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Oragenics

GMO Deliberate Release Notification

Part 1 A

TECHNICAL DOSSIER

A Phase 2, Multi-center, Randomized, Double-blind, Placebo-controlled Study to Assess the Safety and Efficacy of Topically-applied AG013 for the Attenuation of Oral Mucositis in Subjects With Cancers of the Head and Neck Receiving Concomitant Chemoradiation Therapy

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GLOSSARY OF ABBREVIATIONS

AG013	Mouth Rinse formulation of *Lactococcus lactis* strain sAGX0085, deficient in the gene coding for thymidylate synthase and producing human TFF1
BM9T	Buffered minimal culture medium supplemented with 200 µM thymidine
CFU	Colony forming units
CRT	Chemoradiation therapy
CT	Chemotherapy
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Em	Erythromycin
GCP	Good Clinical Practice
GI	Gastrointestinal
GLP	Good laboratory practices
GM	Genetically modified
GMO	Genetically Modified Organism
GMP	Good manufacturing practices
HNC	Head and neck cancer
hTFF1	Human Trefoil Factor 1
hTTF2	Human Trefoil Factor 2
hTTF3	Human Trefoil Factor 3
IMP	Investigational Medicinal Product
kg	Kilogram
Laff	Lactococcal fertility factor
L. lactis	*Lactococcus lactis*
LLOQ	Lower Limit of Quantification
MG1363	*L. lactis* subspecies *cremoris,* pLP712 cured (encodes genes for lactose and casein utilisation)
ml	Millilitre (10^{-3} litre)
MPHD	Maximum proposed human dose
MR	Mouth rinse
mTFF	Murine Trefoil Factor
NCDO	National collection of dairy organisms
ng	Nanogram (10^{-9} gram)
OM	Oral Mucositis

GLOSSARY OF ABBREVIATIONS

pAGX0076	Plasmid carrying the hTFF1 expression cassette to modify *L. lactis*
PCR	Polymerase chain reaction
PK	Pharmacodynamics
pg	Picogram (10^{-12} gram)
PCR	Polymerase chain reaction
PthyA	Promoter of the thymidylate synthase A gene
Q-PCR	Quantitative polymerase chain reaction
QP	Qualified Person
QPS	Qualified Presumption of Safety
RDT	Repeat-dose toxicity
RT	Radiation therapy
sAGX0085	*L. lactis* subsp. *cremoris* strain MG1363 genetically engineered to express hTFF1, deficient in the gene coding for thymidylate synthase A
SOP	Standard Operating Procedure
SR	Solution for reconstitution
Thy12	*Lactococcus lactis* strain producing humanised Interleukin 10, deficient in the gene coding for thymidylate synthase
thyA	Gene encoding thymidylate synthase A
ThyA- / ThyA+	ThyA-negative / positive strain
TFF	Trefoil Factor
usp45	Gene encoding a protein belonging to the nuclear receptor super family
UV	Ultraviolet light

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STATEMENT OF DATA CONFIDENTIALITY CLAIM

This document is submitted by Oragenics as part of a notification for a deliberate release of a GMO.

1 INTRODUCTION

AG013 is a mouth rinse (MR) formulation of living GM *Lactococcus lactis* (*L. lactis*) bacteria, containing the human trefoil factor (*htff1*) gene being developed as a therapeutic option and gain marketing approval for reduction in signs and the symptoms of radiation therapy (RT) and /or chemotherapy (CT) induced oral mucositis (OM).

Oral mucositis (OM) is a common, devastating toxicity of both drug and RT used for the treatment of cancer. The mucosal ulcerations associated with OM are excessively painful and debilitating and have profound clinical and economic implications. Clinically, the severity of OM ranges from focal areas of mild erythema and/or soreness reminiscent of a food burn, to diffuse erythema and full-thickness mucosal ulceration that are only marginally palliated with opioid-based analgesics (Villa and Sonis, 2015; Elting, 2008). Severe OM is associated with significant morbidities including reduced oral intake and consequent weight loss, increases in use of narcotic pain medications and antibiotics, risk of local and systemic infections and febrile days, frequency of hospitalization and length of hospital stay, and unplanned and emergency room visits (Vera-Llonch *et al.,* 2007; Vera-Llonch *et al.,* 2006, Epstein, 2007). Significantly, the development of OM has a markedly negative impact on patients' quality of life (Mirabile et al., 2016; Trotti, 2003; Sonis, 2010; Elting 2008).

AG013 is composed of genetically modified (GM) *L. lactis* bacterium that stably expresses the *htff1* gene. *L. lactis* are non-pathogenic, non-invasive, non-colonizing Gram-positive bacteria, critical in manufacturing dairy products such as buttermilk and cheese. In spite of the widespread use and massive discharge in the environment, *Lactococci* have not been identified as invasive or disruptive. Prior to the industrial use in the manufacture of dairy products, *L. lactis* may have been a commensal to specific plants. No *de novo* colonization of any other ecological niche has ever been reported.

The laboratory strain, MG1363 is designated as the recipient/parental organism for AG013 and is further restricted in its capacity for normal growth than naturally occurring *L. lactis* due to the removal of five plasmids by protoplast-induced curing. One of the plasmids plays a critical role in the bacterium's ability to process nutrients present in milk that are essential for *L. lactis* growth: lactose and caseins, providing a source for sugars (glycolysis) and amino acids respectively. MG1363 can therefore no longer survive in the natural niche of *L. lactis* and is confined to artificially supplemented culture conditions (Gasson, 1983).

AG013, a GM version of MG1363, has been further restricted in its growth capacity due to the replacement of the *thyA* gene with the target therapeutic gene, *htff1* resulting in strict thymine/thymidine dependency, not only for growth but also for survival (thymine-less death).

The Trefoil Factor (TFF) family, which comprises TFF1, TFF2 and TFF3, is involved in protection of the gastrointestinal (GI) tract against mucosal damage and plays an important role in its subsequent repair. All three TFF peptides have shown a therapeutic effect in experimental models and are rapidly up regulated and secreted in an autocrine fashion in response to GI injury. Oral TFFs bind to salivary mucins and form a mucus layer over the epithelia of the mouth, acting

as a physical barrier against bacteria and noxious environmental agents. Moreover, TFF peptides have wound-healing properties and are important in protecting and healing mucosal tissues. The available non-clinical and clinical data suggest that hTFF1 provides a novel pharmacological tool for the prevention and treatment of human GI diseases. In addition, AG013 was well tolerated in rat, dog and hamsters (Appel *et al*., 2008a, 2008b, Prinsen *et al.,* 2012, Caluwearts *et al*., 2012, Sonis *et al.,* 2012) as well as human's studies. Two Phase 1 studies have been conducted to date, one in the USA and one in Belgium (Coulie 2012 and 2013). They both demonstrated the safety, tolerability, pharmacokinetics and efficacy of AG013 applied topically by repeat dosing. AG013 is expected to be present in the oral cavity in saliva, up to 24 hours after dosing and was not detected in fecal samples.

In summary, AG013 is a GM *L. lactis* bacterium being developed to stably express the hTFF1 protein for the treatment of oral mucositis in patients treated with radiotherapy and/or CT. Phase 1 clinical studies to date have demonstrated that AG013 is non-pathogenic, has restricted dispersal potential and survival and no replicating capacity when administered to humans. The risk to personnel and the environment coming into contact with the Genetically Modified Organism (GMO) is expected to be very low when AG013 is administered for use in proposed clinical studies prior to product marketing.

2 INFORMATION REQUIRED IN NOTIFICATIONS CONCERNING RELEASES OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS*

2.1 GENERAL INFORMATION

2.1.1 Name and address of the notifier (company or institute)

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2.1.2 Name, qualifications and experience of the responsible scientist(s)

Dr. Alan Joslyn
CEO of Oragenics, Inc.

2.1.3 Title of the project

A phase 2, multi-center, randomized, double-blind, placebo-controlled study to assess the safety and efficacy of topically-applied AG013 for the attenuation of oral mucositis in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy (CRT).

* According to annex IIIA of the Royal Decree of 21 February 2005 on deliberate release in the environment and placing on the market of genetically modified organisms or of products containing such organisms (MB/BS-24.02.2005-p. 7129).

2.2 INFORMATION RELATING TO THE GMO

2.2.1 Characteristics of (a) the donor, (b) the recipient or (c) (where appropriate) parental organism(s):

AG013 is the lyophilised powder of bacterial strain sAGX0085 mixed with cryoprotectants[†], formulated for oral administration. Strain sAGX0085 was derived from *L. lactis* subsp. *cremoris* strain MG1363 (recipient) and genetically engineered to express hTFF1. *L. lactis* sAGX0085 is a GM *L. lactis* strain, engineered to secrete hTFF1. The gene for *htff1* has been stably inserted in the bacterial chromosome, replacing the *thyA* gene and promoter encoding thymidylate synthase. The accompanying regulatory sequences are aimed at secreting hTFF1. Deleting the *thyA* gene resulted in strict thymine/thymidine dependency, not only for growth but also for survival of the GMO (thymine-less death). Importantly, *L. lactis* sAGX0085 can no longer propagate outside of artificially-supplemented laboratory cultures, and will die quickly once released due to a combination of restrictions:

- *L. lactis* is a poor competitor and has a limited ecological niche.
- *L. lactis* sAGX0085 is devoid of the metabolic pathways that enable the use of milk carbohydrate and amino acid sources and can therefore no longer grow in its ancestral ecological niche.
- *L. lactis* sAGX0085 is dependent on external supplementation of thymine or thymidine for growth and survival. Indeed, the thymidylate synthase gene was removed. When deprived of thymine and thymidine, an inherent suicidal system, “thymineless death” is induced.

Taken together, these restrictions ensure a fail-safe environmental containment strategy.

Donors of different components of the inserted sequences are specified in Section 2.2.2 of this application.

This whole section will be dedicated to the recipient organisms: *L. lactis* strain MG1363

2.2.1.1 Scientific name

Lactococcus lactis
L. lactis is a species of non-sporulating, non-motile, Gram-positive bacterial species.

2.2.1.2 Taxonomy

Kingdom: Bacteria
Division: Firmicutes
Class: Bacilli

[†] Sodium glutamate, sorbitol, dextrin from maize starch

Order: Lactobacillales
Family: *Streptococcaceae*
Genus: *Lactococcus*
Species: *L. lactis*
Subspecies: *cremoris*

2.2.1.3 Other names (usual name, strain name, etc.)

Formerly named "*Streptococcus lactis*".

The particular recipient *L. lactis* strain was designated *L. lactis* subsp. *cremoris* strain MG1363 (Gasson, 1983).

L. lactis subsp. *cremoris* strain MG1363, hereafter referred to as 'MG1363' or '*L. lactis* MG1363' is a derivative of the natural isolate *L. lactis* National Collection of Dairy Organisms (NCDO) 712. Gasson described the removal of all of the 5 different plasmids that were present in NCDO 712 by protoplast-induced curing (Gasson, 1983). One of these plasmids – the 33 MDa plasmid pLP712 – was found to encode genes for lactose and casein utilisation. This plasmid proved essential for normal growth and acid production in milk; the remaining 4 plasmids appeared to be cryptic.

2.2.1.4 Phenotypic and genetic markers,

L. lactis are cocci, typically 1 µm in diameter, that group in pairs and short chains. When fermenting milk, *L. lactis* bacteria produce large quantities of lactic acid. Cultured in the laboratory, *L. lactis* colonies appear white on nutrient agar.

Due to the deletion of essential functions, MG1363 can only survive in supplemented culture media. The removal of pLP712 made it impossible for MG1363 to access nutrients present in milk that are essential for its growth: lactose and caseins, providing a source for sugars (glycolysis) and amino acids respectively. MG1363 can therefore no longer survive in the natural niche of *L. lactis* and is confined to artificially supplemented culture conditions.

To produce sAGX0085, the *L. lactis* thyA coding sequence of MG1363 was replaced by the hTFF1 expression cassette, and therefore sAGX0085 carries the hTFF1 expression cassette flanked by 5' and 3' thyA target regions. The expression cassette consists of the Pxxx promoter of a MG1363 gene encoding a structural protein, upstream of a fragment encoding the *Lactococcus* usp45 secretion leader (SSusp45) and the *htff1* gene [Pxxx>>SSusp45>>hTFF1].

2.2.1.5 Degree of relatedness between donor and recipient or between parental organisms

Not related.

2.2.1.6 Description of identification and detection techniques

L. lactis can be identified using standard microbial techniques (most accurate: API 20 STREP, bioMérieux, France: https://www.mediray.co.nz/media/15816/om_biomerieux_test-kits_package_insert-20600.pdf In addition, more reliable molecular techniques based on next generation sequencing and/or polymerase chain reaction (PCR), possibly combined with sequencing, are routinely used.

For both the recipient wild type strain MG1363 and sAGX0085 full genome sequences were determined and compared, On a 2528862 bp genome, 110 SNP were detected when comparing a predicted sequence based on the published MG1363 sequence (Wegmann *et al.*, 2007) (NCBI Reference Sequence: NC_009004.1) and genetic engineering performed thereon. From these 110, 90 SNP were specific to the MG1363 sibling used for genetic engineering while 20 were specific to sAGX0085. None of these SNP are expected to impact the replication capacity of the GMO, as can be seen from its growth curve in the presence of thymidine (Vandenbroucke, 2008b). No large unmapped contigs were identified that span segments of the 5 plasmids from the wild type.

2.2.1.7 Sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques,

While the identification techniques are highly specific, detection is largely dependent on the possibility to isolate a limited number of bacteria out of a complex microflora. The fact that *L. lactis* -and strain MG1363 in particular- require a specific medium, needs to be taken into account.

2.2.1.8 Description of the geographic distribution and of the natural habitat of the organism including information on natural predators, preys, parasites and competitors, symbionts and hosts

L. lactis is one of the most important micro-organisms in the dairy industry. It is critical for manufacturing dairy products like buttermilk, yogurt and cheese, and thus is distributed globally. When *L. lactis* subspecies *lactis* is added to milk, the bacterium uses lactose as an energy source and produces lactic acid as by-product. The lactic acid coagulates the milk, forming curds that are subsequently used to produce cheese and whey. *L. lactis* is also used to prepare pickled vegetables, beer, wine, some types of bread and sausages and other fermented foods. More recently *L. lactis*, has also been explored as a drug delivery vehicle.

