated to be low for both the environment and human health.

Growth in the market for "greener" microbial-based products may increase human exposure to the DSL *B. subtilis/licheniformis* group which have potential applications in these products (Spök and Klade, 2009), however, the risk from foreseeable future uses is also expected to be low, given the low hazard associated with *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *B. subtilis* subsp. *inaquosorum* ATCC 55406 associated with both human and environmental health.

Masked DSL *Bacillus* Strains

Hazard has been estimated for *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 to be low for both the environment and human health based on laboratory results specific to the masked DSL strains and a history of safe use. Environmental exposure to *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4 and *Bacillus* species 7 18129-3 is expected to be medium based on the wide range of uses reported in response to the section 71 Notice. Human exposure is expected to be medium for direct use of consumer products and low for indirect exposures subsequent to environmental release based on the wide range of uses reported in response to the section 71 Notice. The risk associated with current uses is estimated to be low for both the environment and human health.

Growth in the market for "greener" microbial-based products may increase human exposure to the DSL *B. subtilis/licheniformis* group which have potential applications in these products (Spök and Klade 2009), however, the risk from foreseeable future uses is also expected to be low, given the low hazard associated with these strains.

4. Conclusions

Based on information presented in this Screening Assessment, it is concluded that *Bacillus amyloliquefaciens* 13563-0, *Bacillus atrophaeus* 18250-7, *Bacillus licheniformis* ATCC 12713, *Bacillus subtilis* ATCC 6051A, *Bacillus subtilis* ATCC 55405, *Bacillus subtilis* subsp. *subtilis* ATCC 6051, *Bacillus subtilis* subsp. *inaquosorum* ATCC 55406, *Bacillus* species 16970-5, *Bacillus* species 2 18118-1, *Bacillus* species 4 18121-4,

Bacillus species 7 18129-3 are not entering the environment in a quantity or concentration or under conditions that:

- have or may have an immediate or long-term harmful effect in the environment or its biological diversity;
- constitute or may constitute a danger to the environment on which life depends; or
- constitute or may constitute a danger in Canada to human life or health.

Therefore, it is proposed that the DSL *Bacillus licheniformis/subtilis* group strains do not meet the criteria as set out in section 64 of CEPA 1999.

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A. Appendices

Appendix 1: Colony morphologies of DSL *B. licheniformis/subtilis* group members

Table A-1: Colony morphologies *of B. amyloliquefaciens* 13563-0

Characteristic	TSB agar after 7 days of growth at room temperature[a]	Spizizen potato agar or broth (ATCC Medium 423) at 37°C verified at 24 hours (for solid medium, add 1.5% agar) [a]
Shape	Irregular	Irregular
Size (mm) diameter	5	5
Margin	Undulate	Spreading, irregular edge
Elevation	Flat	No data
Colour/pigment	Off-white	White
Texture	Dull	Smooth, dull
Opacity	Opaque	No data

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch

Table A-2: Colony morphologies of *B. atrophaeus* 18250-7

Characteristic	TSB agar after 24 hours of growth at room temperature [a]	TSB agar after 7 days of growth at room temperature [a]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C after 24 hours under aerobic conditions [b]	Nutrient agar or broth (ATCC medium #3) at 30°C after 24 hours under aerobic conditions [b]	Nutrient agar or broth (ATCC medium #3) at 30°C after 24 hours under aerobic conditions [b]
Shape	Circular	Irregular	Circular	Circular	Circular	Irregular
Size (mm) diameter	2	5-10	0.5	No data	No data	No data
Margin	Entire	Undulate	Entire	Entire	Entire	Undulate
Elevation	Flat	Flat	No data	Low convex	Low convex	Flat
Colour/ pigmentation[a]	White	Off-white/beige	White	Orange	No data	White
Texture	Smooth, moist	Smooth, moist	No data	Glistening	Glistening	No data
Opacity	Opaque	Opaque	No data	No data	Opaque	No data

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] ATCC description, multiple colony morphologies

2068 **Table A-3: Colony morphologies *of B. licheniformis* ATCC 12713**

Characteristic	TSB agar after 7 days of growth at room temperature[a, b]	TSB agar after 7 days of growth at room temperature[a, b]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C for 24 hours under aerobic conditions[c]	Nutrient agar or broth (ATCC medium #3) at 30°C for 24 hours under aerobic conditions[c]
Shape	Circular	Irregular	Irregular	No data	Irregular
Size (mm) diameter	5-7	5-7	2	No data	No data
Margin	Undulate	Undulate-lobate	Undulate, filiform	No data	No data
Elevation	Flat	Umbonate	Raised	Raised	Convex
Colour/pigment	Beige/off-white	Beige/off-white	No data	No data	No data
Texture	Moist, smooth	Wrinkled, dry	Dry	Dry, wrinkled	Mucoid
Opacity	Semi-translucent	Opaque	Opaque	No data	No data

2069 [a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
2070 [b] Colonies stick to agar, multiple colony morphologies
2071 [c] ATCC description, multiple colony morphologies

2072 **Table A-4: Colony morphologies of *B. subtilis* ATCC 6051A**

Characteristic	TSB agar after 7 days of growth at room temperature[a]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]
Shape	Circular to irregular	Irregular	Irregular and spreading with age	Irregular and spreading with age
Size (mm) diameter	6 to 25	2	Larger	Smaller
Margin	Undulate	Entire	Erose	Entire
Elevation	Flat	Flat	Flat	Flat
Colour/pigment	Off-white	Off-white	Beige/cream	Beige/cream
Texture	Moist	Dry	Dull, rougher	Dull, smoother
Opacity	Translucent	Semi-translucent	Opaque	Opaque

2073 [a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
2074 [b] ATCC description, multiple colony morphologies

Table A-5: Colony morphologies of *B. subtilis* ATCC 55405

Characteristic	TSB agar after 7 days of growth at room temperature[a, b]	TSB agar after 7 days of growth at room temperature[a, b]	TSB agar after 7 days of growth at room temperature[a, b]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[c]
Shape	Irregular to circular	Circular	Irregular to circular	2	Circular
Size (mm) diameter	4	4	4	No data	No data
Margin	Entire	Undulate	Lobate	Entire	Entire
Elevation	Convex	Flat	Raise	Convex	Convex
Colour/pigment	Colourless to off-white	Off-white	White	Colourless	No data
Texture	Glossy, mucoid	Matte, dry	Flat, brittle, dry	Mucoid	Mucoid, glistening
Opacity	Opaque	Translucent to opaque	Opaque	No data	Opaque

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] Multiple colony morphologies
[c] ATCC description

Table A-6: Colony morphologies of *B. subtilis* subsp. *subtilis* ATCC 6051

Characteristic	TSB agar after 7 days of growth at room temperature[a]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]
Shape	Irregular	Irregular	Circular	Irregular
Size (mm) diameter	20	No data	No data	No data
Margin	Undulate	Undulate	Entire	No data
Elevation	Raised	ND	Low convex	Flat
Colour/pigment	Off-white	Off-white	No data	No data
Texture	Dry	No data	Shiny	Rough
Opacity	Opaque	Opaque	Opaque	Opaque

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] ATCC description, multiple colony morphologies

Table A-7: Colony morphologies of *B.subtilis* subsp. *inaquosorum* ATCC 55406

Characteristic	TSB agar after 7 days of growth at room temperature[a,b]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar (ATCC medium #3) at 30°C verified at 24 hours[c,d]	Nutrient agar (ATCC medium #3) at 30°C verified at 24 hours[c,d]
Shape	Circular	Irregular	Circular	Circular
Size (mm) diameter	5	1	No data	No data
Margin	Undulate	No data	Erose	No data
Elevation	Flat	No data	Flat	No data
Colour/pigment	White	Colourless	Cream	White
Texture	Dull	No data	No data	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] Colonies grow into agar
[c] ATCC description, multiple colony morphologies
[d] Colonies dig into agar; this organism grows better on solid media than in a broth

Table A-8: Colony morphologies of *Bacillus* species 16970-5

Characteristic	TSB agar after 7 days of growth at room temperature[a]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]
Shape	Irregular	Irregular	Circular
Size (mm) diameter	12 to 20	2	No data
Margin	Undulate	No data	Some with lobate margins
Elevation	Flat	Flat	No data
Colour	Off-white	Off-white	No data
Texture	Dull	Dry	Shiny, smooth
Opacity	Opaque	Opaque	Opaque

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] ATCC description

Table A-9: Colony morphologies of *Bacillus* species 2 18118-1

Characteristic	TSB agar after 7 days of growth at room temperature[a]	Spizizen potato agar or broth (ATCC Medium 423) at 37°C verified at 24 hours (for solid medium, add 1.5% agar)[a]	Spizizen potato agar or broth (ATCC Medium 423) at 37°C verified at 24 hours (for solid medium, add 1.5% agar)[b]	Spizizen potato agar or broth (ATCC Medium 423) at 37°C verified at 24 hours (for solid medium, add 1.5% agar)[b]
Shape	Irregular	Circular	Circular	No data
Size (mm) diameter	5-12	5	No data	No data
Margin	Undulate	Slightly irregular edge	Slightly irregular	Dull spreading irregular edge
Elevation	Umbonate, raised	No data	No data	No data
Colour/pigment	Whitish	No data	No data	No data
Texture	Moist, shiny and dull	ND	Smooth, glistening	Glistening, smooth center
Opacity	Opaque	Opaque	Opaque	No data

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] ATCC description, multiple colony morphologies

Table A-10: Colony morphologies of *Bacillus* species 4 18121-4

Characteristic	TSB agar after 7 days of growth at room temperature[a]	TSB agar after 7 days of growth at room temperature[a]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 37°C for 24 hours under aerobic conditions[b]	Nutrient agar or broth (ATCC medium #3) at 37°C for 24 hours under aerobic conditions[b]
Shape	Circular	Elliptical	Irregular	Irregular	Rhizoid
Size (mm) diameter	5-7	5-7	2	No data	No data
Margin	Undulate	Undulate	Undulate, filiform	Undulate	Filamentous
Elevation	Imperfect-umbonate	Flat	Raised	Convex	Raised
Colour	Beige/off-white	Beige/off-white	No data	Translucent	No data
Texture	Moist	Matte	Dry	Smooth, glistening	Rough
Opacity	Opaque	Opaque	Opaque	No data	Opaque

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] ATCC description, multiple colony morphologies

Table A-11: Colony morphologies of *Bacillus* species 7 18129-3

Characteristic	TSB agar after 7 days of growth at room temperature[a]	Nutrient agar at 30°C for 24 hours[a]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]	Nutrient agar or broth (ATCC medium #3) at 30°C verified at 24 hours and up to one week under aerobic conditions[b]
Shape	Irregular	Irregular	Circular	Irregular
Size (mm) diameter	20	No data	No data	No data
Margin	Undulate	Undulate	Entire	No data
Elevation	Raised	No data	Low convex	Flat
Colour	Off-white	Off-white	No data	No data
Texture	Dry	No data	Shiny	Rough
Opacity	Opaque	Opaque	Opaque	Opaque

[a] Data generated by Health Canada's Healthy Environments and Consumer Safety Branch
[b] ATCC description, multiple colony morphologies

Appendix 2: Characteristics of DSL *B. licheniformis/subtilis* group members – 16S ribosomal RNA gene sequence analysis

16S ribosomal RNA gene sequence data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Restriction fragment length polymorphisms from within the V3 region and between the V4 and V5 region were identified according to Jeyaram *et al.* 2011. The 16S ribosomal RNA gene sequences were compared to the Ribosomal Database project release 11 (https://rdp.cme.msu.edu/) and top 10 matches are shown. The match hit format is: identification code, similarity score (if reference strain is specified), S_ab score, unique common oligomers and sequence full name.

