COLORSPORE™

ABSTRACT

Colorspore™ *Bacillus indicus* HU36 (*Bacillus* HU36) is a unique patented strain of Gram-positive spore forming bacterium that produces a distinct yellow-orange pigmentation. The pigmentation is due to the synthesis of carotenoids, which are gastric stable, bio-accessible, and significantly more bioavailable than carotenoids from other sources. The *Bacillus* HU36 spores can survive the harsh conditions associated with food processing and may be incorporated into a range of food matrices without the need for refrigeration. This probiotic is also uniquely stable in liquids at room temperature and will remain dormant in non-refrigerated beverages. *Bacillus* HU36 was developed at London University as a part of the Colorspore Project—a consortium funded by the European Union—and is the first ingredient to encompass a market worth more than $75 billion. The project has comprehensively annotated *Bacillus* HU36, developed optimal bioprocess conditions for the production of carotenoid containing bacterial spores, demonstrated bio-accessibility and bioavailability, completed in-vivo and in-vitro toxicity, safety studies, and developed formulations for different food prototypes. *Bacillus* HU36 is the new paradigm in probiotic therapy and opens up a new range of delivery formats for companies in the food and dietary supplement markets.

VIRIDIS

Nu Science Trading, LLC

A Viridis BioPharma project presented by Nu Science Trading, LLC

COLORSPORE™

Carotenoid Producing Probiotic *Bacillus indicus* HU36 and its Use as a Functional Food and Dietary Supplements

Abstract

Colorspore™ *Bacillus indicus* HU36 (*Bacillus* HU36) is a unique patented strain of Gram-positive spore forming bacterium that produces a distinct yellow-orange pigmentation. The pigmentation is due to the synthesis of carotenoids, which are gastric stable, bio-accessible, and significantly more bioavailable than carotenoids from other sources. The *Bacillus* HU36 spores can survive the harsh conditions associated with food processing and may be incorporated into a range of food matrices without the need for refrigeration. This probiotic is also uniquely stable in liquids at room temperature and will remain dormant in non-refrigerated beverages. *Bacillus* HU36 was developed at London University as a part of the Colorspore Project—a consortium funded by the European Union—and is the first ingredient to encompass a market worth more than $75 billion. The project has comprehensively annotated *Bacillus* HU36, developed optimal bioprocess conditions for the production of carotenoid containing bacterial spores, demonstrated bio-accessibility and bioavailability, completed in-vivo and in-vitro toxicity, safety studies, and developed formulations for different food prototypes. Bacillus HU36 is the new paradigm in probiotic therapy and opens up a new range of delivery formats for companies in the food and dietary supplement markets.

VIRIDIS BioPharma Pvt. Ltd.
Door No. 6/ 10, Jogani Industrial Complex
V. N. P Marg, Chunabhatti,
Mumbai - 400022 INDIA
022 24055607 | viridis@vsnl.com
www.viridisbipharma.com

Nu Science Trading, LLC
428 E.THUNDERBIRD RD. # 510
PHOENIX, AZ 85022
PHONE: 602-375-7951
FAX: 602-375-7965

Table of Contents

Executive Summary

Colorspore™, *Bacillus indicus* HU36 is a unique strain of Gram-positive spore forming *Bacillus* species that produces a distinct yellow-orange pigmentation. Developed at London University as part of the Colorspore Project, funded with over €3 million by the European Union, this patented strain is a unique and valuable nutritional supplement and functional ingredient with a number of attributes, including:

- Rich pigmentation due to in-vivo synthesis of a number of carotenoids
- Carotenoids that are gastric stable and offer a unique source of nutritious compounds
- Proven immune stimulation and competitive exclusion of intestinal pathogens
- Robust spores that can be incorporated into food matrices without the need for refrigeration and stable in a variety of beverages including fruit and dairy
- Ability to withstand heating at 235°C up to 8 minutes, which allows for incorporation into a number of cooked and baked food products
- Stable as a nutritional supplement and food coloring
- Proven safe in extensive toxicology and feeding studies
- A fully sequenced genome
- Patent protection for the use of *Bacillus indicus* HU36 to deliver gastric stable carotenoids

From a commercial perspective, a probiotic supplement that can be stored at room temperature in a desiccated form, which does not need freeze- or spray-drying or complicated encapsulation methods, is operationally attractive and economically advantageous. The ability of *Bacillus* HU36 spores to survive harsh food processing conditions and the gastric environment, without loss of function or viability to colonize the gut, and the significant antioxidant potency of the carotenoids produced, offers new opportunities in human and animal nutrition frontiers and a competitive advantage with a significant barrier to entry. *Bacillus HU36* is a new paradigm in the use of probiotics for health and nutrition as it goes beyond the standard expectation of immune stimulation and digestive health, since it acts as a potent, nutritional source of key antioxidants that are more bioavailable than supplements and purified versions.

The in-vivo production along with the robustness of both the probiotic and the carotenoids, which can withstand not only processing conditions but also gastric juices and still offer high bio-accessibility and bioavailability that is superior to its counterparts in the marketplace, offers a bottom-line savings to every node in the value chain. *Bacillus* HU36 offers, for the first time, a technology and product combination that can serve a total market of over $75 billion dollars, which encompasses ingredients and finished products including supplements, foods and feeds at the intersection of carotenoids, antioxidants and probiotic fortification.

Tasks completed under the Colorspore Project include: comprehensively annotated genome of *Bacillus* HU36, optimization of bioprocess conditions for the production of carotenoid containing bacterial spores, demonstrations of carotenoid bio-accessibility and bioavailability, in-vivo and in-vitro toxicity and safety studies, and formulation studies of application of *Bacillus* HU36 in several food prototypes.

THE *BACILLUS* HU36 ADVANTAGE

Feature	Typical Probiotic (Lactobacillus/Acidophilus)	Other Spore Probiotics	Bacillus HU36
Helps with digestive health	√	√	√
Provides Immune stimulation	√	√	√
Competitively excludes pathogens	√	√	√
Proven Safety	√	√	√
Gastric Stable		√	√
Stable in Desiccated Form		√	√
Doesn't require enteric coating		√	√
Process Stable - high sheer, high pressure, harsh ingredients, etc.		√	√
Thermo Stable to baking and pasteurizing temperatures		√	√
Stable in room temperature liquids - will remain dormant			√
Does not require refrigeration even in high carbohydrate beverages			√
Produces high bioavailable carotenoid in vivo			√
Rich source of powerful antioxidants			√

Introduction

The human intestinal tract hosts approximately 100-trillion microorganisms, which help promote normal gastrointestinal function, systemic metabolism and immune function. Extensive processing of food, sterilization of the food supply, and depletion of nutrients from the soil, have rendered most modern diets deficient in these essential microorganisms or healthy bacteria. Emerging knowledge of the benefits of probiotic consumption has propelled consumer interest in effective and consumer-friendly formats of these mighty bacteria.

Probiotics - Probiotics are bacteria used largely for immune stimulation and competitive exclusion. Most bacteria are highly sensitive to pH, light, heat, and harsh environmental conditions. In nature, some species produce spores as a means to survive extreme environmental conditions to enable long-term survival in conditions that could otherwise kill vegetative bacteria. Spores are gaining significant attention today on account of their stability in high acid and high temperature environments. Spore-forming *Bacillus indicus* HU36 are able to differentiate into an endospore (spore), a quiescent cell form characterized by several protective layers surrounding a dehydrated cytoplasm. This structural organization makes the spores extremely resistant to external physical and chemical insults and able to survive almost indefinitely in the absence of water and nutrients and to revive upon encountering the appropriate environment. The current probiotic market is just shy of $20 billion with spores quickly gaining market share.

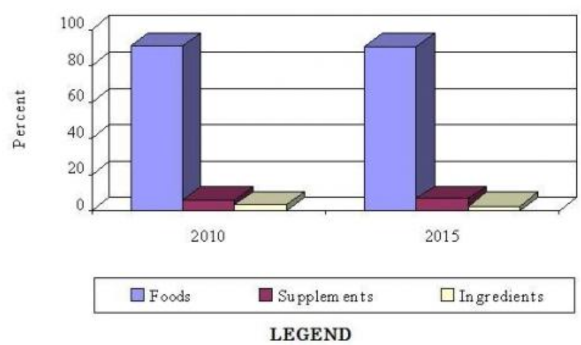
Carotenoids - The growing body of scientific evidence of the health benefits of fruits and vegetables has heightened consumer awareness about the health properties of carotenoids. Consumers understand that a diet rich in foods that contain yellow, orange and pink/red carotenoid pigments can reduce the risk of cardiovascular disease and improve mortality rates (Kohlmeier et al. 1997, Kristenson et al. 1997, Rissanen et al. 2000, IFIC Food Insights 2011). The most popular carotenoid, lycopene in tomatoes, is known for its prostate cancer risk reducing abilities (Giovannucci 1999, Giovannucci 2002, Giovanucci et al. 1995). Other popular carotenoids are beta-carotene, astaxanthin, lutein, etc. The health properties of carotenoids can be explained by antioxidant-activity in-vitro assays. However, there are limitations currently to their use as a natural coloring for foods and beverages, and, as a nutrient for humans and animals. Carotenoids are hydrophobic in nature and are therefore, poorly soluble in aqueous solutions and unstable in the digestive system; therefore bioavailability of supplemented or fortification carotenoids tends to be quite low.

The New Paradigm - *Bacillus* HU36, a spore-forming *Bacillus* species, capable of synthesizing carotenoid pigments, offers a viable alternative bolstered further by its stability and absorption characteristics. The patented HU36 spores are heat stable, making them suitable for applications in heated foods and baked goods. The yellow and orange spores also may be incorporated into other food matrices as a coloring and as a nutrient, without the need for refrigeration. This new technology opens new opportunities for human and animal nutrition, especially as interest in the health benefits of carotenoids continues to grow. A large portion of research has repeatedly shown that individuals who consume a variety and quantity of fruits and vegetables (rich in carotenoids) and those with higher serum β-carotene levels show lower risk of cancer, particularly lung cancer (Mayne 1996, Krinsky 1992). Just as there are studies showing the benefits of carotenoids consumed in its natural state, as a food, there are also inconclusive and conflicting studies of carotenoid use in a pill form (Bjelakovic 2008, Koo 1997).

As such, there are no official recommendations for carotenoid dosage in a supplement form, which varies widely from 1.5mg to 800mg. In a supplement form, carotenoids have limited bioavailability due to gastric degradation.

Studies of β-carotene supplements, by the Royal Holloway University of London (RHUL), show that in as little as 10 minutes after consumption, virtually all the β-carotene is undetectable. The patented technology of *Bacillus* HU36 removes the current barriers of gastric degradation. The HU36 spores survive the gastric barrier without the loss of function, colonize in the gut and show significantly more antioxidant activity than lycopene and other popular carotenoids. *Bacillus HU36* offers a first-to-market opportunity for a product that serves both the probiotic and carotenoid markets in very diverse and unique delivery formats.

In recent years, probiotics have entered the mainstream market and rapidly gained momentum in both dietary supplements and functional food applications. Global sales of probiotic ingredients, supplements, and foods are forecasted to reach $31.1 billion by 2015 (CAGR of 7.6%) with probiotic foods accounting for the largest share (90.1%) of total sales ($19.6 billion in 2010) and expected to reach $28.1 billion by 2015 (CAGR of 7.5%). Probiotic supplements, on the other hand, netted $1.3 billion in 2010, and are predicted to reach $2.07 billion by 2015 (CAGR of 9.6%) (Figure 1, BCC Research).



Probiotics	2010	2015
Foods	90.7	90.3
Supplements	6.1	6.7
Ingredients	3.2	3.0
Total	100.0	100.0

Figure 1: Probiotic foods, supplements & ingredients (BCC Research, 2011)

Bacillus Probiotics

Probiotics are live microbes, which when administered in adequate amounts confer a health benefit to the host (Araya et al 2002). *Bacillus* species has been used as probiotics for at least 50 years starting with an Italian product known as Enterogermina® which was registered in 1958 in Italy as an over-the-counter medicinal supplement. The scientific interest in *Bacillus* species as probiotics though, has only occurred recently in the last 15 years, and three principal reviews have covered the field: Hong et al 2005, Mazza et al 1994, Sanders et al 2003. The species that have been most extensively examined are: *Bacillus subtilis, Bacillus clausii*, *Bacillus* cereus, *Bacillus coagulans* and *Bacillus licheniformis*.

Bacillus species have been well characterized with regard to their probiotic potential as a means to stimulate immunity, exclude harmful pathogens, secrete antimicrobials, and produce beneficial nutrients. These attributes prove remarkably beneficial in enhancing the health properties of other nutrients and aid in removing many of the barriers to efficacy, bioavailability and ultimately commercial applications.

Additionally, *Bacillus* species are Gram-positive bacteria that produce endospores, which are uniquely robust entities that are able to survive temperature extremes, irradiation and long-term storage (Nicolson, 2002). A second advantage is that the spore is capable of surviving the low pH of the gastric chamber (Spinosa et al 2000, Barbosa et al 2005), which is not the case for all species of Lactobacillus. So in principle, a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact (Tuohy et al 2007).There is sufficient scientific evidence to support *Bacillus* species as safe for human consumption and substantive data to support possible health claims related to digestive health, vascular health, joint health and immunity. Studies in human nutrition show the following benefits from *Bacillus* spores[1]:

- Immune modulation for childhood allergies
- Immune stimulation of peripheral T-lymphocytes and B-lymphocytes
- Decrease in frequency of urinary tract infections and positive cultures
- Reduction in side effects related to anti-H. pylori antibiotic therapy
- Effective treatment for small intestinal bacterial overgrowth (SIBO)
- Diminished duration of diarrhea in children 3 to 36 months of age
- Reduced incidence of irritable bowel syndrome diarrhea
- Immune response to adenovirus and influenza-A in-vitro
- Improvement in pain scale in Rheumatoid arthritis patients

[1] *Sources: Marseglia et al. 2007; Ciprandi et al. 2005, Caruso et al 1993, Fiorini et al 1985, Coppi et al 1985, Nista et al 2004, Canani et al 2007, Gabriellu et all 2009, Hun 2009, Baron, 2009, Mandel et al. 2010).*

Bacillus Indicus HU36

Bacillus indicus HU36 is a well annotated and understood microbe and has been well characterized with regard to its probiotic potential as a means to stimulate immunity, exclude harmful pathogens, secrete antimicrobials, and produce beneficial nutrients.

