Heat-killed *M. aurum* Aogashima

(Brand name: Au+)

Novel food Ingredient Application

By Solution Sciences Ltd.

1. Contents

Table 1: Abbreviations

ACNFP	Advisory Committee on Novel Foods and Processes
M. aurum	*Mycobacterium aurum*
ATCC	American Type Culture Collection
CFU	Colony-Forming Units
DNSIYC	Diet and Nutrition Survey of Infants and Young Children
EC	European Community
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organisation
GLP	Good Laboratory Practice
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
GMP	Good Manufacturing Practice
GVP	Good Pharmacovigilance Practice
HACCP	Hazard Analysis & Critical Control Points
HSE	Health and Safety Executive
NCTC	National Collection of Type Cultures (UK)
NDNS	National Diet and Nutrition Survey
NDNS1-3	National Diet and Nutrition Survey - Years 1-3
NF	Novel Food
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
OECD	Organisation for Economic Co-operation and Development
OTC	Over The Counter
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
QC	Quality Control
QPSNCTC	Qualified Presumption of Safety National Collection of Type Cultures (UK)
RASFFATCC	Rapid Alert System For Food American Type Culture Collection
TAMC	Total Aerobic Microbial Counts
TOC	Total Organic Carbon
TYMC	Total Yeast & Mould Counts
WGS	Whole Genome Sequencing
WHO	World Health Organisation

Table 2: List of Annexes

Annex	Authors	Year	Full Title	Study Code/Site	Further Details
Annex 1 CONFIDENTIAL	BioElpida / Bactup	2014	RNA16s tests results	BEA-*M.aurum*-P14-14-04_27F	Strain identification
Annex 2 CONFIDENTIAL	Dr. L Rosa Brunet Dr. D Simpson	2015	Mycobacterial genome assembly and comparison: *Mycobacterium aurum* NCTC10437/ATCC23366 vs *Mycobacterium aurum* Aogashima	Report *Aurum* CH-R&D-01	Strain identification
Annex 3	*Aurum* Switzerland	2015	Mycobacteria in water	*Aurum* Switzerland	Review of the natural environments of mycobacteria
Annex 4 CONFIDENTIAL	BioElpida	2014	Manufacturing and quality control processes for Au+	BioElpida	Manufacturing&QC processes
Annex 5 CONFIDENTIAL	BioElpida	2015	Certificates of analysis of Au+	BioElpida	Certificates of analysis of 3 batches of Au+
Annex 6 CONFIDENTIAL	Sequani	2015	14-Day oral dose range finding test	BIT0001	Toxicological data on Heat killed *M. aurum* Aogashima
Annex 7 CONFIDENTIAL	Sequani	2015	Subchronic 90-day oral toxicity study	BIT0002	Toxicological data on Heat killed *M. aurum* Aogashima
Annex 8 CONFIDENTIAL	Sequani	2015	Bacterial reverse mutation test	BIT0003	Toxicological data on Heat killed *M. aurum* Aogashima
Annex 9 CONFIDENTIAL	Covance	2015	Human Lymphocyte Micronucleus test	BIT0004	Toxicological data on Heat killed *M. aurum* Aogashima
Annex 10	*Aurum* Switzerland	2015	Hypersensitivity reactions induced by environmental mycobacteria	*Aurum* Switzerland	Review of evidence that mycobacteria encountered in the natural environment do not cause hypersensitivity reactions
Annex 11 CONFIDENTIAL			References		All scientific literature used in the dossier

Table 3: List of References

Reference	Authors	Year	Full Title	Study Code/Site	Further Details
Allergome database	Allergome database		allergome.org, consulted on June 2015	http://www.allergome.org	
Brown et al. 2012	Brown K, DeCoffe D, Molcan E, Gibson DL.	2012	Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease	Nutrient. 2012, 4, 1095-1119	
Cambier *et al.* 2014	Cambier CJ, Falkow S, Ramakrishnan L.	2014	Host evasion and exploitation schemes of *Mycobacterium* tuberculosis.	Cell. 2014 Dec 18;159(7):1497-509	
Dir. 2003/89	EU Commission	2003	Directive 2003/89/EC of the European Parliament & of the Council as regards indication of the ingredients present in foodstuffs		
Gey van Pittius *et al.* 2006	Gey van Pittius NC, Sampson SL, Lee H, *et al.*	2006	Evolution and expansion of the *Mycobacterium* tuberculosis PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions.	BMC Evol Biol. 2006 Nov 15;6:95.	
Gomez-Smith *et al.,* 2015	Gomez-Smith CK., LaPara TM., Hozalski RM,	2015	Sulfate Reducing Bacteria and Mycobacteria Dominate the Biofilm Communities in a Chloraminated Drinking Water Distribution System.	Environ Sci Technol. 2015 Jul 21;49(14):8432-40	
Grange 2014	Grange JM.	2014	*Mycobacterium* tuberculosis – the organism.	In Davies PDO, Gordon S, Davies G. eds. Clinical Tuberculosis, 5th Edition. London: CRC Press. Chp. 3, pp. 39-53. 2014	
HSE	Advisory Committee n Dangerous Pathogens	2013	The Approved List of Biological Agents		Classifies *Mycobacterium aurum* as Hazard Group 1 – unlikely to cause human disease