Table A-12: Results of 16S Ribosomal RNA Gene Sequence Analysis of *B. amyloliquefaciens* 13563-0[a]

Short identification	Similarity score	S_ab score	Unique common oligomers	Sequence full name
S001153538	1.000	1.000	1364	*Bacillus* sp. XI; EU779996
S001550906	1.000	1.000	1371	*Bacillus subtilis*; Y2; GQ148813
S001588402	1.000	1.000	1374	*Bacillus amyloliquefaciens*; IMAU80205; GU125623
S001745899	1.000	1.000	1393	*Bacillus amyloliquefaciens*; PBT; FJ169495
S002038639	1.000	1.000	1354	*Bacillus amyloliquefaciens*; HK1; AB279736
S002222255	1.000	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002222257	1.000	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002222259	1.000	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002222261	1.000	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002222263	1.000	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644

[a] The *HinfI* site is present and the *RsaI* sites are absent indicating the micro-organisms is *B. amyloliquefaciens* as opposed to *B. subtilis* to which it is closely related (Jeyaram et al. 2011).

Table A-13: Results of 16S Ribosomal RNA Gene Sequence Analysis of *B. atrophaeus* 18250-7[a]

Short identification	Similarity score	S_ab score	Unique common oligomers	Sequence full name
S000382399	Not calculated	1.000	1432	*Bacillus* sp.; SSA3; AB017587
S000644416	Not calculated	1.000	1436	*Bacillus atrophaeus*; SCH0408; AY881241
S000980555	Not calculated	1.000	1337	*Bacillus atrophaeus*; K01-03; EU326483
S001872424	Not calculated	1.000	1411	*Bacillus subtilis*; JAM A-6-10; AB542912

S002035172	Not calculated	1.000	1375	*Bacillus atrophaeus*; NMTD54; GU568183
S002035195	Not calculated	1.000	1379	*Bacillus atrophaeus*; GBSC56; GU568206
S002166857	Not calculated	1.000	1376	*Bacillus atrophaeus*; RJGP16; GU969134
S002167105	Not calculated	1.000	1372	*Bacillus atrophaeus*; LSSC3; GU994860
S002221550	Not calculated	1.000	1464	*Bacillus atrophaeus* 1942; CP002207
S002221552	Not calculated	1.000	1464	*Bacillus atrophaeus* 1942; CP002207

[a] *B. atrophaeus* 18250-7 ribosomal RNA gene sequence matches *B. atrophaeus* and *Bacillus* sp. sequences *and the HinfI* site is present for the *B. subtilis/licheniformis* group (Jeyaram et al. 2011).

Table A-14: Results of 16S Ribosomal RNA Gene Sequence Analysis of *B. licheniformis* ATCC 12713[a]

Short identification	Similarity score	S_ab score	Unique common oligomers	Sequence full name
S000392549	Not calculated	1.000	1427	*Bacillus licheniformis*; Mo1; AF372616
S000615411	Not calculated	1.000	1409	*Bacillus licheniformis*; ACO1; DQ228696
S000647676	Not calculated	1.000	1421	*Bacillus licheniformis*; K19; DQ351932
S000736754	Not calculated	1.000	1448	*Bacillus licheniformis*; BCRC 15413; DQ993676
S000752038	Not calculated	1.000	1409	*Bacillus licheniformis*; EF059752
S000824918	Not calculated	1.000	1422	*Bacillus licheniformis*; BCRC 12826; EF423608
S000843501	Not calculated	1.000	1442	*Bacillus* sp. J24; EF471917
S000901702	Not calculated	1.000	1389	*Bacillus licheniformis*; NBRC 12107; AB354236
S000941823	Not calculated	1.000	1389	*Bacillus licheniformis*; NBRC 12202; AB363734
S001153503	Not calculated	1.000	1319	*Bacillus licheniformis*; SVD1; EU770587

[a] *Bacillus licheniformis* ATCC 12713 16S ribosomal RNA gene sequence matches mainly *B. licheniformis* ribosomal RNA gene sequences. The RFLP pattern (RsaI sites in V3; *Hinf*I and *Cfo*I site between V4 and V5) is consistent with *B. licheniformis* sp. (Jeyaram et al. 2011).

Table A-15: Results of 16S Ribosomal RNA Gene Sequence Analysis of *B. subtilis* ATCC 6051A[a]

Short identification	Similarity score	S_ab score	Unique common oligomers	Sequence full name
S000003473	Not calculated	1.000	1423	*Bacillus subtilis* (T); DSM10; AJ276351
S000365537	Not calculated	1.000	1446	*Bacillus* sp. TUT1206; AB188212

S000383767	Not calculated	1.000	1412	*Bacillus* sp. CH4-4; AB055846
S000383768	Not calculated	1.000	1412	*Bacillus* sp. CH4-5; AB055848
S000383769	Not calculated	1.000	1412	*Bacillus* sp. CH15-2; AB055849
S000383770	Not calculated	1.000	1412	*Bacillus* sp. CH19-3; AB055850
S000383771	Not calculated	1.000	1412	*Bacillus* sp. CH20-1; AB055851
S000383772	Not calculated	1.000	1412	*Bacillus* sp. CH7-1; AB055852
S000383773	Not calculated	1.000	1412	*Bacillus* sp. CH10-1; AB055853
S000434646	Not calculated	1.000	1401	*Bacillus subtilis*; KL-073; AY030330

[a] *B. subtilis* ATCC 6051A 16S ribosomal RNA gene sequence matches *Bacillus subtilis and Bacillus sp.* ribosomal RNA gene sequence. The RFLP pattern *(RsaI sites in V3; HinfI site between V4 and V5)* is consistent for *B. subtilis* sp. (Jeyaram et al. 2011). However, the first putative RsaI site requires verification as it contains an ambiguous base (the dominant peak appears to correspond to A).

Table A-16: Results of 16S Ribosomal RNA Gene Sequence Analysis of B. subtilis ATCC 55405[a]

Short identification	Similarity score	S_ab score	Unique common oligomers	Sequence full name
S000870716	Not calculated	1.000	1391	*Bacillus amyloliquefaciens*; NBRC 14141; AB325582
S001745899	Not calculated	1.000	1393	*Bacillus amyloliquefaciens*; IMAU80205; GU125623
S002038639	Not calculated	1.000	1354	*Bacillus amyloliquefaciens*; HK1; AB279736
S002222255	Not calculated	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002222257	Not calculated	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002222259	Not calculated	1.000	1447	*Bacillus amyloliquefaciens* DSM 7; DSM7; FN597644
S002228859	Not calculated	1.000	1224	*Bacillus amyloliquefaciens*; BAC3048; HM355639
S003280603	Not calculated	1.000	1329	*Bacillus amyloliquefaciens*; BSS5; JQ407053
S003285855	Not calculated	1.000	1305	*Bacillus* sp. SE18; JQ714100
S003313087	Not calculated	1.000	1342	*Bacillus amyloliquefaciens*; KU-8; JQ696827

[a] *B. subtilis* ATCC 55405 16S ribosomal RNA gene sequence matches mainly *B. amyloliquefaciens* sequences and the *HinfI* RFLP identified by for *B. amyloliquefaciens* is present (Jeyaram et al. 2011).

Table A-17: Results of 16S Ribosomal RNA Gene Sequence Analysis of B. subtilis subsp. subtilis ATCC 6051[a]

Short	Similarity	S_ab	Unique	Sequence full name

identification	score	score	common oligomers	
S000398967	1.000	1.000	1383	*Bacillus subtilis*; BHP6-1; AY162131
S001020073	1.000	1.000	1422	*Bacillus subtilis*; B1-33; EU435361
S001096330	1.000	1.000	1435	*Bacillus* sp. zh161; EU526087
S002038710	1.000	1.000	1388	*Bacillus* sp. PT401; AB374305
S002199724	1.000	1.000	1420	Uncultured *Bacillus* sp.; CapF3B.16; HM152583
S002199742	1.000	1.000	1386	Uncultured *Bacillus* sp.; Filt.13; HM152601
S002199744	1.000	1.000	1386	Uncultured *Bacillus* sp.; Filt.15; HM152603
S002199761	1.000	1.000	1386	Uncultured *Bacillus* sp.; Filt.32; HM152620
S002199769	1.000	1.000	1385	Uncultured *Bacillus* sp.; Filt.40; HM152628
S002199775	1.000	1.000	1386	Uncultured *Bacillus* sp.; Filt.46; HM152634

[a] *Bacillus* species 6051 16S ribosomal RNA gene sequence matches *Bacillus subtilis and Bacillus sp.* ribosomal RNA gene sequence. The RFLP pattern (two *RsaI* sites in V3; *HinfI* site between V4 and V5) is consistent *B. subtilis* sp. (Jeyaram et al. 2011).

Table A-18: Results of 16S Ribosomal RNA Gene Sequence Analysis of B. subtilis ATCC 55406[a]

Short identification	Similarity score	S_ab score	Unique common oligomers	Sequence full name
S001020073	Not calculated	0.946	1422	*Bacillus subtilis*; B1-33; EU435361
S001096330	Not calculated	0.946	1435	*Bacillus* sp. zh161; EU526087
S002038710	Not calculated	0.946	1388	*Bacillus* sp. PT401; AB374305
S002199724	Not calculated	0.946	1420	Uncultured *Bacillus* sp.; CapF3B.16; HM152583
S002199860	Not calculated	0.946	1421	Uncultured *Bacillus* sp.; Filt.132; HM152719
S002199880	Not calculated	0.946	1421	Uncultured *Bacillus* sp.; Filt.152; HM152739
S002410934	Not calculated	0.946	1413	*Bacillus subtilis*; MB5 NIOT; HQ858061
S003257857	Not calculated	0.946	1446	*Bacillus subtilis* subsp. *inaquosorum*; type strain: DSM 22148; HE582781
S003261902	Not calculated	0.946	1388	*Bacillus subtilis*; NBRC 3108; AB680011
S003264071	Not calculated	0.946	1388	*Bacillus subtilis*; NBRC 104440; AB682180

[a] The 16S matches for *B. subtilis subsp. inaquosorum* ATCC 55406 included both *Bacillus sp.* and *B. subtilis subsp. inaquosorum.* The pattern of restriction sites within *B. subtilis subsp. inaquosorum* ATCC 55406 is different compared to *B. licheniformis* strengthening the argument that it was misnamed previously.