Carbohydrate Active Enzymes (CAZymes): The genome annotation of HU36 shows a high total number of CAZymes, which are categorized in the Carbohydrate-active Enzymes Database (www.cazy.org)database into five families: glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and carbohydrate binding modules (CBMs). Comparison of the CAZymes identified in the genomes of HU36 with other *Bacillus* species and validation by experimental data on carbohydrate utilization, levan-based biofilm formation and mucin-binding and mucin-degradation, suggests that HU36 is adapted to the intestinal environment and suited to grow in and colonize the human gut (Figure 1) and degrade mammalian glycans.

Species	GH[a]	GT[b]	PL[c]	CE[d]	CBM[e]	Total
***Bacillus firmus* GB1**	**58**	**42**	**2**	**14**	**24**	**140**
***Bacillus indicus* HU36**	**33**	**48**	**0**	**11**	**27**	**119**
Bacillus clausii KSM-K16	43	30	4	14	11	102
Bacillus cereus ATCC14579	28	48	0	15	13	104
Bacillus cereus ATCC10987	20	42	0	17	14	93
Bacillus cereus AH187	26	40	0	18	16	100
Bacillus cereus G9842	28	48	0	18	15	109
Bacillus pumilus SAFR-032	35	34	2	19	4	94
Bacillus subtilis subsp. spizizenii str.W23	42	37	6	13	27	125
Bacillus subtilis subsp. natto BEST195	55	38	5	13	34	145
Bacillus subtilis subsp. subtilis str.168	48	40	6	13	24	131
Bacillus amyloliquefaciens DSM7	41	36	3	10	25	115
Bacillus pseudofirmus OF4	22	22	0	9	10	63
Geobacillus kaustophilus HTA426	19	28	0	8	15	70
Geobacillus thermodenitrificans NG80-2	29	24	0	12	10	75
Alicyclobacillus acidocaldarius subsp. acidocaldarius DSM446	29	31	0	9	13	82

[a]GH: Glycoside Hydrolases; [b]GT: Glycosyl Transferases; [c]PL: Polysaccharide Lyases; [d]CE:Carbohydrate Esterases; [e]CBM: Carbohydrate Binding Modules

Table 1: Comparative analysis of the number of putative genes for the five CAZyme categories in selected spore forming Bacilli (Manzo et al., 2011)

Probiotic Effect: There is mounting evidence of the probiotic effect of *Bacillus indicus* HU36 on immune stimulation and the importance of *Bacillus indicus* HU36 for the development of a robust gut-associated lymphoid system (GALT). Toll-like receptors (TLRs) are integral to the initial recognition of organisms during infection; TLR2 is a known Gram-positive pathogen recognition receptor. Studies of RAW264.7 macrophage cultures incubated with *Bacillus* subtilis HU36 showed that spores (Sp), vegetative cells (Cell) and even autoclaved (AC) and UV-irradiated (UV) spores of *Bacillus indicus* HU36 can stimulate/activate the TLR2 which is the first step to cytokine induction. These results were compared against the backdrop of PGN, the peptidoglycan and internal control, and MED, the medium and baseline (Figure 2).

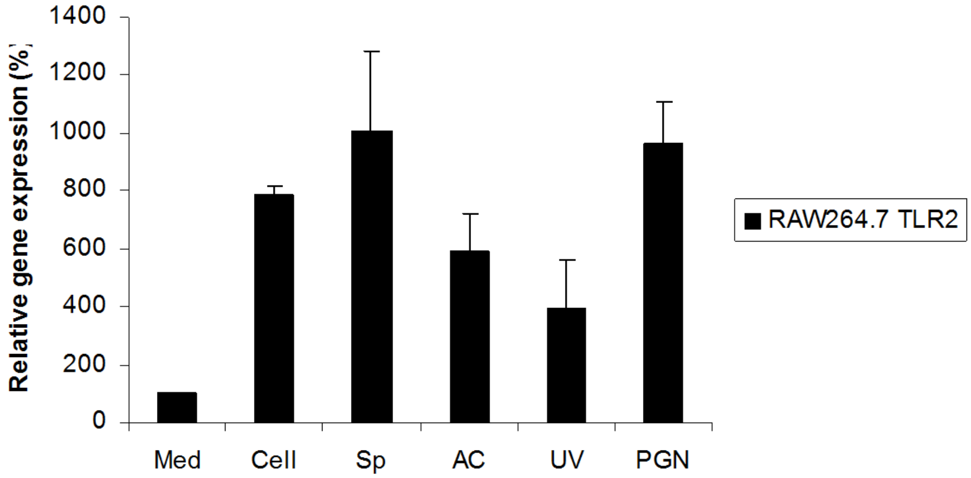


Figure 2: RAW264.7 macrophages incubated with Bacillus subtilis HU36 for 4hrs before RNA extraction (TLR2 gene expression)

The *Bacillus indicus* HU36 spores (Sp) and vegetative cells (Cell) can also stimulate expression of TNFa—a proinflammatory cytokine (Figure 3). The induction of cytokines is required to activate the innate immune response and supports the role of HU36 spores as signalers of immune stimulation.

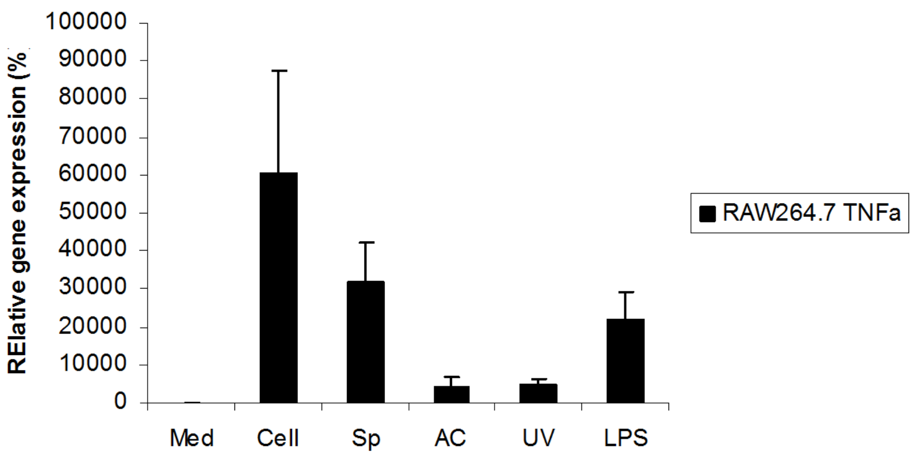


Figure 3: RAW264.7 macrophages incubated with Bacillus subtilis HU36 for 4hrs before RNA extraction (TNFa gene expression)

These studies have shown that *Bacillus indicus HU36* does provide immune stimulation as is expected from probiotics in its class. Thus, it does function as an effective probiotic.

Genetic Composition of *Bacillus indicus* HU36

Bacillus HU36 is a Gram-positive filamentous, rod-shaped organism that appears as individual rods or occasionally in short chains.

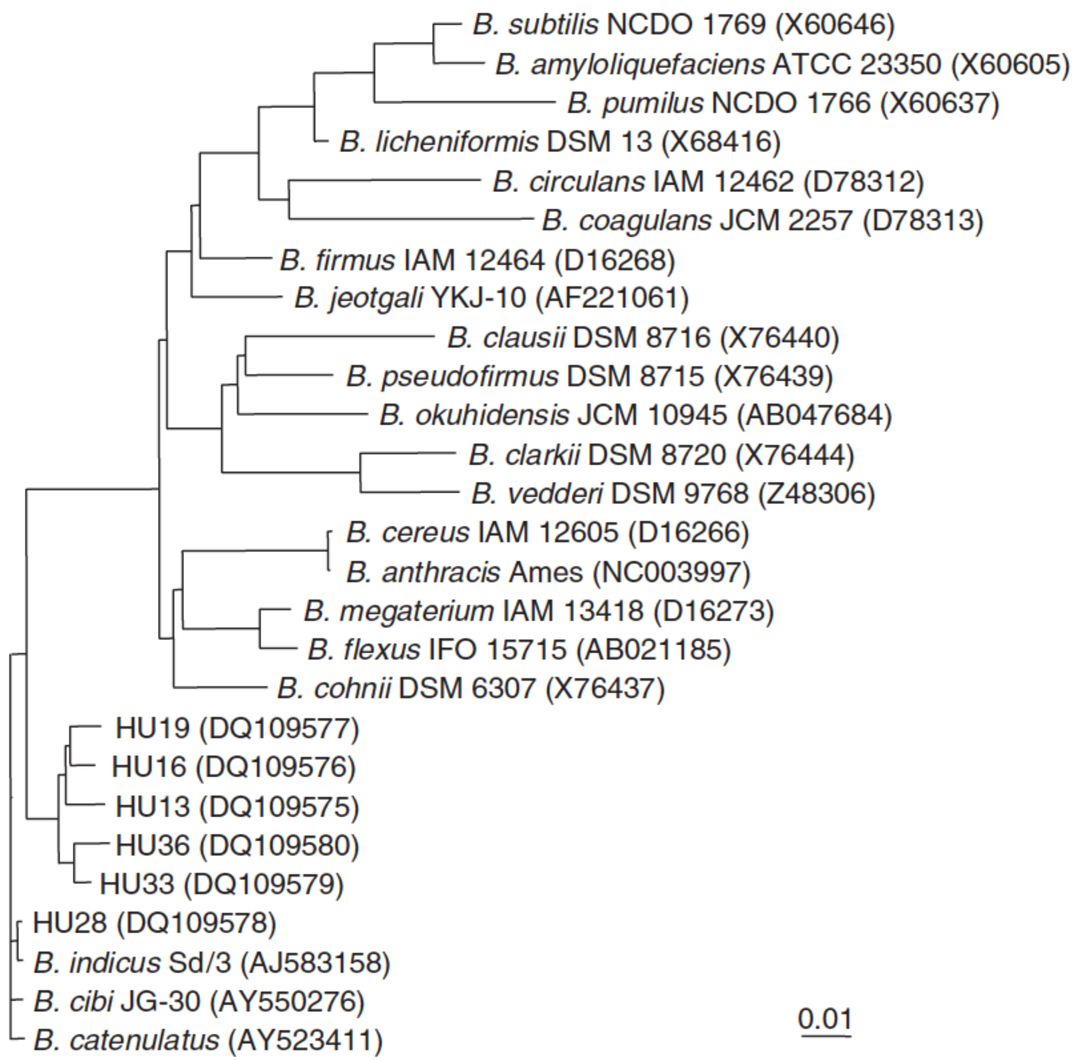


Figure 24: Phylogenetic relationship of six pigmented isolates

This phylogenetic illustration shows the origin of *Bacillus HU36,* which is well known and documented. The organism is natural with no genetic modification.

Colonization

Bacillus indicus HU36 was isolated from freshly voided human feces and described by Duc et al in 2006. Thus it is a natural variant of the human intestinal tract and is well suited for colonization. *Bacillus HU36* has been in regular use as a probiotic in South East Asia supplied by AnaBio out of Vietnam (they have patent protection from The London University and non-exclusive use license for South East Asia only) and is also naturally present in seafood, which is a part of a regular diet of a higher percentage of the world's population.

Beyond the fact that *Bacillus* HU36 was isolated from the human colon, there are in vivo and in vitro studies to demonstrate its ability to colonize the gut. Hong et al (2008) studied the adhesion of *Bacillus* HU36 to Caco-2 and HEP-2 cells in addition to a mucus-secreting cell line, HT29-16E, using in vitro methods. The researchers chose to add HT29-16E cell lines because HT29-16E cells exhibit differentiation features characteristic of mature intestinal cells, including the secretion of mucin, and are therefore presumably more informative than Caco-2 cells. These cell lines are widely accepted models for gut colonization and are well documented in the literature. The researchers took a suspension of c. 10^7–10^8 vegetative cells of the strain and it was incubated for 2 h with the cultured cell line, after which the number of adhering cells was measured. The Bacillus HU36 spores showed positive adhesion that would infer colonization over a 7 day period. A further study on the ability of *Bacillus* HU36 to colonize was performed at Royal Holloway London University using live mice in an in vivo model. The researchers had groups of 4 mice each dosed with 1 X 10^9 cfu of a pure suspension of spores. At the indicated days thereafter, fecal pellets were sampled. The pellets were homogenized in PBS buffer, heated at 65^{o}C for 45min and serial dilutions plated on DSM agar for plate counting (cfu/g of feces). The detection limit was ~1-2 X 10^2 cfu/g feces.

Table 1: Colonization in mice

Time (days)	Naïve - Control	*Bacillus Subtilis*	*Bacillus* HU36
0	1 X 10^2	1 X 10^9	1 X 10^9
2	2 X 10^2	4 X 10^7	4 X 10^8
4	7 X 10^2	5 X 10^6	3 X 10^8
6	5 X 10^2	3 X 10^5	5 X 10^7
8	4 X 10^2	3 X 10^4	1 X 10^7
10	8 X 10^2	2 X 10^3	5 X 10^6
12	1 X 10^2	2 X 10^2	3 X 10^6
18	3 X 10^2	3 X 10^2	7 X 10^5
20	2 X 10^2	3 X 10^2	3 X 10^4
22	5 X 10^2	3 X 10^2	3 X 10^3
36	1 X 10^2	1 X 10^2	2 X 10^2

The data showed clear and strong colonization in mice by *Bacillus* HU36 even as compared to a very common and widely used probiotic spore, *Bacillus Subtilis*. This model studies the persistence of the spore in the gastrointestinal tract of the mouse model. The weaker a colonizer, the faster the cells are shed. Bacillus HU36 showed slow shedding and thus strong colonization especially when compared to a commonly used spore probiotic. *Bacillus* HU36 cells were detectable in fecal samples up to 22 days.

Background on Carotenoids

Carotenoids are lipophilic antioxidants. There are more than 800 carotenoids described in nature to date. These compounds are synthesized mainly in plants, but have also been isolated from yeasts, fungi, marine algae, micro-algae and some species of bacteria. Carotenoids are recognized widely as giving many plants, fruits, flowers and vegetables their red, orange and yellow colors; but it is through diet that many animals such as salmon, trout and flamingos obtain their flesh and feather coloring.

There is growing evidence that people consuming diets rich in carotenoids from natural foods, such as fruits and vegetables, are healthier and have lower mortality from a number of chronic illnesses (Diplock et al. 1998). The health-promoting properties of carotenoids have intensified interest in natural carotenoid sources and their utilization in human nutrition (Rao & Rao, 2007). All carotenoid structures are derived from C30 or C40 hydrocarbon chains. These are highly unsaturated with up to 15- conjugated-double bonds. Most of them carry cyclic end groups such as alpha- or beta-ionone rings. Typically, these ionone rings are modified by oxo groups, like 3-hydroxy, 4-keto and 5,6-epoxy. Carotenoids containing oxygen atoms are called xanthophylls; those containing only carbon and hydrogen atoms are carotenes.