L. lactis was originally isolated from raw milk and this niche remains one of the few that can sustain *Lactococcus* cultures (Hirsch, 1952). They can be found in man (Elliott et al., 1991) and in animals (Klijn et al., 1995a). *Lactococci* can be isolated from various environmental sources such as soil, effluent water and the skin of cattle, but none of these represent ecological niches. *Lactococcus* bacteria are thought to have plants as their natural habitat (Stark and Sherman, 1935) and have occasionally been identified in invertebrates and fish, although they are certainly not widely present there (Bahrndorff et al., 2017; de Lacerda et al., 2016; Opazo et al., 2016; Rungrassamee et al., 2014). Environmental strains of *L. lactis* differ in a number of properties

from those found in industrial starter cultures, indicating that the majority of the industrially produced *Lactococci* do not survive outside of the dairy environment (Stark and Sherman, 1935).

L. lactis lacks the ability to multiply *in vivo* in mammals, except in gnotobiotic mice (Gruzza et al., 1994). When live *L. lactis* were fed to animals and human volunteers, they passed rapidly through the GI tract without colonisation. Drouault and colleagues investigated the survival, physiology and lysis of *L. lactis* in the digestive tract of rats, in order to understand the fate of ingested lactic acid bacteria after oral administration (Drouault et al., 1999). *Lactococci* that transited with the diet proved surprisingly resistant to gastric acidity (90-98% survival). In contrast, only 10-30% of bacteria survived in the duodenum. Viable cells were metabolically active in each compartment of the digestive tract, whereas most dead cells appeared to be subject to rapid lysis.

Klijn and co-workers reported a human feeding study using genetically marked *L. lactis* (Klijn et al., 1995b). Cells could only be recovered from the faeces of the volunteers if they had passed the GI tract within 3 days of ingestion, accounting for approximately 1% of the total number of cells consumed. The presence of related Deoxyribonucleic acid (DNA), extracted from faeces, could be detected up to 4 days, when viable cells were no longer present.

Broad *L. lactis* multiplication can only be sustained in a select number of nutritionally favourable areas such as milk, specifically prepared meats, vegetable fermentations and of course laboratory culture broths. *L. lactis* cannot successfully propagate outside these very specific ecological niches, which is underscored by the fact that, despite ample opportunity, globally, live *Lactococci* are consumed and released in soil and sewage waters in tremendous amounts– no *de-novo* colonisation of any such niches has ever been reported.

As stated before, *L. lactis* strain MG1363 can no longer grow in milk or any other natural environment. The carbon source for *L. lactis* in milk is lactose. In order to utilise this carbon source, wild type *Lactococci* are equipped with lactose utilisation genes as present on the *lac* operon. Furthermore, casein is used as amino acid source and *PrtP* protease is required to break the protein down to short oligopeptides for uptake (de Vos et al., 1989; Kunji et al., 1995). In wild type *L. lactis,* the genes for both essential functions are carried by the pLP712 plasmid. As all plasmids were removed during the isolation of MG1363, the strain has essentially lost the capacity to access its main energy and amino acid sources. In consequence, the habitat of MG1363 is confined to artificially supplemented culture conditions.

L. lactis can grow at temperatures between 10°C and 45°C, with an optimum at 30°C. It is susceptible to infection by bacteriophages, the most important being *936*, *c2* and *P335*. Pedersen et al., (2002) however showed that thyA deficient *L. lactis* are not susceptible to phage infection. In the very unlikely event that the thyA deficient sAGX0085 would be infected by phage, this would eliminate the bacterium and consequently reduce its potential impact.

2.2.1.9 Organisms with which transfer of genetic material is known to occur under natural conditions

Exchange of genetic material between bacteria mostly occurs through plasmids. While different *L. lactis* strains, including those used in the dairy industry, can harbour several plasmids, MG1363 has lost the 5 original plasmids present in the natural isolate NCDO 712.

Conjugative transposons, such as *Tn916,* are elements that transpose during conjugation from a donor cell harbouring the element to a recipient cell. Conjugative transposons have a broad host range: they are not only able to conjugatively transpose with frequencies of 10^{-4} to 10^{-9} among almost all species of gram-positive bacteria that have been investigated but can also transpose among gram-negative bacteria.

The first example of a limitation on the promiscuity of conjugative transposons is presented by *L. lactis* MG1363. In this strain, *Tn916* and *Tn919* do not excise (Bringel et al., 1992). Although the MG1363 strain can act as a recipient for conjugative transposition from another genus, it cannot donate conjugative transposons in plate matings with *Bacillus subtilis*, *Enterococcus faecalis* or *Streptococcus pyogenes*. In intrageneric matings between *L. lactis* MG1363 derivatives, transconjugants can be established but the transposons will be present in the same location in the transconjugant chromosome as in the donor genome, indicating that no transposition has occurred. Consensus is that *L. lactis* MG1363 lacks a factor required for excision of conjugative transposons (Bringel *et al.*, 1991).

By use of, and starting from the hypothetical lactococcal fertility factor (*Laff*), conjugative transfer of selectable, chromosomal markers from MG1363 to MG1363 derivatives has been reported (Bringel et al., 1991). *Laff* is speculated to be identical to Clu/sex-factor (Gasson *et al*., 1983). Stentz et al. (2004) however summarize the general knowledge that a) over a wide range of bacterial genera, cell aggregation provides the first cell-to-cell contact that is necessary for conjugal transfer; and b) cell aggregation has only been observed following sex factor and lactose plasmid cointegration. Having seen the absence of lactose plasmid in MG1363 (Gasson *et al*., 1983), Wegmann *et al.,* 2007), confirmed by full genome sequencing of the MG1363 sibling present in the ActoGeniX/Intrexon ActoBiotics NV culture collection), It is highly unlikely that MG1363 or its derivatives can serve as a conjugative donor, and, especially non-selectable, chromosomal traits could propagate into potential recipient (i.e. MG1363 related) populations.

2.2.1.10 Verification of the genetic stability of the organisms and factors affecting it

L. lactis strains have been used in food production. The growth of *L. lactis*, in particular of MG1363, is largely determined by the specific ecological niche. DNA replication fidelity of *Lactococcus sp.* has not been documented but is anticipated not to differ from that of *E. coli* i.e. as low as 10^{-9} to 10^{-11} errors per base pair (https://www.ncbi.nlm.nih.gov/pubmed/22404288).
For both the recipient wild type strain MG1363 and sAGX0085 full genome sequences were determined and compared. On a 2528862 bp. genome, 110 SNP were detected when comparing a predicted sequence based on the published MG1363 sequence (Wegmann *et al.,* 2007) (NCBI Reference Sequence: NC_009004.1) and genetic engineering performed thereon. From these 110, 90 SNP were specific to the MG1363 sibling used for genetic engineering while 20 were

specific to sAGX0085. None of these SNP are expected to impact the replication capacity of the GMO, as can be seen from its growth curve in the presence of thymidine. No large unmapped contigs were identified that span segments of the 5 plasmids from the wild type. The genetic stability of GM traits in sAGX0085 has been determined as absolute over at least 100 generations.

2.2.1.11 Pathological, ecological and physiological traits:

a) Classification of hazard according to existing Community rules concerning the protection of human health and/or the environment

L. lactis is commonly found in and added to food products. It is not classified as a hazardous organism. The search term "*Lactococcus*" was not found in The European Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work (http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32000L0054&from=EN).

EFSA introduced the concept of "Qualified Presumption of Safety" (QPS) in relation to a generic approach for safety assessment of micro-organisms used in food/feed and the production of food/feed additives. In 2013, a first list of microorganisms with QPS recommendation was published. Since 2013, this list is reviewed by EFSA's Panel on Biological Hazards (BIOHAZ) annually. *Lactococcus lactis* received QPC recommendation in 2013 as a gram-positive non-sporulating bacteria. The latest scientific opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA, published in March 2017, concluded that there is no need to change the QPS recommendation of *L. lactis* (EFSA, 2017).

MG1363 is completely dependent on an artificial growth medium, containing alternative energy and amino acid sources. The specific features of the strain only impair its survival capability.

b) Generation time in natural ecosystems, sexual and asexual reproductive cycle

Asexual multiplication with an average of 30 minutes of generation time in optimal growth conditions.

c) Information on survival, including seasonability and the ability to form survival structures

L. lactis bacteria do not produce survival structures such as spores. They can only survive and reproduce in their ecological niche, milk products, with anticipated survival time to be very dependent on the specificities of the environment and the activity of the autolysin AcmA (Buist *et al.*, 1995). No seasonability has been reported.

d) Pathogenicity: infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organism. Possible activation of latent viruses (proviruses). Ability to colonise other organisms;

L. lactis bacteria are critical for manufacturing dairy products like buttermilk and cheese. The widespread use of these products indicates that they are non-pathogenic. Despite their widespread use and massive discharge in the environment, they have not been identified as invasive or disruptive. Although *L. lactis* can be found in very diverse sources (soil, manure, waste water), the bacteria depend on particular nutritional components for growth. *L. lactis* strain MG1363 is restricted even further to artificially supplemented culture conditions.

As stated above, *L. lactis* is a food-grade micro-organism and has a long history of safe use in the food industry. Therefore, infection is highly unlikely. Nevertheless, an extensive literature search was completed and is repeated on a yearly basis. According to the literature review, bacteria can have some potential for pathogenic interactions in patients with co-morbidities, with consumption of unpasteurized dairy products reported in some cases. Starting from the taxonomic identification of *L. lactis* in the 19th Century, up until 2006, approximately 18 cases of *L. lactis* infection have been reported. The authors of the review indicate that this does not imply specific pathogenic traits attributable to *L. lactis*. All reported cases were proficiently cured by standard antibiotics therapy. Likewise, not a single fatality has ever been attributed to consumption of *L. lactis.*

e) Antibiotic resistance, and potential use of these antibiotics in humans and domestic organisms for prophylaxis and therapy

Some *L. lactis* strains produce nisin, a powerful antibiotic and bacteriocin that inhibits the growth of many other gram-positive organisms. However, MG1363 is a nisin-negative strain (Kuiper *et al.*, 1983, de Ruyter *et al.*, 1996 and Wegmann *et al.*, 2007).

L. lactis bacteria are sensitive to a wide array of antibiotics (De Fabrizio *et al.*, 1994). According to the results of De Fabrizio and colleagues, strains of *L. lactis* were sensitive to ampicillin and other beta-lactams (oxacillin, penicillin, pipericillin), cephalosporin, chloramphenicol, erythromycin (Em), amikacin, gentamicin, tetracycline, sulphonamide, trimethoprim/sulfamethoxazole and vancomycin. Somewhat lowered susceptibility was reported towards carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin, while intrinsic resistance was observed towards colistin, fosfomycin, pipedimic acid and rifamycin.

The following antibiotics susceptibility/resistance profile of MG1363 was determined as part of a nonclinical program:

- MG1363 are resistant to metronidazole (metronidazole), nalidixic acid (first generation quinolone), trimethoprim, sulfamethoxazole and a combination of both in a ratio of 1/19 (sulfonamides).
- MG1363 are sensitive to all other tested antibiotics: gentamicin (aminoglycoside), imipenem (carbapenem), vancomycin (glycopeptide), clindamycin (lincosamide), Em

(macrolide), nitrofurantoin (nitrofuranes), linezolid (ozazolidinones), ampicillin, amoxicillin, penicillin G (penicillins), chloramphenicol (phenicole), bacitracin (polypeptide), ciprofloxacin and levofloxacin (second and third generation quinolones), tetracycline (tetracycline) and cefepime (third generation cephalosporin).

f) Involvement in environmental processes: primary production, nutrient turnover, decomposition of organic matter, respiration, etc.

No involvement in particular environmental processes.

2.2.1.12 Nature of indigenous vectors:

All plasmids have been removed in the isolation of MG1363. The strain is deficient in factors necessary for conjugative transposition.

2.2.1.13 History of previous genetic modifications.

Strain MG1363, used as recipient organism in this study, has not been subject to genetic modifications.

In the past, various *L. lactis* strains have been engineered to express bacterial and viral antigens as well as other bioactive compounds (Chamberlain *et al.,* 1995; Gilbert *et al.*, 2000; Hirt *et al.,* 2000; Robinson *et al.*, 1997; Wells *et al.,* 1993; Xin *et al.*, 2003).

2.2.2 Characteristics of the vector

2.2.2.1 Nature and source of the vector

The modification method makes use of a carrier plasmid, pAGX0076. pAGX0076 has an origin of replication and an Em gene, both derived from pORI19 (Law et al., 1995). pORI19 can replicate in LL108, a specific *repA*+ strain of *Lactococcus*, thereby allowing the preparation of plasmid DNA. When introduced in a *repA- L. lactis* (as in this case MG1363), it cannot replicate.
pAGX0076 was designed in such way that the 5' and 3' target areas, identical to the ones flanking *thyA* on the *L. lactis* chromosome, were positioned 5' and 3' of the hTFF1 expression cassette (for schematic overview of pAGX0076, Figure 1).
The plasmid is not self-mobilizing and conditionally replicative. It was targeted for chromosomal insertion of a recombinant sequence in *L. lactis*.

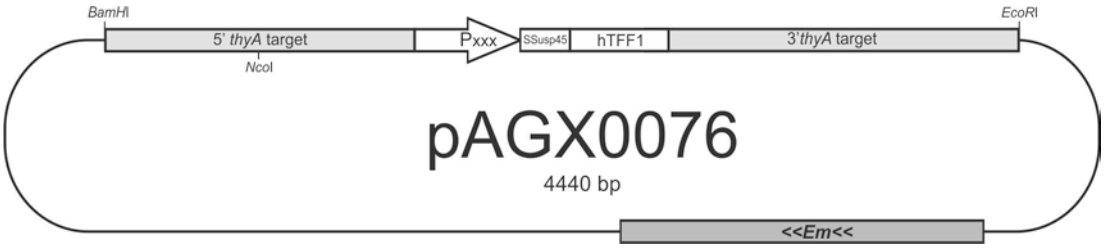


Figure 1: Schematic overview of pAGX0076

2.2.2.2 Sequence of transposons, vectors and other non-coding genetic segments used to construct

The plasmid was designed for the replacement of the *L. lactis thyA* coding sequence by the hTFF1 expression cassette, and therefore carries the hTFF1 expression cassette flanked by 5' and 3' *thyA* target regions. The expression cassette consists of the Pxxx promoter of a MG1363 gene encoding a structural protein, upstream of a fragment encoding the *Lactococcus usp45* secretion leader (*SSusp45*) and the *htff1* gene [Pxxx>>SSusp45>>hTFF1]. Replacing the thyA gene from MG1363 by a heterologous expression cassette renders the strain strictly dependent on thymidine or thymine for growth and survival, as has been described by Steidler et al (2003a).

2.2.2.3. Frequency of mobilisation of inserted vector and/or genetic transfer capabilities and methods of determination,

The plasmid was only required for the introduction of a double homologous recombination in the *L. lactis* chromosome. Once the homologous recombination had occurred, the plasmid was removed. The inserted function-expression of hTFF1- replaced the original wild type function (thymidylate synthase) and was stably integrated. The genetic stability of GM traits in sAGX0085 has been determined as absolute over at least 100 generations.