Reference	Authors	Year	Full Title	Study Code/Site	Further Details
Kishi 2011	Kishi Y.	2011	Chemistry of mycolactones, the causative toxins of Buruli ulcer.	Proc Natl Acad Sci U S A. 2011 Apr 26;108(17):6703-8.	
Rahman *et al.* 2014	Rahman SA, Singh Y, Kohli S, *et al.*	2014	Comparative analyses of nonpathogenic, opportunistic, and totally pathogenic mycobacteria reveal genomic and biochemical variabilities and highlight the survival attributes of *Mycobacterium* tuberculosis.	MBio. 2014 Nov 4;5(6):e02020.	
Rook 2009	Rook GAW.	2009	Review series on helminths, immune modulation and the hygiene hypothesis: The broader implications of the hygiene hypothesis.	Immunology 2009;126(1):3-11.	Definition of pseudocommensal.
Salminen and Deighton, 1992	Saliminen S and Deighton M	1992	Lactic acid bacteria in the gut in normal and disordered states	Digestive Diseases 1992, 10, 227-238	
Simmon *et al.* 2009	Simmon KE, Low YY, Brown-Elliott BA, Wallace RJ Jr, Petti CA.	2009	Phylogenetic analysis of *Mycobacterium aurum* and *Mycobacterium* neo*aurum* with redescription of *M. aurum* culture collection strains.	Int J Syst Evol Microbiol. 2009;59:1371-5.	Phylogenetic analysis of the type strain of *Mycobacterium aurum* and identity of other strains labeled *Mycobacterium aurum*
Skerman *et al.* 1980	Skerman VBD, McGowan V, Sneath PHA.	1908	Approved lists of bacterial names.	Int J Syst bacteriol 1980; 30: 225-420.	The internationally approved bacterial names for future reference purposes.
Smith 2003	Smith I.	2003	*Mycobacterium* tuberculosis pathogenesis and molecular determinants of virulence.	Clin Microbiol Rev. 2003 Jul;16(3):463-96.	
Stone *et al.* 1995	Stone BB, Nietupski RM, Breton GL, Weisburg WG.	1995	Comparison of *Mycobacterium* 23S rRNA sequences by high-temperature reverse transcription and PCR.	Int J Syst Bacteriol. 1995;45:811-9.	
Fall *et al.* 2015	Fall T, Lundholm C, Ortqvist A K,	2015	Early exposure to dogs and farm animals and the risk of childhood	JAMA Pediatr. 2015; 169(11)	

Reference	Authors	Year	Full Title	Study Code/Site	Further Details
	Fall K, Fang F Hedhammar A, Kampe O, Ingelsson E, Almqvist C		asthma		
Tsukamura M. 1966	Tsukamura M	1966	Adansonian classification of mycobacteria.	J Gen Microbiol 1966; 45: 253-273	Description of new species *Mycobacterium aurum*
Tsukamura and Mizuno 1977	Tsukamura M, Mizuno S.	1977	Numerical analysis of relationships among rapidly growing scotochromogenic mycobacteria.	J Gen Microbiol. 1977;98:511-7	
Wang *et al.* 2014	Wang J, Behr MA.	2014	Building a better bacillus: the emergence of *Mycobacterium* tuberculosis.	Front Microbiol. 2014 Apr 3;5:139.	
Weiss *et al.* 2015	Weiss G, Schaible UE.	2015	Macrophage defense mechanisms against intracellular bacteria.	Immunol Rev. 2015 Mar;264(1):182-203.	
Wood *et al.* 2015	Wood C.J., Pretty J., Griffin M.	2015	A case–control study of the health and well-being benefits of allotment gardening	J Public Health (Oxf). 2015 Oct 29	
Yesilkaya *et al.* 2005	Yesilkaya H, Dale JW, Strachan NJC, *et al.*	2005	Natural transposon mutagenesis of clinical isolates of *Mycobacterium* tuberculosis: how many genes does a pathogen need?	J Bacteriol 2005;187:6726–32	

1.Administrative Data

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Notes on confidentiality

According to guidance of the UK ACNFP Secretariat, this dossier and relevant parts of key safety studies and safety-related data are made public. Information and texts for which the applicant claims confidentiality because it pertains to the manufacturing process, business data, marketing know-how, or for which third parties have given limited access permission (i.e. permission to be used for regulatory purposes, but not to be placed in the public domain) are added as annexes to the dossier. Related Annexes are listed as "CONFIDENTIAL" in the index and tables of contents, and are confidential in their entirety, namely:

- Confidential Annex *1*: RNA16s tests results
- Confidential Annex *2*: Mycobacterial genome assembly and comparison: *Mycobacterium aurum* NCTC10437/ATCC23366 vs *Mycobacterium aurum* Aogashima
- Confidential Annex *4*: Manufacturing and quality control processes for Au+
- Confidential Annex *5*: Certificates of analysis of Au+
- Confidential Annex *6*: 14-Day oral dose range finding test
- Confidential Annex *7*: Subchronic 90-day oral toxicity study
- Confidential Annex *8*: Bacterial reverse mutation test
- Confidential Annex *9*: Human Lymphocyte Micronucleus test
- Confidential Annex *11*: References

2. General Introduction and Description of *Mycobacterium aurum* Aogashima

This application provides evidence for the safety of "Heat-Killed *Mycobacterium aurum* Aogashima" (Brand name Au+) as a novel food ingredient, intended for use in foodstuffs (foods or drinks) for the general population. The application has been prepared in accordance with Regulation (EC) Nº 258/97 and Commission Recommendation 97/618/EC. A novel food/ingredient is one that does not have a significant history of consumption within the European Union (EU) before 15 May 1997.

The food ingredient referred to in this dossier is a heat-killed microorganism, a non-pathogenic, non-genetically-modified bacterium, destined for use as a food ingredient in the EU. The microorganism is *Mycobacterium aurum* Aogashima (an isolate proprietary to *Aurum* Switzerland AG), presented as a heat-killed bacterial suspension, under the proposed EU trade name of Au+.

It fulfils the criteria of a novel food ingredient, namely *´foods and food ingredients consisting of or isolated from microorganisms, fungi or alga´*, under the Class 2.2 category: "*complex novel food from non-GM source, the source of the novel food has no history of food use in the Community*".

Mycobacterium aurum (*M. aurum*) is one of over 150 species within the genus *Mycobacterium*. [redacted] The Health and Safety Executive (HSE 2013) does not include *M. aurum* in its Approved Lists of human pathogens and it is therefore classified in Hazard Group 1 – unlikely to cause human disease. *M. aurum* Aogashima is an isolate, proprietary to *Aurum* Switzerland AG. This particular isolate has been selected under specific growth conditions, and appears morphologically different from the type strain. Here we report that the type strain and the proprietary isolate retain a high degree of homology in their genomic sequences.

Heat-killed *M. aurum* has never been used in the EU as a direct ingredient to foods or drinks. *M. aurum* is, however, not novel to the human gastrointestinal tract as it is regularly encountered via consumption of untreated water, except when their numbers are reduced by drinking water treatment or when bottled waters are predominantly consumed.