Carotenoids in animals including humans are widely recognized as having important antioxidant activities: some act as a precursor of vitamin A and some have specific cellular effects (e.g. gap junction communication, stimulation of gene expression). There is also increasing evidence that some of these play an important role in benefiting human health, for like animals, humans are unable to synthesize these molecules and therefore must absorb them from food. To date, all studies showing beneficial health effects imply the carotenoids must be consumed in their natural state as a fruit and vegetable (Bjelakovic 2007, Satia et al. 2009, and Nagel et al. 2010).

Studies in human nutrition show the following benefits from carotenoids[2]:

- α-carotene from fruits and vegetables reduces risk of lung cancer
- β-carotene from fruits and vegetables reduces risk of cancer of the skin, cervix, uterus, mouth, stomach, lung and bladder
- β-cryptoxanthin, high intake from fruits and vegetables, decreases cervical cancer risk
- Lutein and Zeaxanthin, from corn and green leafy vegetables, lowers risk of macular degeneration
- Lycopene, from tomatoes, reduces risk of heart disease and prostate cancer

The specific mechanisms by which carotenoids reduce the risk and development of cancer in humans is not yet fully understood, but carotenoids show several properties that could prevent or slow the progression of cancer (De Flora et al 1999, Burton et al 1984). It is believed that most carotenoids may inhibit growth and malignant transformation and signal apoptosis (Sumantran V.N. et al, 2000). The anti-inflammatory action and anti-carcinogenic health benefits are believed to be a result of gene expression via antioxidant responsive gene elements. The apparent cancer-preventive effects may depend on the enhancement of DNA repair, as well as augmenting cellular antioxidant defenses that help protect cellular components from oxidative damage (Lorenzo et al. 2008).

[2] *Sources: Hinds et al 1984, Stahl et al 2000, MW et al. 2012, Rock 2002, Tan et al. 2008, Giovannucci 2002, Lee et al 1999*

The carotenoids with the most promise of health promoting properties are lycopene, β-carotene, and provitamin A, which is essential for human health (Olsen et al. 1965, Snodderly et al. 1995). Carotenoids with a C40 backbone show the greatest potential in commercial applications given their abundance in nature and current commercial opportunities, particularly in the animal feed sector and for dietary supplement production.

The most economical means of producing carotenoids is through chemical synthesis. However, there are barriers to their success, such as the production of stereo isomers not found in the natural product, contamination with reaction intermediates/products, and lack of potential synergistic nutrients present in the biological mixtures. The byproducts of chemical synthesis do not appeal to growing consumer demand for natural ingredients and coloring, with less processing and environment impact.

Unicellular algae sources of commercially available carotenoids exist, including *Dunaliella salina, Spirulina*, and *Haematococcus*. Carotenoids are also produced commercially from the yeast *Phaffia rhodozyma*, and filamentous fungi *Blakeslea trispora* and *Phycomyces blakesanus*. Unicellular algae present various limitations including slow growth, cooled fermentation requirements and reproductive assistance for high yields. At present there is only one viable plant source—Tagetes flowers (*Tagetes erecta*)—for commercial carotenoid production.

Another option is genetic encoding of biosynthetic enzymes from microbial plant sources, which has been used in Escherichia coli (Misawa et al., 1995) and with food yeast *Candida utilis* (Miura et al., 1998). Metabolic engineering in crops is another alternative, though concern over genetically modified food has prevented utilization of carotenoid crops and commercial production.

Carotenoids are a high-value fine chemical with attractive physiological and therapeutic benefits, but only for the naturally occurring form. Purified and synthetic carotenoids used for fortification and supplementation have poor stability and bioavailability and thus makes it difficult to deliver therapeutic doses. The solution to increased demand and commercial barriers to natural sources of carotenoids clearly lies in *Bacillus HU36*- carotenoids.

Bacillus indicus HU36 Carotenoids

Spore-forming *Bacillus indicus* HU36 produce pigments, mostly carotenoids, with a putative protective role against UV irradiation and oxygen-reactive forms. An exhaustive study by the Royal Holloway University of London (RHUL) of the pigmented *Bacillus* has defined the specific carotenoid pigments and profiles produced by these bacteria. A diverse range of spore-forming *Bacillus* spp. (multiple species) that contains carotenoid pigments has been isolated (Khaneja et al. 2009). These bacteria possess the ability to act as probiotics, with the health benefits and color attributes of carotenoids. The most commonly found pigments were yellow, orange and pink. The carotenoids consist of keto/hydroxyl-carotene derivatives, astaxanthin, 4-ketozeaxanthin, echinenone, a hydroxyl-echinenone derivative, phenicoaxanthin, canthaxanthin, zeaxanthin, and/or α-carotene.

Between 2009 and 2012, the European Union's 7th Framework Programme sponsored a €3.5 million program-grant named COLOSPORE. The project, coordinated by Professor Simon Cutting (RHUL), did an intensive search for new sources of beneficial carotenoids and identified a collection of unique pigmented bacteria each of which was a novel source of carotenoids. These were shown to have the following key attributes:

- Gastric-stable carotenoids that are not destroyed in the GI tract, therefore are far superior to existing sources of carotenoids
- Safe strains with toxicology studies performed (HU36 and GB1)
- Genomes of two prototype strains sequenced (HU36 and GB1)
- Fully characterized biosynthetic pathways (HU36 and GB1)
- Full analysis of the metabolomes of two strains, HU36 and GB1
- Evidence in vitro and in vivo for bioaccessibility and bioavailability

The COLOSPORE strains have been shortlisted to two novel strains for incorporation into foods; these are *Bacillus indicus* HU36 (yellow-orange) and *Bacillus firmus* GB1 (pink-red). Both of these strains have been licensed exclusively to Viridis Biopharma. For the purposes of this paper, the discussion is limited to *Bacillus indicus* HU36.

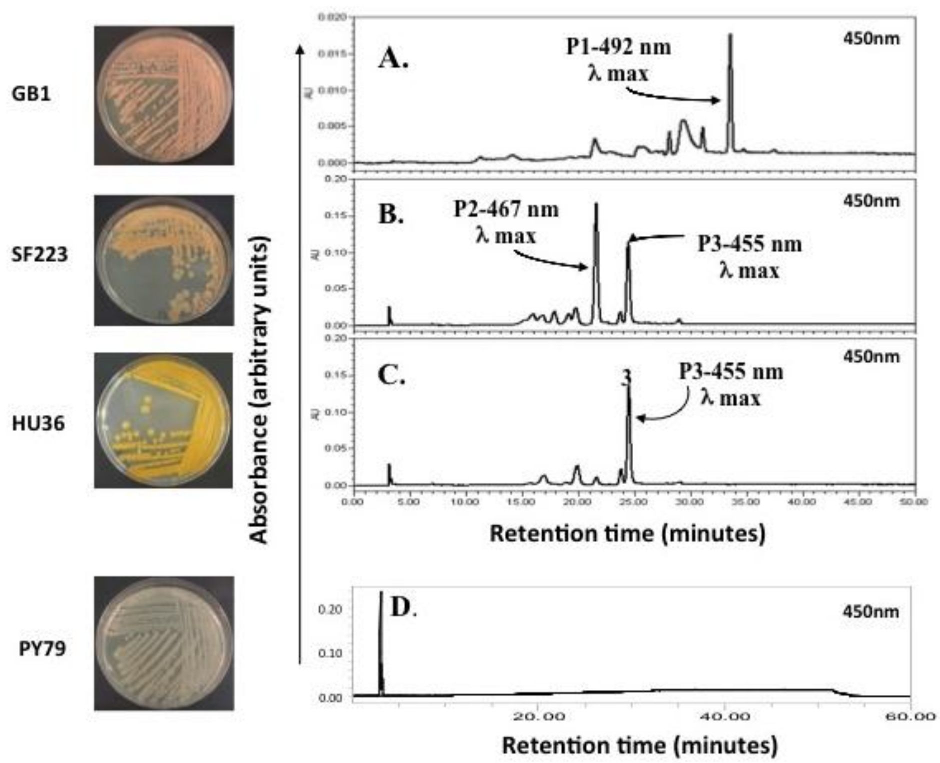


Figure 4: Carotenoid profiling by HPLC.

The exhaustive Colorspore study of pigmented Bacilli undertaken at the RHUL showed the predominance of three types of carotenoids with absorption maxima at 455nm, 467nm and 492nm, corresponding to the visible colors yellow, orange and pink, respectively (Figure 4). Although the presence of other carotenoids cannot be ruled out, these three predominant carotenoids appear to account for the pigments obtained in most pigmented Bacilli. The function of carotenoids is photo-protection, and carotenoid-containing spores exhibited significantly higher levels of resistance to UV radiation than non-carotenoid containing *Bacillus* species. The biochemical analysis of the carotenoids responsible for the yellow/orange pigmentation present in *Bacillus indicus* HU36 has been carried out and the identity of the carotenoids present has been elucidated in detail (Figure 5).

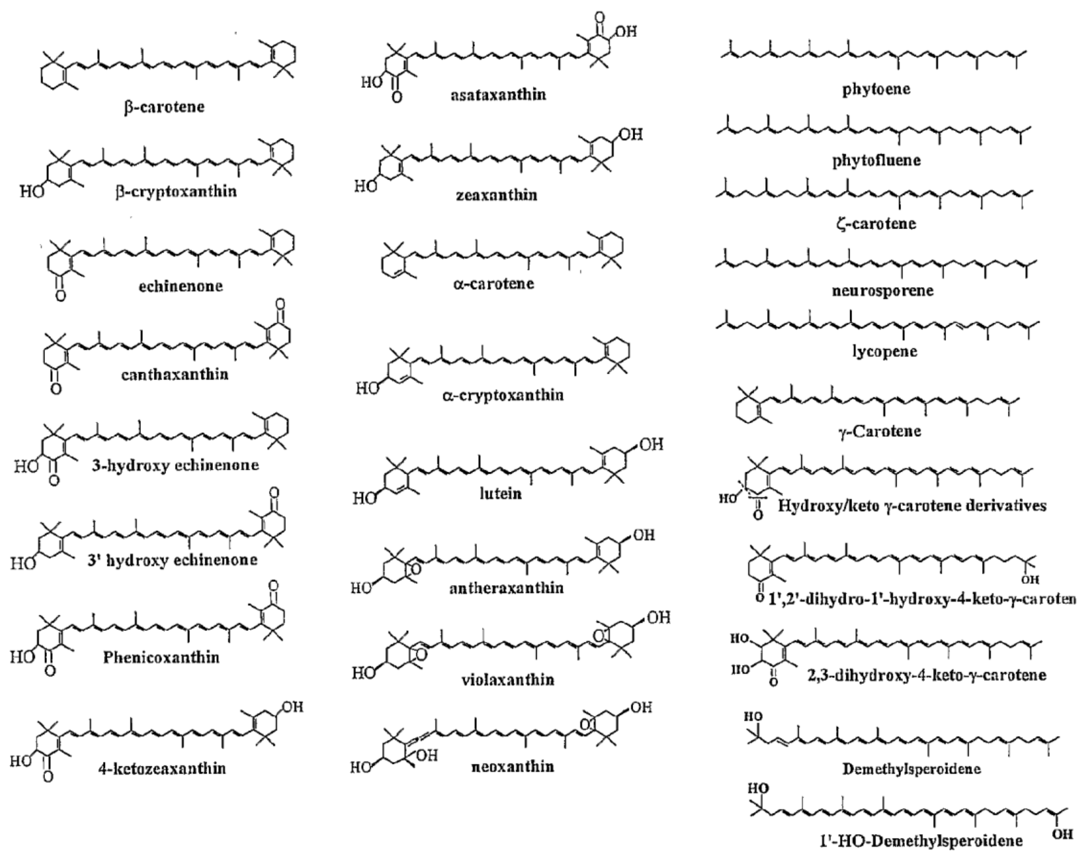


Figure 5: Carotenoids produced by Bacillus indicus HU 36.

Carotenoids are isoprenoids and thus originate from the five-carbon building block, isopentenyl pyrophosphate (IPP), which is the universal precursor of all isoprenoids. Supporting this, squalene has been identified in numerous Gram-positive bacteria including *Bacillus* (Amdur et al 1978). Squalene has been proposed to be an important part of the Mediterranean diet as it may be a chemopreventative substance that protects people from cancer (Smith 2000, Owen et al 2004). Besides a chromophore (which is responsible for the color) another structural feature is their long hydrocarbon chain that conveys a pronounced hydrophobic chemical nature. These lipophilic properties ensure that virtually all *Bacillus*- carotenoids would be located in membranous structures or particular lipid globules.

Chromatographic, UV/Vis and Mass Spectral (MS) data have revealed the exclusive presence of a C30 carotenoid biosynthetic pathway in *Bacillus* species (Perez-Fons et al 2010). Apophytoene was detected representing the first genuine carotenoid formed by this pathway. The most abundant carotenoids present in the *Bacillus* species were oxygenated derivatives of apolycopene, which have either undergone glycosylation and/or esterification. The presence of fatty acid moieties (C9 to C15) attached to the sugar residue via an ester linkage was revealed by saponification and MS/MS analysis. The most abundant apocarotenoids determined were glycosyl-apolycopene and glycosyl-4'-methyl-apolycopenoate esters. Analysis of these carotenoids over the developmental formation of the Bacillus indicus HU36spores revealed that 5-glycosyl-4'-methyl-apolycopenoate synthesis was coupled to sporulation. Potential biosynthetic pathways for the formation of these apocarotenoids in vegetative and spores have been reconstructed from intermediates and the end products have been elucidated. A combination of biochemical techniques have been used to demonstrate the presence of carotenoids and their derivatives in *Bacillus indicus* HU36 and also their growth pattern of the microbe (Figure 5).

