

New type of *Streptococcus agalactiae* serotype Ia bacterium and vaccines thereof

Field of the invention

The invention relates to the field of Aqua culture, especially to the farming of Tilapia. In particular the invention relates to a bacterium of a new type of *Streptococcus agalactiae* serotype Ia (Sa Ia). Further the invention regards preparations, compositions, and vaccines of the new Sa Ia bacteria or of its descendants, regards a diagnostic kit, and regards (medical) uses and methods of preparations, compositions, and vaccines of the new Sa Ia bacteria.

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Background of the invention

Streptococcus is a genus of gram-positive, non-motile cocci in the order Lactobacillales. The genome size is about 2 Mb, and *Streptococci* are categorised by phylogenetic parameters in one of 6 groups based on their 16S rRNA sequences.

15 Previously, phenotypical classification of *Streptococcus* species was based on their haemolytic properties. Also a taxonomic classification based on the carbohydrate composition of cell-wall antigens according to the so-called 'Lancefield grouping' is applied, into (currently) 15 groups.

Further subtyping is based on the serological testing of capsular polysaccharides which are type-specific and important virulence factors. Currently 10 serotypes have been described to occur: Ia, Ib, 20 and II through IX. Such subtyping can also be done using different types of genotyping, e.g. by polymerase chain reaction (PCR) on the genes for capsular polysaccharide synthesis.

The species *Streptococcus agalactiae* (Sa) is taxonomically classified in the group of *S. pyogenes*. It is catalase-negative, and facultative anaerobe.

25 Sa are subclassified in Lancefield group B, hence its colloquial referral as: 'group B streptococci' (GBS). Sa are pathobionts that normally live as commensals, but under certain conditions can be pathogenic and cause invasive infection and disease in humans and in a variety of animals; this may lead to sepsis and several organs can be affected.

30 Sa derived its name from its prominent role in bovine mastitis. However Sa can also be pathogenic to other animals, e.g. Sa is a prominent pathogen for fish, e.g. Tilapia, especially when farmed in large groups in aquaculture. Of the known Sa serotypes, so far four (Ia, Ib, II and III) also occur in fish, and these are zoonotic, making Sa a foodborne human pathogen. A review is given by Zang (2001, Pathogens, vol. 10, p. 558). Sa of serotype Ib are non-haemolytic (a.k.a. gamma-haemolytic), and Sa Ia 35 can be alpha- or gamma-haemolytic.

Tilapias are fish in the family Cichlidae; of main economic relevance are Tilapia, previously in the taxonomic genus Tilapia, and now reassigned to the genera: *Coptodon*, *Oreochromis*, and *Sarotherodon*.

Of these Nile Tilapia (*Oreochromis niloticus*; previously: *Tilapia nilotica*) and various hybrids thereof, are the most farmed. The aquaculture of Tilapia in warm fresh-water is well-developed in several countries in Central- and South America, in Africa, and in Asia. In such fish-farming operations, Sa infection can cause large-scale mortalities and huge economic losses, see: Evans et al. (2006, *Aquaculture Health Int.*, no. 7, p. 10-14).

The disease caused by Sa infection in fish is known as Streptococcosis. Environmental factors, especially temperature, play an important role in the development of this disease: massive increased mortalities typically occur during the high water-temperature season, and a low but persistent level of mortality can occur with decreased water temperature.

Common clinical signs of Streptococcosis include erratic swimming, exophthalmia and affected organs showed haemorrhages, inflammation and ascites. Next to serious effects on animal welfare, the main effects of disease are economic: loss of revenue and increase of costs due to acute disease and mortality.

In aquaculture, the adaptation of husbandry practices is normally insufficient to overcome these negative effects, and the use of antibiotics can be controversial. Therefore vaccines are the preferred way to fight infection and disease caused by Sa. Different types of vaccines against Sa have been developed and were found to be very effective; a review is given in Liu et al. (2016, *Dis. Aq. Org.*, vol. 122, p. 163-170).

Well-known streptococcal vaccines for fish are based on bacterin antigens (formalin killed bacterial cells) that are emulsified with an oil adjuvant. Eldar et al. (1995, *Vaccine*, vol. 13, p. 867-870) describe a bacterin vaccine of *S. difficile*, which bacterium has meanwhile been reclassified as serotype Ib *S. agalactiae*. Such vaccines can be administered by injection e.g. intraperitoneally. Examples of commercially available bacterin vaccines against Sa are from MSD Animal Health: AQUAVAC® Strep Sa-Si, against infection by *Streptococcus agalactiae* serotype Ib, and against *Streptococcus iniae*; and: AQUAVAC® Strep Sa1, against Sa of serotypes Ia and III, which contains formalin-inactivated bacteria (bacterin) of two Sa serotypes: Ia (strain TI 1422), and III (strain TI 1428). The vaccine is a water-in-oil emulsion using a commercial non-mineral oil adjuvant: Montanide™ ISA 763A VG. See also WO 2011/048041.

WO 2008/003734 and WO 2011/048041 describe prior developments in Streptococcal bacterin vaccines.

With global climate changes such as warming and acidification of freshwater habitats, the risk of Sa infection and disease in aquaculture is only increasing (Phuoc et al., 2021, *Aquaculture*, vol. 534, 736256). Consequently, there is a constant need to provide further effective Sa vaccines.

It is therefore an object of the present invention to overcome a disadvantage in the prior art, and to accommodate to this need in the field by providing a vaccine against a new type of Sa Ia bacterium.