I The purpose of the novel food ingredient

The human body contains some 120 trillion cells, but for every one cell we carry around ten microbes. Most are in our intestinal tract but they are also present on the skin and in the upper respiratory and genital tracts. This vast and varied population, over 1,000 species, is termed the microbiome. Although 'germs' are usually associated with infection and disease leading to measures to live 'hygienically', the vast majority of the microbes in the environment are harmless.

Furthermore, there is growing evidence that they are essential to health providing "educational" input to the immune system and have been referred to as our 'Old Friends' (Rook and Rosa Brunet, 2005). Some of these become long-term colonisers of our bodies, notably our intestinal tracts, and are termed commensals. Others that were historically encountered on a regular basis through bathing and drinking untreated water are termed 'pseudo-commensals' as, although they do not colonise the intestinal tract, they were always present in it. We have become therefore particularly deprived of pseudo-commensals by current hygienic measures such as the treatment of drinking water.

It may be said that we all live far too hygienically and at an increasing distance from our natural 'green' environment. For millions of years our ancestors lived in close relationship with an enormous range of micro-organisms and evolution has led our immune systems to 'expect' regular exposure to these microbes and to 'learn' from these encounters (Rook, 2010).

Use of modern molecular techniques to determine the microbial content and diversity of the human microbiome in individual people reveal that, as a community, we suffer from 'biome-depletion', also called dysbiosis, a condition that is postulated to have unfortunate health consequences (Brown *et al*., 2012). A major cause of dysbiosis is the indiscriminate use of antibiotics especially the 'broad-spectrum' antibiotics which not only kill microbes supposedly causing the disease but many, even most, of those in the microbiome, resulting in gross dysbiosis, which takes a long time to recover from.

The evidence for the impact of biome-depletion on health is impressive and growing and has given rise to an important discipline within the health sciences. Originally it was thought that the driving force for 'educating' the immune system was repeated minor viral infections encountered by children living in crowded urban environments but the focus is now much more on the far more ancient 'Old Friends'. Many studies have established that children who regularly come into contact with 'dirt' as, for example, by living on farms or working on an allotment, are much healthier and less prone to the diseases resulting from immune dysregulation, including asthma, allergies and acute leukaemia, than those brought up in modern clean environments (Rook, 2010; Wood *et al.*, 2015). Living with a dog is a way of expanding the biome, as dog-to-human transmission of microbes occurs rapidly, and there is evidence that pre-school children exposed to dogs have a significantly lower incidence of asthma than unexposed children (Fall *et al*., 2015).

Although carrying the risk of water-borne diseases such as typhoid and cholera, untreated water was, and in many parts of the world still is, the main source of many microbes in the biome. Food was another source as, in the days before fridge-freezers, the bacterial content of food rose on storage. Indeed some foods were deliberately fermented in order to preserve them: examples include soured milk, yoghurt and sauerkraut.

Attempts to restore and expand the microbiome are made by the use of 'probiotics' which are living bacteria and/or yeasts. Yoghurt is the most common source of probiotics, although more

expensive food supplements are available. They have been shown to be of use in the prevention and treatment of antibiotic-associated diarrhoea, a condition characterised by gross dysbiosis (Salminen and Deighton, 1992).

Our approach is different. We do not intend to attempt to populate, or repopulate, the microbiome with commensal organisms but, instead, to make up for the presumed deficiency of exposure to an important group of pseudocommensals; namely, the mycobacteria. Historically, populations would have received exposure to such organisms via food and drinking water. In modern societies, the use of treated drinking water, bottled water, domestic water filtration systems and near sterile food production processes has diminished such exposure. The addition of small quantities of dead (heat-killed) *M. aurum* Aogashima may go some way to restore such natural exposure.

The applicant makes no specific or implied medical claims on behalf of this novel food ingredient.

The applicant, Solution Sciences Ltd, has followed the relevant structured schemes established for novel foods (Regulation (EC) Nº 258/97). Solution Sciences Ltd proposes one concentration of heat - killed *M. aurum* Aogashima (Au+) as a novel food ingredient in the EU, manufactured to appropriate quality standards, and compliant with relevant EU food ingredient legislation (Confidential *Annex 4* and *Annex 5*).

In this application, the safety of the Heat-killed *M. aurum* Aogashima for the general population in EU is based on the following:

- Classification of *M. aurum* as a non-pathogenic organism,
- The inactivation process applied during the manufacturing of the product and confirmed by the “sterility” test,
- The results of a series of toxicology studies,
- The anticipated intake of the novel food ingredient by consumers.

3.Identification of essential information requirements

For the safety evaluation of a non-GMM Class 2.2 category novel food product, the following information is required in accordance with Commission Recommendation 97/618/EC.

I. Specification of the novel food,
II. Effect of the production process applied to the novel food,
III. History of the organism used as the source of the novel food,
IX. Anticipated intake/extent of use of the novel food,
X. Information from previous human exposure to the novel food or its source,
XI. Nutritional information on the novel food,
XII. Microbiological information on the novel food,
XIII. Toxicological information on the novel food

4.Consultation of structured schemes

I Structured Scheme I: Specification of the Novel Food ingredient

The novel food ingredient is defined as heat-killed *M. aurum* Aogashima for intended use as a food ingredient in EU (2002/46/CE & 97/618/CE). The brand name proposed for EU use is Au+.

Specified reference material such as samples of Au+ with corresponding Certificates of Analysis are available for use by the EU regulatory authorities on request to BioElpida and *Aurum* Switzerland AG, respectively. The proprietary isolate has not been deposited and is not commercially available.

[redacted]The specifications of heat-killed *M. aurum* Aogashima are summarised in Table 4.