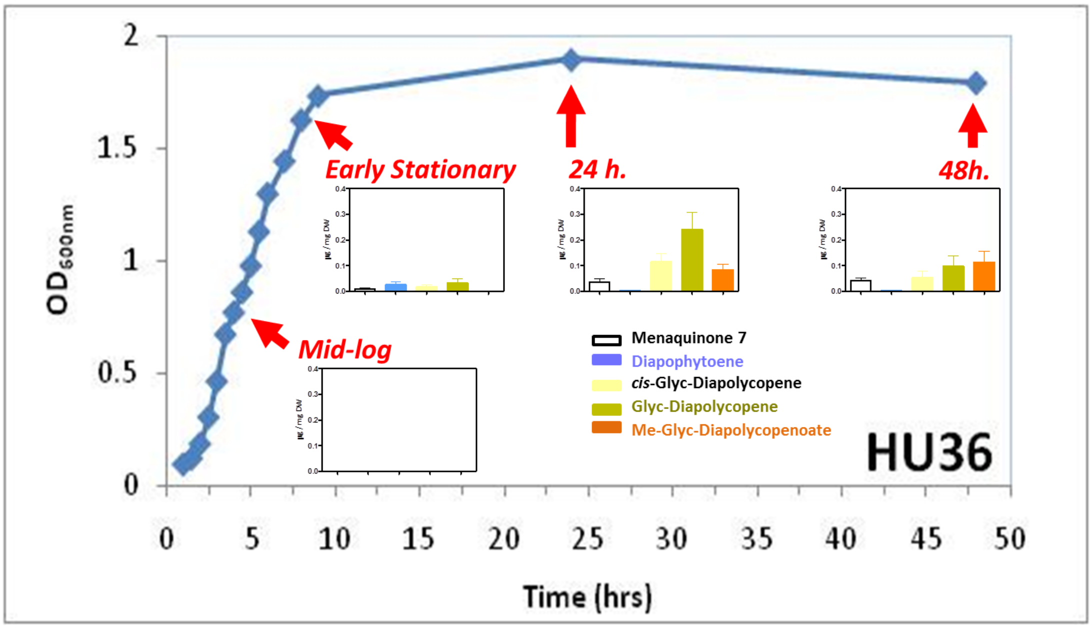
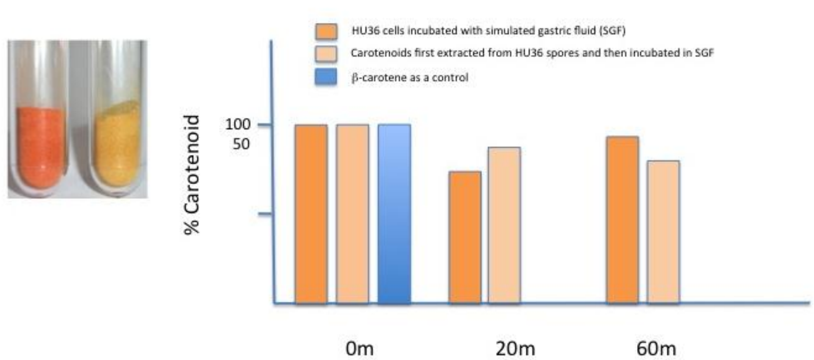


Figure 6: Overview of cellular metabolism of Bacillus indicus HU 36.

The yellow pigment predominates in vegetative cells, while the formation of the orange pigment is enhanced during sporulation. The developmental regulation of the biosynthetic pathway characterized thus far in *Bacillus indicus HU36* is extensive and serves as a platform to help determine the potential biotechnological importance of these molecules in the health, food and feed sectors.

Gastric Stability

A key discovery regarding *Bacillus indicus HU36carotenoids* is their gastric stability because they are di- or mono-acids (Figure 6). The low pH of gastric juice (pH 2) makes it a highly corrosive solution and studies carried out by the RHUL consortium have shown that when ß-carotene, for example, is incubated in simulated gastric juices it is almost completely degraded in as little as 10 minutes with no detectable amounts after 20 minutes. In contrast, *Bacillus indicus HU36*derived carotenoids (whether extracted from the spore or using the whole spore) were essentially unaffected and after 60 minutes of incubation showed no more than 10-20% degradation. The standard transit time for food entering the stomach is 60 minutes, which means that unless carotenoids can evade the action of gastric fluids (for example, by being protected within food particles) a large percentage of the ingested dose will have been degraded by the time it reaches the small intestine. This is one of the reasons why carotenoid supplements carry such a large recommended daily allowance (RDA, 800 mg).



The panel on the left shows dried spores (orange) and cells (yellow) of HU36 (Bacillus indicus). Panel on the right shows stability studies of these carotenoids compared to β-carotene in gastric juices.

Figure 7: Gastric stable Bacillus carotenoids

This type of gastric stability, as seen with the *Bacillus HU36* carotenoids, is of immense value to consumers and a major competitive advantage for food and dietary supplement makers. The Colorspore Project further demonstrated the stability of bacterial carotenoids (against the backdrop of standard carotenoids—lycopene, ß-carotene, and astaxanthin) in conditions encountered in the gastro-intestinal tract, specifically, in the presence of iron, a major cause for instability of carotenoids. A micellar system was used to mimic the low acid environment in the stomach following a meal consisting mainly of meat; oxygen and iron were streamed in at different levels. Analysis of the reaction medium by HPLC/MS showed that the standard carotenoid esters degrade preferentially than the *Bacillus* carotenoids (Figure 7).

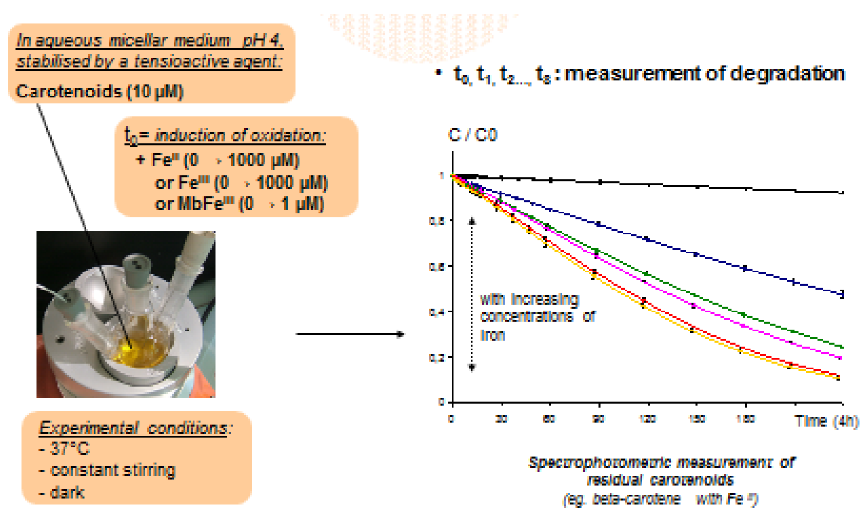


Figure 8: Carotenoid - Iron reaction kinetics in a simulated gut

HU36 carotenoids showed significantly lower degradation rate with Fe(II) and Fe(III) and analysis of the reaction medium by HPLC/MS showed that the bacterial carotenoids were more stable than the standard carotenoids in environments with or without added iron (Figure 8).

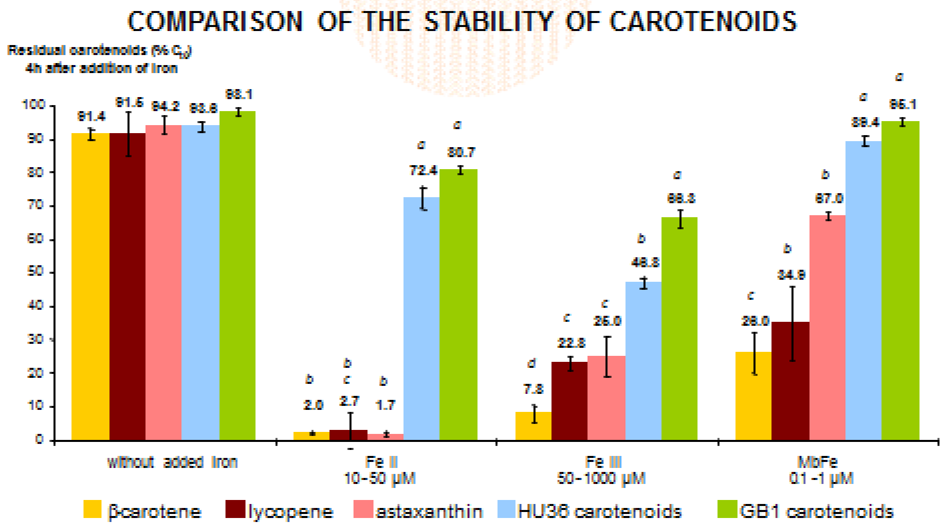


Figure 9: The gastric stability of carotenoids

The more apolar carotenoids and yellow carotenoid esters (standard carotenoids) degraded preferentially versus *Bacillus* carotenoids (Figure 9).

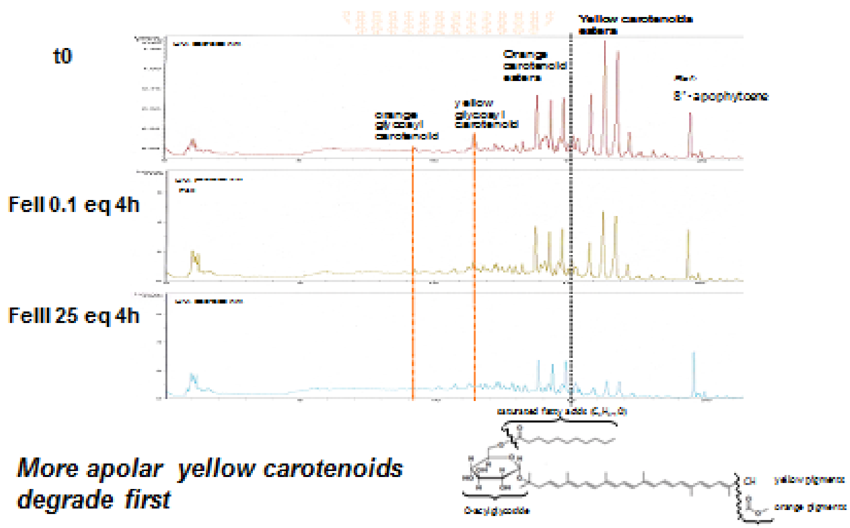


Figure 10: The gastric stability of carotenoids

Antioxidant Activity

The defining features of *Bacillus* HU36 include a defining feature of carotenoids, i.e., their conjugated double bond structure, which is responsible not only for color but also for antioxidant properties. Lycopene is one of the most potent hydrophobic antioxidants known with eleven conjugated double bonds. The *Bacillus*- derived carotenoids also have eleven double bonds (Figure 10) but their antioxidant properties are ten times greater than that of lycopene (Figure 11).

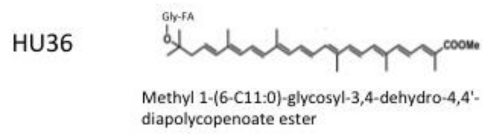


Figure 11: Eleven double bond structure of HU36 carotenoid

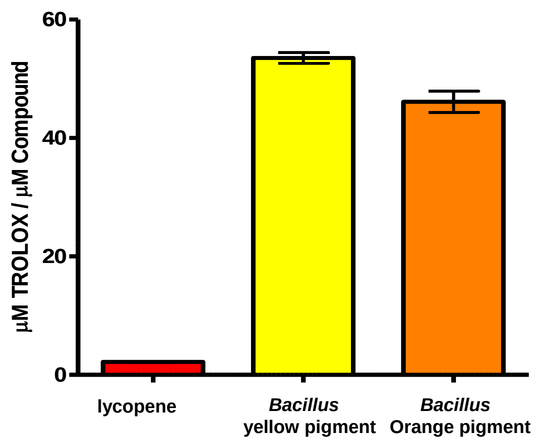


Figure 12: Antioxidant activity: Bacillus carotenoids vs. lycopene

The conjugated double bond structures of *Bacillus HU36* derived carotenoids are responsible for their deep rich colors and also their potent antioxidant properties.

These unique and valuable properties have been attributed to the sugar and/or fatty acid moieties attached to the carotenoid framework. Such additions confer solubility in both hydrophobic and hydrophilic environments and as such are believed to contribute to improved antioxidant properties as a result of these favorable physical characteristics. The discovery of these apocarotenoids is particularly pertinent because there is growing evidence that carotenoids are metabolized in the body to forms with smaller chain lengths. For example, lycopenoids—C20 to C25 molecules derived from carotenes—act as signaling molecules to stimulate the antioxidant response elements to modulate gene expression (Ben-Dor et al, 2005).

The superior antioxidant activity of the *Bacillus HU36* carotenoids is of particular relevance today considering the mounting evidence on the benefits of lowering oxidative stress. For example, iron and oxygen in the digestive system, tend to oxidize any unsaturated lipids present in food and generate potentially toxic compounds such as lipid peroxides and carbonyl compounds. But antioxidants like *Bacillus HU36* carotenoids can help inhibit the heme-iron catalyzed lipid peroxidation and thus avoid the production of harmful peroxides.

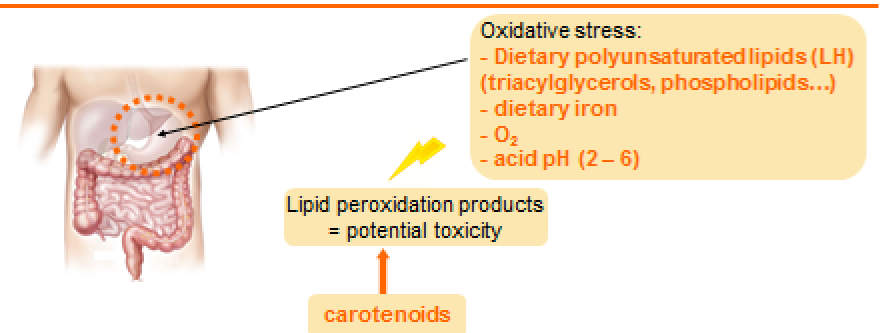


Figure 13: Inhibition of polyunsaturated lipids oxidation before absorption in a model of gastro-intestinal tract

Additional studies to examine the stability and efficacy of Bacillus indicus HU36 carotenoids were conducted on a micellar system and an emulsion system to mimic the low acid environment in the stomach following a meal. The meal would be consisting of unsaturated lipids; oxygen and iron were streamed in at different levels and the inhibition of lipid peroxides were measured spectrophotometrically (Figure 13).