Description of the invention

Surprisingly it was found that this object can be met, and consequently one or more disadvantages of the prior art can be overcome, by providing a new Sa la bacterium of serotype Ia, which is more pathogenic than previous Sa la isolates and can break-through the protection generated by existing vaccines against Sa la infection. This new type of Sa la bacterium can now be used in new and effective vaccines against Streptococcosis in fish.

Several large Tilapia aquaculture operations in freshwater lakes in the Chiapas state of Mexico, routinely applied vaccination of young fish with bacterin vaccines against Sa serotypes Ia, Ib, and III. Nevertheless, in July 2021 sudden and massive mortalities occurred of up to 65 % of the fish. Affected fish displayed gut lesions upon necropsy, whereby the stomach and intestines were highly haemorrhagic, with intestinal intussusception (a.k.a. 'telescoping') as a distinct sign. Toxicological studies of feed and water were performed, but these showed no abnormalities. Over a 3 month period, the symptoms started in the older fish, then transferred to younger fish, and spread also to downstream holding areas. This indicated an infectious nature.

The symptoms observed in the field were not directly indicative of Streptococcosis, therefore tissue samples of dead and diseased fish were isolated from a variety of organs: brain, gills, heart, anterior kidney, liver, spleen, intestine, and stomach. The new isolates were then characterised in a variety of assays.

Based on the initial histopathological observations, an infection with Tilapia lake virus (TiLV) was suspected. However even after testing several mixed tissue samples with a TiLV-specific nested PCR, no TiLV-positive results were found in the test samples from the Mexican outbreak.

A temporary use of antibiotics (Florfenicol - Aquaflor®, MSD Animal Health) in the farms, provided immediate reduction of mortalities during the peak of outbreaks. Consequently the main cause was considered to be bacterial.

Similar vaccine breakthroughs resulting in massive mortalities among Tilapia, also occurred in the fall of 2022 in Tilapia aquaculture farms in freshwater reservoirs in the Cortes department of Honduras. Of the dead fish tested, 27 % showed intestinal intussusception. Sa la bacteria with properties effectively identical to those of the Mexican break-through isolates, were obtained from tissues of diseased fish.

Surprisingly it was found that samples from these outbreaks contained bacteria of a new type of Sa serotype Ia bacterium, which was more pathogenic than known Sa la isolates. Also it was only moderately affected by immunity generated by existing vaccines against Sa la. However, when the new isolates were themselves used as a vaccine, they protected well, both against a homologous challenge infection, i.e. against the new type itself, but also against a heterologous challenge, using previously known Sa la isolates.

When compared to an earlier Sa la isolate, as used in Sa vaccines, and to an earlier Sa la isolate so far used for challenge infections, the new break-through isolates showed essentially the same behaviour in

several ways: during their culturing in vitro, in tests for serological classification (latex agglutination), and in studies for biochemical profiling (API 20™ STREP, bioMérieux SA), and for protein profiling (SDS-PAGE and Western blot).

5 Interestingly, the new isolates differed considerably from known Sa la vaccine- and challenge strains, in that their pathogenicity in naïve fish was clearly higher, and their serological cross-protection showed they were poorly recognised by antibodies against the existing Sa la vaccine strains. In turn however vaccination of fish with a bacterin of the new isolate did protect against the known Sa la strains. Details are described in the Examples hereinafter.

10 Interestingly, when testing infections with the new Sa la isolates in Tilapia under laboratory conditions, the infected fish did display the classical signs of Streptococcosis, such as: spiralling, exophthalmos, meningo-encephalitis, and petechiae of operculum and fins. In addition, when the fish were given feed after challenge infection, they also developed the intestinal intussusception as observed in the field in more than 25 % of the cases.

15 Several bacterial isolates from the outbreaks in Mexico 2021 and in Honduras 2022, were purified and cultured, for further studies. One of the isolates from Mexico: isolate TI 2893 was used to prepare a seed for vaccination studies. This isolate showed a low level of inhibition by antiserum raised against the existing vaccine strain, in a competition-inhibition Elisa, albeit slightly higher than the other isolates from Mexico 2021.

20 A representative sample from the seed that was made of isolate TI 2893 was submitted to the Collection Nationale de Cultures de Microorganismes (CNCM), Institut Pasteur, Paris, France, for a deposit under accession number: CNCM I-5929. The sample was accepted, and was approved as viable, as is demonstrated by the receipt form and the viability statement filed herewith.

25 It is not known exactly how or why the new type of Sa la bacteria are more pathogenic, and why they can break-through the immunity induced by existing Sa la vaccines. This makes that the new Sa la isolates are not at all obvious from any disclosure in the prior art, and bacteria of Sa la that differ from known isolates of Sa la by the specific properties as described herein, were not known before.

30 Therefore in one aspect the invention relates to a *Streptococcus agalactiae* (Sa) bacterium of serotype Ia (Sa Ia), having the characterizing features of the bacterium as deposited at the Collection Nationale de Cultures de Microorganismes (CNCM), Institut Pasteur, Paris, France, under accession number: CNCM I-5929.

35 “*Streptococcus*” is a well-known genus of bacteria. Details on its characterisation and classification can be found e.g. in well-known handbooks such as Bergey’s manuals of: Determinative Bacteriology, and of: Systematic Bacteriology.

40 The species ‘*agalactiae*’, i.e. Lancefield group B, can readily be identified in a similar way. In addition a number of commercial tests are available for making the necessary biochemical- and serological determinations.

As described by the CDC (www.cdc.gov/streplab/groupb-strep/index.html), determining the serotype of an Sa la bacterium, such as serotype la, is well known, and convenient test kits are available from several commercial suppliers, for example by using PCR. Preferably the la serotype is determined using one of several rapid agglutination tests that employ latex particles coated with serotype specific antibodies. An
5 example is the 'ImmuLex™ Streptococcus Group B type la' serological test kit (SSI Diagnostica, Denmark).

