INTRODUCTION

The interaction between the human host and nonpathogenic ubiquitous environmental microorganisms, present throughout human evolution, recently emerged as an area of scientific interest and has evolved into “the Old Friends hypothesis” (Flandroy et al., 2018; Lowry et al., 2016; Rook et al., 2004, 2013). This awareness has reached consumers alike, who are increasingly willing to adjust their dietary habits to achieve improved well-being (Marco et al., 2020).

Indeed, intake of specific food and food supplements is one way to modulate exposure to what have been broadly considered “good” bacteria. The long list of “healthy” foods containing such bacteria includes fermented dairy products like yogurt and kefir, as well as fermented foods such as miso, kimchi, and sauerkraut and beverages such as kombucha tea. Members of the genera Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Saccharomyces, and Streptococcus are most commonly found in these foods, and together they are known as “probiotics” (Di Cerbo et al., 2016; Hori...
et al., 2020). According to the revised definition of the Food and Agriculture Organization (FAO)/World Health Organization (WHO), as well as in the public perception, probiotics are nonpathogenic live microorganisms that, when administered in adequate amounts, confer a health benefit to the host, such as improvement in metabolism and intestinal flora and modulation of immune functions (Aponte et al., 2020; FAO & WHO, 2002; Hill et al., 2014; Wilkins & Sequoia, 2017). The probiotic market is growing rapidly, buoyed by both foods and supplements intended to enhance wellness in healthy individuals, and by preparations for the dietary management of diseases (Grumet et al., 2020).

In addition to probiotics, other environmental nonpathogenic organisms are, or were at some point, commonly present in the human diet, such as environmental saprophytic nontuberculous (NTB) mycobacteria species. Based on recent comparative genomic studies, the genus Mycobacterium (Lehmann, 1896) was divided into an emended genus Mycobacterium, to which pathogenic species belong, and four novel genera: Mycolicibacter (type species: Mycolicibacter terrae), Mycolicibacillus (type species: Mycolicibacillus trivialis), Mycobacteroides (type species: Mycobacteroides obscessus), and Mycolicibacterium (type species: Mycolicibacterium fortuitum) (Gupta et al., 2018). The genus Mycolicibacterium encompasses rapidly growing NTB species, many of which have routinely been isolated from municipal water supplies (Falkinham et al., 2001; Falkinham, 2009; Fernandez-Rendon et al., 2012; Imwidthaya et al., 1989; Kubalek & Mysak, 1996; Le Dantec et al., 2002a, 2002b; Martin et al., 2000; Moghim et al., 2012; Nasr-Esfahani et al., 2012; Pontiroli et al., 2013; Scarlata et al., 1985; Vaerewijck et al., 2005). NTB mycobacteria, which include the mycolicibacteria, are not a permanent constituent of the microbiome, but because they have been regularly encountered in the diet and the environment, there is evidence for their evolutionary adaptedness (Rook, 2010). Interestingly, akin to the bacteria which make up the gut and skin microbiota, researchers have now identified communities of several of these operational mycobacterial taxonomic units in the oral cavity of healthy individuals (Macovei et al., 2015). This is presumably a reflection of the significant exposure to environmental NTB mycobacteria by this route. The extent to which NTB mycobacteria such as mycolicibacteria hold promise, like probiotics, for influencing human well-being is the subject of ongoing research. Nevertheless, “the Old Friends hypothesis” makes a case for their benefit to human health as revealed by the drastic reduction of exposure to saprophytic environmental NTB mycobacteria in modern living conditions (Flandroy et al., 2018; Lowry et al., 2016; Rook et al., 2004, 2013).

Until recently, the assumption has been that probiotics should be viable to exert positive effects. Instead, there is now increasing evidence to show that nonviable probiotics maintain their health-promoting benefits and a new term “postbiotic” has been coined to indicate preparations of inanimate microorganisms and/or their components that confer a health benefit to the host (Aguilar-Toalá et al., 2018; Barros et al., 2020; Seminen et al., 2021; Taverniti & Guglielmetti, 2011). From a commercial standpoint, the use of nonviable bacteria has several advantages, including easing the challenges associated with product storage to maintain viability, reduction of safety concerns arising from horizontal virulence gene transfer from pathogenic bacteria, and the ability to deliver exact numbers of microorganisms per dose. In light of these issues, nonviable bacteria are now under consideration as novel food ingredients. In this report, we present evidence for the safety of heat-killed Mycolicibacterium aurum Aogashima as a novel food ingredient. This is an environmental saprophytic organism which may not have the documented history of safe use that food-associated probiotics have, but nonetheless, is likely to have been evolutionarily present in the diet, through exposure to untreated and even treated water supplies. The safety of this novel food was determined using the decision tree approach developed by Pariza and colleagues which relies on assessment of lack of allergenicity risk, confirmation that resistance to various antimicrobials is intrinsic and nontransmissible and that no harmful effects are detected in standard toxicology testing (Pariza et al., 2015). Our data support the conclusion that heat-killed M. aurum Aogashima is safe as a novel food ingredient.

**Steps for the safety evaluation of Mycolicibacterium aurum Aogashima as a novel food**

1. **Strain characterization and Genome sequence of M. aurum Aogashima DSM 33539**
2. **Safety evaluation study for undesirable physiological effects**
   - Sub-chronic oral toxicity study in rats
3. **Screening for genetic elements associated with pathogenicity and toxicity**
   - Allergenicity assessment
   - Antimicrobial resistance gene assessment
   - Pathogenic gene cluster and virulence factor assessment

**FIGURE 1** Flow chart describing the steps of the safety evaluation process based on Pariza et al., (2015)
2 | MATERIALS AND METHODS

2.1 | Manufacture

M. aurum Aogashima has been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) under the accession number DSM 33539. M. aurum Aogashima is manufactured following Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP) principles. The organism is grown in a bioreactor of either five or twenty-five liters. Once an appropriate biomass is reached, the bacteria are recovered by centrifugation and resuspension in water, before heat inactivation at 121ºC for ≥20min. The resulting M. aurum Aogashima biomass is then further diluted with water and stored prior to use.