Table A-19: Results of 16S Ribosomal RNA Gene Sequence Analysis of *Bacillus* species 16970-5

The results of the 16S rRNA gene sequencing analysis confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of *Bacillus* species 16970-5 and closest similarity matches using the Ribosomal Database cannot be disclosed.

Table A-20: Results of 16S Ribosomal RNA Gene Sequence Analysis of Bacillus species 2 18118-1

The results of the 16S rRNA gene sequencing analysis confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of *Bacillus species* 2 18118-1 and closest similarity matches using the Ribosomal Database cannot be disclosed.

Table A-21: Results of 16S Ribosomal RNA Gene Sequence Analysis of Bacillus species 4 18121-4

The results of the 16S rRNA gene sequencing analysis confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 4 18121-4 and closest similarity matches using the Ribosomal Database cannot be disclosed.

Table A-22: Results of 16S Ribosomal RNA Gene Sequence Analysis of Bacillus species 7 18129-3

The results of the 16S rRNA gene sequencing analysis confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity Bacillus species 7 18129-3 and closest similarity matches using the Ribosomal Database cannot be disclosed.

Appendix 3: Characteristics of DSL *B. licheniformis/subtilis* group members – Fatty acids methyl ester (FAME) analysis

Data generated by Health Canada's Healthy Environments and Consumer Safety Branch shows the best match between the sample and the environmental and clinical MIDI databases and the fatty acid profile similarity index (average of all matches) along with the number of matches (number of matches/total number of tests, parentheses). For methods and additional details, see www.midilabs.com/fatty-acid-analysis. As a general rule of thumb, samples that cluster within a Euclidian distance of 2.5, 6 and 10 represent samples derived from the same strain, subspecies and species, respectively.

Table A-23: FAME analysis of *B. amyloliquefaciens* 13563-0

Environmental		Clinical	
B. amyloliquefaciens (*B. subtilis* group)	0.598 (10/15)	*B. subtilis* GC subgroup A	0.735 (12/17)
B. subtilis	0.441 (3/15)	*B. subtilis* GC subgroup B	0.729 (5/17)
B. atrophaeus GC subgroup A	0.801 (1/15)	Not applicable	
Staphylococcus lutrae	0.490 (1/15) (coag+)	Not applicable	

Table A-24: FAME analysis of *B. atrophaeus* 18250-7

Environmental		Clinical	
B. atrophaeus	0.877 (6/10)	*B. atrophaeus* GC subgroup B	0.814 (5/6)
Analysis not good enough for library search	(4/10)	*B. atrophaeus* GC subgroup A	0.853 (1/6)

Table A-25: FAME analysis of *B. licheniformis* ATCC 12713

Environmental		Clinical	
B. licheniformis (*B. subtilis* group)	0.808 (13/14)	*B. licheniformis* (*B. subtilis* group)	0.674 (6/17)
B. megaterium GC subgroup A	0.719 (1/14)	*Staphylococcus schleiferi*	0.418 (6/17)
Not applicable		*B. pumilis*-GC subgroup A	0.669 (2/17)
Not applicable		*B. pumilis*-GC subgroup B	0.468 (2/17)
Not applicable		*B. subtilis*	0.233 (1/17)

Table A-26: FAME analysis of *B. subtilis* ATCC 6051A

Environmental		Clinical	
B. subtilis	0.876 (16/17)	*B. subtilis*	0.872 (13/13)
B. amyloliquefaciens	0.798 (1/17)	Not applicable	

Table A-27: FAME analysis of *B. subtilis* ATCC 55405

Environmental	Clinical

B. subtilis	0.753 (10/15)	*B. subtilis*	0.662 (12/12)
B. amyloliquefaciens (*B. subtilis* group)	0.736 (5/15)	Not applicable	

2165 **Table A-28: FAME analysis of *B. subtilis* subsp. *subtilis* ATCC 6051**

Environmental		Clinical	
B. subtilis	0.911 (14/14)	*B. subtilis*	0.760 (9/12)
Not applicable		Analysis not good enough for library search	2/12
Not applicable		Micrococcus lylae GC subgroup B	0.292 (1/12)

2166 **Table A-29: FAME analysis of *B. subtilis* subsp. *inaquosorum* ATCC 55406**

Environmental		Clinical	
B. subtilis	0.803 (10/25)	*B. subtilis*	0.727 (12/12)
B. amyloliquefaciens (*B. subtilis* group)	0.793 (9/25)	Not applicable	
B. megaterium GC subgroup A	0.602 (4/25)	Not applicable	
No match	2/25	Not applicable	

2167 **Table A-30: FAME analysis of *Bacillus* species 16970-5**

Environmental	Clinical
The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 16970-5 cannot be disclosed.	The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 16970-5 cannot be disclosed.

2168 **Table A-31: FAME analysis of *Bacillus* species 2 18118-1**

Environmental	Clinical
The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 2 18118-1 cannot be disclosed.	The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 2 18118-1 cannot be disclosed.

2169 **Table A-32: FAME analysis of *Bacillus* species 4 18121-4**

Environmental	Clinical
The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 4 18121-4 cannot be disclosed.	The results generated from the FAME clinical database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 4 18121-4 cannot be disclosed.

2170 **Table A-33: FAME analysis of *Bacillus* species 7 18129-3**

Environmental	Clinical
The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 7 18129-3 cannot be disclosed.	The results generated from the FAME environmental database confirms the identity of this strain as it was purported to be when it was nominated to the Domestic Substances List. However, due to confidentiality claims the identity of Bacillus species 7 18129-3 cannot be disclosed.

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Appendix 4: Cellular content of select fatty acids

Table A-34: Cellular Content of Select Fatty Acids in DSL *B. licheniformis/subtilis* Group Members[a]

DSL Strain	$C_{16:0}$ (%)	Iso-$C_{17:1\omega}$10*c* (%)
B. amyloliquefaciens 13563-0	3.17	2.4
B. atrophaeus 18250-7	3.07	1.5
B. licheniformis ATCC 12713	2.89	1.56
B. subtilis subsp. *subtilis* ATCC 6051	4.34	1.95
B. subtilis ATCC 6051A	2.55	2.65
B. subtilis ATCC 55405	3.05	2.34
B. subtilis subsp. *inaquosorum* ATCC 55406	3.3	1.16
Bacillus species 16970-5	3.52	1.81
Bacillus species 2 18118-1	6.09	1.77
Bacillus species 4 18121-4	3.41	1.11
Bacillus species 7 18129-3	4.34	1.95

[a] Unpublished data generated by Health Canada's Healthy Environments and Consumer Safety Branch

Appendix 5: List of some mobile elements and associated traits identified in certain isolates of the *B. subtilis* complex

Table A-35: List of Some Mobile Elements and Associated Traits Identified in some strains of *B. licheniformis*

Element Name	Associated Traits	References
Plasmid (unknown name)	Dimethoate resistance and additional genes for antibiotic and heavy metal resistance (Na, Er, Ch, Cz, Cf, Ba^{2+} and Zn^{2+})	(Mandal et al. 2005)
Plasmid (pBL1, pBL10, pBL2)	Not specified	(Zawadzki et al. 1996)
Insertion element (*IS3Bli1*)	Encodes two predicted overlapping protein coding sequences, designated *orfA* and *orfB* in relative translational reading frames of 0 and 1. Eight of these elements lie in intergenic regions and one interrupts the *comP* gene	(Rey et al. 2004)
Prophase sequences (NZP1 and NZP3)	Codes for the large subunit of terminase, a signature protein that is highly conserved among prophages	(Rey et al. 2004)

Table A-36: List of Some Mobile Elements and Associated Traits Identified in some strains of *B. subtilis*

Element Name	Associated Traits	References
Transposon (Tn917)	Used in transposition mutagenesis (rapid cloning and construction of transcriptional gene fusions and the characterization of genes which are over-expressed)	(Pragai et al. 1994)
Plasmid (pLS20)	Promotes transfer of tetracycline resistance plasmid pBC16 from *B. subtilis* (natto) to the Bacillus species *B. anthracis, B. cereus, B. licheniformis, B. megaterium, B. pumilus, B. subtilis and B. thuringiensis.*	(Koehler and Thorne, 1987)
Transposon (Tn5)	Neomycin phosphotransferase gene	(Sprengel et al. 1985)
Rolling circle replication (RCR) plasmid pTA family (pTA1015, pTA1040, pTA1050 and pTA1060)	Contains genes encoding a type I signal peptidase and genes encoding proteins belonging to the family of response regulator aspartate phosphatases likely to be involved in the regulation of post-exponential phase processes	(Meijer et al. 1998)
Plasmid (pBS4, pBS12, pBS7, pBS8, pBS6)	Not specified	(Zawadzki et al. 1996)
Transposon (integrative and conjugative elements class: ICEBs1)	• Integrated into the trnS-leu2 gene is regulated by the SOS response and the RapI-PhrI cell-cell peptide signaling system • When DNA damage occurs or high concentrations of potential mating partners that lack the element, ICEBs1 is excised from the chromosome and transferred to recipients.	(Auchtung et al. 2005)
Tn916 (transposon)	• Implicated in the horizontal transfer of antibiotic resistance genes in many species of Gram positive bacteria • Transfer of this element may increase the presence of tetracycline	(Celli and Trieu-Cuot, 1998; Marra and Scott, 1999)
Tn5397 (transposon)	• Originates from *Clostridium difficile*; element transfers to and from *B. subtilis*	(Roberts et al. 2001; Wang

	• Encodes a conjugation system that is similar to that of Tn916 • Contains a group II intron	and Mullany, 2000)
Tn5398 (transposon)	• Originates from *C. difficile* • Facilitates the transfer of an MLS resistance gene (*ermBZ*)	(Mullany et al. 1995)
I*S4Bsu*1 (mobile element)	Spontaneously translocates to the *swrA* gene in *B. subtilis natto*; causes a defect in poly-gamma- glutamic acid (gamma-PGA) synthesis	(Kimura et al. 2011)

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Appendix 6: Virulence genes

Table A-37: List of Some Virulence Genes Identified in Certain Isolates of the *B. subtilis* Complex

Species	Virulence Gene(s)	Associated Traits	References
B. amyloliquefaciens	*HblC*, *HblD*, *HblA*, *NheB*, *NheA*	Enterotoxin production and discontinuous beta-hemolysis	(Phelps and McKillip, 2002)
B. licheniformis	*cesA*	Cereulide synthase	(Nieminen et al. 2007)
B. licheniformis	*lchAA*, *lchAB*, *lchAC*	Lichenysin synthase	(Nieminen et al. 2007)
B. licheniformis	*bceT, hblC, hblA, hblD*	Hbl enterotoxin, *Bacillus* hemolytic enterotoxin	(Oguntoyinbo and Sanni, 2007; Rowan et al. 2001)
B. licheniformis	*lchAA, lchAB* and *lchAC* (lichenysin synthase genes)	• Surfactant lichenysin • Heat-stable cyclic lipopeptide toxins; immobilizes boar sperm • Structurally similar to cereulide, but the toxic activity appears to be different; it has the potential to form ion channels in host cell membranes and has a surfactant effect	(Logan, 2012; Mikkola et al. 2000; Nieminen et al. 2007; Peypoux et al. 1999)
B. licheniformis	*bacA, bacB, bacC* (bacitracin synthetases genes)	• Cyclic polipeptides; interferes with cell wall and peptidoglycan synthesis of Gram positive and negative bacteria Possibly/indirectly related to erythromycin resistance	(Ishihara et al. 2002)
B. licheniformis and *B. subtilis*	*bceT*, *hblC*, *hblA*, *hblD*	Diarrhoeagenic enterotoxin production	(Rowan et al. 2001)

Appendix 7: Virulence and pathogenicity testing of DSL *B. licheniformis/subtilis* strains: Hemolytic activity

Data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Strains were streaked onto 5% sheep blood agar and incubated for 37^{o}C for 24 hours. Hemolysis was judged by clearing zones around colonies which indicate lysis of red blood cells.