Table 4: Key specifications of Heat killed *M. aurum* Aogashima

Concentration of Heat-Killed *M.aurum* in water	[redacted]
Appearance	Suspension of red to orange to yellow particles, which settle on the bottom of the container and are easily resuspended.
Total aerobic count	Absent
Escherichia coli	Absent
Salmonellae	Absent
Staphylococccus aureus	Absent
Pseudomonas aeroginosa in 1 g sample	Absent
Yeasts & moulds	Absent
Live *M. aurum*	Absent

i. Isolate origin, identity and physical properties

[redacted]

M. aurum is a rapidly growing, non-motile, aerobic, non-spore-forming mycobacterial species producing a bright yellow pigment on incubation in the dark. On the basis of pigment formation in the dark it is included in the group of mycobacteria known as scotochromogens. In common with other mycobacterial species, *M. aurum* is “acid fast”, i.e. it retains the red colour imparted by staining with hot carbol fuchsin following treatment with weak mineral acids and/or alcohol. *M.*

aurum, in common with all other members of the genus *Mycobacterium*, does not produce spores and is therefore always in the vegetative form. Being non-sporing, mycobacteria are as susceptible to killing by heat as other non-sporing bacteria. Isolates produce predominantly smooth colonies with a low rate of one-way smooth to rough variation on a variety of solid culture media.

Strains of *M. aurum* were originally identified and characterised on the basis of a range of growth characteristics and utilization of nutrients including nitrogen and carbon sources (Tsukamura and Mizuno 1977). Currently they are identified by determination of 16S- and 23S-rRNA gene sequences (Simmon *et al.,* 2009; Stone *et al.,* 1995).

Most importantly, *M. aurum* is a saprophytic mycobacterial and is to be considered non-pathogenic. Indeed, the application of 16S-rRNA gene sequencing to 10 strains of *M. aurum* in the American Type Culture Collection (ATCC) showed that nine of them, including two implicated as human pathogens (but not the type strain ATCC 23366), had been misidentified and mostly belonged to the species *Mycobacterium neoaurum* and *Mycobacterium lacticola* and this finding, together with a review of isolates from two large reference laboratories led to the conclusion that *M. aurum* ATCC 23366 (NCTC 10437) is not a clinically significant isolate (Simmon *et al.,* 2009).

The microorganism is *M. aurum* Aogashima, an isolate proprietary to *Aurum* Switzerland AG. This particular variant has been selected under specific growth conditions, and while appearing morphologically different from the type strain *M. aurum* NCTC 10437 (ATCC 23366), it retains a high degree of homology in their genomic sequences. BioElpida, the manufacturer of the heat-killed *M. aurum* Aogashima suspension, has preserved their collection of *M. aurum* Aogashima isolate by freezing methods. Subculture of the *M. aurum* Aogashima master cell banks and working cell banks is performed according to manufacturing process (Annex *4*). [redacted]

None of the *Mycobacterium* spp listed are considered pathogens. [redacted]

Indeed, there is no known report of *M. aurum* being the causative agent of any human infection.

ii. Isolate identification and characterisation

M. aurum Aogashima (isolate, proprietary to *Aurum* Switzerland AG [redacted] (Confidential Annex 1). A whole genome sequencing (WGS) has also been performed on *M. aurum* type strain (NCTC10437/ATCC23366) and *M. aurum* Aogashima cell

bank in order to determine the extent of the relation between the two and to obtain knowledge of the genome of *M. aurum* Aogashima. The corresponding data are available in Confidential Annex 2 and summarised in the next two paragraphs.

[redacted]

[redacted]

While the identification of the *M. aurum* Aogashima isolate provided by *Aurum* Switzerland AG is made by the whole genome sequencing (WGS) and complemented by 16S-rDNA sequencing, additional characterisation tests are also applied to the bulk heat killed bacteria used for the manufacture of the novel food ingredient.

iii. Genetic and microbiological identity of Mycobacterium aurum Aogashima

The genetic characterisation of *M. aurum* Aogashima is established by whole genome sequencing (WGS). The genetic stability is then further determined by 16S-rDNA analysis (Confidential Annex 1).

The microbiological identity to the genus is established by the Ziehl–Neelsen stain, used to identify acid-fast organisms, mainly Mycobacteria.

Those tests are considered sufficient for the stability of the isolate used since *M. aurum* Aogashima will be heat-killed during the manufacturing process and the growth medium will be removed.

iv. Assessment of Mycobacterium aurum Aogashima *isolate identity, characterisation and microbial safety by microbiological techniques*

The identification and the biochemical and physiological characteristics of the *M. aurum* Aogashima production isolate, including genetic stability, were established and described by both biochemical and genetic techniques.

The Health and Safety Executive (HSE 2013) does not include *M. aurum* in its Approved Lists of human pathogens and it is therefore in Hazard Group 1 – unlikely to cause human disease.

Additionally, only a very small minority of the vast range of bacterial species cause disease in the immune-competent individual. Those that do, known as pathogens, do so by four principal mechanisms:

- The production and secretion of very powerful toxins, known as exotoxins that, even if the infection is localised, can cause severe and potentially fatal systemic poisoning. Examples include the exotoxins of *Corynebacterium diphtheriae*, *Clostridium botulinum* and *Clostridium tetani* which are among the most poisonous substances known. Accordingly there is a definite need to examine any food ingredient prepared from *Clostridium* species, especially if viable. By contrast, such powerful toxins are not produced by members of the genus *Mycobacterium*. (The only exception to the absence of disease-associated toxins in the genus *Mycobacterium* is mycolactone, produced by a very slow growing species, *M. ulcerans*, the cause of a unique debilitating human skin disease in humans in certain tropical regions (Kishi 2011))
- The production of toxic lipopolysaccharides that remain largely bound to the bacterial cell wall (endotoxins). Typically these are found in the Gram-negative enteric pathogens including *Shigella* and *Salmonella*. The mycobacterial cell wall contains various lipopolysaccharides but these differ structurally and functionally from the endotoxins. For example, the latter activate macrophages and dendritic cells through a cell receptor, Toll-like receptor (TLR)-2, whereas endotoxins utilise TLR-4.
- They inhibit phagocytosis by possession of capsules and/or production of toxins that kill phagocytic cells (leucocidins), and are thus principally extracellular pathogens. Examples include staphylococci and streptococci. Antiphagocytic capsules and leucocidins are not found in pathogenic mycobacteria, which rely on the maintenance of the viability of their host cells.
- The ability to survive and replicate within phagocytic cells, principally macrophages, without destroying them (at least in early disease). This is the key mechanism of virulence in the pathogenic species of the genus *Mycobacterium* (Weiss *et al*., 2015).