2.2 | Safety evaluation process

The safety of M. aurum Aogashima was assessed based on the decision tree approach developed by Pariza and colleagues (2015). A flow chart describing the steps is depicted in Figure 1.

2.3 | Subchronic oral toxicity study

The safety of heat-killed M. aurum Aogashima was investigated in a 90-day toxicity study in rats. Doses tested were selected on the basis of a 14-day oral dose range finding study. Studies were GLP compliant, performed at Sequani Ltd (UK) according to United Kingdom GLP Regulations 1999, SI 1999 No. 3,106, as amended by SI 2004 No. 994, in accordance with the Organization for Economic Cooperation and Development (OECD) Guidelines (OECD, 1998). Briefly, 6- to 8-week-old male (n = 40) and female (n = 40) Crl:WI(Han) rats (Charles River, UK) were divided in groups of 10 males and 10 females and were dosed at 0 (vehicle control), 20, 200, or 2000 μg/ kg/day of heat-killed M. aurum Aogashima, once daily by oral gavage, at a dose volume of 1 mL/kg body weight for at least 90 days, until the day before necropsy. Animals were housed in groups of 5, by sex and provided food and water ad libitum. Animals were examined twice daily for mortality and morbidity. Any clinical signs of toxicity or changes in behavior or appearance were checked for daily. Body weights and food intake were recorded weekly until necropsy. Blood samples were taken for clinical pathology during week 13 according to OECD guidelines (1998). Hematological parameters investigated included changes in immune cell population counts. Blood chemistry parameters measured included markers associated with liver or kidney cellular toxicity, such as alanine and aspartate aminotransferase (ALT and AST, respectively), alkaline phosphatase (AP), urea, and creatinine. Animals were also subjected to a functional observational battery consisting of standard arena observations at predose and once weekly, together with an assessment at week 13 which included grip strength, motor activity, and sensorimotor responses to visual, acoustic, and proprioceptive stimuli according to OECD test guidelines (OECD, 1998). At the end of the treatment period, all animals were subjected to a gross necropsy, internal organs were weighed, and organ tissues from the control and high dose animals were examined microscopically.

2.4 | Genome sequencing and analysis

DNA was extracted from a culture of M. aurum Aogashima as described in Amaro et al., (2008). Genome sequencing was performed using an Illumina MiSeq instrument, as previously described (Sangal et al., 2015). The genomes were assembled into contigs using SPAdes 3.9.0 with a kmer length of 127 and subsequently annotated using the Rapid Annotation of microbial genomes Subsystems Technology (RAST) server (Aziz et al., 2008; Bankevich et al., 2012).

2.5 | Allergenicity assessment

Allergenicity potential of M. aurum Aogashima was assessed by AllerCatPro (https://allercatpro.bii.a-star.edu.sg/), the most up-to-date database, comprising 4,180 unique allergenic protein sequences (Maurer-Stroh et al., 2019). Briefly, linear sequences in the genome of M. aurum Aogashima were first compared to the allergen database to identify sequence windows of 80 residues with at least 35% of identity with proteins known to be allergenic as defined by FAO & WHO (2001). The amino acid sequences of M. aurum Aogashima genome were obtained after translation of nucleotide sequences using Prodigal software v2.6.3. The sequences with an identity above this threshold were then 3D-modeled, and a B-cell epitope-like 3D surface was calculated and compared. Proteins with epitopes presenting an identity level of above 35% were considered allergens as outlined in Maurer-Stroh et al., (2019).

2.6 | Antimicrobial resistance gene assessment

The presence of genes coding for antibiotic resistance (AMR) was assessed in the genome of M. aurum Aogashima. The whole genomic sequence was compared against ResFinder databases and the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020; Zankari et al., 2012). Briefly, in silico genome analysis for the AMR genes was carried out by ResFinder 3.2 webserver which encompasses 15-drug classes in its database: aminoglycoside, beta-lactam, colistin, a fluoroquinolone, fosfomycin, fusidic acid, glycopeptide, macrolide-lincosamide-streptogramin B, nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, tetracycline, and trimethoprim (Zankari et al., 2012). The percent identity and perfect alignment were set at 70% and 60%, respectively. The minimum length or the number of nucleotides that must overlap a resistant gene to count as a hit was set at the default of 60%.
The genome sequence of strain *M. aurum* Aogashima was interrogated for the presence of AMR genes based on CARD and using Resistance Gene Identifier (RGI) software for resistome analysis and prediction (Alcock et al., 2020). Each predicted AMR gene was manually mapped and annotated using the SEED and the RAST server (Aziz et al., 2012). Protein domains of AMR genes were confirmed after comparison with those available in the Conserved Domains Database (CDD) of NCBI (Marchler-Bauer et al., 2015). Any hits were reported and analyzed.

### 2.7 Pathogenic gene clusters and virulence factors assessment

The draft genome sequence of *M. aurum* Aogashima was screened for pathogenic island and virulence factors using the Virulence Factor database (VFDB) (Liu et al., 2019). Experimentally validated virulence factors of major medically important bacterial pathogens belonging to 24 genera were considered. In addition, predicted coding sequences were identified using the GLIMMER3 system (system for finding genes in microbial DNA) prior to using the VFanalyzer tool (Virulence Factor analyzer tool). Lastly, blastp and Conserved Domain tools of NCBI were used to identify the virulence factors associated amino acid sequences of *M. aurum* and determine their functional similarity with those of *Mycobacterium tuberculosis* H37Rv. The established threshold of 60% for functional protein similarity was adopted (Addou et al., 2009).