Table A-38: Hemolytic activity of DSL *B. licheniformis/subtilis* strains

DSL Strain	Hemolytic Activity
B. amyloliquefaciens 13563-0	+[a,b]
B. atrophaeus 18250-7	-[c]
B. licheniformis ATCC 12713	+[d]
B. subtilis ATCC 6051A	w[e]
B. subtilis ATCC 55405	w
B. subtilis subsp. *subtilis* ATCC 6051	w
B. subtilis subsp. *inaquosorum* ATCC 55406	+
Bacillus species 16970-5	w
Bacillus species 2 18118-1	w
Bacillus species 4 18121-4	-
Bacillus species 7 18129-3	w

[a] +, hemolytic activity
[b] Hemolysis was seen in 5 to 10% of colonies
[c] -, no hemolytic activity
[d] Hemolysis was seen in 70 to 80% of colonies
[e] w, weak hemolytic activity – clearing zones do not extend past colony margin

Appendix 8: Virulence and pathogenicity testing of DSL *B. licheniformis/subtilis* strains: Catalase production

Data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Bacteria were propagated on TSB agar at 28^{o}C for 48 hours. Hydrogen peroxide dropped on to colony to determine conversion to water and hydrogen. Catalase positive reaction indicated that a given bacteria has the capacity to protect itself from reactive oxygen-induced killing from immune cells.

Table A-39: Catalase production of DSL *B. licheniformis/subtilis* strains

DSL Strain	Catalase Production
B. amyloliquefaciens 13563-0	+
B. atrophaeus 18250-7	+
B. licheniformis ATCC 12713	+
B. subtilis ATCC 6051A	+
B. subtilis ATCC 55405	+
B. subtilis subsp. *subtilis* ATCC 6051	+
B. subtilis subsp. *inaquosorum* ATCC 55406	+
Bacillus species 16970-5	+
Bacillus species 2 18118-1	+
Bacillus species 4 18121-4	+
Bacillus species 7 18129-3	+

Appendix 9: Antimicrobial compound, other metabolites and toxins produced by certain isolates of the *B. subtilis* complex

Table A-40: Antimicrobial compounds produced in some strains of *B. amyloliquefaciens*

Substance Name	Activity	References
Bacteriocin-like peptides	Broad antibacterial spectrum with activity against Gram positive bacteria	(Reviewed in Abriouel et al. 2011; Smitha and Bhat, 2012)
BLIS[a] 5940, BLIS RC-2, BLIS 5006	Antibacterial and antifungal activity	(Reviewed in Abriouel et al. 2011)
Surfactin, iturin, bacillomycine, azalomycin F, acivicin, arthrobactin, rhodutorola acid, valinomycin, stenothricin, enterochelin, nocardamin	Antibacterial and antifungal activity, inhibition of growth	(Wulff et al. 2002)
Subtilosin A	• Bacteriocin with bactericidal activity against Gram negative bacteria • Spermicidal activity against boar, bovine, horse, rat and human spermatozoa	(Reviewed in Abriouel et al. 2011)

[a] BLIS: bacteriocin-like inhibitory substances

Table A-41: Antimicrobial compounds produced in some strains of *B. atrophaeus*

Substance Name	Activity	References
Subtilosin A	• Bacteriocin with bactericidal activity against Gram negative bacteria • Spermicidal activity against boar, bovine, horse, rat and human spermatozoa	(Reviewed in Abriouel et al. 2011)

Table A-42: Antimicrobial compounds produced in some strains of *B. licheniformis*

Substance Name	Activity	References
Amoebicins (A12-A and A12-B)	• Amoebolytic activity against *Naegkriafowkri* • Antibiotic activity against yeasts (*Saccharomyces heterogenicus* and *Cryptococcus neoformans*) and several fungal species	(Galvez et al. 1993)
Amoebicin (m4-A)	Bactericidal and bacteriolytic activity on *Bacilus megaterium* GR10	(Lebbadi et al. 1994)
Antibiotics (bacitracin, licheniformin, proticin)	Antibiotics which are secreted and may inhibit competing organisms including Gram positive and Gram negative bacteria, yeasts and molds	(Reviewed in Katz and Demain, 1977)
Bacillocin, BLIS P40, BLIS ZJU12, BLIS MKU3, peptide A-12 C	Antibacterial and antifungal activity	(Reviewed in Abriouel et al. 2011)
Bacteriocin BL8	• Thermostable, broad pH range • Antibacterial against Gram positive bacteria	(Smitha and Bhat, 2012)
Bacteriocin-like peptide	Broad spectrum antagonistic activity activities	(He et al. 2006)

Substance Name	Activity	References
	against fungal pathogens and Gram positive bacteria but not most Gram negative bacteria	
Ieodoglucomides A and B	• Cytotoxic activity against lung and stomach cancer cells lines • Moderate antimicrobial activity	(reviewed in Tareq et al. 2012)
Lichenicidin (α, β)	Antibacterial activity associated with the cell surface	(Reviewed in Abriouel et al. 2011; Begley et al. 2009; Dischinger et al. 2009)
Lichenin	Bacteriocin produced under anaerobic conditions	(Reviewed in Abriouel et al. 2011)
Lichenysin	Lipopeptide that acts as an anionic biosurfactin as well as an antimicrobial	(Li et al. 2010; Nerurkar, 2010; Nieminen et al. 2007)
Surfactin, iturin, acivicin, arthrobactin, rhodutorola acid, valinomycin	Antibacterial and antifungal activity, inhibition of growth	(Wulff et al. 2002)

2216 **Table A-43: Antimicrobial compounds produced in some strains of *B. subtilis***

Substance Name	Activity	Reference
Anti-bacterials (ericin S and A, sublancin 168, subtilin B, subtilosin A and A1, mersacidin, betacin, MJP1, Bac 14B, LFB112)	Bacterial activity against bacterial pathogens	(Reviewed in Abriouel et al. 2011)
Antibiotics (mycobacillin, subtilin, bacilysin, bacillomycin, fungistatin, bulbiformin, bacillin, subsporin, bacillocin, mycosubtilin, fungocin, iturin, neocidin, eumycin)	Antibiotics which may inhibit competing organisms including Gram positive and negative bacteria as well as yeasts and molds	(reviewed in Katz and Demain, 1977)
Antibiotics (subtilin, ericin, mersacidin, sublancin 168, subtilosin, surfactin, iturin, bacillomycin, mycosubtilin, fengycin plipastatin, corneybactin, bacilysin, bacilysocin, amicoumacin, mycobacillin, TL-119, rhizocticin, difficidin, 3,3'-neotrehalos-adiamine 168)	Anti-microbial activity, biofilm and swarming development, pheromones in quorum sensing and 'killing factor'.	(reviewed in Stein, 2005)
Heat-stable, protease resistant antimicrobial substance	Inhibits growth of many bacteria	(reviewed in Sorokulova et al. 2008)
Subtilin	• Antimicrobial peptide • Affects pore formation in the cytoplasmic membrane • Produced in higher amounts under starvation	(Reviewed in Abriouel et al. 2011)

Substance Name	Activity	Reference
	conditions to eliminate competing species and increase available nutrients	
Surfactin	• Lipopeptide that acts as a biosurfactin and a potent antimicrobial	(Li et al. 2010)

2217 **Table A-44: Toxic metabolites produced by some strains of *B. amyloliquefaciens***

Substance Name	Activity	References
Amylosin	• Inhibits boar sperm motility Cytotoxic to feline lung cells	(Mikkola et al. 2007)

2218 **Table A-45: Toxic metabolites produced by some strains of *B. licheniformis***

Substance Name	Activity	References
Non-emetic heat stable cytotoxic component	Cytotoxicity activity	(De Jonghe et al. 2010)
Heat labile cytotoxic substance	Cytotoxicity activity	(De Jonghe et al. 2010)
B. cereus-like protein toxin	• Reduction in cellular metabolic activity • Cytotoxic activity	(Beattie and Williams, 1999)
Emetic toxin	• Induces vomiting if ingested • Ionophoric uptake of K+ resulting in the dissipation of the transmembrane potential, stimulating swelling and respiration in mitochondria which leads to their inactivation	(Biesta-Peters et al. 2010; Reviewed in From et al. 2005)
Hemolysin BL (Hbl) enterotoxin	Causes diarrhea	(Rowan et al. 2001)
Heat labile *B. cereus* diarrheal-like toxin	• Cytotoxic to McCoy cells causing leaky membranes, disrupts cell surfaces and decreases metabolic activity	(Lindsay et al. 2000)
Lichenysin	• Inhibits sperm motility • Synthesized in both aerobic and anaerobic condition during growth • Species specific variations (A, B, C, D, G and surfactant BL86)	(Li et al. 2010; Nerurkar, 2010; Nieminen et al. 2007)
Lichenysin A	• Causes loss of motility, damage to plasma membrane and acrosome, loss of cellular NADH and ATP in boar spermatozoa • Toxic towards natural (non-malignant) mammalian cells • May be produce aerobically and anaerobically • More powerful compared to surfactin and lichenysin B	(Mikkola et al. 2000; Yakimov et al. 1996)
Non-proteinaceous, heat-stable, sperm toxic agent	• Inhibits sperm motility and swells acrosome • Damages cell membrane integrity • Depletes cellular ATP • Beta-hemolytic activity	(Salkinoja-Salonen et al. 1999)