As is also known in the field, the classification of a micro-organism in a particular taxonomic group is based on its combined features. The invention therefore also includes variants of the Sa la species that
10 are sub-classified therefrom in any way, for instance as a: subspecies, strain, isolate, genotype, variant, subtype, or subgroup, etcetera. Further, it will be apparent to a person skilled in the art in the field of the invention that while a particular Sa la bacterium for the invention may currently be assigned to this species or subtype, however that is a taxonomic classification that could change in time as new insights can lead to reclassification into a new- or different taxonomic group. For example some groups of Sa
15 serotype lb were previously classified as *S. difficile/difficilis*.

However, as this does not change the bacterium itself, or its antigenic repertoire, but only it's scientific name or classification, such re-classified bacteria remain within the scope of the invention.

The Sa la bacterium according to the invention is comprised in or on a suitable carrier.

20 For the invention the carrier can be liquid or (semi-)solid, and is for example a liquid such as water, glycerol, a buffer, or a medium; a semi-solid such as a gel; a solid such as a freeze-dried body, or a physical structure such as a paper or polymer sheet, or a shaped article.

An Sa la bacterium according to the invention may be obtained directly or indirectly from the deposited
25 bacterium for the invention: directly is by way of obtaining a deposited sample; indirectly is by way of obtaining the bacterium from a descendant (as defined herein) of the deposited bacterium for the invention. Such descendants and indirectly obtained bacteria retain the characterizing features of the deposited bacterium.

The directly or indirectly obtained bacteria can be amplified by one or more passages, in vivo or
30 in vitro.

Next to being infectious and pathogenic for fish, in particular for Tilapia, the deposited bacterium has a number of features in common with known Sa bacteria, such as that the new bacteria are of serotype la. This serotype can be determined with a variety of well-known tests such as using latex agglutination for
35 bacteria culture samples, or using a (multiplex) PCR test on capsular antigen genes of the bacteria's genetic material

Also, the new type of Sa la bacteria were not found to be haemolytic after 3 days incubation at 26 °C on sheep-blood agar plates, so they are: gamma-haemolytic, as are many of the Sa la bacteria.

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Further, the new isolates were found to be of 'sequence type' (ST) 7, as determined by multi-locus sequence typing (MLST) using standard procedures; details are in the examples. This was remarkable as many known Sa la are also ST7, including the existing Sa la vaccine strain. Therefore this is not a distinguishing indicator of serologic- and pathologic variations in Sa la bacteria.

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For the invention, the "characterising features" of the deposited bacterium relate to the various elements and properties of the deposited bacteria's genotype and phenotype, such as the morphologic and genomic characteristics, as well as the biological characteristics such as its physiologic, biochemical, immunologic, and/or pathologic behaviour, which set it apart from known Sa la bacteria.

10 For a future isolate of Sa bacteria, a match with or a difference from the characterising features of the deposited sample, can readily be determined by a skilled person, using routine methods and procedures.

A characterising feature that distinguishes the new type of bacteria according to the invention from known
15 Sa la bacteria, is that the new type of bacteria causes pathological signs in Tilapia at much lower inoculum doses: as described herein, at a dose that was 4000 times lower than for known Sa la isolates, the new bacteria still caused at least an equal level of mortality in a comparative inoculation trial.

Another characterising feature of the new type of Sa la bacteria according to the invention, is that they
20 are able to break-through the vaccine-protection induced by existing Sa la vaccines: as described herein, existing vaccines only protected 20 - 50 % of the vaccinated fish against a challenge infection with the new type. On the other hand, and in the reversed situation: a vaccine -as described herein- based on the new type of Sa la protected at least 81 % of the fish against infection and disease with a known Sa la challenge strain.

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Further characterising features of the new type of Sa la bacteria according to the invention, can be observed by testing their genetic material in PCR assays, with specific primer sets as defined herein. These will generate PCR products with nucleotide fragments of sizes that differ significantly from the size of the fragments obtained when testing the genetic material from known Sa la bacteria using those same
30 primer sets. This applies even to Sa la that were isolated from the same region of Mexico several years earlier. Details are provided in the Examples, and in Table 1 below.

These combinations of properties of the new type of Sa la bacteria, all work together and are supplemental to characterise the bacteria according to the invention: the deposit, the description of the
35 biological-, immunological-, and genetic properties of the bacteria, the pathology symptoms caused by their infection, the vaccine break-through, and the PCR fragments of unique sizes generated from their genetic material with the use of specific primer sets.

Together these properties provide a complete picture to a skilled person that characterise the new type of Sa la bacteria according to the invention in a complete way, and differentiate them from
40 known Sa la bacteria.

An Sa la bacterium according to the invention can readily be amplified, by culturing under suitable conditions, in vivo or in vitro, for one or more passages, by methods as described herein and well-known to the skilled person. Culturing in vivo involves the inoculation into a susceptible animal, such as a fish (as defined herein below). For culturing in vitro appropriate culture media and culturing vessels are widely available. Suitable culture media are for example agar or broth of trypticase soy, or brain heart infusion, with NaCl content in the medium from 0 to 5.5 % w/v. Suitable in vitro culture conditions are e.g.: culture for 16 to 24 hours, at 25 to 37 °C, under facultative anaerobic conditions, and with shaking (for a flask culture) at 100 - 160 rpm.

Details of embodiments and of further aspects of the invention will be described below.

In an embodiment, the Sa la bacterium according to the invention is comprised in a carrier.