### 2.8 Statistical Analysis

In vivo data were analyzed using Graph Pad Prism to give group mean values and standard error. Where appropriate and within each sex, one-way ANOVA followed by Sidak’s multiple comparisons test was used to determine statistical differences upon comparison of groups receiving different doses of heat-killed *M. aurum* Aogashima versus the control group.

### 3 RESULTS

#### 3.1 Subchronic oral toxicity study

A 14-day oral dose range finding study in Crl:WI(Han) rats was performed to determine doses to be tested further in a 90-day oral toxicity study. This dose range study showed that administration of both 200 and 2000 μg/kg/day was well tolerated and 2000 μg/kg/day was selected as the highest dose level in a subchronic oral toxicity study. Eighty Crl:WI(Han) rats (40 males and 40 females) were allocated into different dose groups receiving heat-killed *M. aurum* Aogashima orally at 0 (vehicle control), 20, 200, and 2000 μg/kg/day (10 males and 10 females per group) for 90 days. Daily visual examinations from the start of treatment showed no deaths, no treatment-related clinical signs of morbidity, toxicity, nor changes in behavior. Weekly measurements of body weight and food intake revealed no significant differences between groups. All groups gained a similar amount of weight (Figure 2) and ate a similar amount of food (data not shown) when compared to control groups. Animals showed no evidence for treatment-related neurotoxicity based on functional observation battery assessments. Indeed, there were no effects on functional arena observations or on grip strength or motor activity and sensorimotor responses to visual, acoustic, and proprioceptive stimuli (data not shown). At the end of the treatment period, all animals were subjected to a gross necropsy where organs were weighed and examined macroscopically. We detected no effect on organ weights in the male groups regardless of treatment. In the female group, we observed only a significant decrease in the liver weight and only in the group receiving 20 μg/kg/day (Table 1). Nevertheless, there were no differences detected in the percentages of organ weight in relation to body weight in the 20 μg/kg/day dose group compared to control group (3.32 ± 0.067 versus 3.39 ± 0.037%, respectively). Moreover,
TABLE 1  Effects of feeding different doses of *M. aurum* Aogashima on selected tissue organs. Data are shown as mean (*n* = 10) ± SEM. No significant differences were observed compared to relevant vehicle control group in the males; * indicates significant differences in the female group

| Group          | Heart gr | Kidneys gr | Liver gr | Spleen gr | Thyroid mg | Adrenal mg |
|----------------|----------|------------|----------|-----------|------------|------------|
|                | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ |
| 0 μg/Kg/day    | .85 ± .04 | 1.15 ± .04 | 1.64 ± .05 | 2.41 ± .05 | 8.47 ± .28 | 13.58 ± .44 | .57 ± .03 | .65 ± .02 | 18.80 ± 1.11 | 22.2 ± .44 | 73 ± 4.33 | 60 ± 3 |
| Vehicle Control |          |            |          |          |            |            |          |          |            |            |           |       |
| 20 μg/Kg/day   | .77 ± .03 | 1.26 ± .04 | 1.57 ± .05 | 2.51 ± .09 | 7.54 ± .26 | 13.79 ± .43 | .56 ± .03 | .68 ± .03 | 16.70 ± 1.32 | 23.1 ± .82 | 80 ± 3.67 | 57 ± 1.67 |
| 200 μg/Kg/day  | .82 ± .02 | 1.17 ± .04 | 1.61 ± .05 | 2.46 ± .09 | 8.02 ± .22 | 13.49 ± .45 | .56 ± .02 | .65 ± .02 | 17.90 ± .88 | 21.70 ± 1.11 | 75 ± 2.67 | 57 ± 3.33 |
| 2000 μg/Kg/day | .86 ± .02 | 1.22 ± .04 | 1.66 ± .05 | 2.56 ± .07 | 8.42 ± .26 | 13.82 ± .54 | .54 ± .04 | .69 ± .02 | 17.10 ± 1.42 | 22.2 ± 1.39 | 73 ± 2.33 | 59 ± 3.33 |

TABLE 2  Effects of feeding different doses of *M. aurum* Aogashima on selected blood chemistry parameters. Data are shown as mean(*n* = 10) ± SEM. No significant differences were observed compared to relevant vehicle control group

| Group          | Alanine Aminotrasferase U/l | Aspartate Aminotransferase U/l | Alkaline Phosphatase U/l | Urea mg/dl | Creatine mg/dl |
|----------------|----------------------------|-------------------------------|--------------------------|------------|----------------|
|                | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ | ♀ | ♂ |
| 0 μg/Kg/day    | 29.40 ± 2.70 | 36.90 ± 2.08 | 71.70 ± 11.02 | 61.50 ± 2.69 | 49.90 ± 4.09 | 114.30 ± 8.31 | 34.77 ± 1.32 | 37.30 ± 1.61 | .26 ± .01 | .26 ± .01 |
| Vehicle Control |          |            |            |          |            |            |          |          |           |           |
| 20 μg/Kg/day   | 28.20 ± 1.30 | 33.50 ± 2.28 | 64.30 ± 3.45 | 60.10 ± 2.50 | 57.80 ± 4.52 | 105.60 ± 6.06 | 34.38 ± .96 | 37.55 ± 1.16 | .25 ± .004 | .25 ± .01 |
| 200 μg/Kg/day  | 28.10 ± .81 | 31.80 ± .62 | 59.00 ± 2.83 | 53.10 ± 1.24 | 51.60 ± 4.53 | 105.30 ± 7.89 | 34.5 ± 1.27 | 35.55 ± 1.11 | .26 ± .01 | .25 ± .01 |
| 2000 μg/Kg/day | 29.10 ± 1.37 | 31.80 ± 1.69 | 62.30 ± 5.77 | 60.80 ± 3.59 | 53.50 ± 4.26 | 104.30 ± 4.69 | 37.53 ± 2.03 | 34.63 ± .73 | .26 ± .03 | .22 ± .01 |
found no evidence for changes in the percentages of these populations or in any other immune cell populations (Table 4). Moreover, only in the group receiving 200μg/Kg/day (Table 3). However, we observed a significant decrease in white blood cell, neutrophils, and lymphocytes absolute counts received (Table 4). In females, we observed a significant decrease in any cell populations, including monocytes, regardless of dose.