Substance Name	Activity	References
NucB	• Degrades extracellular DNA that is an essential building block of both single species and mixed biofilms • Nontoxic deoxyribonuclease • Sporulation-specific enzyme	(Rajarajan et al. 2013; Shakir et al. 2012)
Surfactin	Inhibits phytopathogenic fungi	(Nerurkar, 2010)

2219 **Table A-46: Toxic metabolites produced by some strains of *B. subtilis***

Substance Name	Activity	References
Hemolysin BL (Hbl) enterotoxin	Causes diarrhea	(Rowan et al. 2001)
Non-emetic heat stable cytotoxic component and a heat labile cytotoxic substance	Cytotoxicity activity	(De Jonghe et al. 2010)
Protolytic and lipolytic substances	Lysis of proteins and lipids	(De Jonghe et al. 2010)
Putative emetic toxin	Causes nausea and vomiting	(From et al. 2005)

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Appendix 10: Virulence and pathogenicity testing of DSL *B. licheniformis/subtilis* strains: Cytotoxicity

Data generated by Health Canada's Healthy Environments and Consumer Safety Branch. The MTT Assay was used to determine the cytotoxic potential of the strains towards HT29 (colonic epithelial cells) and J774A.1 (macrophage cells). MTT is a yellow, soluble bromide salt which is reduced to a purple, insoluble formazan crystal by dehydrogenase enzymes of living cells (indicating mitochondrial activity). In the crystal state after reduction, it is trapped inside the cell. DMSO or another solvent such as isopropanol or mineral oil can be used to solubilize the formazan, which can then exit the cell, turning the solvent a purple colour that is detectable with a spectrophotometer. This assay is suitable for animal cells that are adherent. Metabolically active bacterial cells can also reduce MTT also. Since most animal cells are not adherent bacteria and their formazan contribution can be rinsed away with PBS prior to solubilisation.

HT29 and J774A.1 were incubated at 37^{o}C in the presence of 5% carbon dioxide. Mammalian cells were dosed with 10^6 CFU/well of vegetative bacteria for 2, 4 and 24 hours. Dose cells were washed twice with PBS before adding MTT.

Loss in bioreduction activity of the cell lines toward MTT was measured to determine the cytotoxic potential of the DSL *B. licheniformis/subtilis* group strains. Cytotoxicity is related to increased losses in bioreduction activity of the cell lines.

Table A-47: Cytotoxic potential of DSL *B. licheniformis/subtilis* strains towards HT29 cells with gentamicin at 2, 4 and 24 hours

DSL Strain[a]	2 hours	4 hours	24 hours
B. amyloliquefaciens 13563-0	w[b]	w	w
B. atrophaeus 18250-7	w[c]	w	w
B. licheniformis ATCC 12713	w	w	w
B. subtilis subsp. *inaquosorum* ATCC 55406	w	-	-
Bacillus species 16970-5	w	-	w
Bacillus species 2 18118-1	-	-	-
Bacillus species 4 18121-4	w	w	w

[a] No data available for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *Bacillus* species 7 18129-3
[b] w, weak cytotoxic activity (5-50% bioreduction loss)
[c] Related to structural components

Table A-48: Cytotoxic potential of DSL *B. licheniformis/subtilis* strains towards HT29 cells without gentamicin at 2, 4 and 24 hours

DSL Strain	2 hours	4 hours	24 hours
B. amyloliquefaciens 13563-0	w[a]	w	w
B. atrophaeus 18250-7	w[b]	w	w
B. licheniformis ATCC 12713	w	w	+[c,d]
B. subtilis ATCC 6051A	ND[e]	w	w
B. subtilis ATCC 55405	ND	-[f]	-
B. subtilis subsp. *subtilis* ATCC 6051	ND	w	w

B. subtilis subsp. *inaquosorum* ATCC 55406	w	w	+[c]
Bacillus species 16970-5	w	w	+
Bacillus species 2 18118-1	w	w	+
Bacillus species 4 18121-4	w	w	w
Bacillus species 7 18129-3	ND	w	w

[a] w, weak cytotoxic activity (5-50% bioreduction loss)
[b] Related to structural components
[c] +, cytotoxic activity (>50% bioreduction loss)
[d] Growth-related
[e] ND, no data
[f] -, no cytotoxic activity (< 5% bioreduction loss)

Table A-49: Cyototoxic potential of DSL *B. licheniformis/subtilis* strains towards J774A.1 cells with gentamicin at 2, 4 and 24 hours

DSL Strain[a]	2 hours	4 hours	24 hours
B. amyloliquefaciens 13563-0	-[b]	-	-
B. atrophaeus 18250-7	w[c,d]	w	w
B. licheniformis ATCC 12713	-	-	w
B. subtilis subsp. *inaquosorum* ATCC 55406	w	w	w
Bacillus species 16970-5	w	w	w
Bacillus species 2 18118-1	-	-	-
Bacillus species 4 18121-4	-	w	w

[a] No data available for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *Bacillus* species 7 18129-3
[b] -, no cytotoxic activity (< 5% bioreduction loss)
[c] w, weak cytotoxic activity (5-50% bioreduction loss)
[d] Related to structural components

Table A-50: Cyototoxic potential of DSL *B. licheniformis/subtilis* strains towards J774A.1 cells without gentamicin at 2, 4 and 24 hours

DSL Strain	2 hours	4 hours	24 hours
B. amyloliquefaciens 13563-0	-[a]	-	-
B. atrophaeus 18250-7	w[b,c]	w	w
B. licheniformis ATCC 12713	w	-	w
B. subtilis ATCC 6051A	ND[d]	w	w
B. subtilis ATCC 55405	ND	-	w
B. subtilis subsp. *subtilis* ATCC 6051	ND	-	w
B. subtilis subsp. *inaquosorum* ATCC 55406	-	-	+[e]
Bacillus species 16970-5	-	-	w
Bacillus species 2 18118-1	-	-	+[f]
Bacillus species 4 18121-4	-	-	w
Bacillus species 7 18129-3	ND	-	w

[a] -, no cytotoxic activity (< 5% bioreduction loss)
[b] w, weak cytotoxic activity (5-50% bioreduction loss)
[c] Cytotoxic activity related to structural components
[d] ND, No data
[e] +, cytotoxic activity (>50% bioreduction loss)
[f] Cytotoxic activity related to growth

Appendix 11: Pathogenicity, toxicity and irritation testing results for strains of the *B. subtilis* complex on terrestrial and aquatic vertebrates, invertebrates and plants

Table A-51: Pathogenicity, toxicity and irritation testing results for *B. amyloliquefaciens* strain FZB24[a]

Study Type	Target organism	Dose concentration	Outcome
Acute oral toxicity and infectivity	CD rats	1.3×10^8 CFU/animal	Low toxicity, not pathogenic $LD_{50} > 1.3 \times 10^8$ CFU/animal
Acute pulmonary toxicity and infectivity	CD rats	0.1 mL of at least 1.4×10^8 viable CFU/animal	Low toxicity, not pathogenic $LD_{50} > 1.4 \times 10^8$ CFU/animal
Acute pulmonary toxicity and infectivity	Rats	1.3×10^8 CFU	Not toxic/ Not pathogenic
Acute intravenous infectivity	CD rats	0.5 mL of at least 1.0×10^7 CFU/animal	Not pathogenic $LD_{50} > 1.0 \times 10^7$ CFU/animal
Acute intravenous infectivity	Rats	1.7×10^8 CFU	Not toxic/ Not pathogenic
Acute dermal toxicity	Rabbits	2000 mg/kg BW[b] (1.5 to 2×10^{12} CFU/animal)	Low toxicity but severely irritating $LD_{50} > 2000$ mg/kg
Primary dermal irritation	Rabbits	0.5 g granular test substance (7.0×10^{10} CFU/g) and 0.5 mL of 1.5% w.v suspension	No irritation observed
Primary eye irritation	Rabbit	3.6×10^{10} CFU	Eye irritant
Acute dermal irritation	New Zealand white rabbit	2000 mg/kg/BW (1.5 to 2×10^{12} CFU/animal)	Low toxicity and severely irritating $LD_{50} > 2000$ mg/kg/BW
Avian oral toxicity	Northern bobwhite (*Colinus virginianus*)	10 mL/kg BW or 1.0×10^9 CFU/g BW	NOEL[c:] 1.0×10^9 CFU/animal
Terrestrial arthropod toxicity	Adult bees (*Apis meelifera*)	10^5 CFU/mL	No signs of toxicity or pathogenicity $LC_{50} > 1.0 \times 10^6$ CFU/mL
Terrestrial arthropod toxicity	Larva (*Apis meelifera*)	6.0×10^3 CFU/larva	$LC_{50} > 6.0 \times 10^3$ CFU/larva
Terrestrial non-arthropod invertebrates	Worm (*Eisenia fetida*)	6.0×10^{11} CFU/kg soil	NOEC[d]: 6.0×10^{11} CFU/kg soil
Freshwater fish toxicity/pathogenicity	Rainbow trout (*Oncorhynchus mykiss)*	1.85×10^9 CFU/kg to 1.85×10^{10} CFU/L (active ingredient)	$LC_{50} > 1.85 \times 10^{10}$ CFU/L NOEC: 1.85×10^{10} CFU/L
Freshwater invertebrate toxicity/pathogenicity	*Daphnia magna*	Up to 1.85×10^{10} CFU/L	$LC_{50} > 1.85 \times 10^{10}$ CFU/L NOEC: 1.85×10^{10} CFU/L

[a] Studies done with the technical grade active ingredient and not the end-use product containing the micro-organism, (PMRA-HC, 2012; U.S. EPA, 2012)

[b] BW, body weight

[c] NOEL, no observed effect level is the highest dose of the test substance in the test substrate at which no statistically significant effect on the test organism was observed, relative to the control

[d] NOEC, no observed effect concentration is the highest concentration of the test substance in the test substrate at which no statistically significant effect on the test organism was observed, relative to the control

Table A-52: Pathogenicity, toxicity and irritation testing results for *B. amyloliquefaciens* strain D747[a]

Study Type	Target organism	Dose concentration	Outcome
Acute oral toxicity	Sprague-Dawley rats (5 week old, male and female)	10^8 CFU/animal	Not toxic, infective or pathogenic LD_{50}> 5000 mg/kg
Acute pulmonary toxicity	Sprague-Dawley rats (5 week old, male and female)	10^7 CFU/animal	Not toxic or pathogenic LC_{50}> 2.18 mg/L
Acute injection	Sprague-Dawley rats (5 week old, male and female)	10^7 CFU/animal	Not toxic, infective, or pathogenic LD_{50}> 5050 mg/kg
Acute eye irritation	New Zealand white rabbits	0.1 mL of the end-use product	Eye irritant
Primary dermal irritation	New Zealand white rabbits	500 mg of the end-use product	No evidence of irritation
Avian oral toxicity	Northern bobwhite quail (*Colinus virginianus*)	8.9×10^9 spores/bird	Not toxic LD_{50}> 4.5×10^{11} spores/kg BW or > 8.0×10^{10} spores/bird
Freshwater fish toxicity/pathogenicity	Rainbow trout (*Oncorhynchus mykiss*)	1.7×10^8 CFU/L	LC_{50}: 8.1×10^{10} CFU/L NOEC: 1.44×10^{10} CFU/L
Freshwater invertebrate toxicity/pathogenicity	*Daphnia magna*	1.7×10^8 CFU/L	EC_{50}: 3.7×10^{10} CFU/L NOEC: 2.84×10^8 CFU/L