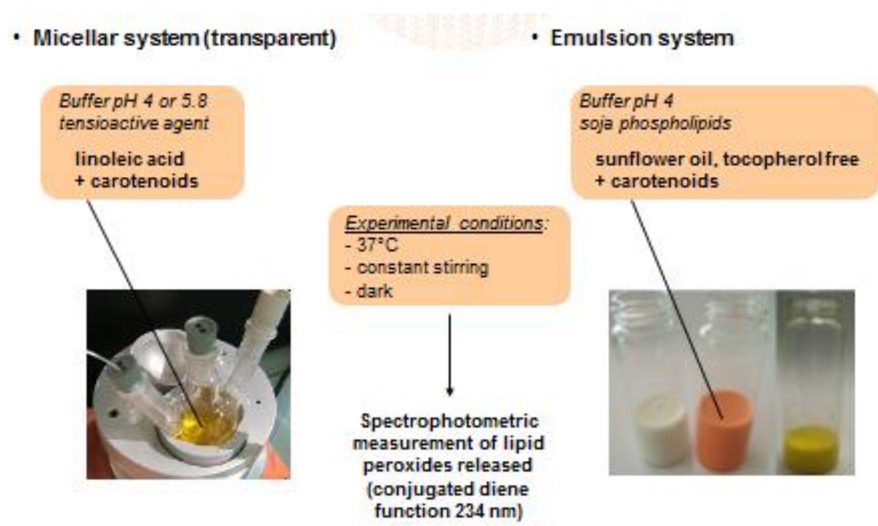


Figure 14: Carotenoid-inhibited peroxidation in a simulated gut

Upon examination of the resulting levels of lipid peroxides released, it was clear that the bacterial antioxidants were several times more potent than the standard carotenoids at two different pH levels, which are generally encountered at two different stages of digestion (Figure 15). This in vitro digestion model is an effective method of comparing the antioxidant power of carotenoids and demonstrated, clearly, the superiority of the *Bacillus indicus HU36* carotenoids when compared to current industry standards (beta-carotene, lycopene and astaxanthin).

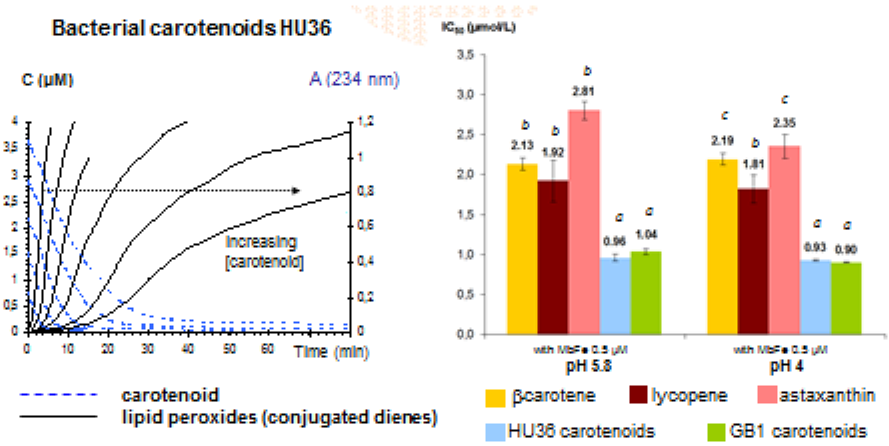


Figure 15: Bacterial carotenoids were more effective inhibitors of peroxidation

Further comparison of the reaction kinetics of standard carotenoids (lycopene) and *Bacillus indicus HU36* carotenoids showed a significant difference in their antioxidant potencies (Figure 16). Lipid peroxides accumulated after the consumption of lycopene but not after the consumption of the *Bacillus* carotenoids hinting that even the catabolites of *Bacillus* carotenoids could also have antioxidant activity.

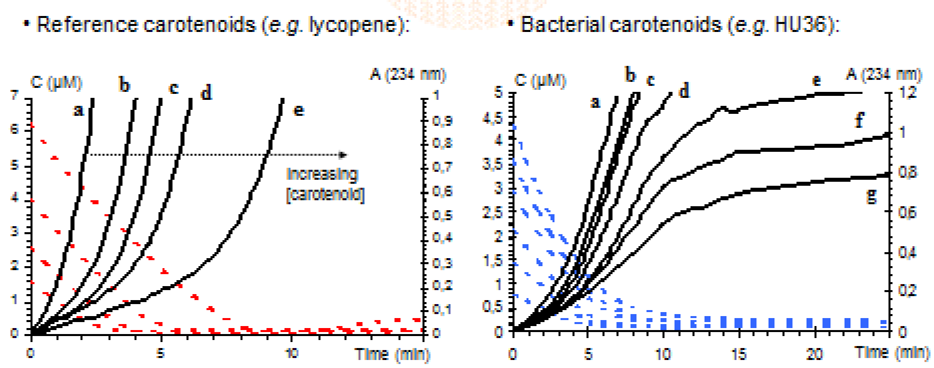


Figure 16: Bacterial carotenoids were more effective inhibitors of peroxidation (micellar system)

Lipid peroxidation was slower in the emulsion system (Figure 17) than in the micellar system. The reaction kinetics of lycopene and *Bacillus HU36* carotenoids were followed for more than 6 hours instead of for just one hour. The area under the curve quantifies the antioxidant potential; lower the area, the lower the antioxidant potential as shown in Figure 18.

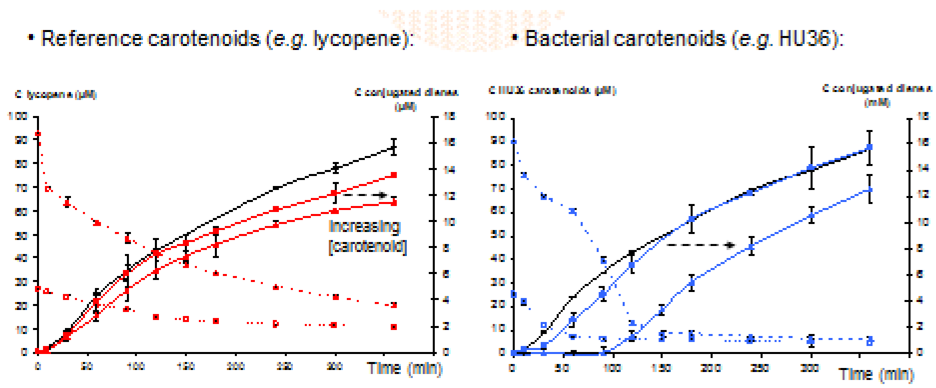


Figure 17: Bacterial carotenoids were more effective inhibitors of peroxidation (emulsion system)

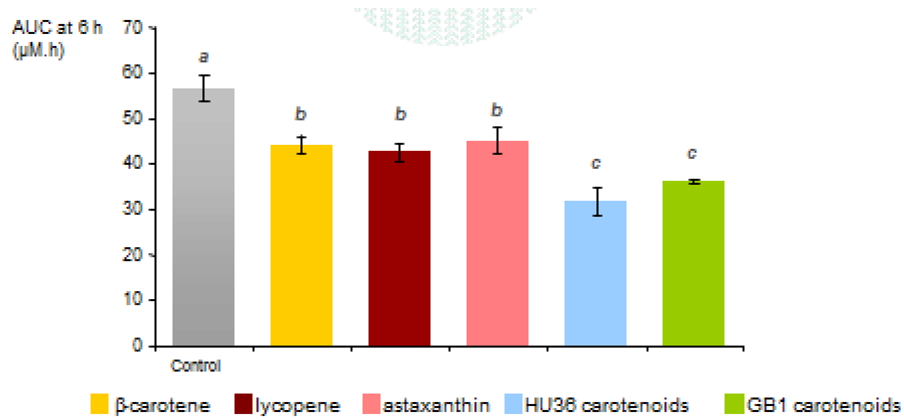
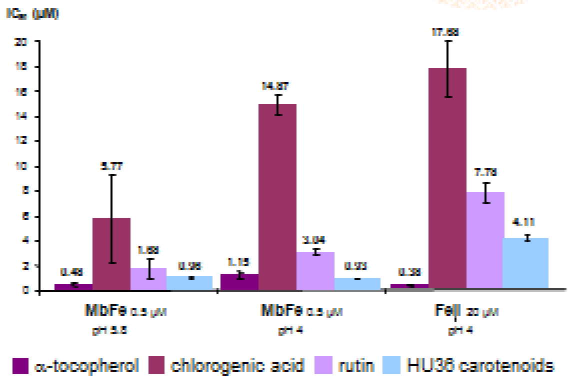


Figure 18: Bacterial carotenoids were better antioxidants (emulsion system)

Next, the researchers created combinations of *Bacillus HU36* carotenoids with known antioxidant of phenolic types to simulate a typical meal where different types of antioxidants are often ingested simultaneously; these combinations showed a synergy. The results showed an additive effect of antioxidant potentials and synergy between *Bacillus HU36* carotenoids and chlorogenic acid followed by synergy with rutin when the oxidative stress is generated by metmyoglobin. The synergy between the *Bacillus HU36* carotenoids and other antioxidants may depend on the initiator of the oxidative stress. (Figure 19)

• Antioxidant activity of 3 phenolic antioxidants



• HU36 with phenolic antioxidants:

	Aditivity (=), Synergy (+), Antagonism (-)		
	$MbFe^{III}$ 0.5 µM pH 5.8	$MbFe^{III}$ 0.5 µM pH 4	Fe^{II} 20 µML pH 4
HU36 1 µM + α-tocopherol 0.4 µM	=	=	-
HU36 1 µM + chlorogenic acid 15 µM	+	+	-
HU36 1 µM + rutin 5 µM	+	+	-

Figure 19: Synergistic effect between Bacillus- carotenoids and phenolic antioxidants

Bioaccessibility

A key concern in nutrition is 'bioaccessibility'—whether the nutrient is released from the food matrix and absorbed by the individual. In vitro bioaccessibility/absorption and in vivo bioavailability studies in rats showed that the *Bacillus*- derived carotenoids were detected in mixed micelles and thus bioaccessible (Figure 20).

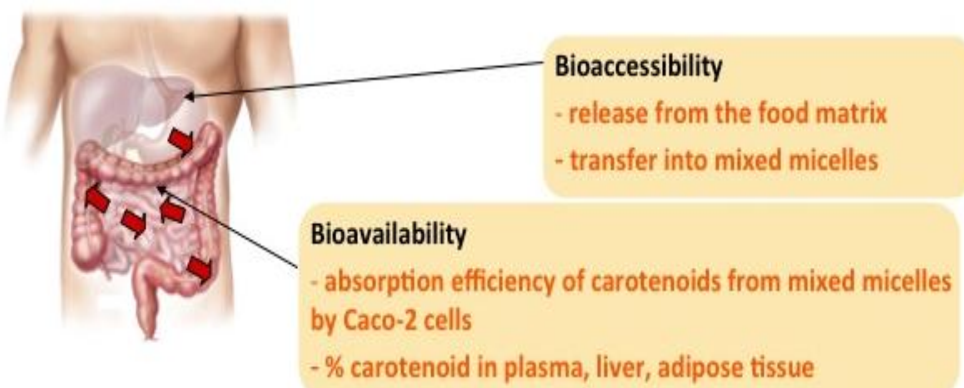


Figure 20: Release from the food matrix and adsorption into host cells

The bio-accessibility of carotenoids from *Bacillus* HU36 and new isolates was determined using *in vitro* digestion models and the absorption of HU36 carotenoids by intestinal cells were determined using animal models (Figure 21). The percentage of *Bacillus HU36* carotenoids recovered in micelles was higher than that of 3 out of the 4 common carotenoids (lutein and astaxanthin) tested confirming the bacterial carotenoids are readily bio-accessible (Figure 22).

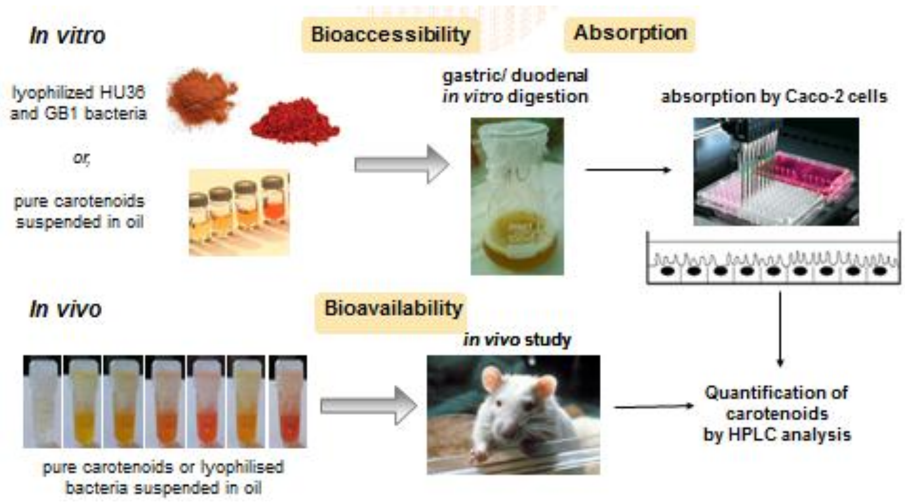


Figure 21: In vivo/in vitro procedures for bioaccessibility, bioavailability & adsorption

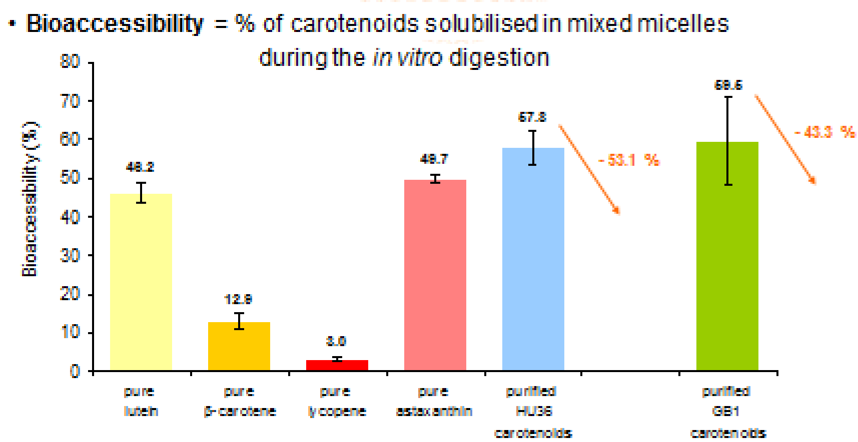


*Figure 22: In vitro results for bioaccessibility of Bacillus-*carotenoids

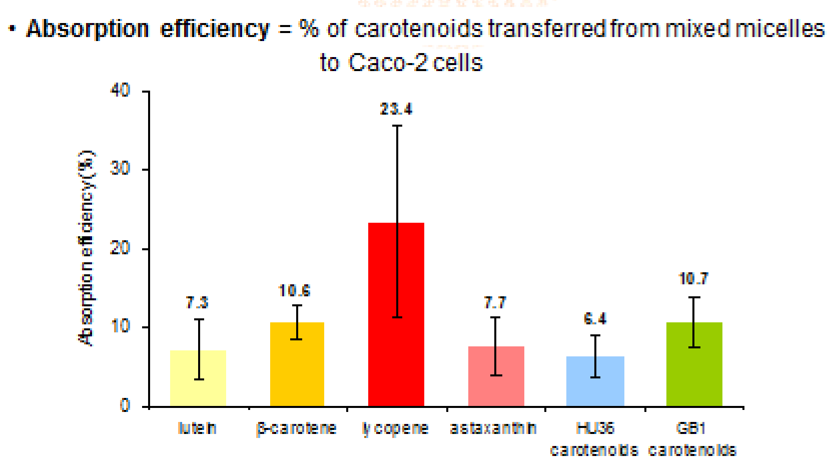


Figure 23: In vitro results for absorption efficiency of Bacillus-carotenoids

As pure compounds, *Bacillus HU36* carotenoids are as bioaccessible as the most bioaccessible carotenoids tested, i.e., lutein and astaxanthin. In general, Bacillus indicus HU36 carotenoids are more powerful and functional under physiological conditions when compared to purified controls.