Statistically significant changes in total white blood cell and immune cell population specific counts in the female groups, aside from a significant decrease in monocytes in the group receiving 200 μg/Kg/day (191.7 ± 6 and 190.3 ± 5.2, respectively). As glucose levels were already higher than normal (106–184 in males and 89–163 mg/dl in females) in the control groups and rats were not comparable to levels in rats receiving 20 μg/Kg/day (179.8 ± 6.7) but significantly lower than those in rat receiving 200 and 2000 μg/Kg/day (191.7 ± 6 and 196.3 ± 7, respectively) but significantly lower than levels in rats receiving 200 μg/Kg/day (191.7 ± 6 and 190.3 ± 5.2, respectively). As glucose levels were already higher than normal (106–184 in males and 89–163 mg/dl in females) in the control groups and rats were not comparable to levels in rats receiving 20 μg/Kg/day (179.8 ± 6.7) but significantly lower than levels in rats receiving 200 and 2000 μg/Kg/day (191.7 ± 6 and 196.3 ± 7, respectively) but significantly lower than levels in rats receiving 200 μg/Kg/day (191.7 ± 6 and 190.3 ± 5.2, respectively).

Similarly, levels in control female rats (163.7 ± 5.2, respectively) but significantly lower than those in rats receiving 200 and 2000 μg/Kg/day (191.7 ± 6 and 196.3 ± 7, respectively) but significantly lower than levels in rats receiving 200 μg/Kg/day (191.7 ± 6 and 190.3 ± 5.2, respectively) but significantly lower than levels in rats receiving 200 μg/Kg/day (191.7 ± 6 and 190.3 ± 5.2, respectively).

In the male group, the only statistically significant changes were calculated, no significant differences were detected in the highest dose tested of 2000 μg/Kg/day. Hence, the report data concluded no observed adverse effect level (NOAEL) and with increases in liver enzymes indicative of hepatocellular or renal toxicity (Table 2). Among all blood chemistry parameters measured, we observed an out of "normal" range significant difference (*p < 0.05) in glucose levels compared to control animals. Levels (mg/dl) in control male rats (191.8 ± 6.5) were comparable to levels in rats receiving 20 and 2000 μg/Kg/day (191.7 ± 6 and 196.3 ± 7, respectively) but significantly lower than levels in rats receiving 200 μg/Kg/day (191.7 ± 6 and 190.3 ± 5.2, respectively).

Prior to necropsy, blood samples were taken for clinical pathology analysis at week 13. Statistical analysis revealed no significant differences in any groups. Also, the carried out microscopic analysis did not report any abnormal macroscopic observations related to the observed difference in the weights of livers was not dose-dependent, did not translate into changes in the weights of organs, and did not increase in liver enzymes indicative of hepatocellular or renal toxicity.
3.2 | Allergenicity

The allergenicity potential of *M. aurum* Aogashima was assessed by an innovative 3D-modeling-based analysis, using the AllerCatPro database. Only fifteen potentially allergenic protein sequences were detected with linear sequence window identity above the thresholds of 35% (Table 5). Most of the detected amino acids sequences in the genome of *M. aurum* Aogashima corresponded to allergenic proteins previously found in fungi (56%), while 26%, 15%, and 3% of the predicted proteins belong to foods, arthropods, and mammals (just one sequence), respectively. None of these proteins showed a 3D epitope identity, and therefore, we concluded that there was no evidence for allergenicity following consumption of heat-killed *M. aurum* Aogashima (Table 5).

3.3 | Antimicrobial resistance gene assessment

We found no hits between the genome of *M. aurum* Aogashima and the AMR genes included in ResFinder databases. Instead, 3 hits above 70% identity: *rbpA* (RbpA bacterial RNA polymerase-binding protein), *mtrA* (resistance-nodulation-cell division antibiotic efflux pump), and *murA* transferase (*Mycobacterium tuberculosis* intrinsic *murA* conferring resistance to fosfomycin) were reported using CARD webserver. These genes confer resistance to rifampicin, penam, and fosfomycin, respectively. However, as reported in Table 6, all hits were below the general reference value for gene homology (97%). Furthermore, these genes have been reported to be widely present in the *Mycobacteriaceae* and, therefore, not surprisingly also in the *M. aurum* type strain DSM 43999T (Table 6).

3.4 | Pathogenic gene clusters and virulence factors assessment

The whole genome sequence of *M. aurum* Aogashima was screened to identify genetic element sequences that encode for virulence factors or protein toxins. We found no evidence for pathogenic islands. Screening of the genome of *M. aurum* Aogashima for all known virulence factors associated genes showed that most of the predicted genes were found in nonpathogenic or commensal bacteria and are involved in host interaction, survival, and maintenance of basic functions (Table 7). As shown in Table 7, several proteins with experimentally verified virulence factors were present in the genome of *M. aurum* Aogashima but their amino acid sequence similarity with that of the pathogenic *M. tuberculosis* is below the 60% cutoff value for functional homology (Table 7).