[a] Studies done with the technical grade active ingredient and not the end-use product containing the micro-organism, (U.S. EPA, 2011)

Table A-53: Pathogenicity, toxicity and irritation testing results for *B. licheniformis* strain SB3086[a]

Study Type	Target organism	Dose concentration	Outcome
Acute oral toxicity/pathogenicity	Rats	1.0×10^8 CFU/animal[b]	Not toxic, infective, or pathogenic LD_{50}> 5000 mg/kg
Acute pulmonary toxicity/pathogenicity	Rats	1.1×10^8 CFU/animal[b]	Not toxic, infective, or pathogenic
Acute intravenous toxicity/pathogenicity	Rats	1.0×10^7 CFU/animal[b]	Not toxic, infective, or pathogenic
Acute dermal toxicity	New Zealand white rabbits	ND[c,d]	LD_{50}> 5050 mg/kg
Primary eye irritation	New Zealand white rabbits	0.1 mL/animal (concentration not provided)[d]	Non-irritating
Delayed contact hypersensitivity	Guinea Pigs	ND[d]	Not a dermal sensitizer
Avian oral toxicity/pathogenicity	Young mallards (*Anas platyrhynchos*)	4.5×10^{10} CFU/kg of BW[b]	No signs of illness or abnormal behaviour observed
Fresh water fish toxicity/pathogenicity	Rainbow trout (*Oncorhynchus mykiss*)	LC_{50} (of Formula 710-132) > 1.1×10^6 CFU/mL[b]	No effects as the result of the active microbial agent observed

Study Type	Target organism	Dose concentration	Outcome
Fresh water aquatic invertebrate toxicity/pathogenicity (21-day renewal life-cycle)	*Daphnia magna*	1×10^4 CFU/mL[b]	LC_{50}: 1.8×10^6 CFU/mL NOAEC[d]:1.2×10^6 CFU/mL[b,e] 2 daphnids died at the end of the test at 1×10^7 CFU/mL (1000 times the expected environmental concentration)
Invertebrate toxicity/pathogenicity	Honeybee larvae (*Apis mellifera* L.)	1.6×10^6 CFU/mL (of Formula 710-132)[f]	No statistically significant effects on larvae survival, adverse behaviour or developmental abnormalities observed

[a] (U.S. EPA, 2001)
[b] TGAI, technical grade active ingredient
[c] ND, no data
[d] NOAEC, no observed adverse effect concentration
[e] Formulation ingredients are known aquatic toxicants at high concentrations
[f] EP, end product

Table A-54: Pathogenicity, toxicity and irritation testing results for several strains of *B. licheniformis* and *B. subtilis*

Study Type	Target organism	Dose concentration	Outcome
Experimental infection (intravenous inoculation)[a]	Immune depressed BALB/c mice	4×10^7 CFU	Numerous and larger lesions in many organs and more severe infection with lesions occurring in more organs compared to healthy/normal mice used in the study
Experimental infection (intravenous inoculation)[a]	Normal mice	4×10^7 CFU	Lesions observed in liver and kidneys
Experimental infection (intravenous inoculation)[b]	Immune depressed BALB/c mice	Intravenous doses of $<10^6$ to 10^{10} CFU	Only brain and pulmonic lesions could be definitely attributed to *B. licheniformis.* Mice were able to clear high numbers of bacteria within 1 week
Experimental infection (intravenous inoculation)[c]	Pregnant crossbred Red Danish Dairy X American Brown Swiss cows (6-8 months of gestation, n=8) and their calves	Intravenous doses of $<10^9$ to 10^{12} CFU (once or on 4 consecutive days)	Demonstration of the abortifacient potential of *B. licheniformis* and the tropism for the bovine placenta. Lesions in the fetal membranes, the fetal side of the placentomes, necrosis in the fetal compartment of the placenta and inflammation in some calves. Two abortions were observed
Cytotoxic Activity[d]	Boar sperm motility inhibition	1-10 µg/mL	EC_{50}: 20-30 µg/mL
Acute eye irritation study[e]	Male albino rabbits	0.1 g of 1.1×10^{11} CFU/kg BW	No irritation or negative symptoms in the cornea or iris
Acute skin irritation study[e]	Male albino rabbits	0.5 g of 1.1×10^{13} CFU/kg BW	No clinical signs of erythema or oedema
Acute oral toxicity (14-day) [e]	Adult male albino Wistar rats	1.1×10^{11} CFU/kg BW	No treatment-related changes
Subchronic oral	Male and female	1.1×10^{11} CFU/kg	NOAEL[f]>1.1×10^{11} CFU/kg BW

Study Type	Target organism	Dose concentration	Outcome
toxicity (13-week) [e]	Wistar rats	BW and 1.1×10^{11} CFU/kg BW	
Micronucleus assay (2 days at 24 hour intervals) [e]	Adult male and female Swiss albino mice (CFT strain)	1.1×10^{10} CFU/kg BW and 1.1×10^{11} CFU/kg BW	No signs of bone marrow cytotoxicity and no observed genocyctotoxicity
Oral Pathogenicity and Toxicity Study[g]	Bobwhite quails (*Colinus virginianus*)	3,333 kg/mg daily for 5 days	LD_{50}> 2,000 mg/kg

[a] *B. licheniformis* ATCC 14580, (Agerholm et al. 1995)
[b] 13 strains of *B. licheniformis,* (Agerholm et al. 1997)
[c] *B. licheniformis* strain DVL 9315323, (Agerholm et al. 1999)
[d] *B. licheniformis* strains NR 5160 and NR 6768 (toxic heat-stable non-protein substance), (Nieminen et al. 2007)
[e] *B. licheniform*is strain Me I, The concentration used in the study corresponds to 77×10^{11} CFUs for an average 70 kg human being and thus the concentration used can be considered to be 2566 to 77000 times safe for human consumption (suggested human dose range: 1×10^8 to 3×10^9 CFUs) (Nithya et al. 2012)
[f] NOAEL, no observed adverse effect level
[g] *B. subtilis* strain GB03, (U.S. EPA, 1993)

Table A-55: Pathogenicity, toxicity and irritation testing results for a mixture containing two strains of *B. licheniformis* and *B. subtilis*[a]

Study Type	Target organism	Dose concentration	Outcome
Acute toxicity	BALB/c mice	5×10^7 to 2×10^{11} CFU/mouse (oral administration)	No changes in tissues, organs or weight
Acute toxicity	BALB/c mice	5×10^7 to 5×10^9 CFU/mouse (IP[b] or IV[c] administration)	No changes in tissues, organs or weight
Chronic toxicity	Mice	1×10^6 CFU/day	No effect on health status
Chronic toxicity	Rabbits	1×10^9 CFU/day	No effect on health status
Chronic toxicity	Piglets	1×10^9 CFU/day	No effect on health status

[a] *B. licheniformis* strain VKPM B2336 and *B. subtilis* strain VKPM B2335, (Sorokulova et al. 2008)
[b] IP, intraperitoneal
[c] IV, intravenous

Table A-56: Pathogenicity, toxicity and irritation testing results for *B. subtilis* strain QST 713[a]

Study Type	Target organism	Dose concentration	Outcome
Acute oral toxicity and infectivity	CD rats	1.13×10^8 CFU/animal	Non-toxic and not infective LD_{50}> 1.13×10^8 CFU/animal
Acute pulmonary toxicity and infectivity	CD rats	1.2×10^8 CFU/animal	Non-toxic and not infective LD_{50}> 1.2×10^8 CFU/animal
Intravenous Infectivity	CD rats	9.4×10^8 CFU/animal	Non-infective
Acute dermal toxicity	CD rats	2 g/kg BW (2.3×10^{11} to 2.73×10^{11} CFU/animal	Low toxicity LD_{50}> 2g/kg BW
Eye irritation	Rabbits	0.1 ml (4.8×10^9 CFU/animal)	Minimally irritating
Dermal irritation	Rabbits	500 mg (2.4×10^{10} CFU/animal)	Slightly irritating
Avian oral toxicity	Northern bobwhite quail (*Colinus virginianus*)	1×10^8 CFU/g BW/day (5000 mg/kg BW/day)	LD_{50}> 5000 mg/kg BW

Study Type	Target organism	Dose concentration	Outcome
Freshwater fish	Rainbow trout (*Oncorhynchus mykiss*)	Max dose: 1.4×10^7 CFU/mL	LC_{50}: 1.4×10^7 CFU/mL
Freshwater aquatic invertebrate (48-hour)	*Daphnia magna*	5×10^5 CFU/mL, 1×10^6 CFU/mL, 2×10^6 CFU/mL and 4×10^6 CFU/mL	Mortality of 15, 15, 45 and 85% respectively and lethargy of surviving daphnids in the 100mg/L treatment
Freshwater aquatic invertebrate (21-day)	*Daphnia magna*	5×10^5 CFU/mL, 1×10^6 CFU/mL, 2×10^6 CFU/mL and 4×10^6 CFU/mL	LC_{50} ~ 3×10^5 CFU/mL NOEC: 7.5×10^3 CFU/mL
Freshwater aquatic invertebrate (21-day)	*Daphnia magna*	7.9×10^5 CFU/mL, 1.8×10^6 CFU/mL, 3.4×10^6 CFU/mL, 7.3×10^6 CFU/mL and 2.0×10^7 CFU/mL	LC_{50} ~ 1.6×10^6 CFU/mL NOEC: 7.9×10^5 CFU/mL.
Freshwater aquatic invertebrate	Grass shrimp (*Palaemonetes pugio*)	4.0×10^6 CFU/g	LC_{50} > 4.0×10^6 CFU/mL
Aqueous plant	Single cell green alga (*Scenedesmus subspicatus*)	Max dose: 5.1×10^5 CFU/mL	NOEC≥ 100 mg/L LOEC> 100 mg/L
Non-target insect study (oral/dietary)	Honey Bee -*Apil mellifera* L.	Max dose: 100,000 ppm	LD_{50}> 100,000 ppm
Dietary toxicity/ pathogenicity	Honey Bee -*Apil mellifera* L.	600, 6,000 and 60,000 ppm	LC_{50}: 5663 ppm
Non-target insect study (oral/dietary)	Green lacewing (*Chrisoperla carnea*)	Max dose: 60000 ppm	LC_{50}> 60,000 ppm
Non-target insect study (oral/dietary)	Ladybird beetle - *Hippodamia convergens*	Max dose: 1.2×10^9 CFU/mL (60000 ppm)	LC_{50}> 60,000 ppm NOEC: 60,000 ppm (1.2×109 CFU/g)
Toxicity and pathogenicity test (30 days)	Parasitic Hymenoptera - *Nasonia vitripenis*	600, 6,000 and 60,000 ppm	LC_{50}: 28,000 ppm (15 days)
Non-target insect study (oral/dietary)	Parasitic Hymenoptera - *Nasonia vitripenis*	Max dose: 3.2×10^9 CFU/mL (60000 ppm)	LC_{50} ~ 24,739 ppm

[a] Studies done with the technical grade active ingredient (Mendelsohn and Vaituzis, 1999; U.S. EPA, 2006; U.S. EPA, 2010; PMRA-HC, 2007b).