Bioavailability

Although the concept of bioavailability in nutritional sciences lacks the well-defined standards associated with the pharmaceutical industry, consumers of supplements understand and use bioavailability as a screen when selecting nutritional supplements. Bioavailability for dietary supplements is defined loosely as the proportion of the administered substance capable of being absorbed and available for use or storage. Despite the complexity of the subject, people understand and rate probiotics and carotenoids on the basis of their bioavailability characteristics.

Bacillus HU36 carotenoids are readily bioavailable, even more so than standard carotenoids as demonstrated by recovery in blood and tissues of rats following gastric/ duodenal in vitro digestion and in-vivo digestion by rats (Figure 24). The total amounts of carotenoids recovered in the rat body, assuming that plasma+liver+adipose tissue contain most of the carotenoids, were 0.92 (±0.14) nmol for β-carotene, 3.44 (±0.94) nmol for lycopene, 0.65 (±0.38) nmol for lutein, 0.30 (±0.24) nmol for astaxanthin, 8.89 (±4.35) nmol for HU36 carotenoids from a purified solution, and 8.67 (±4.15) nmol for HU36 carotenoids from lyophilized cells. Purified bacterial carotenoids from HU36 were recovered for about two-thirds in the liver and one third in the adipose tissue (Figure 23). This distribution was similar to that of the xanthophyll lutein suggesting a close metabolism.

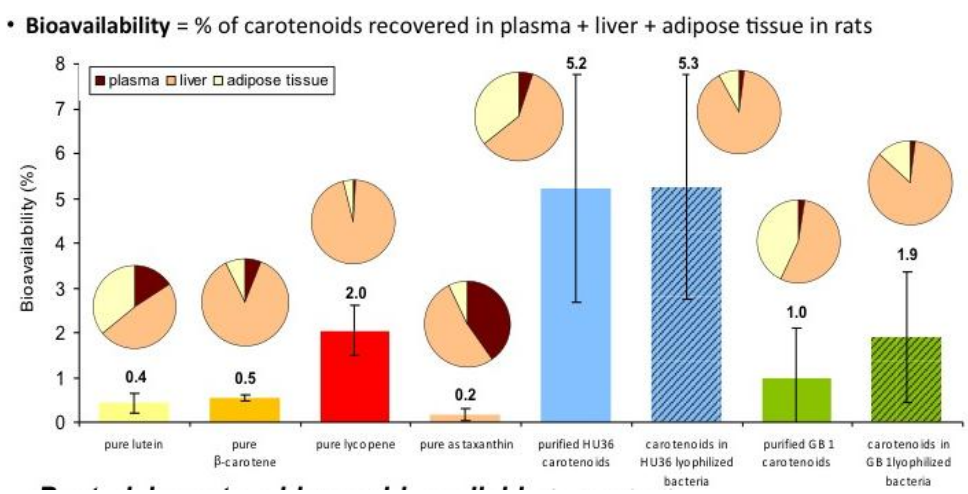


Figure 24: Bioavailability of Bacillus-carotenoids

In-vivo experiments were carried out at Good Laboratory Practice (GLP)-compliant, Quality Control department of the National Institute of Vaccines and Biological Substances (IVAC) in Nha Trang, Vietnam. The in-vivo accumulation of carotenoids from *Bacillus* HU36 was monitored in male Swiss albino mice following a daily dose of $2x10^8$ spores for a period of 60 days.

Bioavailability of carotenoids from HU36 was measured including: survival rates in gastrointestinal tract, carotenoid blood levels, accumulation of carotenoid in organs and observable health effects. All of the test mice were scored as being bright-eyed, alert, having a smooth coat with sheen, responsive to stimuli, interested in the environment and with no significant difference in weight gain between the test and the control groups. Carotenoid concentrations were negligible in the blood, but were detected at 6.40 ng/g liver—a level significantly greater than the 0.15 ng/g liver for standard carotenoids. Daily fecal count for spores showed survival in mice and proves *in vivo* survival. *In vivo* assessments of acute and chronic dosing in guinea pigs and rabbits also showed survival without any toxicity in these animals under the study conditions and supports the use of *Bacillus indicus* HU36 as food supplements.

Advantages of *Bacillus Indicus HU36* Carotenoids

Gastric stability	They survive transit through the GI-tract unlike other commercial carotenoid supplements that are rapidly degraded when ingested.
Heat stability	As spores, *Bacillus* strains that produce carotenoids can be stored indefinitely. This enables them to be incorporated into food matrices, a process not possible with other sources of carotenoids. Further, spores can survive pasteurization and short-term exposure to high temperatures of baking. The spores produce these highly bioavailable carotenoids in the gut itself where absorption rates are quite high.
Bioavailability	*Bacillus HU36* carotenoids appear to be absorbed in the GI-tract. These pre-publication results arise from studies conducted by the COLORSPORE project and demonstrate the potential of *Bacillus indicus HU36* carotenoids. This is a key aspect of the project and necessary to fully substantiate health claims. The studies also indicate that *Bacillus indicus HU36* carotenoids are more stable, under physiological conditions, than standard purified products.
Antioxidant activity	*Bacillus HU36* carotenoids have superior antioxidant activity compared to lycopene (see Figure 7). Activity is shown in both hydrophilic and hydrophobic phases promising potential health benefits and applications. *Bacillus indicus HU36* carotenoids were shown to be more powerful antioxidants under physiological conditions.
Existing use as food supplement	*Bacillus* spores are in widespread use as probiotic supplements. They have attracted growing interest as probiotics and have a number of health related benefits ascribed to them in vitro and also *in vivo* including human studies. Conceptually, at least, this may support their use as food supplements.
Safety	*Bacillus* HU36 has been subjected to toxicology studies and no adverse effects have been determined. The Bacillus HU36 carotenoids have a long history of human exposure as the strain is a human isolate. This has a favorable safety profile over synthetic carotenoids.
Potential health benefits	The carotenoids found in *Bacillus* HU36 are apolycopene derivatives and the same class that has been shown to play a role in reducing the risk of certain cancers, notably prostate cancer.
Production	Bacterial spore production is commercially viable as demonstrated by the number of companies that produce them as probiotics for food and supplement applications. *Bacillus* HU36 offers a live bacterial product that is straightforward and attractive, operationally, in contrast to purifying or synthesizing carotenoids. In addition, *Bacillus HU36* is the only ingredient on the market that can penetrate the probiotic and antioxidant markets with a single and cost effective ingredient. Based on the nutritional benefits of *Bacillus HU36*, matching its value by mixing a standard spore forming probiotic strain with purified carotenoids would prove to be a cost barrier.

Safety

Bacillus HU36 has been evaluated for safety in murine, rabbit and guinea pigs. Acute and sub-chronic toxicity testing revealed no indicators of concern. *Bacillus* HU36 was shown to be devoid of enterotoxin genes and is a natural variant isolated from humans.

References citing *Bacillus indicus* HU36 include:

- Duc et al (Duc et al., 2006) reported the initial isolation and preliminary characterization of pigmented Bacilli including HU36.
- Khaneja et al (Khaneja et al., 2010) reported the characterization of Bacillus- carotenoids.
- Perez-Fons et al (Perez-Fons et al., 2011) reported the carotenoid pathways found in HU36.
- Hong et al (Hong et al., 2008) reported on the safety of HU36 based on animal studies.
- Manzo et al (Manzo et al., 2011) reported on the production of potentially useful enzymes produced by HU36.

This organism has been studied in depth and *Bacillus indicus* does not show any sign of chronic toxicity or virulence in *in vivo* assessments (Hong et al, 2008). This confirms that *Bacillus indicus* HU36 has good potential for use as a food supplement. Moreover, the organisms entire genome has been sequenced, labeled and all biochemical pathways have been characterized.

Food Formulations Studies

Bacillus indicus HU36 may be used as such: as spores or as an extract to produce colorings and dye; food and food additives; food supplement or a cosmetic. The spores and vegetative cells of HU36 are of different colors because of differential presence of at least one carotenoid in the spore and vegetative cells forms which therefore offer a variety of aesthetic (color, flavor, and aroma) and nutritional implications to formulators. In addition to being incorporated into human foods, it has also been tested and proven effective in feeds for farmed land animals and aquatic animals including fish and shell-fish.

The Colorspore Project evaluated the spores of *Bacillus* HU36 for their food ingredient potential and viability in a number of product systems. The organism showed several traits that could prove advantageous in food processing including: resistance to elevated temperatures in baked products, compatibility with several foods including fruits, dairy, yogurt and chocolate and full resistance to gastric fluids. The Colorspore Project bacterial production process can produce 0.1 mg of carotenoids from 1 g of dried biomass. *Bacillus* HU36 was tested in several food product types for functionality, taste, nutrition, and viability.

Probiotic and Synbiotic Chocolate

Dark chocolate was enriched with *Bacillus indicus* HU36 and the formulation was optimized using response surface methodology (RSM). Two dietary fibers—maltodextrin and lemon fiber—were added for their effects on color and organoleptic quality properties of dark chocolate. The process consisted of melting dark chocolate couverteurs containing 50% cocoa in a water bath at 45°C and adding lyophilized *Bacillus indicus* HU36 spores to yield 10^6 cfu/g of chocolate. The fibers, whose particle size was 40 micron at most, were added to and mixed with the couverteurs and bacteria. The mixture was tempered manually according to the Tabliering method. After cooling and packaging, the tempered chocolate samples were stored at 18°C until testing against a control that contained no fiber and no bacteria and a second control containing only the probiotic but without any dietary fiber.

Ten trained panelists, consisting of graduate students and faculty members of ITU Food Engineering Department, Goethe University, Germany, participated in sensory evaluation. Samples were scored for appearance, aroma, mouthfeel, texture and overall acceptability using a 9-point scale ranging from 1 to 9 (dislike extremely to like extremely).

The viability of *Bacillus indicus* HU36 in dark chocolate was also examined and analysis showed that at least 10^5 cfu/g bacteria survived in all samples. The probiotic and the fibers did not significantly affect the color of the top and bottom surface of the chocolates analyzed according to L*, a*, b* and WI. The optimum formulations generated by the RSM model identified 1.5% and 3.2 – 3.9% as optimum for the lemon fiber and maltodextrin, respectively.

Probiotic Yogurt

A study investigated the viability of *Bacillus indicus* HU36 in set-type plain yogurt made with starter cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) and its effects on yogurt quality during 21 days of storage at 4 °C in terms of color attributes, sensorial, rheological and textural properties. The process consisted of heating reconstituted skimmed milk (13% total solids) at 85°C for15 minutes, inoculation with 10^8 cfu/mL of *Bacillus indicus* HU36 and pre-fermentation at 37°C for 2 hours, inoculation with the commercial starter inoculum preparation

(0.4% v/v), and incubation at 42°C until the pH 4.6 end point. The finished products were stored at 4°C and tested on the 1st, 7th, 14th, and 21st days. Fermentation kinetics, monitored by measuring the pH at 15 minute intervals during fermentation until the pH 4.5 was reached, showed no significant changes due to HU 36.

Viscoelastic properties of the yogurt samples were determined by dynamic oscillation tests.

Rheological properties of yogurt samples, monitored using a Haake RheoStress Rheometer, showed no significant difference from the control in the hardness, cohesiveness, springiness, chewiness, gumminess, and adhesiveness of the finished product. The color of yogurts made with HU36, as measured by Chroma Meter, were more yellow in the first few days of storage and then evened out to match the color of the control (Danone Activia Probiotic Yogurt). Bacterial counts, carried out one day after the fermentation was completed and once a week during the storage of yogurt at 4°C for 21 days in triplicate, showed *Bacillus indicus* HU36 at the adequate level (10^5 cfu/ mL). ~5 log cfu mL-1) at the end of storage. Yogurts supplemented with prebiotics (maltodextrin or inulin at 3, 4, and 5%) enhanced the viability of HU36 to show 10^6 cfu/mL levels of HU36 at the end of storage.

Flavored Dairy Beverage

Bacillus HU36 was tested at 10^6 cfu/mL in probiotic fruit flavored dairy beverages. Two formulations were developed:

- Mango formula made with milk, yogurt and mango (in syrup)
- Apricot formula made with milk, cream cheese and apricot (in syrup)

The beverages were produced in a laboratory and the production consisted simply of mixing and homogenizing the ingredients. The samples were analyzed for microbial content, color, sensory attributes, and pH over a period of 7 days. Microbiological analysis results showed a 10^5 cfu/g survival after 7 days storage. The HU36 samples were deeper in hue compared to the control samples, but there were no differences in terms of pH, aroma, texture, and overall quality of the mango and apricot beverages compared to the respective controls.

Value-added Oils

Refined olive oil was enriched with *Bacillus HU36* carotenoids at various levels ranging from 2ppm to 5 ppm and the protective effect of the carotenoids was measured over a period of time. The enriched oils were placed in tubes and stored at 60°C in a dark oven to promote autoxidation and the peroxide value of the oils was measured after 0, 3, 6, 9, and 12 days. Oils containing bacterial carotenoids delayed the onset of oxidative degradation; increasing the concentration afforded more protective effect. Overall, the bacterial carotenoids were not significantly different from the standard carotenoids.