A 'carrier' for the invention can be solid or liquid, either at room temperature or below zero °C. For example it can be a liquid comprising the Sa la bacterium according to the invention. Said liquid can e.g. be water, glycerol, a physiological buffer, a culture medium, or a stabiliser. The carrier may contain further excipients, e.g. to stabilise the bacterium, such as e.g. proteins, sugars, amino acids, or polymers.

When (semi-)solid, the carrier can e.g. be a gel, a frozen liquid, or a freeze dried body, a semi-solid such as a gel; a solid such as a freeze-dried body, or a physical structure such as a paper or polymer sheet, or a shaped article.

In an embodiment the carrier according to the invention is one or more selected from: a physiological buffer, glycerol, a stabiliser, and preservatives.

A stabiliser for the inventions is e.g. selected from polysaccharides, glucose polymers, chemicals (e.g. DMSO), and proteins (e.g. skimmed milk, or serum).

In an embodiment, the Sa la bacterium according to the invention is an isolated bacterium.

For the invention 'isolated' indicates: taken out of its natural surroundings, and free -including partially free and essentially free- from certain contaminants. Isolated for the invention does not rule out the use of a carrier as described herein above for the invention. For example, a sample taken from an infected fish or from a fish tissue, from water in which infected fish were present, or a sample taken from a bacterial culture in liquid-, semi-solid-, or solid medium, can constitute an isolated Sa la bacterium according to the invention.

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One of the characterising features of the new type of Sa la bacteria according to the invention thus becomes apparent when testing its genetic material in a PCR assay, using a set of specific primers. This way the Sa la bacteria according to the invention can be distinguished from known Sa la bacteria.

Therefore, in an embodiment the *Sa la* bacterium according to the invention is characterised in that in a polymerase chain reaction (PCR) with genetic material from said bacterium, a nucleotide (nt) fragment of a specific size is produced, when using a specific primer set, as follows:

- a fragment of 500 nucleotides (nt), with the primer set of SEQ ID NO: 1 and SEQ ID NO: 2; or
- 5 – a fragment of 1672 nt, with the primer set of SEQ ID NO: 3 and SEQ ID NO: 4; or
- a fragment of 1772 nt, with the primer set of SEQ ID NO: 5 and SEQ ID NO: 6; or
- a fragment of 1912 nt, with the primer set of SEQ ID NO: 7 and SEQ ID NO: 8; or
- a fragment of 2016 nt, with the primer set of SEQ ID NO: 9 and SEQ ID NO: 10; or
- a fragment of 876 nt, with the primer set of SEQ ID NO: 11 and SEQ ID NO: 12.

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In a preferred embodiment the *Sa la* bacterium according to the invention is characterized in that in a PCR with genetic material from said bacterium a nucleotide (nt) fragment is produced of 500 ± 5 nt, with the primer set of SEQ ID NO: 1 and SEQ ID NO: 2.

- 15 In another preferred embodiment the *Sa la* bacterium according to the invention is characterized in that in a PCR with genetic material from said bacterium a nt fragment is produced of 1672 ± 5 nt, with the primer set of SEQ ID NO: 3 and SEQ ID NO: 4.

- 20 In yet another preferred embodiment the *Sa la* bacterium according to the invention is characterized in that in a PCR with genetic material from said bacterium a nt fragment is produced of 1772 ± 5 nt, with the primer set of SEQ ID NO: 5 and SEQ ID NO: 6.

- 25 In still another preferred embodiment the *Sa la* bacterium according to the invention is characterized in that in a PCR with genetic material from said bacterium a nt fragment is produced of 1912 ± 5 nt, with the primer set of SEQ ID NO: 7 and SEQ ID NO: 8.

- 30 In even another preferred embodiment the *Sa la* bacterium according to the invention is characterized in that in a PCR with genetic material from said bacterium a nt fragment is produced of 2016 ± 5 nt, with the primer set of SEQ ID NO: 9 and SEQ ID NO: 10.

- In still even another preferred embodiment the *Sa la* bacterium according to the invention is characterized in that in a PCR with genetic material from said bacterium a nt fragment is produced of 876 ± 5 nt, with the primer set of SEQ ID NO: 11 and SEQ ID NO: 12.

- 35 The indicated PCR product sizes for bacteria according to the invention, were obtained using the indicated primers sets on the genomic DNA, not only from the deposited bacterium (isolate TI 2893), but also from the other co-isolates from Mexico 2021, and from isolates of the *Sa la* outbreak in Honduras in 2022.

For the invention, the PCR can be performed using common methods and materials for e.g. sample preparation, cycling conditions, and PCR reagents. Details, and preferred PCR conditions for each of the primer-sets to be used, are described in the Examples.

As the person skilled in the art will understand, the type of PCR and the type of bacterial genetic material to be used for the invention are not critical. For example, the PCR can be an end-point PCR done on DNA, or can be a reverse-transcriptase PCR (RT-PCR) done on RNA. Also the PCR can be of a qualitative- or of a quantitative type. Quantitative PCR (qPCR) is also known as: real-time PCR; all well-known in the art.

Preferably the PCR for the invention is an end point PCR. Preferably the genetic material is bacterial genomic DNA.

As the inventors have found, each of these primer sets will produce PCR products of the indicated size for the Sa la bacteria according to the invention, which sizes are clearly different from the sizes obtained when using those same primer sets on the genetic material from known Sa la bacteria.

For the invention, the 'size' of the nt fragments obtained by PCR for the invention can be determined accurately by computer analysis of digital sequence data, i.e. by doing in silico PCR analysis on long-read sequences from the genomic DNA of Sa la bacteria. However, and as the skilled person will appreciate, when doing such size determination under wet-lab conditions, it may not always be possible to determine that size so exactly and up to the last nucleotide number indicated for the invention.