Table A-57: Pathogenicity, toxicity and irritation testing results for *B. subtilis* strain MBI 600[a]

Study Type	Target organism	Dose concentration	Outcome
Acute oral toxicity and infectivity	CD Rats	2.0×10^8 spores	Low toxicity, not infective LD_{50}> 2×10^8 CFU
Acute pulmonary toxicity and infectivity	CD Rats	3.3×10^8 to 3.7×10^8 spores	Toxic, not infective LD_{50}> 3.5×10^8 CFU
IV infectivity	CD Rats	10^7 spores	No significant signs of toxicity
Acute dermal toxicity	New Zealand White Rabbits	2mL/kg body weight	Low toxicity (slight oedema) LD_{50}> 2mL/kg body weight
Eye irritation	New Zealand White	1.0×10^9 CFU	Minimally irritating

Study Type	Target organism	Dose concentration	Outcome
	Rabbits		
Dermal irritation	New Zealand White Rabbits	2.0×10^{10} CFU	Minimally irritating
Acute Avian Oral Toxicity and Pathogenicity	Bobwhite quails (*Colinus virginianus*)	GUS 378 Concentrate: 4000 mg/kg BW/day	Low toxicity, not pathogenic
Acute Avian Oral Toxicity and Pathogenicity	Bobwhite quails (*Colinus virginianus*)	Water-soluble metabolites: 240 mg/kg BW/day	Low toxicity, not pathogenic
Acute Avian Oral Toxicity and Pathogenicity	Bobwhite quails (*Colinus virginianus*)	Washed spores: 3680 mg/kg BW/day	Low toxicity, not pathogenic
Plant Toxicity and Pathogenicity	Soybean seeds	10^5 to 10^7 viable spores/seed	Not pathogenic
Freshwater Fish Toxicity and Infectivity	Carp	2.0×10^6 CFU/mL, 2.0×10^7 CFU/mL, 2.0×10^8 CFU/mL	No treatment-related toxicity or pathogenicity

[a] Studies done with the technical grade active ingredient and not the end-use product containing the micro-organism, (PMRA-HC, 2007a)

Table A-58: Pathogenicity and toxicity testing results for *B. subtilis* ATCC 6051A and ATCC 55405[a]

Study Type	Target organism	Dose concentration	Outcome
Pathogenicity/toxicity testing	Red fescue (*Festuca rubra*)	10^5 CFU/g soil dry weight	Shoot length significantly affected ($p = 0.03$)[b]
Pathogenicity/toxicity testing	Springtail (*Folsomia candida*)	10^3 CFU[c]/g soil dry weight ; 10^4 CFU[d]/g soil dry weight	• Significant reduction ($p < 0.01$) in juvenile production[c] • No juvenile production (statistical analysis could not be performed)[d] • Adult survival not affected by either strain
Pathogenicity/toxicity testing	Earth worm (*Eisenia andrei*)	10^5 CFU[c]/g soil dry weight; 10^4 CFU[d]/g soil dry weight	No adverse effects reported

[a] Data generated by Environment Canada's Biological Methods Division
[b] The survival, growth and reproduction of test organisms were significantly inhibited in the field-collect soil relative to the artificial soil
[c] *B. subtilis* ATCC 55405
[d] *B. subtilis* ATCC 6051A

Appendix 12: Virulence and pathogenicity testing of the DSL *B. licheniformis/subtilis* strains

Murine exposure data generated by Health Canada's Healthy Environments and Consumer Safety Branch. Female BALB/c mice were exposed to 10^6 CFU/25µL of bacteria (vegetative cells or spores) via an endotracheal nebulizer for pulmonary exposure. Animals were necropsied at 24 hours and 1 week for vegetative cells and spores exposures respectively.

Clearance following endotracheal exposure

Table A-59: Enumeration of vegetative cells (CFU/mg) of DSL *B. licheniformis/subtilis* group strains following endotracheal exposure

Strain[a]	Lung	Trachea	Esophagus
B. amyloliquefaciens 13563-0	468.5	124.1	16.1
B. atrophaeus 18250-7	270.7	96.6	45.0
B. licheniformis ATCC 12713	323.1	31.4	14.6
B. subtilis subsp. *inaquosorum* ATCC 55406	228.4	1.5	4.8
Bacillus species 16970-5	249.2	17.6	30.2
Bacillus species 2 18118-1	235.0	51.8	17.8
Bacillus species 4 18121-4	310.7	47.3	44.3

[a] No data available for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *Bacillus* species 7 18129-3

Figure A-1: Enumeration of vegetative cells (CFU/mg) of DSL *B. licheniformis/subtilis* group strains following endotracheal exposure

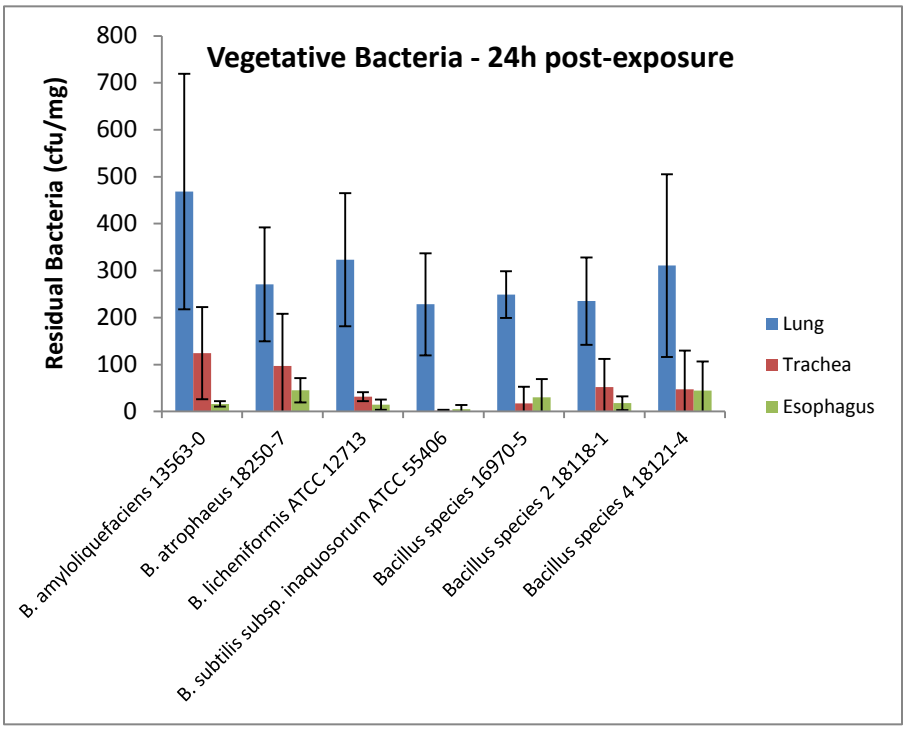
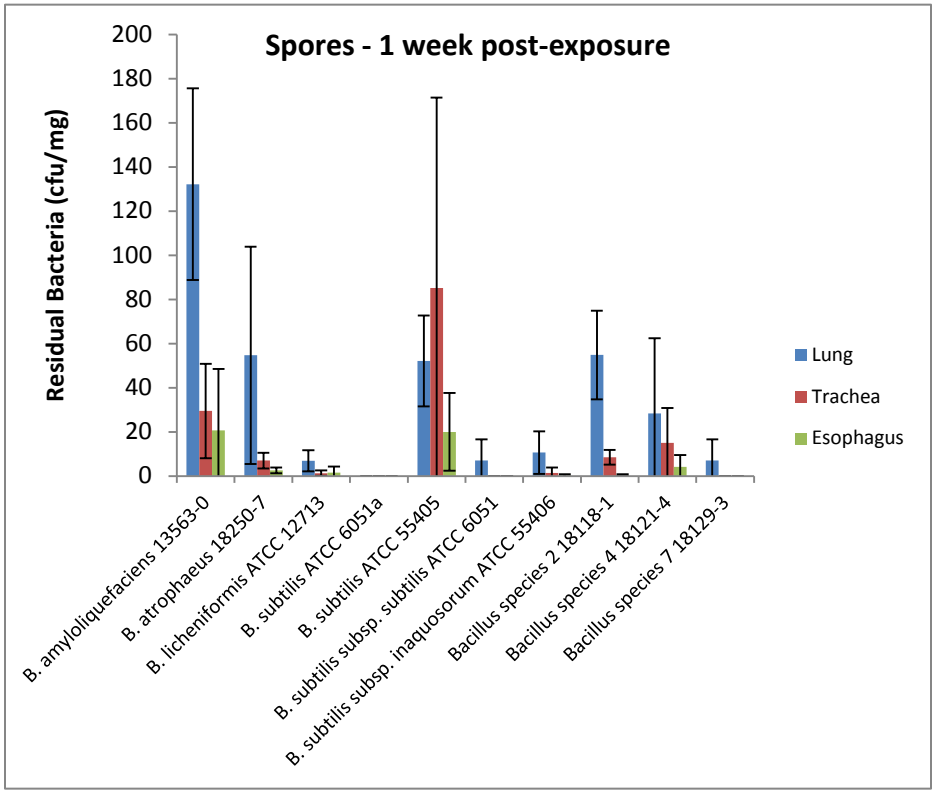


Table A-60: Enumeration of spores (CFU/mg) of DSL *B. licheniformis/subtilis* group strains following endotracheal exposure

Strain[a]	Lung	Trachea	Esophagus
B. amyloliquefaciens 13563-0	132.2	29.5	20.7
B. atrophaeus 18250-7	54.7	7.0	2.6
B. licheniformis ATCC 12713	6.9	1.3	1.6
B. subtilis ATCC 6051A	0.0	0.0	0.0
B. subtilis ATCC 55405	52.1	85.1	20.0
B. subtilis subsp. *subtilis* ATCC 6051	7.0	0.0	0.0
B. subtilis subsp. *inaquosorum* ATCC 55406	10.6	1.4	0.3
Bacillus species 2 18118-1	54.8	8.5	0.4
Bacillus species 4 18121-4	28.4	15.0	4.1
Bacillus species 7 18129-3	7.0	0.0	0.0