2.2.2.4 Information on the degree to which the vector is limited to the DNA required to perform the intended function.

For the plasmid used in the construction of sAGX0085, designed for the stable transfer of sequences to the *L. lactis* chromosome, the payload region was confirmed by DNA sequence determination, antibiotic resistance was present and absence of replication was concluded subsequent to introduction in MG1363. Replacing the thyA gene from MG1363 by a heterologous expression cassette renders the strain strictly dependent on thymidine or thymine for growth and survival, as has been described by Steidler et al (2003a);

2.2.3 Characteristics of the modified organism

2.2.3.1 1. Information relating to the genetic modification:

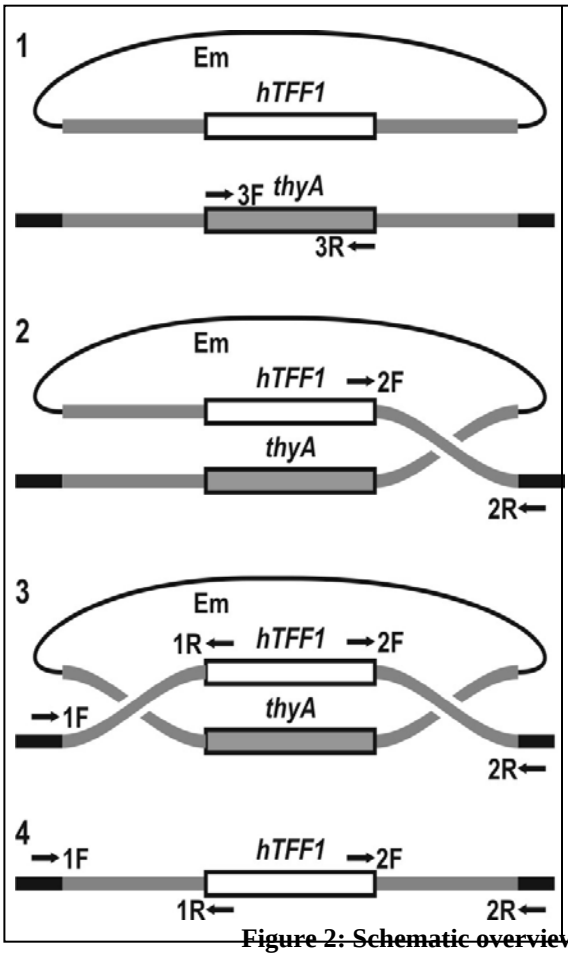
a) Methods used for the modification

The modification was based on targeted gene replacement by double homologous recombination. Replacing the *L. lactis* MG1363 *thyA* gene and promoter by the new hTFF1 expression cassette was performed by double homologous recombination at 1 kb target areas 5' and 3' of *thyA* (5' and 3' target areas) (Steidler et al., 2003b). A schematic overview of the modification strategy is given in Figure 2.

Upon introduction of pAGX0076 in MG1363, Em selection was applied to the culture. Em-resistant colonies were selected on solid agar plates containing Em. Because of the replication incompetence of pAGX0076, Em-resistant bacteria could only have arisen following a first homologous recombination either at the 5' or, as in this particular case, 3' target site. Homologous recombination could be verified by PCR.

Release of Em selection enabled the excision of pAGX0076 from the bacterial chromosome by a second homologous recombination, at either one of the 5' or 3' target site. For some Em-sensitive progeny, the second homologous recombination had occurred at the target site alternative to the one in the first homologous recombination. This event replaced the *thyA* gene with the *htff1* gene on the bacterial chromosome with seamless junctions and was identified by PCR. Adequate subculture rapidly diluted out all remnants of the carrier plasmid.

The resulting *L. lactis* strain, sAGX0085, carries the [Pxxx>>SSusp45>>hTFF1] DNA construct at the place of the *thyA* gene. No other foreign DNA is present in sAGX0085 and the structure of the final strain was completely as designed. Strain sAGX0085 was subject of a full genome sequencing program which confirmed the predicted DNA sequence (Steidler et al., 2003b). Neither residual sequences of the *thyA* gene, nor the integration vector pAGX0076 other than the DNA construct and the *thyA*-flanking regions could be detected. The 180 bp *htff1* is a synthetic gene designed based on the human TFF1 protein sequence (UniProtKB - P04155) with codon optimization for expression in *L. lactis*, showing only marginal homology (94% matches over bp 49-65; 88% over bp 116-132; 90% over bp 160-180; 80% over bp 164-180) with wildtype hftt1 cDNA (GenBank: BC032811.1).



Gray lines represent target areas for recombination, **bold black lines** represent non-target MG1363 chromosome fragments and **thin black lines** represent the integration vector. **1**, **2** and **3** represent PCR primer pairs (forward (**F**) and reverse (**R**)), designed in such way that:

- PCR using primer pair 1 showed co-linearity between chromosomal DNA located 5' of the target area and the *TFF1* gene,
- PCR using primer pair 2 showed co-linearity between chromosomal DNA located 3' of the target area and the *TFF1* gene, and
- PCR using primer pair 3 showed the presence of *thyA*.

Different stages included:

- introduction of the non-replicative vector,
- 3' crossover, forced by Em selection and identified by PCR2,
- second, 5' crossover in the absence of Em and identified through screening by PCR1, and
- acquisition of desired transgenic chromosome organisation.

Figure 2: Schematic overview of targeted gene replacement

b) Methods used to construct and introduce the insert(s) into the recipient or to delete a sequence

The construction of vector plasmid pAGX0076 as well as the targeted gene replacement was described in Sections 2.2.2 and 2.2.3.1

c) Description of the insert and/or vector construction;

Table 1 below lists the components of the insert.

Table 1: List of components of the insert as present in sAGX0085

Size (bp)	Indication	Description/function	Origin [Ref]
848	llmg_0963'	*ThyA* 5' flanking sequence cloned to allow crossover integration.	*L. lactis* MG1363 (Steidler et al., 2003b)
104	Pxxx	A promoter isolated from MG1363 controlling the expression of a bacterial protein, characterized as one of the most prominent protein bands in a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)-analysis of cytoplasmic MG1363 proteins	*L. lactis* MG1363 AGX research
81	SSUsp45	Secretion sequence leader of *usp45*, a gene encoding an extracellular secretory protein.	*L. lactis* MG1363 (van Asseldonk et al., 1990)
183	htff1	Synthetic *htff1* gene with codon-optimisation for *L. lactis*.	Synthetic, based on human sequence. (Gene ID: 7031)
1000	-	Non-coding sequence downstream of *ThyA* coding sequence.	*L. lactis* MG1363 (Steidler et al., 2003b)
	llmg_0965'	*ThyA* 5' flanking sequence cloned to allow crossover integration	*L. lactis* MG1363 (Steidler et al., 2003b)

d) Purity of the insert from any unknown sequence and information on the degree to which the inserted sequence is limited to the DNA required to perform the intended function;

DNA sequence of the insert, as determined on a PCR fragment derived from sAGX0085, was identical to a prediction of the desired sequence(Vandenbroucke 2008b) (Figure 3).

The entire sequence is known and confirmed by DNA sequencing (Vandenbroucke 2008b). The insert was limited to the intended functions.

e) Methods and criteria used for selection;

Initial selection was based on a combination of PCR analysis and Em selection. The selected GM strain, sAGX0085, was confirmed to have the insert and to be sensitive to Em.

The region containing the inserted hTFF1 expression cassette was PCR amplified from the bacterial chromosome of sAGX0085. These DNA fragments were purified and the DNA sequence was determined. The experiment clearly showed that the DNA sequence of sAGX0085 at the *thyA* locus was identical to an *in silico* prediction of the desired sequence (Steidler, 2008) (Figure 3).

The successful removal of [PthyA>>thyA] was documented by DNA analysis (Vandenbroucke, 2008b). Also, the auxotrophic nature was confirmed: the ratio of viability decrease of sAGX0085 in the absence of thymidine is identical to that of other *thyA*-deficient *L. lactis* strains (Steidler et al., 2003a; Vandenbroucke, 2008b).

f) Sequence, functional identity and location of the altered/inserted/deleted nucleic acid segment(s) in question with particular reference to any known harmful sequence

A schematic comparison of the sequences present in the recipient strain MG1363, plasmid pAGX0076 and the bacterial chromosome of GM strain sAGX0085 are provided in Figure 3. The insertion of the new hTFF1 expression cassette inherently resulted in the removal of the *thyA* gene and promoter. No other sequences were modified (Figure 4). The predicted amino acid sequence of the gene product of this novel *htff1* gene is identical to that of native hTFF1.

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Figure 3: Complete nucleotide sequence of the transgenic insert of sAGX0085

The transgenic insert [Pxxx>>SSusp45>>hTFF1], was positioned in between 5' and 3' *thyA* flanking regions (*i.e.* regions flanking the *thyA* the gene in the ancestral *L. lactis* strain MG1363). Alignment of the predicted (line 1) and experimentally determined (lines 2 and 3) DNA sequences.

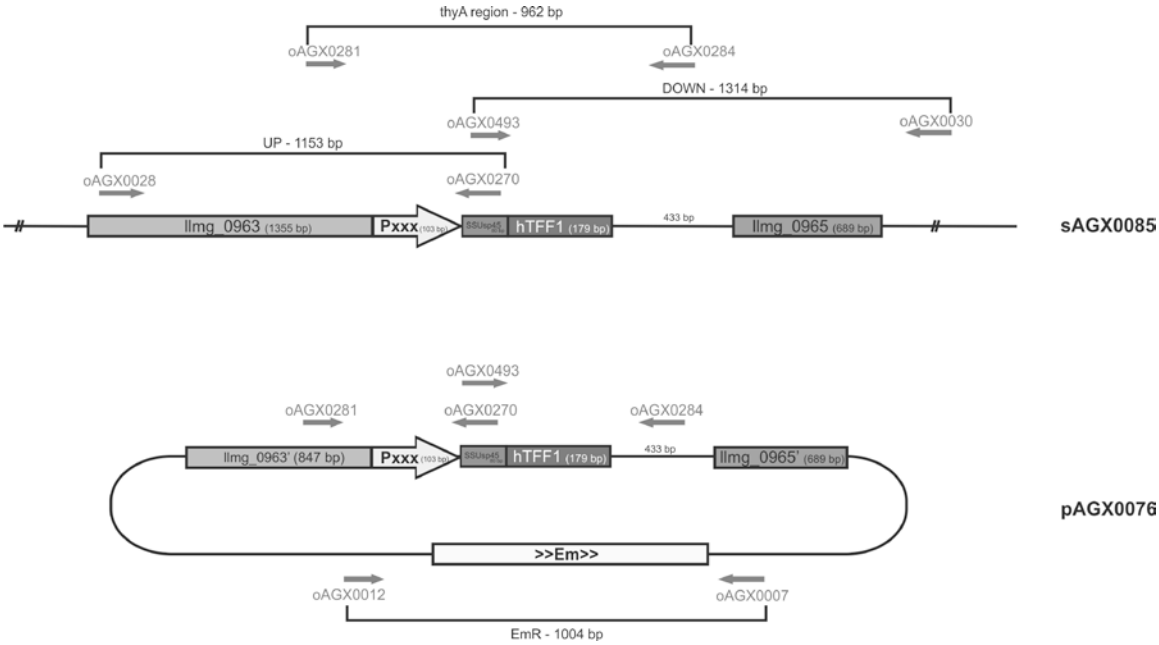

Figure 4: Schematic overview of the *thyA* locus of MG1363, sAGX0085 and pAGX0076, with indication of the oligonucleotide binding sites and PCR amplification products

2.2.3.2 Information on the final GMO:

a) Description of genetic trait(s) or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed

The targeted gene replacement resulted in 2 new characteristics:

- Production and secretion of hTFF1:

hTFF1 is a member of the Trefoil Factor (TFF) family, which comprises TFF1 (formerly pS2), TFF2 (formerly spasmolytic peptide, SP), and TFF3 (formerly Intestinal Trefoil Factor, ITF). These proteins play an essential role in epithelial restitution and repair within the GI tract (Taupin and Podolsky, 2003).

TFFs are small, non-mitogenic, protease-resistant peptides that share a preserved, distinct motif of six cysteine residues linked by three disulphide bonds, defining a so-called 3-leafed “trefoil domain” or “P domain”.

Human TFF peptides are expressed in several tissues of the human body, most pronouncedly by mucus-secreting cells of the GI tract, including the mouth, oesophagus, stomach and intestines. Under normal circumstances, hTFF1 and hTFF2 are primarily located in the stomach and duodenum, whereas hTFF3 is predominately present in the mucus cells of the small and large intestine (Hanby et al., 1993; Podolsky et al., 1993; Rio et al., 1988).

Vandenbroucke *et al.* engineered *L. lactis* to secrete bioactive murine TFF (mTFF) 1, 2 or 3, and evaluated the prophylactic and therapeutic effects of the GM bacteria in experimentally-induced colitis in mice. Oral administration of the Murine Trefoil Factor (mTFF) secreting *L. lactis* resulted in significant protection against colitis and significantly accelerated the healing process of established intestinal injury (Vandenbroucke et al., 2004). In addition, *L. lactis*-mediated TFF delivery proved to be at least 3 orders of magnitude more efficient than administration of purified TFF protein.

In an established acute radiation hamster model, topical application of GM *L. lactis* strains, engineered to secrete either hTFF1 or hTFF3 to the oral mucosa, favourably affects the severity and course of radiation-induced OM.

The exploratory efficacy results of the Phase 1b study AG013-ODOM-101[‡] show that subjects who received AG013 had a lower percentage of days with ulcerative mucositis, and more subjects who received AG013 on any dosing schedule had no or only 1 day of ulcerative mucositis compared to subjects who received placebo.

Thymine/Thymidine dependency:
Thymidylate synthase is the enzyme used to generate thymidine monophosphate, which is subsequently phosphorylated to thymidine triphosphate for use in DNA synthesis. In sAGX0085, insertion of the hTFF1 expression cassette removed the *thyA* gene of MG1363, interrupting this essential step in DNA synthesis. As such, not only growth but also survival of sAGX0085 became dependent on the addition of thymine/thymidine to the growth medium, with a strong preference for the utilisation of thymidine (addition of thymine is approximately 1,000-fold less effective than addition of thymidine to culture media (Steidler et al., 2003a)).