Therefore the indicated sizes of nucleotide (nt) fragment lengths for the invention are to be interpreted as being: plus or minus 25 nt around the indicated size values; preferably as being 20, 15, 10, 5, 4, 3, 2, or 1 nt plus or minus around the indicated size values, in that order of preference. Most preferably, there are no nt plus or minus around the indicated size values.

Thus for example, the PCR product of 500 nt obtained by using the primer set of SEQ ID NO: 1 and SEQ ID NO: 2, may be detected as being between 475 and 525 nucleotides in size, preferably 480 - 520 nt in size, etc..

Even when allowing for such a very limited level of inaccuracy, the Sa la bacteria according to the invention can still be distinguished clearly from known Sa la isolates, using the PCR testing as described herein for the invention.

For comparison to the prior art, the primer sets as defined herein for the invention were tested on a wide variety of Sa la samples, isolated from Tilapia with Streptococcosis, from between 2001 and 2022, and isolated in a variety of countries from Asia and from Central America.

Table 1 displays the comparative results from the PCR product sizes of the deposited isolate TI 2893, versus the PCR results of known Sa la isolates.

As shows from Table 1, using either of the primer sets of SEQ ID NOs 9 and 10, or of SEQ ID NOs 11 and 12, on an Sa la bacterium other than according to the invention, can result in PCR fragments of different sizes, depending on the specific isolate tested. Nevertheless, for a bacterium according to the invention a fragment of clearly distinguishable size is obtained with these primer sets.

Table 1: Comparative PCR results

SEQ ID NO	Primer nt sequence 5' -> 3'	PCR fragment length in TI 2893 (nt)	PCR fragment length in other Sa la (nt)	Coding region in genome of TI 2893
1	TCAATTGCATGAACTGGTAGC	500	1699	pepN
2	GGATAGATGACGGACCATTTTCG			
3	TTGATGAGAATTGGGGTGGTAC	1672	406	DUF1310
4	CTTTTGCCTCATCACTATGTGC			
5	GACCTATAGCAGCATACTGTCC	1772	506	DUF3307
6	TCTCTTGATTGTTTTCTCCCC			
7	CTATGCTTCATTACAACGTGGC	1912	811	yjdM
8	AGGCTACAACAATACCAAGTGG			
9	TTGCTGTGAGTTTAAACCTGTC	2016	941 or 2207	abiH
10	GCTCCTCACTGGTAGAGTAATC			
11	GTATCTGCTATGGATAGTGTG	876	828, 972 or 1020	fbsA
12	AAAGGAATCTTGTATCCGATTC			

- 5 For the invention, the last column of Table 1 describes the region of the (putative) gene in the genome of TI 2893 from which the PCR fragments are derived; the genomic sequence of TI 2893 is presented in SEQ ID NO: 13 herein. The names indicated refer to the corresponding (hypothetical) genes as could be annotated by different analyses, and comparisons to known (Sa) bacterial genomes:
- pepN: a well-known bacterial gene encoding an aminopeptidase, that in some Streptococci is a virulence factor. In SEQ ID NO: 13 it is located in the region of nt 1034500;
 - DUF1310: encodes a conserved domain found in a family of hypothetical proteins of around 125 amino acids in size, that seem to be specific for *Listeria* and *Streptococcus* species. The function of this family is unknown. DUF1310 is also known as: PF07006 in the InterPro database. It is located in the region of nt 1935000 in SEQ ID NO: 13;
 - DUF3307: encodes a conserved protein domain in a family of bacterial proteins of unknown function. DUF3307 is a.k.a. PF11750. It is located in the region of nt. 1028000 in SEQ ID NO: 13;
 - yjdM: encoding a protein initially considered to be involved in alkylphosphonate uptake. In *E. coli* the yjdM gene is regulated by an SOS-box-containing promoter. It is located in the region of nt 991000 in SEQ ID NO: 13;
 - abiH: abortive infection gene, related to stress response such as from phage infection. It is located in the region of nt 1274000 in SEQ ID NO: 13; and
 - fbsA: encodes a fibrinogen binding protein, which is a known virulence factor. It is located in the region of nt 1094000 in SEQ ID NO: 13.
- 25 As is well-known in the art, the indication 'DUF' stands for: 'Domain of Unknown Function', and refers to conserved protein domains encoded by (putative) genes that are so far without assigned function. The

domains are derived from large numbers of multiple alignments of known sequences. A list of DUF domains is publicly available from the protein domain databases 'Pfam' and 'InterPro', accessible e.g. via the EMBL-EBI website: 'www.ebi.ac.uk'.

- 5 In a more preferred embodiment the *Sa* la bacterium according to the invention is characterised in that in a PCR with genetic material from said bacterium, a nt fragment is produced of the sizes as defined for the invention, with two or more of the primer sets as defined for the invention.

10 In an embodiment of the two or more primer sets as defined for the invention, the two or more primer sets are selected from:

- SEQ ID NO: 1 and 2, and SEQ ID NO: 3 and 4;
- SEQ ID NO: 1 and 2, and SEQ ID NO: 5 and 6;
- SEQ ID NO: 1 and 2, and SEQ ID NO: 7 and 8;
- SEQ ID NO: 3 and 4, and SEQ ID NO: 5 and 6;
- 15 - SEQ ID NO: 3 and 4, and SEQ ID NO: 7 and 8;
- SEQ ID NO: 5 and 6, and SEQ ID NO: 7 and 8;
- SEQ ID NO: 1 and 2, SEQ ID NO: 3 and 4, and SEQ ID NO: 5 and 6;
- SEQ ID NO: 1 and 2, SEQ ID NO: 3 and 4, and SEQ ID NO: 7 and 8;
- SEQ ID NO: 1 and 2, SEQ ID NO: 5 and 6, and SEQ ID NO: 7 and 8;
- 20 - SEQ ID NO: 3 and 4, SEQ ID NO: 5 and 6, and SEQ ID NO: 7 and 8; and
- SEQ ID NO: 1 and 2, SEQ ID NO: 3 and 4, SEQ ID NO: 5 and 6; and SEQ ID NO: 7 and 8.