There are around 150 named species of mycobacteria. The great majority live harmlessly in the environment (see Annex 3). Very occasionally, some species of environmental mycobacteria, notably slow-growing species, cause opportunist disease in immunocompromised subjects or can colonise diseased tissue. The very few mycobacterial pathogens causing transmissible disease in immunocompetent human beings and/or animals are *M. leprae* and members of the *M. tuberculosis* and *M. avium* complexes.

Pathogenicity in these mycobacteria is not due to any single gene or molecule. Several reviews have listed many molecules that are thought, in concert with many other molecules, to play a role in mycobacterial pathogenicity (Grange *et al*., 2014; Smith 2003; Cambier *et al*., 2014).

Interestingly, mycobacterial species that have become pathogens have devolved to the parasitic state by shedding many genes and causing duplication and expansion of some other genes coding for certain determinants of virulence (Yesilkaya *et al*., 2005; Wang *et al*., 2014; Rahman *et al.,* 2014). For example, in the *M. tuberculosis* complex there has been a huge expansion of the genes coding for PE/PPE proteins and the associated ESAT-6 protein for which a role in virulence has been ascribed (Gey van Pittius *et al.,* 2006).

Figure 1. *Mycobacterium* Phylogenic Tree

A phylogenic tree for 27 different species of Mycobacteria is given in Figure 1 above (taken from Phelan *et al.,* 2015) and shows the clear phylogenetical distance between *M. aurum* and the two highly pathogenic members of the mycobacterial genus, namely *M. leprae* and *M. tuberculosis*.

In summary, the pathogenic mycobacteria do not owe their virulence to toxic substances but to their ability, by complex genetic rearrangements that distinguish them from saprophytic mycobacteria, to survive and replicate within cells that have internalised them. Thus, in contrast to studies of genera such as *Clostridium*, which have completely different virulence mechanisms, we claim that a search for toxins and other determinants of virulence in a saprophytic organism, such as *M. aurum*, especially when killed by heat, would not be productive.

The data summarised above indicate that *M. aurum* Aogashima is a well-characterised isolate, adequately and correctly identified, biochemically and genetically stable, that is believed not to possess genes coding for toxins or virulence factors.

Moreover, in the heat-killed *M. aurum* Aogashima suspension, the bacterial cells are inactivated by the heat-killing process as demonstrated by the absence of growth of any bacteria (sterility test performed on the bulk material, see section II).

Plasmids can survive the heating process, but current knowledge suggests that they are not relevant to pathogenicity. Work conducted in the 1970s-80s provided inconclusive evidence. No plasmids were found in *M. tuberculosis*, and while present in *M. avium*, they appear to mediate only minor properties such as hydrophobicity, resistance to heavy metal salts and the temperature range of growth - characteristics with no known relevance to toxicity and virulence. A study in the USA showed that *M. avium* strains from HIV patients all had a plasmid but a study in the UK did not confirm this. Likewise some environmental mycobacteria contain bacteriophages but they have not been shown to be associated with virulence. The possibility that the *M. aurum* Aogashima bacteria could be transfected with plasmids post-production may be entirely excluded because the organisms are killed by autoclave heat treatment as part of the production process.

v. Specification and composition of Heat-killed M. aurum Aogashima ingredient

[redacted]

M. aurum Aogashima has been grown by aerobic cultivation in liquid medium, then killed by autoclaving and stored at 4°C in the dark to avoid any variation due to temperature or light exposure (Confidential Annex 4).

Specifications of heat-killed *M. aurum* Aogashima are summarised in Table 4 and in Confidential *Annex 4*. Certificates of analysis of three batches (P14-15-01; P14-15-02; P14-15-03) are supplied as Confidential Annex 5.

Heavy metals analysis and microbiological analysis have been undertaken and the results are summarized in the Table *5*.

Table 5: Heavy metals and microbiological analysis on three batches

Certificates	**Batch P14-15-01**	**Batch P14-15-02**	**Batch P14-15-03**
Heavy metals (ppm)			
Arsenic	<0.25	<0.25	<0.25
Cadmium	<0.25	<0.25	<0.25
Chromium	<0.25	<0.25	<0.25
Mercury	<0.25	<0.25	<0.25
Nickel	<0.25	<0.25	<0.25
lead	<0.25	<0.25	<0.25
Selenium	<0.25	<0.25	<0.25
barium	<0.25	<0.25	<0.25
Microbial analysis			
Total aerobic count	Absent	Absent	Absent
Escherichia coli	Absent	Absent	Absent
Salmonellae	Absent	Absent	Absent
Staphylococccus aureus	Absent	Absent	Absent
Pseudomonas aeroginosa in 1 g sample	Absent	Absent	Absent
Yeasts & moulds	Absent	Absent	Absent
Live *M. aurum*	Absent	Absent	Absent

Foodstuffs containing heat-killed *M. aurum* Aogashima as an ingredient will be labelled in accordance with relevant provisions in Council Directive 90/496 on foodstuffs labelling and Regulation (EU) N°1169/2001 on the provision of food information to consumers. Proposed label text related to heat-killed *M. aurum* Aogashima to be added on the concerned foodstuff are as follows:

"Heat-Killed *Mycobacterium aurum* Aogashima. "

Heat-killed *Mycobacterium aurum* Aogashima will be marketed under the 'Au+' trade name.

II Structured Scheme II: Effect of the production process applied to the novel food ingredient.

The basic production process and quality control procedures are summarized in Figure 2 and detailed in Confidential Annex 4. The basic production process is aerobic cultivation. The *M. aurum* isolate used in heat-killed *M. aurum* Aogashima suspension has not been modified genetically, and is genetically stable.

Production process & quality control:

Please refer to Figure 2 and Confidential Annex 4 for information on the manufacturing process and quality control procedures applied to every batch of Au+. Production controls (Sterility, purity, Identity (PCR), heavy metals) guarantee the safety of Au+ and control the concentration of heat-

killed *M. aurum* Aogashima (OD, viability, dry residue) as detailed in the Au+ certificates of analysis (Confidential Annex 5). Appearance, pH and particles distribution are given as information for industrial uses.