Depletion of thymidine or thymine is toxic to virtually all actively growing cells. Originally described by Barner and Cohen in 1954 (Barner and Cohen, 1954), *thymine-less death* has been reported in bacteria, yeast and mammalian cells. This effect is unusual in that deprivation of many other nutritional requirements has a biostatic, but not lethal, effect. Studies of numerous microbes have indicated that thymine starvation has direct and indirect effects. The direct effects involve both single- and double-strand DNA breaks and while the former may be repaired effectively, the latter inevitably lead to cell death.

As a consequence, the removal of thymidylate synthase has not only made sAGX0085 fully growth-dependent on external supplementation of thymidine or thymine (further limiting its ecological niche to laboratory cultures), the *thymine-less death* mechanism provides a natural, self-limiting system that eliminates sAGX0085 when exposed to non-optimal environments.

[‡] ClinicalTrials.gov Identifier: NCT00938080 at http://clinicaltrials.gov/ and RAC protocol #0810-942

b) Structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified organism

The structure has been previously described in Section 2.2.2. Complete DNA sequencing of the sAGX0085 genome has been performed (Steidler, 2008). Results from this complete genome sequence demonstrated that:

- The experimentally-determined DNA sequence of strain sAGX0085 showed at least 99,995% homology to the predicted DNA sequence.
- None of the obtained contigs showed any significant homology to the backbone part of integration plasmid [pAGX0076] (containing an Em resistance marker and origin of replication), nor to *thyA*

c) Stability of the organism in terms of genetic traits

The sAGX0085 has been maintained in culture since January 2007.

Analysis of genetic stability of *L. lactis* strain sAGX0085 was performed after a minimum of 100 generations of growth, obtained by repeated sequential dilution and growth to saturation (Vandenbroucke, 2008c).

The genetic stability was analysed by four parameters:

1. Inability of sAGX0085 to grow in thymidine-deficient medium
2. Unchanged hTFF1 secretion by sAGX0085
3. PCR analysis of the modified *thyA* locus of sAGX0085
4. DNA sequence verification of the [Pxxx>>SSusp45>>hTFF1] expression cassette of *L. lactis* strain sAGX0085

The quality of the material is monitored during storage and production. As the insertion occurred on the bacterial chromosome, the stability of the genetic trait is expected to be similar to any other chromosomal trait. No instability created by transposons is anticipated.

d) Rate and level of expression of the new genetic material. Method and sensitivity of measurement

The expression level of hTFF1 from sAGX0085 was documented in a standardised *in vitro* setting (Vandenbroucke, 2008b). The experiment was conducted according to internal Standard Operating Procedures (SOPs). These SOPs describe standardised growth conditions to allow for comparison of expression between similar numbers of bacterial cells from different *L. lactis* strains, detail the analysis of secreted proteins from *L. lactis* and hTFF1 sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA). The lower limit of quantification (LLOQ) of the assay is 5 pg/ml. This assay has been validated and is a release test for AG013 for use as an Investigational Medicinal Product (IMP) in clinical studies.

An sAGX0085 culture, grown under optimal conditions for 3 hours in BM9T growth medium (culture medium supplemented with 200 µM thymidine), produces approximately 188 ng hTFF1 per ml.

e) Activity of the expressed protein(s)

The only protein that is produced is the hTFF1. The *htff1* gene used in the genetic engineering of *L. lactis* sAGX0085 is a synthetic gene that was codon-optimised for use in *L. lactis*. The predicted amino acid sequence of this novel gene product is identical to that of native hTFF1.

The protein is expressed/measured by ELISA. A protein activity assay is currently under development.

f) Description of identification and detection techniques including techniques for the identification and detection of the inserted sequence and vector

- Through PCR amplification of 16sRNA and subsequent sequencing of the PCR fragment, the species identity of sAGX0085 was established as *Lactococcus lactis* subspecies *cremoris* MG1363 during the manufacturing of the master cell bank. In addition, the presence of the *htff1* gene and the absence of the *thyA* gene was also demonstrated by sequencing.
- ELISA was used to quantify the levels of hTFF1 secreted by the sAGX0085.

g) Sensitivity, reliability (in quantitative terms) and specificity of detection and identification techniques;

This GMO is an IMP which will be released by a qualified person for use in a clinical trial. Test will have been validated and sensitivity of assays defined along with specifications and acceptance criteria. Specificity and detection limits are provided below:

- PCR and sequencing methods result in clear-cut identifications. Specificity of the primers and method have been validated.
- The detection limit of the hTFF1 ELISA is 5 pg/ml. The assay is specific with respect to other TFF peptides, growth factors, etc.
- The detection limit for Western blot analysis of hTFF1 is 25 ng hTFF1 per lane (*i.e*. concentrated expression samples).
- The auxotroph sAGX0085 only grows on thymidine or thymine containing culture media.

h) History of previous releases or uses of the GMO;

To date, AG013 has been studied in humans in a Phase 1b study (AG013-ODOM-101) in the US and a Phase 1 pharmacokinetic (PK) study in healthy volunteers (AG013-CSM-MU-004) in Belgium.

The Phase 1b study was a multicenter, single-blinded, placebo-controlled, sequential dose-escalation study that evaluated the safety, tolerability, and PK profile of AG013 in subjects experiencing OM during induction CT for the treatment of Head and Neck Cancer (HNC) [ClinicalTrials.gov Identifier: NCT00938080].

A total of at least 21 subjects were planned to be enrolled in 3 successive groups of at least 7 subjects each (at least 5 subjects were assigned to AG013 and at least 2 subjects were assigned to placebo). An independent data and safety monitoring board (DSMB) reviewed the safety results from each group prior to dose escalation. The study achieved its primary objective by demonstrating that AG013 was generally safe and well tolerated. The incidence of sepsis due to AG013 was followed as an event of special interest in this clinical study and no subjects experienced an adverse event of this type.

The second objective of the study was to evaluate the pharmacokinetics of AG013. In general, live bacterial levels of AG013-sAGX0085 were high immediately following dosing and decreased by 90 minutes post-dose. No differences were noted among the active treatment groups; no dose relationship was seen. In all treatment groups, AG013-sAGX0085 levels were 0 by the End Of Study visit. AG013-sAGX0085 could not be detected in blood. No dose frequency-related differences in hTFF1 levels could be detected in saliva or oral mucosa amongst the active treatment groups. Levels of hTFF1 in serum were not significantly different between treatments groups at all time points measured.

The Phase 1 PK study in healthy volunteers was a single-center, open-label Phase 1 study to assess the effect of food/beverage and to characterize the pharmacokinetics of single and multiple oral doses of AG013 in healthy subjects. Ten subjects were enrolled in the study.

AG013 was generally safe when applied by mouth rinse once or three times on one day. Overall, consistent levels of AG013 (bacterial count and protein) could be recovered from the different sample sites in the oral cavity, up to 24 hours after dosing. Furthermore, live AG013 bacteria levels coincided with protein levels. These data demonstrate that live AG013 bacteria adhere to the oral mucosa and actively secrete protein at the mucosal surface. This results in homogeneous exposure to the entire mucosal surface. There was no evidence for systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum) and there was no recovery of live AG013 bacteria in feces.

Overall, the *in vivo* safety pharmacology studies, and the 2 completed phase I studies support safe administration of AG013 for attenuation of OM in patients with cancer of head and neck receiving concomitant chemoradiation therapy.

i) Considerations for human health and animal health, as well as plant health:

- Toxic or allergenic effects of the GMOs and/or their metabolic products

There is no indication that the GMO itself is either toxic or allergenic. *L. lactis* bacteria have a long history of safe use in food products and the changes that were induced in the recipient strain MG1363 as well as in the GMO sAGX0085 do not affect the basic toxic or allergenic features.

AG013 strain sAGX0085 produces hTFF1, that is naturally produced by human salivary glands and is present in whole saliva. The predicted amino acid sequence is identical to the native hTFF1. No allergenic potential is identified, seen the fact that TFF1 is omnipresent in the mouth and serum.

In order to examine the potential toxicity of AG013, the repeat-dose toxicity (RDT) studies consisted of three GLP studies in two healthy animal species for up to 3 month duration (*i.e.*, in rat for 14 days and for 3 months and dog for 14 days) (Appel, 2008; Prinsen, 2012; Vanhoenacker, 2009).

These GLP studies were designed to mimic, as closely as possible, the proposed clinical trials with respect to dose and dosing regimen. Oral gavage was chosen as the best suited route of administration, as this represents the case where human subjects may either spit or swallow the product. In addition, this route allows for more reliable and controllable dosing of the product to animals and maximizes exposure.

Overall, no treatment-related effects were found for up to 3-month exposure to AG013. Importantly, results from genotoxicity testing, included in the 14-day toxicology study in healthy rats, confirmed that AG013 did not induce primary DNA damage. Therefore, the overall results of these toxicology studies support safe administration of AG013 to human subjects as outlined in the proposed clinical program, indicating at least 100-fold (95 and 111 for 14 days dogs and 3 months rats) respectively safety margin compared to the maximum proposed human dose (MPHD) as per the proposed Phase 2 study.

A dose of 2×10^{11} CFU per oral rinse in the proposed Phase 2 study is expected to be the minimal dose needed to obtain a clinically meaningful reduction in severity of OM in participants.

- Comparison of the modified organism to the donor, recipient or (where appropriate) parental organism regarding pathogenicity;

AG013 strain sAGX0085 is non-pathogenic, just like the recipient strain MG1363. The expressed hTFF1 is not toxic. TFF1 is omnipresent in the mouth and serum. Furthermore, *L. lactis* and MG1363 are under strict biological containment, non-pathogenic and non-invasive. Systemic effects are therefore not expected.

- Capacity for colonisation

L. lactis has a poor capacity of colonisation and as such does not normally colonize human tissues (Cho et al., 2012, Todor, On line textbook of bacteriology. http://textbookofbacteriology.net/featured_microbe.html).

The niche of MG1363 was severely restricted due to the elimination of all plasmids, negating the possibility of using its natural energy and amino acid sources. sAGX0085 is furthermore biologically contained due to the removal of thymidylate synthase, which resulted in thymine/thymidine dependency and introduced the *thymine-less death* mechanism.

The organism is not expected to be pathogenic for humans.

As part of the safety assessments, the potential survival of *L. lactis* sAGX0085 in complement-preserved human serum was investigated *in vitro*. The test demonstrated that *L. lactis* sAGX0085 is unable to survive in complement-preserved human serum (Vandenbroucke, 2009).

In vivo safety pharmacology studies have been performed in neutropenic rats and neutropenic hamsters with AG013. Although limited systemic absorption was noticed after topical administration of the bacteria to the ulcerated cheek pouch of severely neutropenic hamsters, there were no indications for clinical infection. The bacteria could survive neither in systemic circulation, nor in the peripheral tissues. Furthermore, IV injection of *L. lactis* into neutropenic rats demonstrated that there was no indication of sepsis, and the bacteria were rapidly cleared from the blood stream.

In addition, the antibiotic susceptibility/resistance profile of sAGX0085 was established and demonstrated that sAGX0085 is susceptible to clinically relevant, commonly used antibiotics, (Vandenbroucke, 2008a). Hence, in the unlikely event of clinically significant systemic exposure of sAGX0085, the infection can be treated quickly and easily. The antibiotic susceptibility/resistance profile of *L. lactis* sAGX0085 was determined as part of the nonclinical program for AG013. In conclusion, sAGX0085 shows a comparable antibiotic susceptibility/resistance profile to its non-GM parent strain MG1363:

- sAGX0085 is resistant to metronidazole (metronidazole), trimethoprim and sulfamethoxazole
- sAGX0085 is sensitive to ciprofloxacin, levofloxacin, cefdinir, amoxicillin, vancomycin, penicillin G, amoxicillin + clavulanate and sulfamethoxazole + trimethoprim.

Briefly, the results from these safety pharmacology studies support safe administration of AG013 to potentially immunocompromised OM patients, whose non-intact oral mucosa could represent an increased risk for bacteraemia. In the proposed phase 2 study special attention is given to clinically significant bacteraemia and clinical sepsis, which should be recorded as AE or SAE. In the event that a subject develops symptoms suggesting clinically significant bacteraemia or sepsis, the subject should be treated per the site's standard of care, including commonly used antibiotics.
A disposition plan for the management of clinical sepsis is provided in the clinical study protocol.

- Other product hazards

No product hazards have been identified.

2.3 INFORMATION RELATING TO THE CONDITIONS OF RELEASE AND THE RECEIVING ENVIRONMENT

2.3.1 Information on the release

It should be noted that the information on the intended release is based on the planning of the notifier and might be subject to possible modifications arising from the indications during the clinical trial. Whenever such modification would arise the GMO competent authorities will be informed in particular if the modification might affect the GMO risk assessment.

2.3.1.1 Description of the proposed deliberate release, including the purpose(s) and foreseen products

Oragenics' overall objective of the development program is to establish AG013 as a therapeutic option, and gain marketing approval, for reduction of the signs and the symptoms of RT and/or CT induced OM.

The proposed clinical trial is a Phase 2, multi-center, randomized, double-blind, placebo-controlled study to assess the safety and efficacy of topically-applied AG013 for the attenuation of oral mucositis in subjects with cancers of the head and neck receiving concomitant chemoradiation therapy.

This is a study with 4 periods:

- The screening phase: this will be no longer than 4 weeks
- The active treatment phase: will be between 7 and 9 weeks depending on the participant's prescribed CRT plan (during CRT + 2 weeks).
- The short term follow-up phase: will be four weeks in duration, and
- The long term follow-up: will continue until 12 months post CRT.

The clinical formulation of AG013, the lyophilised powder of bacterial strain sAGX0085, is an oral, topical administration in the form of a MR. This MR suspension is prepared by adding a solution containing water, an aroma and a sweetener to the AG013 powder mixed with cryoprotectants.
The AG013 powder is packed in clear glass vials with tamper-evident, child-resistant screw caps. This vial is in turn packed into a sealed aluminium bag. The solution for re-suspension (reconstitution) is delivered in a dark glass bottle with tamper-evident, child-resistant screw caps.

In the proposed phase 2 study a fixed dose of IMP will be administered at a dose frequency of three times daily (Table 2). The study population consists of patients with high risk for OM associated with CRT. The dose level and dose frequency selected for evaluation in this study is expected to be safe and to show efficacy based on the results of the Phase 1b clinical study. This new study will evaluate the dose frequency of AG013 three times daily oral rinses versus placebo. The selection of the dose frequency of AG013 three times daily is based on the positive efficacy trends noted in the Phase 1b trial AG013-ODOM-101, and on the results of the Phase 1

PK study in healthy volunteers AG013-CSD-MU-004 demonstrating that three times daily dosing result in a clinically relevant 24-hour exposure period of AG013.