In a further embodiment of the two or more primer sets as defined for the invention, the two or more primer sets are selected from the set of SEQ ID NO: 9 and 10, and from one or more of the sets of:

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- SEQ ID NO: 1 and 2;
 - SEQ ID NO: 3 and 4;
 - SEQ ID NO: 5 and 6;
 - SEQ ID NO: 7 and 8; and
 - SEQ ID NO: 11 and 12.

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In still a further embodiment of the two or more primer sets as defined for the invention, the two or more primer sets are selected from the set of SEQ ID NO: 11 and 12, and from one or more of the sets of:

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- SEQ ID NO: 1 and 2;
 - SEQ ID NO: 3 and 4;
 - SEQ ID NO: 5 and 6;
 - SEQ ID NO: 7 and 8; and
 - SEQ ID NO: 9 and 10.

- 40 In an even more preferred embodiment the *Sa* la bacterium according to the invention is characterised in that in a PCR with genetic material from said bacterium, a nt fragment is produced of the sizes as defined for the invention, with all six of the primer sets as defined for the invention.

In a still even more preferred embodiment the Sa la bacterium according to the invention is characterised in that the bacterium is the bacterium as deposited under accession number: CNCM I-5929, or is a descendant from said deposited bacterium.

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For the invention, a 'descendant' of the deposited bacterium for the invention, is an Sa la bacterium that is derived from the deposited bacterium by passageing. This passageing can be done in vitro or in vivo, and can be by one, or by more than one passage.

As is well-known, a 'passage' of a bacterium regards the incubation of that bacterium under conditions conducive to its amplification, and for a certain duration. In this context that duration is at least one generation time (a.k.a. division cycle, or generation) of the bacterium, but preferably a passage will have a duration of more than one generation time. For the new type of Sa la bacteria according to the invention, one generation time is about 30 minutes.

15 The descendant according to the invention, has all the characterising features of the deposited Sa la bacterium according to the invention.

In an embodiment the Sa la bacterium, or the descendant, both according to the invention, has a genome having 90 % nucleotide sequence identity with the sequence of SEQ ID NO: 13.

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SEQ ID NO: 13 presents the 2.1 million nucleotides of the genome of the bacterium of isolate TI 2893, which is deposited as CNCM I-5929. In the Examples is described how the genome was isolated, sequenced, and analysed.

25 In a preferred embodiment the Sa la bacterium, or the descendant, both according to the invention, has a genome having 91 % nucleotide sequence identity with the sequence of SEQ ID NO: 13; more preferably has a genome having 92, 93, 94, 95, 96, 97, 98, or even 99 % nucleotide sequence identity with the sequence of SEQ ID NO: 13, in this order of preference.

30 In an embodiment the Sa la bacterium, or the descendant, both according to the invention, has a genome that comprises the sequence of SEQ ID NO: 13.

In an embodiment the Sa la bacterium, or the descendant, both according to the invention, has a genome that consists of the sequence of SEQ ID NO: 13.

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For the invention, a percentage of nucleotide sequence identity relative to the sequences of the SEQ ID NO's as disclosed in the present application, is to be calculated by making nucleotide sequence alignments using the NCBI's Blast™ computer program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), selecting the options "blastn" and "Align two or more sequences" with standard settings and default parameters. In these alignments the sequences of the SEQ ID NO's from the present application are to be used as the subject (a.k.a. as the target).

40

In our analyses of the Sa la bacterium according to the invention, the inventors found that the bacterium can contain a plasmid. SEQ ID NO: 14 presents the 4.4 kb of this extrachromosomal plasmid sequence, as detected in the isolate TI 2893, that was deposited as CNCM I-5929.

5

In an embodiment the Sa la bacterium, or the descendant, both according to the invention, can contain a plasmid comprising the sequence of SEQ ID NO: 14.

Preferably, the Sa la bacterium, or the descendant, contain a plasmid consisting of the sequence of SEQ ID NO: 14.

10

The Sa la bacterium or the descendant, both according to the invention, can advantageously be employed in a wide variety of medical- or non-medical uses, including in vaccination and diagnostics. However, for vaccinations the live wildtype versions of such bacteria are pathogenic to fish, therefore those are preferably employed in an attenuated- or in an inactivated (i.e. non-live) form of such bacteria in the further uses, methods and compositions. Also a mixture of live-attenuated and non-live bacteria may be employed.

15

A use as a live-attenuated vaccine, may also refer to a use of the wild type live bacteria according to the invention as a vaccine for an animal target that is less sensitive to its pathogenicity than are Tilapia.

20

Therefore, in a further aspect the invention regards a composition comprising a preparation of the Sa la bacterium or of the descendant, both according to the invention.

A 'composition' for the invention can be solid, semi-solid, or liquid, and can contain a carrier as described herein for the invention.

25

The term 'comprising' (as well as variations such as 'comprises', 'comprise', and 'comprised') as used herein, intends to refer to all elements, and in any possible combination conceivable for the invention, that are covered by or included in the text section, paragraph, claim, etc., in which this term is used, even if such elements or combinations are not explicitly recited; and not to the exclusion of any of such element(s) or combinations.