Figure 2: Summary of the Production Flow Chart and quality control (QC) of Au+

Au+ production Flow Chart	**QC – every lot**
Au+ cell bank	Species and isolate characterization by classic culture, biochemical and molecular microbiological techniques.
Cultivation process	↓
Centrifugation process	
Harvesting and dilution in water	
Heat-Killing process	
Au+	Appearance of Au+ Microbial purity and efficiency of the heat-killing process ("sterility test") Safety tests Heavy metals and arsenic Loss on drying

Sterility:

M. aurum Aogashima is completely killed by autoclaving [redacted] as demonstrated by the absence of growth of any bacteria in the sterility test performed on the bulk material (Confidential Annex 4 and Annex 5).

Stability:

As Au+ packaging (sterile sealed glass bottle) does not allow exchange with the environment, safety parameters and microorganism mass are considered to be unchanged over time. The expected evolution of Au+ is a modification of the color over time. The color alteration will be due to pigment structure modification. This pigment is used for Au+ final bulk product as a biomarker for chemical stability. Therefore, the Au+ manufacturing process guarantees the safety over time on unopened vials in terms of microbiological and chemical contaminants, if the manufacturer preservation recommendations are followed by the user. The chemical stability can be checked by the user using the colorimetric range mentioned in the Au+ certificate (Confidential Annex 4) and is shown in Figure 3 below.

Figure 3: Colour range within key specification requirement for *M. aurum* Aogashima

For clarification purposes, the key specifications of heat-killed *M. aurum* Aogashima included in Table 4 and in the confidential certificates of analysis of the three batches; the description of the pigment of the suspension. The suspension is described as “red to orange to yellow particles”, which settle on the bottom of the container and are easily resuspended. This description of the specification implies that the acceptable colour of the product ranges from red to orange to yellow. A colour chart is included in Figure 3 to depict this range. Any product that falls out of the range of colour described (red to orange to yellow) does not meet key specification. On the same basis, any product whose pigment is included in the range described, for example orange, meets the specifications.

The bulk product is relatively stable and the killed bacteria will be too dilute in the final consumer products to enable colorimetric observations to be made. It is considered that, after dilution in final consumer products, the stability will not be an issue in those products with a shelf–life measured in days or weeks and those products with longer shelf-lives will generally utilise packaging that will protect the diluted Au+ product from light. Any degradation products that may be formed over time will occur only in trace amounts, be equivalent to those encountered in normal life, and without concern to health.

Manufacturer storage recommendation

[redacted]

Recommendations of use:

[redacted]

III Structured Scheme III: History of the organism used as the source of the novel food ingredient

M. aurum is one of over 150 species within the genus *Mycobacterium* that are found free-living in watery environments, including marshland, rotting vegetation, wet soil and free water and have been shown to colonise pipes supplying water for industrial and domestic use (Annex 3). A relative

amount of *M. aurum* in natural water cannot be given as the quantity of mycobacteria in water varies greatly from region to region, even locations within regions. Also, the species present vary over time in a given location. Furthermore, most studies on mycobacteria presence in water focus on those, such as members of the *M. avium* complex that can cause disease in immunocompromised persons, especially those with HIV. The non-pathogenic species are not usually identified as there is no public health need to do so and, in this respect, *M. aurum* is one of a group known as "tap water scotochromogens" - a term emphasising their clinical unimportance and their usual source. Also, mycobacteria in water are usually found in large clumps so that colony counts give a gross underestimate of the actual numbers.
As an example, a study provides novel insights into the quantity and composition of biofilms in full-scale drinking water distribution systems (Gomez-Smith et al., 2015). In this case, the analytical method (real-time PCR targeting the 16S rRNA gene and next-generation, high-throughput Illumina sequencing) showed that bacteria from the genus *Mycobacterium* dominated all communities at the main wall-bulk water interface (25-78% of the community) and that 6.5% of mycobacteria present were *M. aurum*.
Mycobacteria are not permanent commensals of the human intestine but historically they were regularly encountered in untreated drinking water. In modern living conditions, exposure has been reduced as their numbers are reduced by water treatment or when bottled waters are predominantly consumed. In common with lactobacilli and many other genera that likewise do not efficiently replicate in the human host, they have been termed 'pseudocommensals' (Rook 2009).

The genus *Mycobacterium* is divided into two sub-genera, the slow and rapid growers. Although the genus contains two major human pathogens, *Mycobacterium tuberculosis* and *Mycobacterium leprae*, only a few other species, mostly slow growers, have been associated with disease in immune compromised persons. The great majority are non-pathogenic environmental saprophytes, especially the rapid grower group to which *M. aurum* belongs. Although the literature contains two reports of bacteraemia due to *M. aurum* in immunocompromised persons, these strains were subsequently shown to have been misidentified (Simmon *et al.,* 2009) and the species is therefore considered to have no clinical significance.

The Health and Safety Executive (HSE 2013) does not include *M. aurum* in its Approved Lists of human pathogens and it is therefore in Hazard Group 1 – unlikely to caused human disease. (The website of the American Biological Safety Association (www.absa.org/riskgroups/) shows that it is not included in any higher Hazard or Risk Groups, including that of the World Health Organization). Furthermore, cultures of *M. aurum* type strain NCTC 10437 can be obtained from the Department for Public Health in England and are exempt from the safety measures applied to other mycobacteria, viruses etc., as detailed on the PHE website:
http://www.phe-culturecollections.org.uk/products/bacteria/detail.jsp?refId=NCTC+10437&collection=nctc#medDoc

IV– VIII Structured Schemes: Not relevant to Heat killed *M. aurum* Aogashima (non-GMM)

The *M. aurum* isolate used to manufacture heat-killed *M. aurum* Aogashima suspension is not genetically modified and therefore these sections are not applicable.

IX Structured Scheme IX: Anticipated intake/extent of use of the novel food ingredient

The heat-killed *M. aurum* Aogashima suspension is to be marketed for use in a range of foods and food supplements at a maximal concentration of [redacted] This low dose was selected after considering the variety of food and drink products to which heat-killed *M. aurum* Aogashima is expected to be added, the anticipated maximum consumption of such products, and the NOAEL of 2000 µg/kg bw/day of heat-killed *M. aurum* Aogashima, which was based on the results of a sub-chronic 90-day oral toxicity study in rats.

The proposed uses and maximum levels of heat-killed *M. aurum* Aogashima are presented in Table 6.