Table 2: Overview of the study doses

	Treatment frequency	AG013 1 dose	Daily dose	Daily dose/kg
AG013	3	2 x 10^{11} CFU	6 x 10^{11} CFU	8.6 x 10^{9} CFU

Approximately 200 subjects will be enrolled in the study to obtain 160 evaluable subjects (i.e., those who receive at least 4 weeks of IMP and a cumulative radiation dose of at least 50 Gy). This study will be conducted in approximately 49 sites in the United States and Europe.

AG013 will be delivered to the clinical trial centre pharmacy as single dose packages that do not need further manipulation. Direct contact with the lyophilised powder is excluded until opening of the single doses. Only at the moment of administration, exposure to the suspended powder is possible.

A study site will receive an initial shipment of AG013 kits, placebo kits and kits with Solution for Reconstitution (SR) as soon as the site is ready for activation. Re-supply will be automatically placed through Interactive Web Response System (IWRS) as subjects are being enrolled. As soon as shipment arrives at the site, the responsible site personnel need to check for completeness and integrity of the shipment and check the monitoring device for potential temperature excursion. The study site will ensure storage prior to dispatch to the participants enrolled in the clinical study. There is no need for any additional manipulation, as the package contains all doses ready for application by the participant. The AG013/Placebo components of the mouth rinse formulation should be stored refrigerated (2°C to 8°C) in their original package. The SR can be stored at room temperature (15°C to 25°C) in its original package. The IMP must be kept in a secure area at the study site. Participants will be supplied weekly with IMP during the active phase of the study. Each time they will receive two kits (one AG013/placebo and one SR kit) that contain enough IMP for 7 days plus 1 spare day.

The pre-packaged doses will include everything that is required (Figure 5):

- Solution for reconstitution (SR), → Bottle 1.
- AG013 (or Placebo) in a powder form, → Bottle 2 in an aluminium sachet.

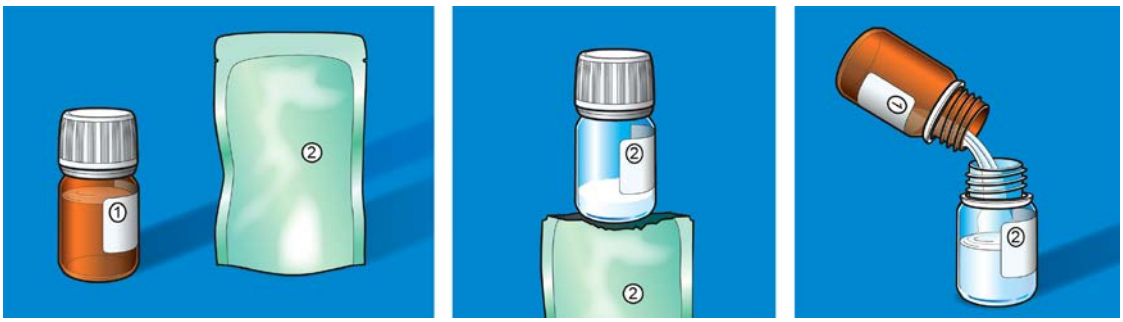

Figure 5: Summary of the different steps to reconstitute the MR suspension

Briefly, the SR needs to be added to AG013 or Placebo and shaken for at least 30 seconds to obtain a homogeneous white opaque suspension, ready for use. The MR should be applied for 30 seconds and then the suspension should be expectorated into a sink or toilet. As a result, the GMO will be primarily released into the environment at the study subjects home via the sewer system. It is likely to be shed in saliva but is not expected to be shed in stools (based on Phase 1 data in healthy volunteers) as the MR is not swallowed by study subjects The impact to the environment is considered negligible, as the GMO cannot survive outside the target ecosystem, cannot replicate, and is non-pathogenic to humans and other organisms in the environment. The gene of interest is non-toxic.

The subjects must return all used and unused bottles to the study site even in the case when a subject discontinues the study.

The study is conducted on an ambulatory/outpatient basis, *i.e.* subjects do not reside in the clinical study centre during the study. Detailed instructions for dose preparation and dispensing as well as a questions and answers booklet with detailed instructions on the actions to be taken in case of spillage or accident will be provided to subjects. However, the first dose is administered in the clinical trial centre witnessed by a trained person to check that everything is done correctly. This will be done at the moment of applying the first dose on Day 1 of CRT (prior to CT or RT, whichever comes first). During the active treatment phase the participants will come for observations and sampling twice weekly. This frequency is reduced to once weekly in the short-term follow-up phase. During study visits, the participants' condition will be evaluated. Observations include documentation of any adverse effect, physical examination (OM and pain assessment etc.) vital signs, and clinical laboratory evaluations.

In case of spillage the affected area can be decontaminated with a standard detergent (soap) or bleach.

The mouth rinse that is being discarded into the sink or toilet is expected to enter the sewage system. Any impact on personnel, animals or the environment is expected to be very low due to the non-pathogenic, non-replicating nature and reduced survival capacity of the GMO. Additional treatment and containment are not required. Moreover, the sewage treatment system is designed to eliminate bacteria.

In the long-term follow-up participants are assessed for tumour status.

Table 3 lists materials that may possibly contain sAGX0085, stating the fate of each of them.

Table 3: Fate of materials possibly containing sAGX0085

Materials	Fate
Unused doses	Used in study or returned by a subject
Blood and mucosal samples	- Sampled by the study site staff in closed tubes at the day of a hospital visit. - The tubes are stored at the clinical site until shipment to a central laboratory for further analysis. - After analysis the samples will be destroyed per procedures applied to hazardous medical waste (incinerated).
Used MR (spat out)	- Evacuation via sewage system
Faeces	- Evacuation via sewage system

2.3.1.2 Foreseen dates of the release and time planning of the experiment including frequency and duration of releases

Recruitment of the first participants is expected to start in July 2018. Completion will depend on availability of participants fulfilling the selection criteria and could take until June 2019. For each individual participant, a treatment period of 7 to 9 weeks, with three daily doses, is envisaged.

2.3.1.3 Preparation of the site previous to the release,

No specific preparation of the investigational site (hospital) is foreseen. The actual release, *i.e.* the moment where the GMO is released from its bottle and may be in contact with the environment, is restricted to the application by the subject. Also, since the study subject is required to spit out the MR after the required contact time, live bacteria are released. As the trial is on an ambulatory/outpatient basis, this is likely to occur at the clinical trial centre and at the subjects' home. While the location of the clinical study centre will be known, the identity and coordinates of the study subject will not be known to the notifier.

2.3.1.4 Size of the site

Not relevant

2.3.1.5 Method(s) to be used for the release

AG013 will be administered as a mouth rinse.

2.3.1.6 Quantities of GMOs to be released

A single MR dose contains 2 x 10^{11} CFU of AG013 sAGX0085 strain. During the active treatment phase, subjects will rinse with the suspension three times each day and this 7 to 9 weeks depending on the duration of the radiotherapy. it can be estimated that a maximum quantity of $3.8x10^{13}$ colony forming units (CFU) will be released per subject on active treatment (supposing that every AG013 bacteria in the mouth rinse is released viable, which is impossible and a worst case scenario) The suspension is to be spat out into a sink or toilet.

In the Phase 1 healthy volunteer study, no live bacteria were recovered in faeces after a single dose.

Actual release is limited to:

- spat out MR,
- shedding via saliva
- contact with empty bottle, or
- accidental disruption of a bottle.

In all of these cases, survival of the bacteria will be extremely limited in time and during this period the very limited amount of bacteria potentially present will not be metabolically active.

In the clinical trials, the drug product (containing the bacteria) is available as a powder, to be reconstituted in a liquid. In the event that the packaging is disrupted, the powder quickly degrades after being in contact with moisture and warmth. The microorganism is sensitive to temperatures above 40°C, low pH, air drying, direct sunlight, UV, soap, bleaching agents, antibiotics and high salt concentration solutions. The quantity of a spillage will be limited (one dose). The affected area can be decontaminated with a standard detergent (soap) or bleach.

Brief contact with the powder and the solution is possible at time of reconstitution of the mouth rinse and when administered. The subject only receives the necessary material for a one-week treatment period. At the same time, instructions are provided and explained in order to ensure compliance to treatment. Other family members may be exposed when handling empty containers and possibly material with shed bacteria. Standard hygienic practices should be sufficient to limit or prevent significant exposure.

Accidental spillage: AG013 could leak into the environment due to accidental spillage during reconstitution or during administration, or due to rupture of the pre-packed product. AG013 strain sAGX0085 cannot survive outside of artificially-supplemented laboratory conditions and will be rapidly eliminated. The quantity of such spillage will normally be limited to one treatment dose, but the environmental containment system is robust, and its efficiency will not be influenced by the quantity of spillage that might occur (e.g. a large spillage consisting of a complete one-week treatment package). The affected area can be decontaminated with a standard detergent (soap)

or bleach. Detailed instructions on the actions to be taken in case of spillage or accident are presented in a Q&A document, which will be provided and explained to the subject.

2.3.1.7 Disturbance on the site (type and method of cultivation, mining, irrigation, or other activities)

Not applicable. AG013 will be released as part of a clinical study.

2.3.1.8 Worker protection measures taken during the release

Investigators and other clinical staff can only be exposed during the handling of a treated subject or of specific material that has been in contact with particular parts of the clinical study subject. Normal hygiene conditions for clinical staff handling biological samples (in particular spat out MR and saliva) should be sufficient. All waste material should be handled as hazardous medical waste.

Only the removal of blood samples requires the use of syringes. Based upon previous experience with AG013, it is anticipated that the blood will not contain any AG013 bacteria, therefore syringes can be handled as per normal procedure.

To date, AG013 has been studied in humans in 2 studies: a Phase 1b clinical study conducted in the US (AG013-ODOM-101) and a Phase 1 PK study in healthy volunteers conducted in Belgium (AG013-CSD-MU-004).There was no evidence for systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum) and there was no recovery of live AG013 bacteria in feces (healthy volunteers study only).

2.3.1.9 Post-release treatment of the site

In other similar studies using *L. lactis* strains, viable bacteria has been detected after administration, up to 3 days in stools samples (Braat et al., 2006; Drouault et al., 1999; Klijn et al., 1995b).

AG013 is being developed for the treatment of the oral cavity and not the GI tract. Study subjects are not required to swallow the mouth rinse and therefore the risks of shedding in stools is very low. In addition, if this was to occur the dose would be considerable low. Shedding in saliva is expected immediately after the use of the mouth rinse but the dose is low compared to that in the oral cavity and drops after 90 minutes. It is expected that any *L. lactis* AG013 entering the immediate environment and the sanitary sewer system will be inactivated and/or removed by the physical, biological, and/or chemical treatments in place in wastewater treatment plants. Therefore, there are no specific post-release treatment of the sites foreseen.

2.3.1.10 Techniques foreseen for elimination or inactivation of the GMOs at the end of the experiment

As discussed in Section 2.2.1, given the strict biological containment, combining several inherent inactivation factors, no additional inactivation is foreseen. If required, a standard antibiotic treatment would suffice to inactivate the bacteria.

The clinical study under consideration is run under the auspices of Good Clinical Practice (GCP). GCP guidelines stipulate that:
"The investigator/institution and/or a pharmacist or other appropriate individual, who is designated by the investigator/institution, should maintain records of the product's delivery to the trial site, the inventory at the site, the use by each subject, and the return to the sponsor or alternative disposition of unused product(s). These records should include dates, quantities, batch/serial numbers, expiration dates (if applicable), and the unique code numbers assigned to the investigational product(s) and trial subjects. Investigators should maintain records that document adequately that the subjects were provided the doses specified by the protocol and reconcile all investigational product(s) received from the sponsor. Any unused investigational medicinal product shall be returned by the patient to the dispensing unit for reconciliation."

Once returned, any used and unused IMP containing the GMO will be appropriately destroyed by set guidelines and according to the institutional standards. Any material that is collected during the study and that has potentially been in contact with the bacteria, will be disinfected or inactivated as hazardous medical waste (such as disposable materials, packages).

2.3.1.11 Information on, and results of, previous releases of the GMOs, especially at different scales and in different ecosystems

In the pre-clinical trials healthy and diseased hamsters, rats and dogs have been administered AG013 without adverse effects. No local or systemic pathogenic effects were documented after chronic administration of AG013.

Safety pharmacology,pharmacodynamic (PD) and PK studies using AG013 established that:

- That limited systemic absorption of AG013 is likely as confirmed by in vivo study to determine the risk for systemic absorption of live *L. lactis* bacteria in hamsters in which concomitant OM and severe neutropenia (induced by myelosuppressive agents) were present. There was no indication of clinical infections, i.e. bacteria could survive neither the systemic circulation, nor in the peripheral tissues.
- Systemic absorption of the bacteria did not cause systemic infection, even under neutropenic circumstances in an *in vivo* study in severely neutropenic rats (induced by myelosuppressive agents). IV inoculation was used which represents the worst-case scenario where AG013 would enter the blood stream in neutropenic human subject.

- The engineered bacteria are metabolically active in the oral cavity (hamsters) and GI tract (rats and dogs), and that neither the bacteria, nor the secreted hTFF1, enter into the systemic circulation.
- that the hTFF1 expression cassette in healthy hamsters is cleared from the oral cavity and oropharyngeal-GI tract with similar kinetics as the bacteria and confirmed that the expression cassette does not accumulate in tissues and organs that are predisposed by the route of administration.

The repeated dose toxicology studies (3 GLP studies in 2 healthy animal species) up to 3-month duration did not indicated any treatment-related effects. All safety pharmacology and toxicology studies were found to be negative for side effects of administration of *L. lactis* and therefore support safety administration of AG013 to OM patients at risk of developing RT and/or CT induced neutropenia, whose non-intact oral mucosa might represent additional risks for bacteremia.

To date, AG013 has been studied in humans in a Phase 1b study (AG013-ODOM-101) in the US and a Phase 1 pharmacokinetic (PK) study in healthy volunteers (AG013-CSM-MU-004) in Belgium.

The Phase 1b study was a multicenter, single-blinded, placebo-controlled, sequential dose-escalation study that evaluated the safety, tolerability, and PK profile of AG013 in subjects experiencing OM during induction CT for the treatment of HNC [ClinicalTrials.gov Identifier: NCT00938080].

A total of at least 21 subjects were planned to be enrolled in 3 successive groups of at least 7 subjects each (at least 5 subjects were assigned to AG013 and at least 2 subjects were assigned to placebo). An independent data and safety monitoring board (DSMB) reviewed the safety results from each group prior to dose escalation. The study achieved its primary objective by demonstrating that AG013 was generally safe and well tolerated. The incidence of sepsis due to AG013 was followed as an event of special interest in this clinical study and no subjects experienced an adverse event of this type.