30

Therefore any such text section, paragraph, claim, etc., can also relate to one or more embodiment(s) wherein the term 'comprising' (or its variants) is replaced by terms such as 'consist of', 'consisting of', or 'consist essentially of'.

35

A 'preparation' of the Sa la bacterium or of the descendant, both according to the invention, regards a form of such bacterium wherein it has been subjected to a treatment, e.g. a chemical-, biological-, physical-, or mechanical treatment. Examples of preparations for the invention, are: extracts, lysates, and sonicates; all well-known in the art.

The treatment applied to manufacture the preparation for the invention, may result in the partial- or the complete inactivation of the Sa la bacterium or the descendant, both according to the invention. Such (partial) inactivation may be an intended- or an unintended consequence of that treatment.

- 5 Therefore in an embodiment the composition according to the invention is characterised in that the preparation comprises the Sa la bacterium or the descendant, both according to the invention, in inactivated form.

A composition comprising inactivated bacteria is colloquially called: a bacterin.

10

'Inactivated' for the invention means: persistently incapable to replicate, even when under otherwise optimal conditions; also: non-live.

The inactivation may be partial or complete, so that in the composition according to the invention many, some, or no live cells remain from the live cells of the Sa la bacterium or the descendant, both
15 according to the invention, that were present before the inactivation treatment was applied.

In a preferred embodiment of the composition according to the invention, the inactivation is complete, i.e. no live cells of the Sa la bacterium or the descendant, both according to the invention, are present anymore.

20

A wide variety of treatments is known and available for the inactivation of bacteria, such as: chemical-, physical-, biological-, or mechanical treatments. Examples of physical- and mechanical inactivation are: heating, high shear, high pressure, freeze-thawing, or exposure to ionising radiation, e.g. UV light, X-rays, or gamma rays. An example of biological inactivation is incubation with an enzyme. Examples of chemical
25 inactivation are: exposure to high- or low pH, to a detergent, an organic- or an inorganic solvent, a chaotropic agent, formaldehyde, a lactone, or an aziridine.

In an embodiment of the composition according to the invention comprising the Sa la bacterium or the descendant, both according to the invention, in inactivated form, the bacteria were inactivated using
30 formaldehyde.

Formaldehyde (methanal) for the invention is preferably a compound with CAS registry number: 50-00-0. Formaldehyde is typically and more conveniently employed as an aqueous solution, known as formalin.

- Conditions for formalin-inactivation of Sa bacteria are well-known, and comprise e.g. the use of
35 0.01 - 5 % v/v formalin, at a certain temperature (e.g. room temperature), for 30 minutes to several hours, depending on the temperature of the inactivation incubation.

- The Sa la bacterium, the descendant, and/or the composition, all according to the invention, can advantageously be used in a variety of ways, in particular for the purpose of veterinary healthcare in
40 aquaculture, such as in diagnostics and in vaccination.

Therefore, in a further aspect the invention regards a use of the Sa la bacterium, the descendant, or the composition, all according to the invention, in a diagnostic test.

Diagnostic tests are well-known in this field, and for the invention can refer e.g. to uses in which the bacterium, or a preparation thereof, is employed in a test to determine whether an animal has antibodies specific for the Sa la bacterium or for the descendant, both according to the invention, or not.

Alternatively, antibodies can be generated against the Sa la bacterium, or the descendant, or the composition, all according to the invention, and these antibodies can e.g. be used in a test to determine if an animal comprises (antigens of) the Sa la bacterium or of the descendant, both according to the invention, or not.

Antibodies that are 'specific' are well-known as immunoglobulins which can bind to a Sa la bacterium or the descendant, both according to the invention, or to a part or preparation thereof in a composition according to the invention, and such binding has a strength (i.e. affinity and/or avidity) that is higher than that against another antigen. Such specific binding can readily be distinguished from any non-specific- or background binding for example in an in vitro binding assay, by gradually diluting-out either the antibody or the antigen; a non-specific binding is typically lost rapidly, e.g. at 1:10 or 1:100 dilution, while specific binding remains even with higher dilutions.

In a further aspect the invention regards a diagnostic test kit, characterised in that said kit comprises a container comprising the Sa la bacterium, the descendant, or the composition, all according to the invention.

In a further aspect the invention regards a diagnostic test kit, characterised in that said kit comprises a container comprising an antibody that binds specifically with the Sa la bacterium or with the descendant, both according to the invention.

As described, especially advantageous are methods for and uses of the bacteria of the invention, in vaccination of fish against Streptococcosis.

Therefore, in a further aspect the invention regards the use of an antibody that binds specifically with the Sa la bacterium or with the descendant, both according to the invention, for use as a vaccine for fish against Streptococcosis.

In a further aspect the invention regards the Sa la bacterium, the descendant, or the composition, all according to the invention, for use as a vaccine for fish against Streptococcosis.

Also, in a further aspect, the invention regards a vaccine for fish against Streptococcosis, characterised in that said vaccine comprises the Sa la bacterium, the descendant, or the composition, all according to the invention, and a pharmaceutically acceptable carrier.

A "vaccine" is well-known to be an immunogenic composition, which comprises an immunologically active compound in a pharmaceutically acceptable carrier. The vaccine can induce the immune system of a target inoculated therewith to launch a protective immunological response against a pathogen by the specific- and/or a specific activation of the target's humoral- and/or cellular immune response.

5

The vaccine and the use as a vaccine, both according to the invention, serve to induce in an inoculated fish protection against infection and/or disease caused by infection with Sa bacteria, and specifically with Sa la bacteria. This protection is obtained by preventing or reducing the establishment or the proliferation of a productive infection of such Sa bacteria in the target's organs. This is achieved for example by

10 reducing the bacterial load, or interfering with bacterial amplification, in the vaccinated target. In turn this leads to a reduction in the target fish of the number, the intensity, and/or the severity of clinical signs of Streptococcosis.