4 | DISCUSSION

Humans have evolved in a microbial world. The resulting evolutionary adaptedness is based on microbes’ colonization of human skin.
and mucosal surfaces as well as regular microbial contact in the air, surfaces, and in food and beverages (Rook, 2010). Until very recently, diet provided the most exposure through raw, minimally processed or fermented foods and beverages and through untreated water. A link between consumption of live microbes—such as those found in fermented food—and health has been reported in both intervention and associative studies as well as randomized controlled trials (Marco et al., 2017; Sanlier et al., 2019). As health evidence is mounting, there have been calls to include recommendations for the consumption of microbes in dietary guidelines, akin to the ones related to dietary fibers (Marco et al., 2020). For these reasons, microbes have gained increasing interest as potential novel food ingredients. Numerous microbes are currently being investigated for their safety as novel food ingredients and for their potential benefit to human health. These include novel probiotics such as Clostridium butyricum CBM588, as well as postbiotics, defined as inanimate bacterial preparations which confer health benefit to the host. The latter would include Yarrowia lipolytica, Mycobacterium setense strain Manresensis, and pasteurized Akkermansia municiphila among others (Aguilar-Toalá et al., 2018; Akter et al., 2020; Barros et al., 2020; Cani & de Vos, 2017; EFSA Panel on Nutrition et al., 2019a; 2019b; Kanai & Mikani, 2015; Salminen et al., 2021; Taverniti & Guglielmetti, 2011).

The food use of dead microbes has several advantages compared to live organisms: the difficulties of ensuring cell viability at the levels reported in the product description and for the duration of their shelf-life are avoided, for example. Similarly, using heat-killed organisms limits concerns arising from use of these products in vulnerable groups such as the very young and immunosuppressed individuals and allows for more widespread use (Piqué et al., 2019).

Interestingly, the organism under study in this report, heat-killed M. aurum Aogashima, may fall within the definition of postbiotic, should a health benefit for this preparation be shown in separately presented studies. The purpose of the work described here, however, is solely to present and assess the evidence for the safe use of heat-killed M. aurum Aogashima as a novel food ingredient.

This environmental saprophytic organism is likely to have been long present in the diet as a harmless water contaminant (Falkinham et al., 2001; Le Dantec et al., 2002a, 2002b; Vaerewijck et al., 2005). Safety of heat-killed M. aurum Aogashima as a novel food ingredient was assessed according to the decision tree approach developed by Pariza and colleagues (2015). The interest in expanding the number of microbes being considered as novel food, beyond the current standardized cultures and probiotics supplements, has driven a new approach to assess safety. This new framework is also pertinent to those cultures that are perceived to lack an established history of safe use for their intended application. We provide evidence that

| Known allergen hit name | % identity linear 88aa window | % identity 3D epitope |
|-------------------------|------------------------------|----------------------|
| Alcohol Dehydrogenase   | Candida albicans             | 41.1 – 67.5          |
| ALF_CANAL fructose-bisphosphate aldolase | Candida albicans | 55 |
| Aldehyde dehydrogenase Clath 10 | Cladosporium herbarum | 48.8 |
| Mannitol dehydrogenase Cla h 8 | Cladosporium herbarum | 37.5 – 38.2 |
| NADP-dependent mannitol dehydrogenase | Alternaria alternata | 37.7 – 40.8 |
| Probable beta-glucosidase | Arthroderma benhamiae | 51.2 |
| THIO_COPCM Thiredoxin | Coprinus comatus | 44.9 |
| Asp f IAO | Aspergillus fumigatus | 43 |
| Asp f FDH | Aspergillus fumigatus | 40 |
| Pen C | Penicillum citrinum | 58.8 |
| Seed maturation-like protein precursor | Sesamum | 37.5 – 45 |
| Tri a 34.0101 (glyceraldehyde-3-phosphate dehydrogenase) | Triticum aestivum | 71.2 |
| Aldehyde dehydrogenase-like protein Tyrophagus | Tyrophagus putrescensiae | 56.2 |
| Cul n 8 | Culicoides nubeculosus | 41.1 |
| Cyclophilin, CyP | Mammals | 57.5 |

**TABLE 5** In silico genome analysis for allergenic proteins of *M. aurum* Aogashima using AllerCatPro server
the genome of \textit{M. aurum} Aogashima is free of (1) genetic elements associated with pathogenicity or toxigenicity, (2) transferable antibiotic resistance gene DNA, and (3) genes coding for antibiotics used in human or veterinary medicine. Moreover, our evidence shows that (4) the no observed adverse effect level (NOAEL) was the highest dose tested, 2000 μg/kg BW/day.

Genetic elements associated with pathogenicity or toxigenicity were investigated by extensive in silico analysis and showed no evidence of pathogen-specific virulence factors in \textit{M. aurum} Aogashima. Indeed, the virulence factors associated genes identified were common to both pathogenic and nonpathogenic and commensal bacteria and associated with highly conserved functions such as amino acid and purine metabolism and the catabolism of cholesterol and were not located on pathogenic island (Niu et al., 2013). Hence, these highly conserved coding sequences are not considered appropriate markers of pathogenicity of \textit{M. aurum} Aogashima. In this context, the presence of secreted protein associated genes (e.g., \textit{fbpA}) is expected because they play a fundamental role in cell envelope maintenance (Belisle et al., 1997). The same can be said with respect to the genes \textit{ptpA} and \textit{ptpB} which are widely distributed among pathogenic and nonpathogenic mycobacterial species and also found in the genomes of other prokaryotes, including \textit{Lactobacillus} spp. (Altermann et al., 2005; Boekhorst et al., 2006). It should also be noted that following comprehensive phylogenomics and comparative genomic analysis on 150 genomes of \textit{Mycobacterium} spp, Gupta and his colleagues have reclassified the \textit{Mycobacterium} genus into five distinct monophyletic groups (Gupta et al., 2018). As a result, what was once known as \textit{Mycobacterium aurum} has been reclassified into the novel genus \textit{Mycolicibacterium} ("Fortuitum-Vaccae" clade) which is comprised of rapidly growing environmental species that are divergent from the clinical pathogenic \textit{Mycobacterium} species. Hence, the absence of true virulence genes and pathogenic island in the genome sequence of \textit{M. aurum} Aogashima are in line with its assignment to the species \textit{M. aurum} known for its nonpathogenic trait (Risk group 1).