The second objective of the study was to evaluate the pharmacokinetics of AG013. In general, live bacterial levels of AG013-sAGX0085 were high immediately following dosing and decreased by 90 minutes post-dose. No differences were noted among the active treatment groups; no dose relationship was seen. In all treatment groups, AG013-sAGX0085 levels were 0 by the End of Study visit. AG013-sAGX0085 could not be detected in blood. No dose frequency-related differences in hTFF1 levels could be detected in saliva or oral mucosa amongst the active treatment groups. Levels of hTFF1 in serum were not significantly different between treatments groups at all time points measured.

The Phase 1 PK study in healthy volunteers was a single-center, open-label Phase 1 study to assess the effect of food/beverage and to characterize the pharmacokinetics of single and multiple oral doses of AG013 in healthy subjects. Ten subjects were enrolled in the study.

AG013 was generally safe when applied by mouth rinse once or three times on one day. Overall, consistent levels of AG013 (bacterial count and protein) could be recovered from the different sample sites in the oral cavity, up to 24 hours after dosing. Furthermore, live AG013 bacteria levels coincided with protein levels. These data demonstrate that live AG013 bacteria adhere to the oral mucosa and actively secrete protein at the mucosal surface. This results in homogeneous exposure to the entire mucosal surface. There was no evidence for systemic exposure neither to live AG013 bacteria (blood) nor to hTFF1 secreted (serum) and there was no recovery of live AG013 bacteria in feces.

Overall, the *in vivo* safety pharmacology studies, and the 2 completed phase I studies support safe administration of AG013 for attenuation of OM in patients with cancer of head and neck receiving concomitant chemoradiation therapy.

2.3.2 Information on the environment (both on the site and in the wider environment):

2.3.2.1 Geographical location and grid reference of the site(s)

Following sites will be initially included in the proposed Phase 2 study:

Belgium:

- University Hospitals Leuven,
  Campus Gasthuisberg, Department of Radiotherapy-Oncology
  Herestraat 49, 3000 Leuven, Belgium

- University Hospital Brussels
  Campus Jette,
  Laarbeeklaan 101, 1090 Brussels, Belgium

- Jules Bordet Institute, Department of Radiotherapy,
  Rue Heger-Bordet 1, 1000 Brussels, Belgium

- Charleroi Grand Hospital (GHDC),
  Grand'Rue 3, 6000 Charleroi, Belgium

- University Hospital Antwerp (UZA),
  Department of Oncology
  Wilrijkstraat 10, 2650 Edegem, Belgium

- St. Maarten General Hospital, Rooeinberg Campus
  Rooienberg 25, 2570 Duffel, Belgium

Oragenics, Inc.
4902 Eisenhower Blvd., Suite 125
Tampa, FL 33634 United States

2.3.2.2 Physical or biological proximity to humans and other significant biota,

The subject will be directly exposed to the bacteria. Once administered through oral rinsing and after residing in the mouth for some time, the bacteria will follow the intestinal flow and be present in the GI tract for a short period. Systemic distribution of the GMO is not anticipated.

Other family members may be exposed when handling empty bottles and possibly material with shed bacteria. Standard hygienic practices should be sufficient to limit or prevent significant exposure. Detailed instructions for dose preparation and dispensing as well as a questions and answers booklet with detailed instructions on the actions to be taken in case of spillage or accident will be provided to subjects.

The bacteria will be released via the sewage system directly (mouth rinse spat out in sink or toilet) or possibly via the evacuation of stool.

2.3.2.3 Proximity to significant biotopes, protected areas, or drinking water supplies,

Given the unpredictable nature of the actual location of the releases, the proximity of significant biotopes, protected areas or drinking water supplies cannot be excluded. However, the only route for exposure would be via the sewage system, which would in any event not be expected to reach such areas. AG013 strain sAGX0085 has no additional features that make exposure more likely, on the contrary, the strict dependence on specific culturing components and the self-eliminating thymine/thymidine dependency makes any exposure even more limited in time.

2.3.2.4 Climatic characteristics of the region(s) likely to be affected,

Not relevant. Similar to standard *L. lactis*.

2.3.2.5 Geographical, geological and pedological characteristics,

Not relevant. Similar to standard *L. lactis*.

2.3.2.6 Flora and fauna, including crops, livestock and migratory species,

Not relevant. Similar to standard *L. lactis*.

2.3.2.7 Description of target and non-target ecosystems likely to be affected,

There is no formal target ecosystem. *L. lactis* bacteria provide a unique delivery system for targeted expression in the mouth and throat of humans. Adherence to oral mucosa is limited in time and the therapeutic function is expressed in a transitional way. Upon the application, some material might be released in the home of the participant, via saliva or less likely via the stool of study subjects.

2.3.2.8 A comparison of the natural habitat of the recipient organism with the proposed site(s) of release,

The propagation of *L. lactis* occurs almost exclusively through growth in particular niches such as raw milk.

The special limiting features of recipient strain MG1363 made it unable to access nutrients present in milk that are essential for its growth: lactose, providing sugars for glycolysis and caseins, as a source for amino acids. MG1363 can therefore no longer survive in the natural niche of *L. lactis* and is confined to artificially supplemented culture conditions.

In addition, the engineered homologous recombination made sAGX0085 completely dependent on external supplementation of thymine/thymidine. This has led to the introduction of the phenomenon known as *thymine-less death*.

Whereas the release environment can be concluded to be similar to that normally encountered for *L. lactis*, the modifications characterizing sAGX0085 ensure that the strain cannot survive in such environment leading to death in even the most optimal condition, devoid of thymidine (Steidler *et al* 2003a).

2.3.2.9 Any known planned developments or changes in land use in the region which could influence the environmental impact of the release.

Not relevant.

2.4 INFORMATION RELATING TO THE INTERACTIONS BETWEEN THE GMOs AND THE ENVIRONMENT

2.4.1 Characteristics affecting survival, multiplication and dissemination

2.4.1.1 Biological features which affect survival, multiplication and dispersal

As pointed out before, sAGX0085 has been designed to show a high level of biological containment:

- *L. lactis* is a poor competitor, only capable of replicating in defined ecological niches such as raw milk. Dispersal is passive. While survival and metabolic activity are possible outside of the specific ecological niche, the bacteria are susceptible to a range of environmental factors resulting in limited survival outside of their common niche.
- MG1363 requires the presence of a carbon source other than lactose and an amino acid source other than casein for survival and multiplication.
- sAGX0085 requires an external source of thymine or thymidine. Absence of these not only results in growth arrest, but actively triggers the phenomenon known as *thymine-less death*.

Steidler and colleagues described how growth and survival of the comparable *L. lactis* strain Thy12 was dependent on external energy, amino acid and thymine/thymidine sources (Steidler et al., 2003b). sAGX0085 only differs from Thy12 in the hTFF1 expression cassette. During development, the growth curves of the different strains were compared under different culture conditions. Growth kinetics clearly showed absolute dependency on thymidine for growth of *thyA*-strains sAGX0085. In the absence of thymidine, no growth was observed for sAGX0085, while in the presence of thymidine, sAGX0085 showed an identical growth profile as MG1363.

Thymine-less death was activated in the absence of thymidine: no viable sAGX0085 cells could be observed after 144 hours of incubation at 30°C.

2.4.1.2 Known or predicted environmental conditions which may affect survival, multiplication and dissemination (wind, water, soil, temperature, pH, etc.)

The lyophilised powder formulation is extremely sensitive to air moisture and ambient temperatures. The rehydrated organism is extremely sensitive to temperatures above 40°C, low pH, air drying, direct sunlight, UV and high salt.

2.4.1.3 Sensitivity to specific agents.

The organism is sensitive to soap, bleaching agents (HClO), antibiotics and high salt.

2.4.2 Interactions with the environment

2.4.2.1 Predicted habitat of the GMOs

The only environment that allows multiplication is artificial laboratory culture, supplementing all required factors. sAGX0085 has lost the capacity to survive outside of the laboratory environment. Transiently, the GMO will be metabolically active in the oropharyngeal-GI tract and will be present in shed and waste material. The number of bacteria will however quickly decline, in optimal growth medium, albeit without thymidine approximately 1 log per 10 hours (unpublished data).

2.4.2.2 Studies of the behaviour and characteristics of the GMOs and their ecological impact carried out in simulated natural environments, such as microcosms, growth rooms, greenhouses

In vitro and *in vivo* studies comparing growth characteristics of cultures of wild type *L. lactis*, MG1363 and sAGX0085 have been reported, confirming the dependency on specific factors. Studies in rats, in hamsters, in dogs and in man confirmed the predicted behaviour and characteristics of sAGX0085 (Section 2.3.1.11).

2.4.2.3 Genetic transfer capability

a) Post-release transfer of genetic material from GMOs into organisms in affected ecosystems

Exchange of genetic material between bacteria mostly occurs through plasmids. While different *L. lactis* strains, including those used in the dairy industry, can harbour several plasmids, MG1363 and AG013 have lost the 5 original plasmids present in the natural isolate. In addition, *L. lactis* MG1363 and AG013 do not contain conjugative transposons and AG013 is also thyA deficient, preventing phage replication (Pedersen *et al*., 2002). Therefore, transduction of modified genetic material via phages is very unlikely.

Conjugative transposons, such as *Tn916,* are elements that transpose during conjugation from a donor cell harbouring the element to a recipient cell. Conjugative transposons have a broad host range: they are not only able to conjugatively transpose with frequencies of 10^{-4} to 10^{-9} among almost all species of gram-positive bacteria that have been investigated but can also transpose among gram-negative bacteria.

The first example of a limitation on the promiscuity of conjugative transposons is presented by *L. lactis* MG1363. In this strain, *Tn916* and *Tn919* do not excise (Bringel et al., 1992). Although the MG1363 strain can act as a recipient for conjugative transposition from another genus, it has not been found to donate conjugative transposons in plate matings with *Bacillus subtilis*, *Enterococcus faecalis* or *Streptococcus pyogenes*. In intrageneric matings between *L. lactis* MG1363 derivatives, transconjugants can be established but the transposons will be present in the same location in the transconjugant chromosome as in the donor genome, indicating that no

transposition has occurred. It is thought that *L. lactis* MG1363 lacks a factor required for excision of conjugative transposons (Bringel et al., 1991).

Conjugative transfer of selectable, chromosomal markers from MG1363 to MG1363 derivatives has been reported for lactococcal fertility factor (*Laff*) (Bringel *et al*., 1991). *Laff* is speculated to be identical to Clu/sex-factor Stentz et al., (2004) however summarize the general knowledge that a) over a wide range of bacterial genera, cell aggregation provides the first cell-to-cell contact that is necessary for conjugal transfer; and b) cell aggregation has only been observed following sex factor and lactose plasmid cointegration. The lactose plasmid in MG1363 is absent (Gasson, 1983, Wegmann *et al.,* 2007) as confirmed by full genome sequencing of MG1363. It is highly unlikely that MG1363 or its derivatives can serve as a conjugative donor, and, especially non-selectable, chromosomal traits could propagate into potential recipient (i.e. MG1363 related) populations.

It is possible that genetic elements could be released in the environment upon lysis of *L. lactis* and might be taken up by other bacteria. In the case of the GMO, the likelihood of release of intact naked DNA is reduced as thymine-less death triggers the degradation of DNA before the actual cell lysis.

None of the genetic modifications made to wild type *L. lactis* during construction of AG013 would be expected to enable the transfer or maintenance of genetic material into the environment (outside its obligate host species).

b) Post-release transfer of genetic material from indigenous organisms to the GMOs;

For this particular GMO, the only relevant risk is transfer of an intact *thyA* inwards (Steidler et al., 2003b).

Under artificial laboratory conditions, a vector plasmid carrying an intact *thyA* has been shown to compensate a *thyA* mutation following electroporation into different bacteria (Ross et al., 1990). However, this experimental scenario is unlikely in *L. lactis* bacteria, as they are not known to be naturally competent and therefore will not take up foreign DNA from other bacteria (Wydau et al., 2006).

To the best of our knowledge, in the *Bacteriae* and *Archaeae*, *thyA* genes do not reside on plasmids, so plasmid-borne mobility of *thyA* inwards seems impossible. Closely related *Lactococcus* species could mobilise DNA into the GMO but again, mobile elements are not nearby. Moreover, successful establishment of donor DNA would require double homologous recombination, which would resolve the GM trait.

Steidler and colleagues evaluated the possible integration of an intact wild type *thyA* sequence in strain Thy12, requiring double homologous recombination over the transgene and essentially removing the transgene (Steidler et al., 2003b). Donor bacteria were *L. lactis* MG1363, *L. lactis*

subsp. *lactis* and subsp. *cremoris*, *Lactobacillus casei*, *Escherichia coli* subsp. DH5alpha and O157, and *Salmonella choleraesuis*. In none of the cases, forced acquisition of *thyA* from other microorganisms could be demonstrated, most likely due to high sequence diversity at *thyA* loci.

Compensation of the *thyA* deletion would be insufficient to circumvent the other specific metabolic requirements of *L. lactis* strain sAGX0085 and its parent organism (inability to degrade lactose and casein). Also, because *lactococci* are not documented to multiply in the GI tract, there is no selective pressure on the acquisition of *thyA*.

2.4.2.4 Likelihood of post-release selection leading to the expression of unexpected and/or undesirable traits in the modified organism,

AG013 sAGX0085 strain is equivalent to MG1363 and its wild type non-pathogenic ancestor *L. lactis* subsp. *cremoris* NCDO 712 (Gasson, 1983). There are no indications of expression of unexpected and/or undesirable traits. The introduced as well as the eliminated function are fully documented, and epigenetic effects can be excluded.

2.4.2.5 Measures employed to ensure and to verify genetic stability. Description of genetic traits which may prevent or minimise dispersal of genetic material. Methods to verify genetic stability

The *htff1* gene was stably integrated on the chromosome.

Analysis of the genetic stability of *L. lactis* strain sAGX0085, obtained by repeated sequential dilution and growth to saturation, was performed after a minimum 100 generations of growth. The genetic stability was analysed by four parameters:

- Inability of sAGX0085 to grow in thymidine-deficient medium.
- Unchanged hTFF1 secretion by sAGX0085.
- PCR analysis of the modified thyA locus of sAGX0085.
- DNA sequence verification of the [Pxxx>>SSusp45>>hTFF1] expression cassette of *L. lactis* strain sAGX0085.

The experiment confirmed genetic stability for all of these parameters.

During the preparation of the formulated product, quality control is included to confirm that the material corresponds to the strain description. The IMP is Qualified Person (QP) released for use in the clinical trial when it meets the defined specifications only when manufacturing, Quality Control and packaging has been performed in accordance with the manufacturing instructions, analytical methods and specifications and full compliance with Good Manufacturing Practice (cGMP) requirements.