[a] No data available for *Bacillus* species 16970-5

Figure A-2: Enumeration of spores (CFU/mg) of DSL *B. licheniformis/subtilis* group strains following endotracheal exposure



Pulmonary Cytokines

Table A-61: Pulmonary cytokine expression (pg/mL) from vegetative cell exposures

DSL Strain[a]	IL-1 beta	IL-6	MCP-1	IL-12 (p70)	KC	TNF-alpha
Control (saline)	73.58 ± 13.26	2.02 ± 0.61	1501.94 ± 288.19	18.07 ± 2.26	7.32 ± 1.16	728.57 ± 107.77
B. amyloliquefaciens 13563-0	141.39 ± 60.31	1.46 ± 1.11	1597.70 ± 177.87	13.05 ± 5.77	62.46 ± 16.28	575.77 ± 58.43
Bacillus atrophaeus 18250-7	1619.34 ± 564.47	4.20 ± 0.73	4446.72 ± 1536.15	16.49 ±9.96	452.83 ± 160.76	718.82 ± 135.08
B. licheniformis ATCC 12713	1818.28 ± 573.73	5.80 ± 1.94	6032.81 ± 2094.65	19.08 ± 7.44	387.79 ± 146.88	705.84 ± 228.92
B. subtilis subsp. *inaquosorum* ATCC 55406	262.22 ± 44.63	1.10 ± 0.49	1624.45 ± 242.78	12.23 ± 4.65	34.61 ± 19.19	591.58 ± 87.85
Bacillus species 16970-5	101.13 ± 5.94	1.10 ± 0.53	1651.50 ± 319.11	16.38 ± 5.78	26.29 ± 18.31	667.01 ± 133.49
Bacillus species 2 18118-1	1444.876 ± 778.68	4.53 ± 2.24	5554.10 ± 2162.64	13.95 ± 2.00	660.90 ± 74.56	647.20 ± 205.74
Bacillus species 4 18121-4	7545.602 ± 1988.01	53.43 ± 21.70	20278.06 ± 7401.54	24.40 ± 7.21	2082.50 ± 501.70	983.90 ± 172.32

[a] No data available for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *Bacillus* species 7 18129-3

Table A-62: Pulmonary cytokine expression (pg/mL) from spore exposures

DSL Strain[a]	IL-beta	IL-6	MCP-1	IL-12 (p70)	KC	TNF-alpha
Control (saline)	90.86 ± 13.65	2.29 ± 1.27	837.12 ± 147.20	12.62 ± 8.05	9.13 ± 2.46	484.79 ± 160.58
B. amyloliquefaciens 13563-0	98.07 ± 11.21	2.87 ± 1.75	921.78 ± 187.96	9.82 ± 7.62	11.89 ± 4.39	476.05 ± 167.15
Bacillus atrophaeus 18250-7	120.36 ± 52.91	2.53 ± 1.60	750.55 ± 146.43	14.23 ± 8.82	9.48 ± 2.70	521.90 ± 110.22
Bacillus species 2 18118-1	104.37 ± 9.54	2.13 ± 1.78	843.20 ± 101.71	13.86 ± 6.73	10.0 ± 3.74	526.06 ± 115.86
Bacillus species 4 18121-4	90.31 ± 19.54	3.06 ± 1.47	884.30 ± 105.05	17.10 ± 6.72	7.17 ± 0.74	374.76 ± 91.07

[a] No data available for *B. licheniformis* ATCC 12713, B. *subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *inaquosorum* ATCC 55406, *Bacillus* species 16970-5 and *Bacillus* species 7 18129-3

Acute phase response

Table A-63: Serum Amyloid A (SAA)[a] Levels (µg/mL) in serum samples obtained from BALB/c mice treated with vegetative cells or spores of DSL strains

Strain[b]	Vegetative cells	Spores
Control (saline)	13.80 ± 3.52	14.08 ± 0.63
B. amyloliquefaciens 13563-0	17.87 ± 2.73	16.60 ± 2.09
Bacillus atrophaeus 18250-7	20.73 ± 1.21	21.56 ± 14.0

B. licheniformis ATCC 12713	39.54 ± 28.54	No data
B. subtilis subsp. *inaquosorum* ATCC 55406	17.76 ± 4.34	No data
Bacillus species 16970-5	13.72 ± 2.66	13.88 ± 3.10
Bacillus species 2 18118-1	23.35 ± 3.29	16.04 ± 2.38
Bacillus species 4 18121-4	17.68 ± 7.89	No data

[a] Serum amyloid A, an indicator of systemic effects, was measured using ELISA
[b] No data available for *B. subtilis* ATCC 6051A, *B. subtilis* ATCC 55405, *B. subtilis* subsp. *subtilis* ATCC 6051 and *Bacillus* species 7 18129-3

Figure A-3: Serum Amyloid A (SAA) Levels (µg/mL) in serum samples obtained from BALB/c mice treated with vegetative cells of DSL strains

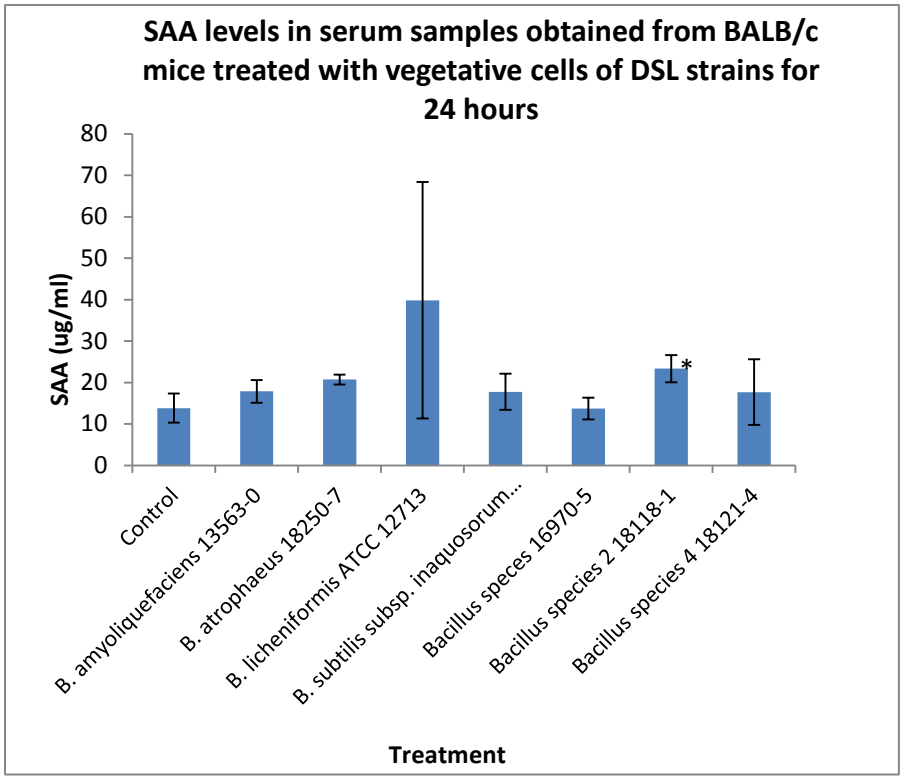
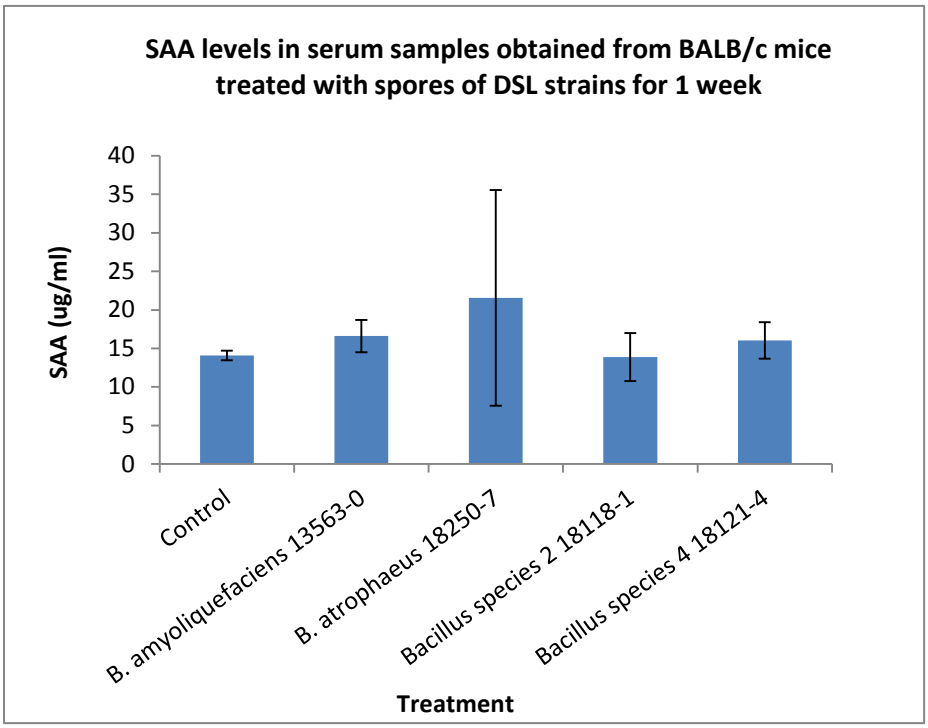


Figure A-4: Serum Amyloid A (SAA) Levels (µg/mL) in serum samples obtained from BALB/c mice treated with spores of DSL strains



Appendix 13: Food poisoning outbreaks

Table A-64: Food poisoning outbreaks involving *B. licheniformis*

Place	Year	# of Cases[a]	Symptoms	Cause	Death(s)
Veterans Administration Hospital, Denver, CO[b]	1959	161	Gastroenteritis including abdominal cramps, diarrhoea and vomiting	Cooked turkey meat that was held at room temperature overnight	1
Australia[c]	1976	49	Abdominal pain, diarrhoea and vomiting	Meals-on-wheels co-contaminated with *Clostridium perfringens* and *B. cereus*	1
Prison in Ohio, USA[d]	1995	165	No data	Turkey and gravy were implicated	No data
Kindergarten in Split, Croatia[e]	2000	12	Nausea, headache and vomiting	Contaminated milk powder that was prepared two hours prior to consumption and not boiled. Co-contamination with *B. subtilis*	0

[a] Case refers to an individual person diagnosed with food poisoning
[b] (Tong et al. 1962), though the authors implicate *B. subtilis* as the causative agent, the food poisoning was likely caused by *B. licheniformis* as the onset and symptoms are more in line with the description by Lund (1990) and biochemical testing results appear to be closer to *B. licheniformis* (e.g. growth in salt and anaerobic growth).
[c] (Jephcott et al. 1977)
[d] (CDC 1995)
[e] (Pavić et al. 2005), contamination of food as the result of toxin-producing isolated of *B. licheniformis* and *B. subtilis* was proven via vacuolation assay and MTT cell culture test.