Biscuits (Cookies)

Lyophilized spores of *Bacillus indicus HU36* were mixed with 1 kg of a commercial biscuit dough using facilities at United Biscuits (High Wycombe, UK) to give a final bacterial count of $\sim 1 \times 10^9$

spores/g of the dough. The mixture3 was blended using a Hobart planetary mixer (Troy, OH, USA) in two stages. The dough (at 14% moisture) was covered, allowed to rest for 25 minutes, then formed into 19 g dough pieces using a bench scale rotary molder, baked at 235°C for 8 minutes and the resulting biscuits (at 2% moisture) were stored in heat-sealed foil bags until analysis. The spores were found to survive in the individual biscuits with no more than a 1-log reductive in viability. The studies also showed that HU36 spores when administered weekly to mice conferred non-specific cellular immune responses, indicative signs of the stimulation of innate immunity. That spores can survive the baking process offers the possibility of using spores as probiotic supplements in a wide range of baked food products.

Examples of unique formulations possible due to the specialized properties of *Bacillus* HU36:

Beverages and Dairy	➢ **Pasteurized milk/ Ultra pasteurized milk**. White and chocolate – *Bacillus* HU36 can be added pre-pasteurization and will survive the pasteurization process. In addition, *Bacillus* HU36 can be made to remain in spore form in the beverage throughout the shelf life of the beverage and thus will not grow in the container. This offers milk producers a unique opportunity to penetrate the probiotic market and a potential for targeting probiotics to children via chocolate milk. ➢ **Non-Refrigerated Beverages** – *Bacillus* HU36 can remain dormant in liquids without refrigeration. Teas, fruit juices, vitamin drinks, energy drinks can all be formulated with *Bacillus* HU36 ➢ **Powdered Beverages** - *Bacillus* HU36 is very stable in its desiccated form and is ideal for powdered beverages. ➢ **Yogurt and Yogurt Based Drinks –** Goethe University completed a successful trial on the incorporation of *Bacillus* HU36 into yogurt and found it to be stable and organoleptically compatible.
Baked Products	➢ **Baked** – cookies, breads, biscuits, cakes, etc. Bacillus HU36 is stable at very high temperatures, as high as 235°C for up to 8 minutes and can be baked into consumer packaged products. ➢ **Baking mixes** – baking premixes can be formulated with *Bacillus* HU36 for home and food service use.
Snacks	➢ **Extruded Products** – *Bacillus* HU36 can survive some extrusion process and thus can be incorporated into cereals, snacks and other similar items. ➢ **Confectionary** – candies, gums, bars, etc.
Soups and Sauces	➢ **Canned or Shelf Stable** – Since *Bacillus* HU36 has the unique ability to remain dormant in liquids without refrigeration, companies can formulate soups fortified with probiotics that are shelf stable and that could survive retort temperatures.
Dietary Supplements	➢ **Liquid Shot Delivery** – As seen with popular energy shots delivery systems. ➢ **Alternative to capsules** - Effervescent tabs, chews, stik packs, etc.

[3] Biscuit formulation: flour (58.6%), fat (18%), sugars (13.6%), salt (0.7%), sodium bicarbonate (1.1%), malic acid (0.4%), ammonium bicarbonate (0.2%) and water (7.4%)

Market Opportunity

Bacillus HU36 offers, for the first time, carotenoids, antioxidants and probiotics with high bio-accessibility and bioavailability in a single ingredient. The technology can serve a total market of over $75 billion dollars encompassing ingredients and finished products including supplements, foods and feeds at the intersection of carotenoids, antioxidants and probiotic fortification. It is the first of its kind: a carotenoid-rich probiotic that is process stable, bake stable and stable in both refrigerated and non-refrigerated beverages, with bio-accessibility and bioavailability that delivers nutritional benefits unmatched by other carotenoid and probiotic ingredients.

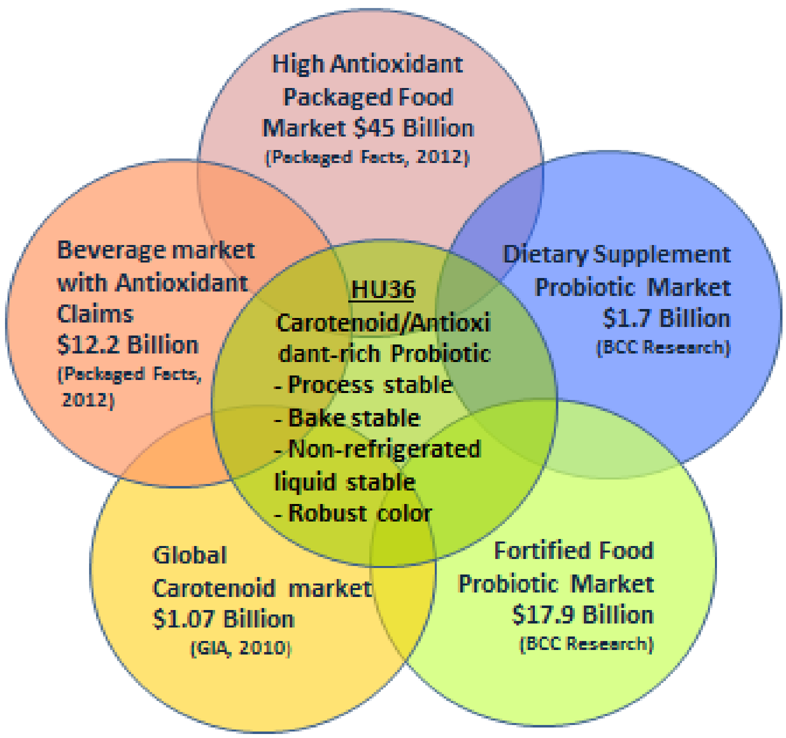


Figure 25: The market for Bacillus indicus HU36

The global market for high-antioxidant packaged foods is currently estimated at has $45 billion dollars (Packaged Facts, 2012) and includes food products such as cereals, bars, snacks, and beverages bearing claims of high antioxidant value. Although antioxidants are naturally present in a number of fruits and vegetables, consumers reach out preferentially for processed foods and beverages with added antioxidants, because they have come to regard fortified foods as even better for them and as extensions of the foods and the nutritional supplements they consume. These products rely on forms of antioxidants with higher stability and bioavailability to deliver the health and beauty promises they make and *Bacillus* HU36 offers just that capability. The current beverage market for beverages that make antioxidant claims is estimated to net $12.2 billion in sales in 2012 (Packaged Facts, 2012). *Bacillus* HU36 offers processors the means to not just satisfy the growing consumer thirst for antioxidant-rich beverages but also an easier way to formulate healthy beverages.

Global carotenoid sales, estimated at $1.07 billion, consist of ß-carotene leading with the largest market share and followed by lycopene, canthaxanthin and astaxanthin (Global Industry Analysts). *Bacillus indicus* HU36 produce some of these carotenoids *in vivo* but in their most stable and bio-available forms and opens the door to natural carotenoids, one of the fasted growing segments of this market.

According to BCC Research, the global food probiotic market, consisting of yogurts, smoothies, etc., is currently worth $17.9 billion; the dietary supplement—capsules and tablets—probiotic market is approximately one-tenth at $1.7 billion. Spores are just entering the fray but are gaining market share steadily due to their gastric stability and strong colonization.

Bacillus HU36 offers the means to design and develop products that fit into all of these market segments with a single product or line of products. Since HU36 provides high-bioavailable carotenoids, high antioxidant activity, is all natural, is a spore probiotic and is bake and liquid stable, it can be used to make:

- Drinks, teas, milk, chocolate milk, yogurt, protein drinks, diet drinks, sodas and enhanced waters that have high bioavailable carotenoids, high antioxidant levels, live-cell probiotics, and are shelf-stable.
- High bio-available carotenoid, high antioxidant, live cell probiotic confectionery items including: chocolates, hard candy, hard-shell candy, fruit roll-ups, lollipops, and candy bars.
- High bioavailable carotenoid, high antioxidant, live cell probiotic baked products such as cakes, cookies, biscuits, muffins, and cereals.

As a dietary supplement, *Bacillus* HU36 offers a new paradigm to present to a very competitive probiotic market place. The notion that beneficial bacteria also have the role of digesting foods we eat and in turn, producing nutritious compounds for our bodies to absorb, is the next evolution in the dietary-supplement market. *Bacillus* HU36, being a spore, offers claims of better colonization and survival through the digestive track when compared to non-spore organisms; it also offers claims related to the most bioavailable form of antioxidants and carotenoids. If one considers the cost of high-quality carotenoids being purchased and added to a probiotic product, the sum of the two would be far greater than the use of a single ingredient with a dual purpose.

Intellectual Capital

Viridis BioPharma has the exclusive rights to three patents that are critical to the commercial success and market realization of *Bacillus indicus* HU36 and *Bacillus*- carotenoids.

US 20080171066A1

(19) **United States**
(12) **Patent Application Publication** (10) **Pub. No.: US 2008/0171066 A1**
Cutting (43) **Pub. Date: Jul. 17, 2008**

Patent 71066 is the invention of an improved method to make non-pathogenic *Bacillus* HU36 spores. It consists of: (i) a polynucleotide sequence encoding a phagosome membrane-rupturing agent; and (ii) a polynucleotide sequence encoding at least one further heterologous polypeptide. These may be used to deliver heterologous polypeptides to cells and in particular to phagocytic cells. The invention of non-pathogenic spore forming Bacilli related to the use of Bacilli in, or as, food, food supplements, probiotics, coloring agents, biosensors, and as sources of carotenoid and isoprenoid derived metabolites, as well as to the Bacilli themselves. The invention also relates to using the Bacilli spores in pharmaceutical compositions, vaccines and medicaments for a variety of purposes including in immunization and vaccination. This patent pre-empts competitors from the economic advantage in the production of *Bacillus* spores and also its use to deliver bioactives.

US 20090175911A1

(19) **United States**
(12) **Patent Application Publication** (10) **Pub. No.: US 2009/0175911 A1**
Cutting et al. (43) **Pub. Date: Jul. 9, 2009**

Patent 75911 is the invention of *Bacillus* spores and vegetative cell forms with carotenoids. The invention affords the owner the means to use the *Bacillus* in detection methods as sensors for bioactives and stimulants, and also as a coloring agent and component of foods, supplements, probiotic compositions, cosmetics, pharmaceuticals and vaccines. An important claim is the use of *Bacillus* for the production of carotenoids, its precursors, and downstream derivatives. The patent covers, besides HU36, also the sequence identity of HU19, HU28, and HU33 – offering the owner the prospect of launching additional *Bacillus*- based production of bioactives and functional ingredients.

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 June 2007 (14.06.2007)

WIPO OMPI

PCT

(10) International Publication Number
WO 2007/066108 A1

The third patent 66108 is an international patent filing and offers the owners protection and competitive advantage across the globe for all matters covered in the patent described above.

Conclusions

Viridis BioPharma offers in its Colorspore™—*Bacillus* HU36—a unique probiotic-carotenoid combination with nonpareil possibilities for the enhancement and production of functional foods and nutritional ingredients. The concept of bioavailable carotenoids through ingestible probiotics that also have other probiotic related therapeutic physiological benefits is truly novel in the food and supplement industry. For consumers, it means a one-stop shop for a combination of physiological effects—one that they can feel and benefit from across all ages.

More stable than standard carotenoids, *Bacillus* HU36 also offers stronger antioxidant potential for a very unique approach to probiotic therapy with the production of highly therapeutic carotenoids and ubiquinones. For food and supplement manufacturers, it means competitive and operational advantage with a unique patented strain of Gram-positive spore forming bacterium that produces distinct bio-accessible carotenoids that are gastric stable, process stable, and significantly more bioavailable than other forms currently available on the market.

Viridis' technology offers additional advantages from the ability of HU36 spores to survive the harsh conditions associated with food processing without the need for refrigeration as a sure barrier to entry for other probiotic and carotenoid technologies. The technology greatly opens up the market potential of common food products that until today could not deliver viable dosages of probiotics, carotenoids, and antioxidant power. The bundling of highly bioavailable carotenoids, potent antioxidants and robust probiotics in a single ingredient can serve a $75 billion market encompassing ingredients and finished products including supplements, foods, feeds, and cosmetics. *Bacillus* HU36 has been proven in several food systems and it shows promise by fitting into virtually any food and beverage product including: drinks, teas, milk, chocolate milk, yogurt, protein drinks, diet drinks, sodas, enhanced waters, chocolates, hard candy, hard-shell candy, fruit roll-ups, lollipops, candy bars, cakes, cookies, biscuits, muffins, and cereals.

References

Agarwal, S. & A. V. Rao, (1998) Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. Lipids 33: 981-984.

Amdur, B. H., E. I. Szabo & S. S. Socransky, (1978) Presence of squalene in Gram-positive bacteria. J Bacteriol 135: 161-163.

Baron, M., (2009) A patented strain of *Bacillus* coagulans increased immune response to viral challenge. Postgrad Med 121: 114-118.BCC Research. The Probiotics Market: Ingredients, Supplements, Foods, June 2011

Ben-Dor, A., M. Steiner, L. Gheber, M. Danilenko, N. Dubi, K. Linnewiel, A. Zick, Y. Sharoni & J. Levy, (2005) Carotenoids activate the antioxidant response element transcription system. Mol Cancer Ther 4: 177-186.

Canani, R. B., P. Cirillo, G. Terrin, L. Cesarano, M. I. Spagnuolo, A. De Vincenzo, F. Albano, A. Passariello, G. De Marco, F. Manguso & A. Guarino, (2007) Probiotics for treatment of acute diarrhoea in children: randomised clinical trial of five different preparations. BMJ 335: 340.

Cartman, S. T., R. M. La Ragione & M. J. Woodward, (2008) *Bacillus* subtilis spores germinate in the chicken gastrointestinal tract. Appl Environ Microbiol 74: 5254-5258.

Caruso, A., G. Flamminio, S. Folghera, L. Peroni, I. Foresti, A. Balsari & A. Turano, (1993) Expression of activation markers on peripheral-blood lymphocytes following oral administration of *Bacillus* subtilis spores. International Journal of Immunopharmacology 15: 87-92.

Ciprandi, G., A. Scordamaglia, D. Venuti, M. Caria & G. W. Canonica, (1986) In vitro effects of *Bacillus* subtilis on the immune response. Chemioterapia 5: 404-407.

Ciprandi, G., M. A. Tosca, M. Milanese, G. Caligo & V. Ricca, (2004) Cytokines evaluation in nasal lavage of allergic children after *Bacillus* clausii administration: a pilot study. Pediatr Allergy Immunol 15: 148-151.