Table 6: Intended uses of heat-killed *M. aurum* Aogashima)

Use group	**Maximum level of heat-killed *M. aurum* Aogashima**
Milk and dairy products	< 0.033 ppm
Soft drinks	< 0.033 ppm
Juice Drinks	< 0.033 ppm
Teas	< 0.033 ppm
Bottled water	< 0.033 ppm
Products for special nutritional use (i.e. probiotics)	< 0.033 ppm
	< 0.033 ppm
Food for sports people	< 0.033 ppm
Mouth spray (sublingual)	< 0.033 ppm
Water based daily shot	< 0.033 ppm
Infant formula (at 50% serving)	< 0.033 ppm
Breads	< 0.033 ppm
Bars	< 0.033 ppm
Nutrition powders	< 0.033 ppm
Confections	< 0.033 ppm
Food supplements	

Estimates of the intake of the novel food ingredient were derived from consumption data of the UK national diet and nutrition survey (NDNS), the UK National Diet and Nutrition Survey - Years 1-3 (NDNS1-3) and the UK Diet and Nutrition Survey of Infants and Young Children (DNSIYC, 2011).

Among the population groups, on a per person basis, the highest mean and 95th percentile intakes of heat-killed *M. aurum* Aogashima of 0.066 and 0.130 mg/day, respectively, were calculated for adults, while toddlers had the lowest intake estimates at 0.035 and 0.065 mg/day (Table 7), respectively.

Table 7: Summary of the estimated daily intakes of heat-killed *M. aurum* Aogashima (calculations as worst case from UK surveys data)

Population group	Age group (years)	Subjects (n)	Total population (mg/day)		Consumers only (mg/day)	
			Mean	P95	Mean	P95
Toddlers	1-3	1314	0.031	0.063	0.035	0.065
Children	3-10	651	0.038	0.065	0.043	0.070
Adolescents	10-18	666	0.045	0.088	0.053	0.094
Adults	18-65	1266	0.060	0.126	0.066	0.130

P95, 95th percentile

On a body weight basis, toddlers were calculated to have the highest intakes, at both the mean and 95th percentile, with heat-killed *M. aurum* Aogashima intakes of 3.29 and 6.16 µg/kg body weight, respectively (Table 8).

Table 8: Summary of the estimated daily intakes of heat-killed *M. aurum* Aogashima per kg body weight (calculations as worst case from UK survey data)

Population group	Age group (years)	Subjects (n)	Total population (µg/kg bw/day)		Consumers only (µg/kg bw/day)	
			Mean	P95	Mean	P95
Toddlers	1-3	1314	2.84	5.97	3.29	6.16
Children	3-10	651	1.67	3.07	1.90	3.29
Adolescents	10-18	666	0.87	1.67	1.01	1.79
Adults	18-65	1266	0.81	1.72	0.89	1.77

The estimated intakes represent an overestimate of the consumption of heat-killed *M. aurum* Aogashima, because the estimates were based on a larger number of food groups (Grains and grain-based products, milk and dairy products, sugar and confectionary, fruit and vegetable juices, non-alcoholic beverages (excepting milk based beverages), drinking water (including tap water and bottled water), food for infants and small children, products for special nutritional use) than those considered in the present application.

For all the population groups, these calculated intakes are greater than one hundred-fold less than the proposed NOAEL of 2000 µg/kg bw/day of Heat killed *M. aurum* Aogashima, which was based on the results of a sub-chronic 90-day oral toxicity study in rats.

This product is to be sold as the concentrate 'Au+' to third parties who will add it to their products (milk, juices, dairy products…). Therefore instructions for use will be provided to the users on how to store and how to dilute the product (see Chapter II).

The stability of the concentrate Au+ has been demonstrated. The stability of this ingredient in the intended final products has not been directly assessed as the product is intended to be sold to various users. However, as Au+ is a suspension of heat-killed *M.aurum* Aogashima, no undesirable

substances are expected to be generated when added to various products such as fruit juices and dairy products, as is also expected with milk or milk products because environmental mycobacteria killed by pasteurization are present in milk and milk products.

X Structured Scheme X: Information from previous human exposure to the novel food ingredient or its source

Despite there being no history of use of *M. aurum* as a novel food or food ingredient, there is evidence that this organism is present in many different environments, as described in Structured Scheme III: "History of the organism used as the source of the novel food ingredient".

Heat-killed *M. aurum* Aogashima has never been used in the EU as a direct ingredient to foods or drinks. *M. aurum* is, however, not novel to the human gastrointestinal tract as, in common with many other environmental Mycobacteria, it is a common environmental organism and may be consumed in tap water and various food types. It is classed as a pseudo-commensal of the gastrointestinal tract.

XI Structured Scheme XI: Nutritional information on the novel food ingredient

Heat-killed *M. aurum* Aogashima is not intended to replace other foods in the diet. In view of the very small amount to be included in the food it will have no nutritional value. The purpose of this novel food ingredient is described in section 2.I.

XII Structured Scheme XII: Microbiological information on the novel food ingredient

Heat killed *M. aurum* Aogashima suspension contains a heat-killed *M. aurum* isolate, a non-GMM (non-Genetically-Modified Micro-organism), originating from the soil. The *M. aurum* Aogashima isolate has been fully described in previous sections and shown to be a non-pathogenic, non-toxigenic isolate of established biochemical and genetic stability.

The inactivation of *M. aurum* Aogashima by the heat killing process [redacted] has been demonstrated by the absence of growth of any bacteria in the sterility test performed on the bulk material.

XIII Structured Scheme XIII: Toxicological information on the novel food ingredient

As the *M. aurum* Aogashima organisms are non-viable in the final product, no influence on the normal gut microflora is expected and it can be anticipated that the heat-killed *M. aurum* isolate will be degraded in the gastrointestinal tract and/or eliminated in the faeces.

The toxicity of the heat-killed *M. aurum* Aogashima has been investigated in a series of studies performed according to OECD test guidelines and GLP (Good Laboratory Practice) compliant (See Confidential Annex 6, Annex 7, Annex 8, and Annex 9).