2.4.2.6 Routes of biological dispersal, known or potential modes of interaction with the disseminating agent, including inhalation, ingestion, surface contact, burrowing, etc.

Following administration, the dispersal of AG013 is essentially passive. The MR will release the bacteria in the oral cavity. When participants spit out the bacterial suspension in the sink or toilet, after the prescribed contact time, some remaining bacteria will follow the digestive route. The majority will however directly enter the sewage system. When swallowed they will then follow the digestive tract moving along with the faecal stream, without intestinal colonisation.

The main route for dispersal is therefore the sewage system, either directly or potentially via disposal of faeces although this is expected to be limited as discussed previously. This environment is very aggressive for AG013 sAGX0085 bacterial strain that lacks all necessary components for survival and multiplication. Furthermore, stability studies have shown that the lyophilised powder needs to be stored in tightly sealed aluminium bags (vapour barrier), protected from moisture and refrigerated (2-8°C) in order to preserve quality. In liquid conditions under room temperature, a log 6 reduction of the number of bacteria is observed within 8 hours.

To a limited extend, contact and thereby dispersal can occur through:

- contact with emptied bottles (MR container) and other waste material,
- contact with clothes and textiles that have been in touch with saliva or potentially faecal material,
- hygienic material,
- hands

Additionally, contact could occur in case of accidental spillage during reconstitution or during administration, or due to rupture of the pre-packed product. The quantity of such spillage will normally be limited to one treatment dose.

In all of these cases, survival of the bacteria will be extremely limited in time and during this period, the very limited amount of bacteria potentially present will not be metabolically active as mentioned previously in Section 2.2.1.

2.4.2.7 Description of ecosystems to which the GMOs could be disseminated,

During the limited survival time of the bacteria, the most likely environment in which the GMO will be present is the sewage system. Given other potential limited exposure routes, the GMO is likely to be present in the normal living environment of the participant. None of these ecosystems promote the survival or multiplication of AG013 sAGX0085 bacterial strain as mentioned previously in Section 2.2.1.

2.4.2.8 Potential for excessive population increase in the environment

Given the inherent limited potential for population increase of *L. lactis* and the additional biological containment features, no excessive population increase is expected.

2.4.2.9 Competitive advantage of the GMOs in relation to the unmodified recipient or parental organism(s)

As pointed out, AG013 sAGX0085 bacterial strain is even more restricted in its capacity to survive and to multiply than the recipient organism. hTFF1 production is not influencing any reaction that affects the recipient.

2.4.2.10 Identification and description of the target organisms if applicable,

The proposed indication is to reduce the signs and the symptoms of radiotherapy (RT) and/or CT induced oral mucositis (OM) in cancer patients.

2.4.2.11 Anticipated mechanism and result of interaction between the released GMOs and the target organism(s) if applicable,

Not applicable.

2.4.2.12 Identification and description of non-target organisms which may be adversely affected by the release of the GMO, and the anticipated mechanisms of any identified adverse interaction

AG013 sAGX0085 bacterial strain will be present in compartments which are natural for *L. lactis,* essentially the human GI tract and sewage system. With the exception of *htff1*, no other change has occurred and therefore it is expected that the impact will be similar to that of *L. lactis.* Again, the possible interactions will be more limited given the specific biological containment features and reduced life expectation of sAGX0085.

hTFF1 binds to salivary mucins and forms a mucus layer over the epithelia of the mouth, acting as a physical barrier against bacteria and noxious environmental agents. Moreover, TFF peptides have wound-healing properties and are important in protecting and healing mucosal tissues. In the pre-clinical trials healthy and diseased hamsters, rats and dogs have been administered AG013 without adverse effects. No local or systemic pathogenic effects were documented after chronic administration of AG013.

2.4.2.13 Likelihood of post-release shifts in biological interactions or in host range,

If there is any shift in interactions as compared to the wild type, the GMO will be reduced in its capacities.

2.4.2.14 Known or predicted interactions with non-target organisms in the environment, including competitors, preys, hosts, symbionts, predators, parasites and pathogens

No specific interactions with non-target organisms have been identified. As discussed in Section 2.4.2.3(b), bacteriophages cannot replicate in *ThyA*-deficient hosts**.**

2.4.2.15 Known or predicted involvement in biogeochemical processes

No involvement in biogeochemical processes has been identified.

2.4.2.16 Other potential interactions with the environment

No other potential interactions with the environment have been identified.

2.5 INFORMATION ON MONITORING, CONTROL, WASTE TREATMENT AND EMERGENCY RESPONSE PLANS

2.5.1 Monitoring techniques

2.5.1.1 Methods for tracing the GMOs, and for monitoring their effects,

To detect sAGX0085, a quantitative method has been developed based on the detection of the synthetic *htff1* gene which uniquely identifies the GMO.
The *htff1* gene allows for the detection and quantification of total numbers of GM bacteria (live and dead), while a viable count assay allows for the detection and quantification of total numbers of live bacteria. These data provide a ratio between live and dead bacteria. The *htff1* gene in the GM *Lactococcus* is a unique, synthetic gene which can be distinguished from native *htff1*.

During the treatment period and in a subset of study subjects, monitoring will be done by taking blood and mucosa samples to be analysed for the presence of viable AG013 bacteria (live and dead), using the viable count assay and the AG013-specific quantitative (Q-)PCR method, and hTFF1 using ELISA.

2.5.1.2 Specificity (to identify the GMOs, and to distinguish them from the donor, recipient or, where appropriate, the parental organisms), sensitivity and reliability of the monitoring techniques

The sensitivity of the method based on *htff1* DNA quantification is $3x10^3$ *Lactococcus lactis* equivalents (LLeq) per ml. The specificity of this assay is guaranteed by using hTFF1-specific primers and probe.

2.5.1.3 Techniques for detecting transfer of the donated genetic material to other organisms

To detect the hypothetical transfer of donated genetic material to other organisms, PCR of the *htff1* gene can be used.

2.5.1.4 Duration and frequency of the monitoring.

During the clinical trial, in the event of symptoms suggesting clinically significant bacteraemia or sepsis, the GMO will be monitored via detection using a qPCR method for *htff1* gene in blood samples. The subject will be treated per the site's standard of care. Prior to treatment of the event, 3 consecutive whole blood samples need to be taken from the subject and aliquots of these samples must be sent immediately for analysis. The first two samples are to be drawn together and the third sample is to be drawn 10-15 minutes after the first tube is drawn.

2.5.2 Control of the release

2.5.2.1 Methods and procedures to avoid and/or minimise the spread of the GMOs beyond the site of release or the designated area for use,

The GMO is highly biologically contained. The lyophilised powder is furthermore sensitive to air moisture and ambient temperatures. The rehydrated organism is extremely sensitive to temperatures above 40°C, low pH, air drying, direct sunlight, ultraviolet light (UV) and high salt. Therefore, when released into the environment, it will not be able to survive.

The AG013 lyophilised powder is packed in child-proof bottles. The study subject will be instructed how to prepare and administer the AG013 MR. The first time AG013 will be administered in the clinical study centre under supervision. Detailed instructions for dose preparation and dispensing as well as a questions and answers booklet with detailed instructions on the study medication. The participant will receive supply of the IMP for only one week (+ 1 spare day), which reduces the amount of GMO held by each patient at one given time.

All empty bottles that may still contain traces of the GM material will be collected by the study subject and returned to the clinical trial centre. This specified in the detailed instructions for dose preparation and dispensing as well as the questions and answers booklet the subjects receive.

Once administered, the bacteria will be spat out in the sink or toilet and follow the faecal flow. At the same time, the bacterial population will not be multiplying and will be eliminated within a period ranging from 8 to 72 hours depending on the circumstances due to the biological containment features. In consequence, no additional measures are foreseen.

The main route for dispersal is therefore the sewage system, either directly or potentially via disposal of faeces. This environment is very aggressive for AG013 bacterial strain sAGX0085, lacking all necessary components for survival and multiplication. Furthermore, stability studies have shown that the lyophilised powder needs to be stored in tightly sealed aluminium bags (vapour barrier), protected from moisture and refrigerated (2-8°C) in order to preserve quality. In liquid conditions under room temperature, a log 6 reduction of the number of bacteria is observed within 8 hours.

2.5.2.2 Methods and procedures to protect the site from intrusion by unauthorised individuals,

Until administration, the material is either stored under controlled conditions at the clinical study centre in a secure area or by the study subject. In both cases, it can be assumed that unauthorised individuals will not have free access to the material.

The product label meets EU GMP Annex 13 labelling requirements and any country specific requirements for an IMP. The patient dosing instructions include reference to the IMP being a

GMO and provides essential information alongside the patient's Q&A booklet to minimise the risk of transmission to an unintended individual.

Risks are mitigated by using glass bottles with tamper-evident, child-resistant screw caps and the need to mix the different components for a given dose.

Once applied, no further protection is foreseen. Material that enters the sewage system is no longer protected, except for the general measures that usually prevent access to the sewage system for the public at large.

The main route for dispersal is therefore the sewage system, either directly or via disposal of faeces. This environment is very aggressive for AG013 bacterial strain sAGX0085, lacking all necessary components for survival and multiplication. Furthermore, stability studies have shown that the lyophilised powder needs to be stored in tightly sealed aluminium bags (vapour barrier), protected from moisture and refrigerated (2-8°C) in order to preserve quality. In liquid conditions under room temperature, a log 6 reduction of the number of bacteria is observed within 8 hours.

2.5.2.3 Methods and procedures to prevent other organisms from entering the site.

At the clinical study centre, standard precautions are in place to avoid other organisms (pets, insects, rodents, etc.) from entering the site and the storage equipment. Once provided to the participant, family members and visitors, pets, insects and rodents might be present in or close to the facility (expected to be the participant's home). During storage, the exposure would be very limited, except for cases of accidental release form the package. Once administered, they might get exposed by contact with contaminated materials or with the sewage system. None of these organisms however are expected to influence the behaviour of sAGX0085 or to act as a vector.

2.5.3 Waste treatment

2.5.3.1 Type of waste generated,

Two types of waste that are expected to carry living sAGX0085 are identified:

- Materials that have been exposed to GMO material (*e.g.* empty bottles, wipes, etc.)
- Spat out MR and faeces, hygienic wipes, disposed of in sewage system.

In addition, other materials could be temporarily holding bacteria, *e.g.* handkerchiefs.

2.5.3.2 Expected amount of waste

The calculation of the waste of 70 (35 on active treatment) participants in Europe is difficult to determine The amount of AG013 is quickly diluted in the specific sewage system. Estimating the total amount of waste generated at all the sites of release (in all the countries where the study will

be conducted) in the clinical study for e.g. empty bottles, one can predict a total of 6,615 empty bottles

2.5.3.3 Description of treatment envisaged

The participant will return all used and unused bottles to the study site during the weekly visits. The bottles will be inactivated as hazardous medical waste according to the institutional standards. It should be noted that this is an ultimate precaution, as it can be expected that by the time of return, no more living sAGX0085 will be present.

Other materials should be disinfected according to standard medical procedures suitable for the equipment or handled according to standard hygienic procedures (*e.g.* washing of exposed textiles using standard household product).

2.5.4 Emergency response plans

2.5.4.1 Methods and procedures for controlling the GMOs in case of unexpected spread

Unexpected spread would mainly be limited to accidental opening of the packaged materials, releasing the lyophilised powder or the suspended liquid. Release of lyophilised powder is very unlikely given the design of the package. Individual bottles may be forced open. In both cases, it would concern a very small dose. Spillage from suspended liquid is probably more likely, but again would only concern a very limited quantity (the total suspension equals 15 ml per dose containing 2×10^{11} CFU). In case of such an accidental spread, use of standard detergent (soap) or bleach ("javel") immediately will completely eradicate the GMOs and decontaminate the affected area. As pointed out before, sAGX0085 is short-lived when dissolved in water at room temperature. Any additional cleaning treatment will ensure that additional spread is prevented.

Although it is expected that this is a standard practice both in the clinical study centre and at the participant's home, special instructions will be provided to this end.

2.5.4.2 Methods for decontamination of the areas affected, for example eradication of the GMOs

In the case of an unexpected spread at the site of release, standard detergent (soap) or bleach ("javel") should be used immediately and will completely eradicate the GMOs and decontaminate the affected area.

2.5.4.3 Methods for disposal or sanitation of plants, animals, soils, etc., that were exposed during or after the spread,

It is expected that the biological containment system will limit the spread is space and time. Material that has been in contact with sAGX0085 will be either disinfected or inactivated as hazardous medical waste. No specific sanitation measures are foreseen.

2.5.4.4 Methods for the isolation of the area affected by the spread,

During application, the participants could be isolated by ensuring that all shed material is collected. However, in the previous Phase 1b study, safety and biological containment was confirmed and as such this approach is therefore not warranted. In addition, this would not be practical given the longer duration and the larger number of participants. However, if a counter indication is observed, the participants might be treated in this way. Once the material is released in the sewage system, isolation would be impossible to achieve and is not necessary, as the risk to personnel and the environment is negligible.

2.5.4.5 Plans for protecting human health and the environment in case of the occurrence of an undesirable effect

The bacteria can be inactivated with several treatments. Furthermore, the biological containment system is expected to eliminate the bacteria in a short period after the release. In addition, there are no indications of possible undesirable effects on the environment.

As pointed out in Section 2.3.1.11, *L. lactis* sAGX0085 is sensitive to most groups of commonly used antibiotics.

2.6 REFERENCES

Alpert, C.A., D.D. Mater, M.C. Muller, M.F. Ouriet, Y. Duval-Iflah, and G. Corthier. 2003. Worst-case scenarios for horizontal gene transfer from Lactococcus lactis carrying heterologous genes to Enterococcus faecalis in the digestive tract of gnotobiotic mice. Environmental biosafety research. 2:173-180.

Appel, M.R., A; Wolthoorn, J. 2008a A subacute (14-day) oral (gavage) toxicity study, including a Comet assay, with a recombinant human Trefoil Factor 1 producing Lactococcus lactis (sAGX0085) in rats. TNO Final report, 7998/02.

Appel, M.R., A; Wolthoorn, J. 2008b A subacute (14-day) oral (gavage) toxicity study, including a Comet assay, with a recombinant human Trefoil Factor 1 producing *Lactococcus lactis* (sAGX0085) in rats. TNO Final report, 7998/02.

Bahrndorff, S., N. de Jonge, H. Skovgard, and J.L. Nielsen. 2017. Bacterial Communities Associated with Houseflies (Musca domestica L.) Sampled within and between Farms. PLoS One. 12:e0169753.

Barner, H.D., and S.S. Cohen. 1954. The induction of thymine synthesis by T2 infection of a thymine requiring mutant of Escherichia coli. Journal of bacteriology. 68:80-88.