Ciprandi, G., A. Vizzaccaro, I. Cirillo & M. A. Tosca, (2005) *Bacillus* clausii exerts immuno-modulatory activity in allergic subjects: a pilot study. Eur Ann Allergy Clin Immunol 37: 129-134.

Coppi, F., M. Ruoppolo, A. Mandressi, C. Bellorofonte, G. Gonnella & A. Trinchieri, (1985) Results of treatment with *Bacillus* subtilis spores (Enterogermina) after antibiotic therapy in 95 patients with infection calculosis. Chemioterapia 4: 467-470.

Cutting, S. M., (2011) *Bacillus* probiotics. Food Microbiol 28: 214-220.

Diplock, AT, Charleux, JL, Crozier-Willi, G, Kok, FJ, Rice-Evans, C, Roberfroid, M, Stahl, W, Vina-Ribes, J. Functional food science and defence against reactive oxidative species, British Journal of Nutrition 1998, 80, Suppl. 1, S77–S112.

Dolin, B. J., (2009) Effects of a proprietary *Bacillus* coagulans preparation on symptoms of diarrhea-predominant irritable bowel syndrome. Methods Find Exp Clin Pharmacol 31: 655-659.

Duc, L. H., P. Fraser & S. M. Cutting, (2006) Carotenoids present in halotolerant *Bacillus* spore formers. FEMS Microbiology Letters 255: 215-224.Eat a Rainbow: Functional Foods and Their Colorful Components. October 2011. Food Insights, International Food Information Council Foundation, Washington, D.C.

Fiorini, G., C. Cimminiello, R. Chianese, G. P. Visconti, D. Cova, T. Uberti & A. Gibelli, (1985) *Bacillus* subtilis selectively stimulates the synthesis of membrane bound and secreted IgA. Chemioterapia 4: 310-312.

Fuhrman, B., A. Elis & M. Aviram, (1997) Hypocholesterolemic effect of lycopene and beta-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. Biochem Biophys Res Commun 233: 658-662.

Gabrielli, M., E. C. Lauritano, E. Scarpellini, A. Lupascu, V. Ojetti, G. Gasbarrini, N. G. Silveri & A. Gasbarrini, (2009) *Bacillus* clausii as a treatment of small intestinal bacterial overgrowth. Am J Gastroenterol 104: 1327-1328.

Giovannucci, E., (1999) Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. J Natl Cancer Inst 91: 317-331.

Giovannucci, E., (2002) Lycopene and prostate cancer risk. Methodological considerations in the epidemiologic literature. Pure Appl. Chem. 74: 1427-1434.

Giovannucci, E., A. Ascherio, E. B. Rimm, M. J. Stampfer, G. A. Colditz & W. C. Willett, (1995) Intake of carotenoids and retinol in relation to risk of prostate cancer. J Natl Cancer Inst 87: 1767-1776.

Heller, F. R., O. Descamps & J. C. Hondekijn, (1998) LDL oxidation: therapeutic perspectives. Atherosclerosis 137 Suppl: S25-31.

Hong, H. A., H. Duc le & S. M. Cutting, (2005) The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 29: 813-835.

Hong, H. A., J.-M. Huang, R. Khaneja, L. V. Hiep, M. C. Urdaci & S. M. Cutting, (2008) The safety of *Bacillus* subtilis and *Bacillus indicus* as food probiotics. Journal of Applied Microbiology 105: 510-520.

Hong, H. A., R. Khaneja, N. M. Tam, A. Cazzato, S. Tan, M. Urdaci, A. Brisson, A. Gasbarrini, I. Barnes & S. M. Cutting, (2009a) *Bacillus* subtilis isolated from the human gastrointestinal tract. Res Microbiol 160: 134-143.

Hong, H. A., E. To, S. Fakhry, L. Baccigalupi, E. Ricca & S. M. Cutting, (2009b) Defining the natural habitat of *Bacillus* spore-formers. Res Microbiol 160: 375-379.

Hosoi, T. & K. Kiuchi, (2004) Production and probiotic effects of Natto. In. E. Ricca, A. O. Henriques & S. M. Cutting (eds). Wymondham, UK: Horizon Bioscience, pp. 143-154.

Hun, L., (2009) *Bacillus* coagulans significantly improved abdominal pain and bloating in patients with IBS. Postgrad Med 121: 119-124.

Khaneja, R., L. Perez-Fons, S. Fakhry, L. Baccigalupi, S. Steiger, E. To, G. Sandmann, T. C. Dong, E. Ricca, P. D. Fraser & S. M. Cutting, (2009) Carotenoids found in Bacillus. J Appl Microbiol.

Khaneja, R., L. Perez-Fons, S. Fakhry, L. Baccigalupi, S. Steiger, E. To, G. Sandmann, T. C. Dong, E. Ricca, P. D. Fraser & S. M. Cutting, (2010) Carotenoids found in Bacillus. Journal of applied microbiology 108: 1889-1902.

Kohlmeier, L., J. D. Kark, E. Gomez-Gracia, B. C. Martin, S. E. Steck, A. F. Kardinaal, J. Ringstad, M. Thamm, V. Masaev, R. Riemersma, J. M. Martin-Moreno, J. K. Huttunen & F. J. Kok, (1997) Lycopene and myocardial infarction risk in the EURAMIC Study. Am J Epidemiol 146: 618-626.

Kristenson, M., B. Zieden, Z. Kucinskiene, L. S. Elinder, B. Bergdahl, B. Elwing, A. Abaravicius, L. Razinkoviene, H. Calkauskas & A. G. Olsson, (1997) Antioxidant state and mortality from coronary heart disease in Lithuanian and Swedish men: concomitant cross sectional study of men aged 50. Bmj 314: 629-633.

Logan, N. A., (2004) Safety of aerobic endospore-forming bacteria. In: Bacterial spore formers: probiotics and emerging applicaions. E. Ricca, A. O. Henriques & S. M. Cutting (eds). Norfolk, UK: Horizon Bioscience, pp. 93-106.

Manzo, N., E. D'Apuzzo, P. M. Coutinho, S. M. Cutting, B. Henrissat & E. Ricca, (2011) Carbohydrate-active enzymes from pigmented Bacilli: a genomic approach to assess carbohydrate utilization and degradation. BMC Microbiology In press.

Mazza, P., (1994) The use of *Bacillus* subtilis as an antidiarrhoeal microorganism. Bollettino Chimico Farmaceutico 133: 3-18.

Meroni, P. L., R. Palmieri, W. Barcellini, G. De Bartolo & C. Zanussi, (1983) Effect of long-term treatment with B. subtilis on the frequency of urinary tract infections in older patients. Chemioterapia 2: 142-144.

Nicholson, W. J., N. Munakata, G. Horneck, H. J. Melosh & P. Setlow, (2000) Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. Microbiology and Molecular Biology Reviews 64: 548-572.

Nicholson, W. L., (2002) Roles of *Bacillus* endospores in the environment. Cellular and Molecular Life Sciences 59: 410-416.

Nista, E. C., M. Candelli, F. Cremonini, I. A. Cazzato, M. A. Zocco, F. Franceschi, G. Cammarota, G. Gasbarrini & A. Gasbarrini, (2004) *Bacillus* clausii therapy to reduce side-effects of anti-Helicobacter pylori treatment: randomized, double-blind, placebo controlled trial. Aliment Pharmacol Ther 20: 1181-1188.

Owen, R W; Haubner, R; Würtele, G; Hull, W E; Spiegelhalder, B; Bartsch, H (2004). "Olives and olive oil in cancer prevention". European Journal of Cancer Prevention 13 (4): 319–26.

Perez-Fons, L., S. Steiger, R. Khaneja, P. M. Bramley, S. M. Cutting, G. Sandmann & P. D. Fraser, (2011) Identification and the developmental formation of carotenoid pigments in the yellow/orange *Bacillus* spore-formers. Biochimica et biophysica acta 1811: 177-185.

Perez-Fons, L., S. Steiger, R. Khaneja, S. M. Cutting, G. Sandmann & P. D. Fraser, (2010) Identification and the developmental formation of carotenoid pigments in the yellow/orange *Bacillus* spore-formers. BBA-Molecular and Cell Biology of Lipids In press.

Rao, A. V. & L. G. Rao, (2007) Carotenoids and human health. Pharmacol Res 55: 207-216.

Rhee, K. J., P. Sethupathi, A. Driks, D. K. Lanning & K. L. Knight, (2004) Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. Journal of Immunology 172: 1118-1124.

Rissanen, T., S. Voutilainen, K. Nyyssonen, R. Salonen & J. T. Salonen, (2000) Low plasma lycopene concentration is associated with increased intima-media thickness of the carotid artery wall. Arterioscler Thromb Vasc Biol 20: 2677-2681.

Sanders, M. E., L. Morelli & T. A. Tompkins, (2003) Sporeformers as human probiotics: Bacillus, Sporolactobacillus, and Brevibacillus. Comprehensive Reviews in Food Science and Food Safety 2: 101-110.

Smith, Theresa J (2000). "Squalene: potential chemopreventive agent". Expert Opinion on Investigational Drugs 9 (8): 1841–8.

Sumi, H., H. Hamada, H. Tsushima, H. Mihara & H. Muraki, (1987) A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. Experientia 43: 1110-1111.

Sumi, H., C. Yatagai, H. Wada, E. Yoshida & M. Maruyama, (1995) Effect of *Bacillus* natto-fermented product (BIOZYME) on blood alcohol, aldehyde concentrations after whisky drinking in human volunteers, and acute toxicity of acetaldehyde in mice. Arukoru Kenkyuto Yakubutsu Ison 30: 69-79.

Tam, N. K., N. Q. Uyen, H. A. Hong, H. Duc le, T. T. Hoa, C. R. Serra, A. O. Henriques & S. M. Cutting, (2006) The intestinal life cycle of *Bacillus* subtilis and close relatives. J Bacteriol 188: 2692-2700.

Witztum, J. L., (1994) The oxidation hypothesis of atherosclerosis. Lancet 344: 793-795.

Appendix: Summary of Completed Tasks of the COLORSPORE EU Project

Identification of carotenoid producing microorganisms

Task 1.1: Isolation of pigmented spore formers

Task 1.4: Physiological and biochemical characterization

40 pigmented strains that produce carotenoids were isolated from a variety samples from marine and shrimp ponds in Vietnam.

Task 1.5: Species Identification

Phylogeny tree of 37 pigmented strains isolated from Vietnam was built based on 16S-rDNA sequences and compared with 100 type strains from The Ribosomal Database Project.

Task 1.6. Initial carotenoid screening

The UV spectrophotometer was used to scan all 40 organic extracts to screen carotenoid initially. The reference carotenoids were scanned to make the comparison.

Task 1.8. Probiotic characteristics

40 isolated strains were tested for enzyme producing ability and salt tolerance.

Bioprocess Production of Prototypes

Task 3.1: Development of optimal bioprocess conditions for the production of carotenoid containing bacterial spores

A. Investigation of optimal temperature, pH and salt concentration for growing selected carotenogenic strains HU36 (*B. indicus*) (dark yellow pigment GB1 (*B. firmus*) (pink pigment) among others.

B. Sporulation characteristics of strain DD1.1 (*Bacillus marisflavi*): Sporulation characteristics of the most potential strains DD1.1 (*Bacillus marisflavi*) were determined.

C. Alternative media: Alternative media from cheap materials to replacing expensive commercial TSA and TSB media were investigated for 6 strains.

D. Optimal bioprocess conditions for HU36 (*Bacillus indicus HU36*), DD1.1 (*B. marisflavi*), GB1 (*B. firmus*) on fermenter: Optimal bioprocess conditions for HU36 (*Bacillus indicus* HU36), DD1.1 (*B. marisflavi*), GB1 (*B. firmus*) were investigated using a fermenter.

E. Sporulation conditions for DD1.1, HU36, GB1: Investigated the suitable trace metal mix supplement for the sporulation

F. Sporulation condition on fermenter of GB1, HU36 and DD1.1: Sporulation conditions and yield of sporulation on fermenter of DD1.1, HU36, GB1 were determined.

Task 3.2: Characterisation of carotenoid formation by spore-forming bacteria under bioprocess conditions

Production of carotenoid during vegetative phase of selected strains: Relationship between growth curves and carotenoid contents of 6 selected strains has been established on Tryptic Soy Broth (TSB) and alternative media. Culture duration for highest carotenoid contents was also determined.

Nutritional Value

Task 4.3: Antioxidant activity of bacterial carotenoids in different tests

The antioxidant activity test using free radical DPPH was used to test the neutralization of free radicals of organic extract of strains. This test was popular for testing the antioxidant activity of compounds or extracts.

Task 4.9: Bioavailability of carotenoids from HU36 and new isolates in an animal model

Bioavailability of carotenoids from HU36, GB1 and DD1.1 strains in a mice model is being carried out, including: survival rates in gastrointestinal tract, carotenoid blood levels, accumulation of carotenoid in organs and observable health effects.

Safety and Risk-Assessments

Task 5.2: Analysis of antimicrobial resistance

Antimicrobial susceptibility test for 73 strains from consortium's collection was done according to CLSI M7-A9 guidelines.

Task 5.3: Antimicrobial production

40 isolated strains were tested for antibiotic producing ability by perpendicular streaking method.

Task 5.6: *In vivo* toxicity studies

Acute and sub-chronic toxicity tests in mice model are being carried out on *Bacillus marisflavi* (DD1.1), *Bacillus firmus* (GB1), *Bacillus indicus* (HU36)

Formulation

Task 6.2: Development of different formulations

Granulated formulation forms of preparations containing *Bacillus marisflavi* (DD1.1), *Bacillus firmus* (GB1), *Bacillus indicus* (HU36) were investigated.

Task 6.6: Stability of spores and carotenoid in different formulations

Stability of spores in biomass and granulated formulation during storage at room temperature were investigated.

Task 6.7: Colouration of farmed shrimps

Shrimp trials of preparations containing HU36, GB1 strains are being carried out, including shrimps colouration and weight gain effects.

Task 6.8: Texture of different milk formulations and food matrix stability

Study of supplementation with pigmented *Bacillus* powder in yogurt was investigated. The final product was pigmented and delicious tasting but did not have objectionable aroma. Pigmented yogurt can be stored at 45 days without changing the spore concentration in comparison to day 1. Work is completed on dark chocolate, fruit beverage, and baked products.

CONTACT FOR LICENSING AND PRODUCT INTEREST:

Kiran Krishnan
Managing Director
Nu Science Trading, LLC

Phone: (630) 699-4311
Fax: (630) 771-1569
Email: kkiran_00@yahoo.com

428 E. Thunderbird Rd.
Phoenix, AZ 85022