Antimicrobial resistance gene assessment was made by screening the genome using both ResFinder and CARD webservers to ensure coverage of all AMR determinants (i.e., acquired resistance genes, resistant mutations of housekeeping genes, efflux overexpression, etc.), drug targets, antibiotic molecules and drug classes, and the molecular mechanisms of resistance (Alcock et al., 2020; Zankari et al., 2012). We found no evidence for any resistance genes associated with the most common antimicrobial compounds of concern in food (namely, Ampicillin, Chloramphenicol, Kanamycin, Streptomycin; Erythromycin, Gentamycin, Tetracyclin, Vanomycin, and Lincomycin). We did, however, detect similarities with \textit{rpbA}, \textit{mtrA}, and \textit{murA}. While these genes are known to confer resistance to rifampicin, penam, and fosfomycin, respectively, their identity values were close to, but still below, the cutoff of 97% homology. Moreover, these genes are commonly present in mycobacteria as they are likely involved in essential cell functions (Maitra et al., 2019; Newell et al., 2006). Finally, there is no evidence for transferability. Hence, the absence of significant resistance genes in \textit{M. aurum}
### Table 7: Virulence factors associated genes detected in the genome sequences of *M. aurum* Aogashima and *M. tuberculosis* H37Rv

| VFclass                                      | Virulence factors            | Related genes | *M. aurum* Aogashima | *M. tuberculosis* H37Rv | Similarity (%) | comments   | e-values |
|----------------------------------------------|------------------------------|---------------|----------------------|-------------------------|----------------|------------|----------|
| Amino acid and purine metabolism             | Glutamine synthesis         | glnA1         | orf04741             | Rv2220                  | 84.3           | <95%       | 0        |
|                                              | Leucine synthesis           | leuD          | orf04409             | Rv2987c                 | 84.2           | <95%       | 6E−127   |
|                                              | Lysine synthesis            | lysA          | orf04608             | Rv1293                  | 79.3           | <95%       | 0        |
|                                              | Nitrate/nitrite transporter | narK2         | orf00718             | Rv1737c                 | 25.6           | <60%       | 1E−14    |
| Catabolism of cholesterol                    | Cyp125                       | cyp125        | orf01099; orf01566;  | Rv3545c                 | 54.4           | <95%       | 4E−172   |
|                                              |                              |               | orf0461              |                         |                |            |          |
|                                              | FadE28                       | fadE28        | orf04037             | Rv3544c                 | 68.5           | <95%       | 1E−164   |
|                                              | FadE29                       | fadE29        | orf04036             | Rv3543c                 | 81.6           | <95%       | 0        |
| Cell surface components                      | Carboxylesterase             | coeA          | orf04747; orf04749   | Rv2224c                 | 53.6           | <60%       | 3E−169   |
|                                              | Exported repetitive protein  | erp           | orf04235             | Rv3810                  | 49.6           | <60%       | 6E−58    |
|                                              |                              | fad23         | orf01422             | Rv1185c                 | 60.2           | <95%       | 0        |
|                                              |                              | fadE5         | orf01415; orf05079   | Rv0244c                 | 82.7 and 66.1  | <95%       | 0        |
|                                              |                              | gtf1          | orf01592             | Rv1526c                 | 48.8           | <95%       | 1.3      |
|                                              |                              | gtf2          | orf01265; orf01577   | Rv1524                  | 58.2 and 53.8  | <95%       | 9E−162   |
|                                              | mmpL10                       | orf01421      | Rv1183               | 57.2                    | <95%           | 0         |
|                                              | mmpS4                        | orf00497; orf04089 | Rv0451c      | 47.5                    | <95%           | 3E−47     |
|                                              | mps1                         | orf02234      | Rv0101               | 49.3                    | <95%           | 0         |
|                                              | papA3                        | orf01420      | Rv1182               | 54.5                    | <95%           | 0         |
|                                              | rmlA                         | orf01604; orf05016 | Rv0334      | 46.7                    | <95%           | 4E−161    |
|                                              | Heparin-binding hemagglutinin| hhbA          | orf03209             | Rv0475                  | 67.9           | >60%       | 5E−64    |
|                                              | Lipoprotein                  | lprG          | orf05150             | Rv1411c                 | 52.3           | <95        | 4E−78    |
|                                              | Methyltransferase            | mmaA4         | orf00819             | Rv0642c                 | 68.1           | <95        | 1.5      |
|                                              | MymA operon                  | adhD          | orf03671; orf04049;  | Rv3086                  | 34.1           | <60%       | .016     |
|                                              |                              |               | orf05070; orf05579   |                         |                |            |          |
|                                              | fadD13                       | orf01899      | Rv3089               | 36.4                    | <60%           | 2E−95     |
|                                              |                                |               | orf05192             | Rv3083                  | 50.0           | <60%       | why not 95% |
|                                              |                                |               |                      |                         |                |            |          |
|                                              |                                | ddrA          | orf00094; orf01270;  | Rv2936                  | 46.4, 51.5, 67.8| <60%       | 1E−79    |
|                                              |                                |               | orf02392             |                         |                |            |          |
|                                              |                                | ddrB          | orf02393             | Rv2937                  | 43.1           | <60%       | 1.00E−76 |
|                                              |                                | ddrC          | orf02394             | Rv2938                  | 46.7           | <60%       | 2E−90    |
|                                              |                                | fadD26        | orf02385; orf02387   | Rv2930                  | 60.2, 55.1     | <60%       | 0        |
|                                              |                                | fadD28        | orf01727; orf01571   | Rv2941                  | 55.3, 63.4     | <60%       | 0        |
|                                              |                                | ppsA          | orf02386             | Rv2931                  | 56.0           | <60%       | 0        |
|                                              |                                | ppsB          | orf02388; orf02389   | Rv2932                  | 54.0, 55.1     | <60%       | 0        |
|                                              |                                | ppsD          | orf02390             | Rv2934                  | 58.8           | <60%       | 0        |
|                                              |                                | ppsE          | orf02391             | Rv2935                  | 34.7           | <60%       | 1E−125   |
|                                              |                                | pcaA          | orf00241; orf00242;  | Rv0470c                 | 60.0, 56.3, 68.9, 96.2| <95         | 2E−123   |
|                                              |                                |               | orf00820; orf01613   |                         |                |            |          |