The mutagenicity of heat-killed *M. aurum* Aogashima suspension has been investigated by *in vitro* reverse mutation and *in vitro* micronucleus tests. The reverse mutation (Ames) test, performed according to OECD Guideline 471 and in compliance with GLP, was carried out on four *Salmonella Typhimurium* strains (TA1535, TA1537, TA98, TA100) and *Escherichia coli* strain (WP2 *uvrA*), in the presence and absence of a metabolic activation system (S-9 mix). No increases in revertant colony numbers were observed at concentrations of the test item ranging from 50 to 5,000 µg/plate, in the presence or absence of S-9 mix, under either plate incorporation or pre incubation conditions. The micronucleus test was performed on cultured human peripheral blood lymphocytes, according to OECD Guideline 487 and in compliance with GLP, in the presence and absence of a metabolic activation system (S-9 mix). Based on the results of a range-finder study, the Micronucleus Experiment was performed at concentrations of Heat-killed *M. aurum* Aogashima suspension between 25 and 1000 µg/ml (limited by solubility in culture medium). Due to precipitation of the test item in the medium observed from 100 µg/ml, micronuclei analysis was carried out at 3 concentrations: 25, 50 and 100 µg/ml (limited by precipitation not by toxicity). No significant or biologically relevant increases in micronuclei were observed in those concentrations. These results indicate that Heat killed *M. aurum* Aogashima suspension has no mutagenicity, clastogenicity or aneugenic effects.

The potential systemic toxicity of heat-killed *M. aurum* Aogashima suspension was studied in a sub-chronic toxicity study. A preliminary study was first performed with heat-killed *M. aurum* Aogashima suspension administered orally, by gavage, to rats once daily for 14 days in order to select dose levels for a subsequent 90-day toxicity study in the rat. Nine male and nine female rats of the Crl:WI(Han) strain were allocated to the study. Groups of three males and three females were dosed with 0 (vehicle), 200 or 2000 µg/kg/day heat-killed *M. aurum* Aogashima suspension, once daily, by oral gavage, at a dose volume of 1 mL/kg body weight for 14 days, until the day before necropsy. All animals were observed daily from the start of treatment and body weights and food intake were recorded at twice weekly intervals until the day of necropsy. Animals were killed by exposure to carbon dioxide gas in a rising concentration and subjected to a gross necropsy. There were no deaths and no treatment-related clinical signs and no adverse effect on body weight or food intake. At necropsy, there were no macroscopic abnormalities related to administration of the test item. Therefore, since the test item administered once daily, by oral gavage, for 14 days to the Crl:WI(Han) rat at dose levels of 200 or 2000 µg/kg/day was well tolerated, 2000 µg/kg/day was considered to be a suitable high dose level for a subsequent 90 day toxicity study in the rat.

A 90 day toxicity study was performed with heat-killed *M. aurum* Aogashima suspension administered orally, by gavage, to the rat once daily for 90 days, according to OECD Guideline 408 and in compliance with GLP. A total of 40 male and 40 female rats of the Crl:WI(Han) strain were allocated to the study. Groups of 10 males and 10 females were dosed with 0 (vehicle), 20, 200 or 2000 µg/kg/day heat-killed *M. aurum* Aogashima suspension, once daily, by oral gavage, at a dose volume of 1 mL/kg body weight for 90 days, until the day before necropsy. All animals were observed daily for clinical signs of toxicity or changes in behaviour and appearance from the start of treatment, twice daily for mortality and morbidity, and body weights and food intake were recorded at weekly intervals until the day of necropsy. Animals were killed by exposure to carbon dioxide gas in a rising concentration and subjected to a gross necropsy. There were no deaths and no treatment-related clinical signs and no adverse effect on body weight or food intake. There were

no adverse haematological effects of treatment. There were no blood chemistry changes that were related to treatment with heat-killed *M. aurum* Aogashima suspension. At necropsy, there were no macroscopic or microscopic abnormalities related to administration of the test item.

These results suggest that the maximum NOAEL (No Observed Adverse Effect Level) oral dose of Heat killed *M. aurum* Aogashima suspension orally is greater than 2000 µg/kg bodyweight/day.

Evaluation of allergenicity potential of heat-killed *M. aurum* Aogashima suspension

M. aurum is not listed as a food allergen by the European Community. Classes of common food allergens have been defined such as cow's milk, fruits, legumes (especially peanuts and soybeans), eggs, crustaceans, tree nuts, fish, vegetables (celery and other foods of the Umbelliferae family), wheat and other cereals, mustard, sulphur dioxide and sulphites (Dir. 2003/89). Microbial proteins, in keeping with food proteins and other potential allergens derived from foods and food ingredients, may provoke allergic reactions in sensitive individuals.

However, there is no evidence that *M. aurum* or other rapid growing mycobacteria cause hypersensitivity reactions; on the contrary, there is evidence that, as mycobacteria are non-sporing, they are less allergenic and that exposure to them down regulates hypersensitivity reactions (Annex 10). Environmental mycobacteria do not induce Type 1, allergic, hypersensitivity reactions. There is no evidence that killed environmental mycobacteria induce Type 3, immune complex-mediated hypersensitivity reactions. Cell-mediated immunity is distinct from delayed hypersensitivity and the latter is not induced by environmental exposure to rapidly growing mycobacteria. Environmental mycobacteria are not long-term commensals of the intestinal tract, but transient 'pseudocommensals' so that sustained environmental exposure to them is required. Disease due to live *M. aurum* is exceedingly rare or non-existent and reported but non-substantiated cases being restricted to those profoundly immunosuppressed.

Moreover, no allergic reactions to *M. aurum* have been reported in the literature or in the Allergome database.

Taking into account the data from the toxicological tests and literature data, the allergenicity and pathogenicity potential of products containing heat-killed *M. aurum* Aogashima suspension is considered to be exceedingly low.

5. Evaluations and conclusion by applicant

This application contains the necessary data requirements in accordance with Regulation (EC) Nº 258/97 and Commission Recommendation 97/618/EC on novel foods, to support the safety of Heat killed *M. aurum* Aogashima for human consumption, based on:

- **Classification of *M. aurum* as a non-pathogenic organism,**
- **The inactivation process applied for the manufacturing of the product confirmed by the "sterility" test,**
- **The results of a series of toxicology studies,**
- **The anticipated intake of the novel food ingredient.**

Based on these data, we can reasonably conclude that heat-killed *M. aurum* Aogashima (Au+) will not endanger human health when used in accordance with this dossier.