Braat, H., P. Rottiers, D.W. Hommes, N. Huyghebaert, E. Remaut, J.P. Remon, S.J. van Deventer, S. Neirynck, M.P. Peppelenbosch, and L. Steidler. 2006. A phase I trial with transgenic bacteria expressing Interleukin-10 in Crohn's disease. Clinical Gastroenterology and Hepatology. 4:754-759.

Bringel, F., G.L. Van Alstine, and J.R. Scott. 1991. A host factor absent from Lactococcus lactis subspecies lactis MG1363 is required for conjugative transposition. Mol Microbiol. 5:2983-2993.

Bringel, F., G.L. Van Alstine, and J.R. Scott. 1992. Transfer of Tn916 between Lactococcus lactis subsp. lactis strains is nontranspositional: evidence for a chromosomal fertility function in strain MG1363. Journal of bacteriology. 174:5840-5847.

Buist G, Kok J, Leenhouts KJ, Dabrowska M, Venema G, Haandrikman AJ. Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of Lactococcus lactis, a muramidase needed for cell separation. J Bacteriol. 1995 Mar;177(6):1554-63.

Cho I, Blaser MJ. 2012. The human microbiome: at the interface of health and disease. Nat Rev Genet. Mar 13;13(4):260-70.

Caluwaerts, S., 2009. *In vivo efficacy of the environmental containment strategy for L. lactis sAGX0085.* Electronic data on file; ActoGeniX Final Report SICAL09001.

Caluwaerts, S., 2012. Pharmacological and Safety Evaluation of a Novel AG013 Formulation in the Acute Radiation Model of Oral Mucositis in Hamsters; Pharmacokinetic report. Electronic data on file; ActoGeniX Final Report SICAL12002.

Coulie B. A Phase 1b, multi-center, single blinded, placebo-controlled, sequential dose escalation study to assess the safety and tolerability of topically applied AG013 in subjects receiving induction chemotherapy for the treatment of cancers of the head and neck. Final Clinical Study Report (CSR) 2012.

Coulie, B., A single-center, open-label Phase 1 study to assess the effect of food/beverage and to characterize the pharmacokinetics of single and multiple oral doses of AG013 in Healthy Subjects. Final Clinical Study Report (CSR) 2013.

Chamberlain, L.M., K. Robinson, J.M. Wells, and R.W.F. Le Page. 1995. Immunogenicity of glutathione S-transferase (P28) from Schistosoma mansoni expressed in Lactococcus lactis. Immunology. 86:139.

De Fabrizio S.V., Parada J.L., and L. R.A. 1994. Antibiotic resistance of Lactococcus lactis : an approach of genetic determinants location through model system. Microbiologie, aliments, nutrition. 12:307-315.

de Lacerda, J.R.M., T.F. da Silva, R.E. Vollu, J.M. Marques, and L. Seldin. 2016. Generally recognized as safe (GRAS) Lactococcus lactis strains associated with Lippia sidoides Cham. are able to solubilize/mineralize phosphate. Springerplus. 5:828.

de Ruyter PG, Kuipers OP, de Vos WM. 1996. Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. Appl Environ Microbiol. Oct;62(10):3662-7.

de Vos, W.M., P. Vos, H. de Haard, and I. Boerrigter. 1989. Cloning and expression of the Lactococcus lactis subsp. cremoris SK11 gene encoding an extracellular serine proteinase. Gene. 85:169-176.

Drouault, S., G. Corthier, S.D. Ehrlich, and P. Renault. 1999. Survival, physiology, and lysis of Lactococcus lactis in the digestive tract. Applied and environmental microbiology. 65:4881-4886.

EFSA. 2017. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA Journal. 15:1-177.

Elting LS, Keefe DM, Sonis ST, Garden AS, Spijkervet FK, Barasch A, TishlerRB, Canty TP, Kudrimoti MK, Vera-Llonch M; 2008. Burden of Illness Head and NeckWriting Committee. Patient-reported measurements of oral mucositis in head andneck cancer patients treated with radiotherapy with or without

chemotherapy:demonstration of increased frequency, severity, resistance to palliation, andimpact on quality of life. Cancer;. 113(10):2704-13.

Elliott, J.A., M.D. Collins, N.E. Pigott, and R.R. Facklam. 1991. Differentiation of Lactococcus lactis and Lactococcus garvieae from humans by comparison of whole-cell protein patterns. Journal of clinical microbiology. 29:2731-2734.

Epstein JB. 2007. Mucositis in the cancer patient and immunosuppressed host. Infect Dis Clin North Am. 21(2):503-22, vii.

Gasson, M.J. 1983. Plasmid complements of Streptococcus lactis NCDO 712 and other lactic streptococci after protoplast-induced curing. Journal of bacteriology. 154:1-9.

Gilbert, C., K. Robinson, R.W. Le Page, and J.M. Wells. 2000. Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in Lactococcus lactis. Infection and immunity. 68:3251-3260.

Gruzza, M., M. Fons, M.F. Ouriet, Y. Duval-Iflah, and R. Ducluzeau. 1994. Study of gene transfer in vitro and in the digestive tract of gnotobiotic mice from Lactococcus lactis strains to various strains belonging to human intestinal flora. Microb Releases. 2:183-189.

Hanby, A.M., R. Poulsom, S. Singh, G. Elia, R.E. Jeffery, and N.A. Wright. 1993. Spasmolytic polypeptide is a major antral peptide: distribution of the trefoil peptides human spasmolytic polypeptide and pS2 in the stomach. Gastroenterology. 105:1110-1116.

Hirsch, A. 1952. The evolution of the lactic streptococci. J. Dairy Research. 19:290-293.

Hirt, H., S.L. Erlandsen, and G.M. Dunny. 2000. Heterologous inducible expression of Enterococcus faecalis pCF10 aggregation substance asc10 in Lactococcus lactis and Streptococcus gordonii contributes to cell hydrophobicity and adhesion to fibrin. Journal of bacteriology. 182:2299-2306.

Klijn, N., A.H. Weerkamp, and W.M. de Vos. 1995a. Detection and characterization of lactose-utilizing Lactococcus spp. in natural ecosystems. Applied and environmental microbiology. 61:788-792.

Klijn, N., A.H. Weerkamp, and W.M. de Vos. 1995b. Genetic marking of Lactococcus lactis shows its survival in the human gastrointestinal tract. Applied and environmental microbiology. 61:2771-2774.

Kunji, E.R., A. Hagting, C.J. De Vries, V. Juillard, A.J. Haandrikman, B. Poolman, and W.N. Konings. 1995. Transport of beta-casein-derived peptides by the oligopeptide

transport system is a crucial step in the proteolytic pathway of Lactococcus lactis. J Biol Chem. 270:1569-1574.

Kuipers OP, Beerthuyzen MM, Siezen RJ, De Vos WM. Characterization of the nisin gene cluster nisABTCIPR of Lactococcus lactis. Requirement of expression of the nisA and nisI genes for development of immunity. Eur J Biochem. 1993 Aug 15;216(1):281-91.

Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts. 1995. A system to generate chromosomal mutations in Lactococcus lactis which allows fast analysis of targeted genes. Journal of bacteriology. 177:7011-7018.

Mirabile A, Airoldi M, Ripamonti C, Bolner A, Murphy B, Russi E, Numico G, Licitra L, Bossi P. 2016. Pain management in head and neck cancer patients undergoing chemo-radiotherapy: Clinical practical recommendations. Crit Rev Oncol Hematol. Mar;99:100-6.

Opazo, R., F. Gajardo, M. Ruiz, and J. Romero. 2016. Genome Sequence of a Lactococcus lactis Strain Isolated from Salmonid Intestinal Microbiota. Genome Announc. 4.

Pedersen, M.B., P.R. Jensen, T. Janzen, and D. Nilsson. 2002. Bacteriophage resistance of a deltathyA mutant of Lactococcus lactis blocked in DNA replication. Applied and environmental microbiology. 68:3010-3023.

Podolsky, D.K., K. Lynch-Devaney, J.L. Stow, P. Oates, B. Murgue, M. DeBeaumont, B.E. Sands, and Y.R. Mahida. 1993. Identification of human intestinal trefoil factor. Goblet cell-specific expression of a peptide targeted for apical secretion. J Biol Chem. 268:6694-6702.

Prinsen, M.W., M. 2012. A sub-chronic (3-month) repeated dose oral toxicity study, including a 4-week recovery period, with a recombinant human Trefoil Factor 1 producing Lactococcus lactis sAGX0085 (AG013_sAGX0085) in rats. TNO Final Report, V 2009/01.

Rio, M.C., J.P. Bellocq, J.Y. Daniel, C. Tomasetto, R. Lathe, M.P. Chenard, A. Batzenschlager, and P. Chambon. 1988. Breast cancer-associated pS2 protein: synthesis and secretion by normal stomach mucosa. Science. 241:705-708.

Robinson, K., L.M. Chamberlain, K.M. Schofield, J.M. Wells, and R.W. Le Page. 1997. Oral vaccination of mice against tetanus with recombinant Lactococcus lactis. Nat Biotechnol. 15:653-657.

Ross, P., F. O'Gara, and S. Condon. 1990. Thymidylate synthase gene from Lactococcus lactis as a genetic marker: an alternative to antibiotic resistance genes. Applied and environmental microbiology. 56:2164-2169.

Rungrassamee, W., A. Klanchui, S. Maibunkaew, S. Chaiyapechara, P. Jiravanichpaisal, and N. Karoonuthaisiri. 2014. Characterization of intestinal bacteria in wild and domesticated adult black tiger shrimp (Penaeus monodon). PLoS One. 9:e91853.

Sonis ST. 2010. New thoughts on the initiation of mucositis. Oral Dis. Oct;16(7):597-600.

Sonis, S. and B. Watkins, Pharmacological and Safety Evaluation of a Novel AG013 Formulation in the Acute Radiation Model of Oral Mucositis in Hamsters. Biomodels Final Report AGX-12, 2012

Stark, P., and J.M. Sherman. 1935. Concerning the Habitat of Streptococcus lactis. Journal of bacteriology. 30:639-646.

Steidler, L., S. Neirynck, N. Huyghebaert, V. Snoeck, A. Vermeire, B. Goddeeris, E. Cox, J.P. Remon, and E. Remaut. 2003a. Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. Nat Biotechnol. 21:785-789.

Steidler, L., S. Neirynck, N. Huyghebaert, V. Snoeck, A. Vermeire, B. Goddeeris, E. Cox, J.P. Remon, and E. Remaut. 2003b. Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. Nat. Biotechnol. 21:785-789.

Steidler, L. 2008. Determination of genome sequence of sAGX0085. Electronic data on file, ActoGeniX Final Report KLVAN08033.

Stentz R, Jury K, Eaton T, Parker M, Narbad A, Gasson M, Shearman C. 2004. Controlled expression of CluA in Lactococcus lactis and its role in conjugation. Microbiology. Aug;150(Pt 8).

Taupin, D., and D.K. Podolsky. 2003. Trefoil factors: initiators of mucosal healing. Nat Rev Mol Cell Biol. 4:721-732.

Trotti A, Bellm LA, Epstein JB, Frame D, Fuchs HJ, Gwede CK, Komaroff E, Nalysnyk L, Zilberberg MD. 2003. Mucositis incidence, severity and associated outcomes in patients with head and neck cancer receiving radiotherapy with or withoutchemotherapy: a systematic literature review. Radiother Oncol. 66(3):253-62

van Asseldonk, M., G. Rutten, M. Oteman, R.J. Siezen, W.M. de Vos, and G. Simons. 1990. Cloning of usp45, a gene encoding a secreted protein from Lactococcus lactis subsp. lactis MG1363. Gene. 95:155-160.

Vandenbroucke, K. 2008a. Antibiotic susceptibility - resistance profile of sAGX0085. Electronic data on file; ActoGeniX Final Report KLVAN08032.

Vandenbroucke, K. 2008b. Construction and selection of strain sAGX0085- AGX n°654. . Electronic data on file; ActoGeniX Final Report KLVAN08028.

Vandenbroucke, K. 2008c. Genetic stability of sAGX0085. Electronic data on file, ActoGeniX Final Report KLVAN08036.

Vandenbroucke, K. 2009. Viability of MG1363, sAGX0037, sAGX0085 and Staphylococcus aureus in pooled, complement-preserved human serum. Electronic data on file; ActoGeniX Final Report KLVAN09001.

Vandenbroucke, K., W. Hans, J. Van Huysse, S. Neirynck, P. Demetter, E. Remaut, P. Rottiers, and L. Steidler. 2004. Active delivery of trefoil factors by genetically modified Lactococcus lactis prevents and heals acute colitis in mice. Gastroenterology. 127:502-513.

Vanhoenacker, P. 2009. A toxicity study with sAGX0085 in Beagle dogs and rats (TNO study V7998/01 and V7998/02): additional data on exposure. Electronic data on file; ActoGenix Final Report PEVAN09001.

Vera-Llonch M, Oster G, Hagiwara M, Sonis S. 2006. Oral mucositis in patients undergoing radiation treatment for head and neck carcinoma. Cancer. Jan 15;106(2):329-36.

Vera-Llonch M, Oster G, Ford CM, Lu J, Sonis S. 2007. Oral mucositis and outcomes of autologous hematopoietic stem-cell transplantation following high-dose melphalan conditioning for multiple myeloma. J Support Oncol. May;5(5):231-5.

Villa A, Sonis ST. Mucositis: 2015. Pathobiology and management. Curr Opin Oncol. May;27(3):159-64.

Wegmann U, O'Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C, Ventura M, Goesmann A, Gasson MJ, Kuipers OP, van Sinderen D, Kok J. 2007 Complete genome sequence of the prototype lactic acid bacterium Lactococcus lactis subsp. cremoris MG1363. J Bacteriol. Apr;189(8).

Wells, J.M., P.W. Wilson, P.M. Norton, M.J. Gasson, and R.W. Le Page. 1993. Lactococcus lactis: high-level expression of tetanus toxin fragment C and protection against lethal challenge. Mol Microbiol. 8:1155-1162.

Wydau, S., R. Dervyn, J. Anba, S. Dusko Ehrlich, and E. Maguin. 2006. Conservation of key elements of natural competence in Lactococcus lactis ssp. FEMS Microbiol Lett. 257:32-42.

Xin, K.Q., Y. Hoshino, Y. Toda, S. Igimi, Y. Kojima, N. Jounai, K. Ohba, A. Kushiro, M. Kiwaki, K. Hamajima, D. Klinman, and K. Okuda. 2003. Immunogenicity and protective efficacy of orally administered recombinant Lactococcus lactis expressing surface-bound HIV Env. Blood. 102:223-228.