(Continues)
| VFclass                  | Virulence factors                     | Related genes                  | M. aurum Aogashima | M. tuberculosis H37Rv | Similarity (%) | comments e-values |
|-------------------------|---------------------------------------|-------------------------------|--------------------|----------------------|----------------|-------------------|
| Sulfolipid−1 biosynthesis and transport | mmpL8 orfo1266                         | Rv3823c                       | 50.4               | <95                  | 0              |
|                         | papA1 orfo1262                         | Rv3824c                       | 51.0               | <95                  | 2E-170         |
|                         | lipY orfo3429                          | Rv1235                        | 28.3               | <60%                 | 2E-53          |
|                         | sugA orfo3428                          | Rv1236                        | 53.0               | <60%                 | 3E-91          |
|                         | sugB orfo3427                          | Rv1237                        | 57.1               | <60%                 | 3E-109         |
|                         | sugC orfo3426                          | Rv1238                        | 53.9               | <60%                 | 8E-142         |
| Copper uptake           | ctpV orfo3576                          | Rv0969                        | 54.3               | <60%                 | 0              |
|                         | fadD33 orfo2653                        | Rv1345                        | 61.7               | <95%                 | 0              |
|                         | MbtA orfo0510                          | Rv2384                        | 64.3               | <95%                 | 0              |
|                         | MbtB orfo0509                          | Rv2383c                       | 60.9               | <95%                 | 1E-23          |
|                         | MbtC orfo0507                          | Rv2382c                       | 75.0               | <95%                 | 0              |
|                         | MbtD orfo0506                          | Rv2381c                       | 51.6               | <95%                 | 0              |
|                         | MbtE orfo0505                          | Rv2380c                       | 67.4               | <95%                 | 0              |
|                         | MbtF orfo0504                          | Rv2379c                       | 55.6               | <95%                 | 0              |
|                         | MbtG orfo0503                          | Rv2378c                       | 76.9               | <95%                 | 0              |
|                         | MbtH orfo0502 orfo1576                 | Rv2377c                       | 73.5               | <95%                 | 6E-41          |
|                         | MbtJ orfo4363                          | Rv2385                        | 60.6               | <95%                 | 4E-131         |
|                         | MbtK orfo5262                          | Rv1347c                       | 62.7               | <95%                 | 4E-88          |
|                         | PanC orfo5603                          | Rv3602c                       | 70.5               | <95%                 | 6E-138         |
|                         | PanD orfo0584                          | Rv3601c                       | 63.1               | <95%                 | 5E-56          |
| Pantothenate synthesis  | NuoG orfo2508                          | Rv3151                        | 73.1               | >60%                 | 0              |
| Antiapoptosis factor    | NuoG orfo2508                          | Rv3151                        | 73.1               | >60%                 | 0              |
| Mammalian cell entry (mce) operons | Mce1 mce1A orfo1133                  | Rv0169                        | 53.3               | -                    | 1E-150         |
|                         | mce 1B -                               | Rv0170                        | -                  | -                    | -              |
|                         | mce 1C -                               | Rv0171                        | -                  | -                    | -              |
|                         | mce 1D orfo4143                        | Rv0172                        | 67.6               | 0                    |                |
|                         | mce 1E -                               | Rv0173                        | -                  | -                    | -              |
|                         | mce 1F orfo4141                        | Rv0174                        | 68.1               | 0                    |                |
|                         | mce 2A orfo4716                        | Rv0589                        | 66.5               | 0                    |                |
|                         | mce 2B orfo4717                        | Rv0590                        | 72.4               | 3E-129               |                |
|                         | mce 2C -                               | Rv0591                        | -                  | -                    | -              |
|                         | mce 2D -                               | Rv0592                        | -                  | -                    | -              |
|                         | mce 2E orfo4142                        | Rv0593                        | 67.8               | 0                    |                |
|                         | mce 2F -                               | Rv0594                        | -                  | -                    | -              |
|                         | mce 3A orfo3117                        | Rv1966                        | 65.3               | >60%                 | 6E-180         |
|                         | mce 3B orfo3118 orfo5701               | Rv1967                        | 68.1               | >60%                 | 1E-163         |
|                         | mce 3C orfo3119                        | Rv1968                        | 61.5               | >60%                 | 4E-164         |
|                         | mce 3D orfo3120                        | Rv1969                        | 63.6               | >60%                 | 2E-04          |
|                         | mce 3E orfo3121 orfo4341               | Rv1970                        | 63.6               | >60%                 | 0              |
|                         | mce 3F orfo3122 orfo4340               | Rv1971                        | 58.9               | >60%                 | 1E-179         |

(Continues)
| VFclass | Virulence factors | Related genes | M. aurum Aogashima | M. tuberculosis H37Rv | Similarity (%) | comments | e-values |
|---------|------------------|---------------|-------------------|---------------------|----------------|----------|----------|
| Mce4    | mce4A            | orf01047; orf01461 | Rv3499c          | 66.7                | >60%         | 0        |
|         | mce4B            | orf01048; orf01460 | Rv3498c          | 69.1                | >60%         | 1E−168   |
|         | mce4C            | orf01049; orf01459 | Rv3497c          | 67.7                | >60%         | 2E−171   |
|         | mce4D            | orf01050; orf01458 | Rv3496c          | 63.8                | >60%         | 0        |
|         | mce4E            | orf01051; orf01457 | Rv3495c          | 64.4                | >60%         | 6E−179   |
|         | mce4F            | orf01052; orf01456 | Rv3494c          | 69.6                | >60%         | 0        |
| Phagosome arresting | Nucleoside diphosphate kinase | ndk | orf05384 | Rv2445c | 80.7 | >60% | 2E−81 |
|         | PE family protein | PE_ PGRS30    | -                 | Rv1651c            |               |          |
|         | Tyrosine phosphatase | ptpA | orf00358 | Rv2234    | 70.0 | >60% | 3E−82 |
| Secreted proteins | 19-kD protein | lpqH | orf04515 | Rv3763 | 60.1 | <95% | 3E−59 |
|         | Alpha-crystallin | hspX | orf00236; orf05393 | Rv2031c | 39.0 | <60% | 3E−18 |
|         | Antigen 85 complex | eis | orf02486 | Rv3804c | 70.6 | >60% | 4E−150 |
|         | fbpB             | -              | Rv1886c          |                    |               |          |
|         | fbpC             | orf01653; orf02135; orf04229; orf04918 | Rv0129c | 76.1 | >60% | 5E−175 |
|         | Enhanced intracellular survival protein | eis | orf05310 | Rv2416c | 56.1 | <60% | 4E−150 |
| ESX-1 (T7SS) | PE35 | orf05503 | Rv3872 | 47.7 | <60% | 3E−26 |
|         | PPE68            | orf05504 | Rv3873 | 41.5 | <60% | 7E−63 |
|         | eccA1            | orf05499 | Rv3868 | 76.7 | >60% | 0     |
|         | eccB1            | orf05500 | Rv3869 | 64.2 | >60% | 0     |
|         | eccCa1           | orf05501 | Rv3870 | 80.0 | >60% | 0     |
|         | eccCb1           | orf05502 | Rv3871 | 73.3 | >60% | 0     |
|         | eccD1            | orf05508 | Rv3877 | 66.6 | >60% | 0     |
|         | eccE1            | orf04675 | Rv3882c | 67.5 | >60% | 0     |
|         | espI             | orf03155; orf05507 | Rv3876 | 34.9 | <60% | 6E−49 |
|         | espJ             | orf05509 | Rv3878 | 32.2 | <60% | 2E−12 |
|         | espK             | orf05512 | Rv3879c | 55.0 | <60% | 5E−88 |
|         | espL             | orf05699 | Rv3880c | 53.8 | <60% | 4E−31 |
|         | espR             | orf05183 | Rv3849 | 80.1 | >60% | 4E−80 |
|         | esxA             | orf05506 | Rv3875 | 54.4 | <60% | 5E−31 |
|         | esxB             | orf05505 | Rv3874 | 43.4 | <60% | 2E−19 |
|         | mycP1            | orf04676 | Rv3883c | 71.8 | >60% | 0     |
| ESX-3 (T7SS) | PE5 | orf01528 | Rv0285 | 69.8 | >60% | 7E−31 |
|         | PPE4             | orf01529 | Rv0286 | 58.4 | <60% | 4E−79 |
|         | eccA3            | orf01525 | Rv0282 | 73.1 | >60% | 0     |
|         | eccB3            | orf01526 | Rv0283 | 63.0 | >60% | 0     |
|         | eccC3            | orf01527 | Rv0284 | 74.7 | >60% | 0     |
|         | eccD3            | orf01533 | Rv0290 | 62.8 | >60% | 9E−176 |
|         | eccE3            | orf01535 | Rv0292 | 50.8 | <60% | 1E−80 |
|         | espG3            | orf01532 | Rv0289 | 55.3 | <60% | 3E−115 |
M. aurum Aogashima was also evaluated for allergenic potential using the traditional FAO/WHO issued guidelines as well as an innovative 3D-modeling-based analysis (AllerCatPro database). It was concluded that M. aurum Aogashima would not trigger any allergenic or hypersensitivity reactions in humans. Based on the low number of the predicted allergenic protein sequences detected in the genome by the traditional methodology, and the absence of 3D epitope similarity, it is highly probable that this organism does not produce any true allergenic proteins. Indeed, only fifteen protein sequences were deemed as potentially allergenic based on linear sequence window identity (80 residues) above the thresholds of 35% (traditional methodology). Of those, only two predicted allergenic proteins in M. aurum Aogashima related to food. None of those showed 3D epitope identity, strongly suggesting that the predicted protein sequence matches might be false positives.

Safety of heat-killed M. aurum Aogashima was further assessed by toxicology testing, including a subchronic (90 day) oral challenge using male and female Crl:WI(Han) adult rats. All doses tested, including the highest doses of 2000 μg/Kg/day, had no treatment-related adverse effects. No relevant abnormalities between groups receiving M. aurum Aogashima and the control group were detected upon statistical analysis in a variety of parameters evaluated. Indeed, statistical differences were limited to differences in specific immune cell counts, but did not apply to differences in percentages of the same cell population. Furthermore, all cellular values remained well within the natural healthy range for adult rats (Giknis & Clifford, 2008). In the case of the reported glucose levels, we consider these may be normal biological variations due to continuous access to food and the effects of circadian rhythms (Kohsaka & Bass, 2007). For these reasons, and because of the absence of a dose relationship (given no differences were detected in the highest dose groups which received doses 10 times of those where differences were observed), we consider these differences part of normal biological variation rather than any effect of consumption of heat-killed M. aurum Aogashima.

Based on the findings of the work and analysis described here, our conclusion is that the use of heat-killed M. aurum Aogashima in food products is safe and that it is suitable for being evaluated as a novel food ingredient.

**ETHICAL APPROVAL**

All animal work performed at Sequani Ltd was conducted conforming to the UK legislation under the Animal (Scientific Procedures) Act 1986 (ASPA) Amendment Regulations (SI 2012/3039). Sequani Ltd is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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**CONFLICT OF INTEREST**

TD is a senior executive and holds stock in Aurum Switzerland AG. IN has no conflict of interest to declare.

**AUTHOR CONTRIBUTION**

Imen Nouioui: Conceptualization (supporting); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Project administration (supporting); Resources (lead); Supervision (supporting).
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