For Sa la infection, the main signs of infection and disease in fish are uni- or bi-lateral exophthalmia (also known as "pop-eye"), distended abdomen, petechiae of operculum and fins, and

15 erratic swimming (spiralling). Upon section, meningo-encephalitis is displayed, and in affected organs: haemorrhages, inflammation, and ascites

The application of a vaccine and of a use as a vaccine, both according to the invention, in the vaccination of fish against infection and/or disease caused by Sa la bacteria, is well within the skills of the routine

20 practitioner.

Similarly, the efficacy of a vaccination for the invention is prominent from the immunological response following vaccination, e.g. as can be determined in an experimental setting from the reduction of clinical symptoms or mortality after a challenge infection, and from scoring the vaccinated fish's signs of disease, clinical scores, serological parameters, or by re-isolation of the challenge pathogen; and

25 comparing these results to the response to the same challenge in unvaccinated animals.

For the invention, the protection against Streptococcosis by the vaccine and by the use as a vaccine, both according to the invention, results in the vaccinated fish targets in an improvement of health and of economic performance. This can for instance be assessed from parameters such as increase of well-being, survival, and growth rate, reduction of feed conversion rate, reduction of costs for veterinary

30 healthcare, and increase of the economy of operation.

For the invention 'protection' is determined in vaccination-challenge trials in Tilapia, and is expressed as a value of 'relative percentage survival' (RPS), which is calculated by dividing the percentage mortality in vaccinates, by that in controls. In this system, higher RPS values indicate a better protection. Details of

35 the challenge model to be used, and of RPS calculations, are also described in WO 2011/048041.

The vaccine according to the invention can induce protection against Sa la infection and disease of at least 50 % RPS; preferably of at least 60, 70, 80, or 90 % RPS; more preferably of at least 95 % RPS.

40 Various embodiments, preferences and examples of the vaccine, the use as a vaccine, and of the vaccination, all according to the invention, are outlined below.

A "pharmaceutically acceptable carrier" is intended to aid in the stabilisation and administration of the vaccine, while being harmless and well-tolerated by the target. Such a carrier can for instance be sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer,
5 which can comprise further additives, such as stabilisers or preservatives.

In an embodiment, the vaccine and the use as a vaccine, both according to the invention, comprise the Sa la bacterium or the descendant, both according to the invention, in inactivated form.

10 In an embodiment, the vaccine and the use as a vaccine, both according to the invention, comprise an adjuvant.

In a preferred embodiment, the vaccine and the use as a vaccine, both according to the invention, comprise the Sa la bacterium or the descendant, both according to the invention, in inactivated form, and
15 comprise an adjuvant.

An "adjuvant" is a well-known component of a vaccine that stimulates the immune response of a target in a non-specific manner. Many different adjuvants are known in the art. Examples of adjuvants are: complete- or incomplete Freund's adjuvant, vitamin E or alpha-tocopherol, non-ionic block polymers and
20 polyamines such as dextran sulphate, Carbopol™, pyran, Saponin, such as: Quil A™, or Q-vac™. Saponin and vaccine components may be combined in an ISCOM™. Also, aluminium salts, such as aluminium-phosphate or an aluminium-hydroxide which is available for example as: Alhydrogel™ (Brenntag Biosector), Rehydrigel™ (Reheis), and Rehsorptar™ (Armour Pharmaceutical).

A much used adjuvant is an oil, e.g. a mineral oil such as a light (white) mineral (paraffin) oil; or a
25 non-mineral oil such as: squalene; squalane; vegetable oils or derivatives thereof, e.g. ethyl-oleate. Also combination products such as ISA™ (Seppic), or DiluvacForte™ and Xsolve™ (both MSD Animal Health) can advantageously be used.

A handbook on adjuvants and their uses and effects is: "Vaccine adjuvants" (Methods in molecular medicine, vol. 42, D. O'Hagan ed., 2000, Humana press, NJ, ISBN: 0896037355).
30

The adjuvant can be comprised in the vaccine or in the use as a vaccine, both according to the invention, in several ways. When the adjuvant comprises an oil, the vaccine can be provided in aqueous form, and can be formulated as an emulsion with the oil, in different ways: as a water-in-oil (W/O), an oil-in-water (O/W), or as a double emulsion, either W/O/W or O/W/O.
35

An "emulsion" is a mixture of at least two immiscible liquids, whereby one is dispersed in another. Typically the droplets of the dispersed phase are very small, in the range of micrometres or less. Procedures and equipment for the preparation of an emulsion at any scale are well-known in the art. To stabilise an emulsion, one or more emulsifiers can be used.

40 An "emulsifier" is a molecule with amphiphilic properties, having both a hydrophobic- and a hydrophilic side. Many emulsifiers are known in the art with their various properties. Most are readily

available commercially, and in several degrees of purity. Common emulsifiers for vaccines are sorbitan monooleate (Span® 80) and polyoxyethylene-sorbitan-monooleate (Polysorbate 80, or Tween® 80). Also an emulsion-stabiliser can be added; examples are benzyl alcohol, and triethanolamine.

- 5 In a preferred embodiment of the vaccine and of the use as a vaccine, comprising an adjuvant according to the invention, the adjuvant comprises an oil. In an embodiment the oil is a non-mineral oil, e.g. as in the commercial products: Montanide™ ISA 763A or 763B.

More preferably the oil is a mineral oil. Even more preferably the mineral oil comprises a light (or white) liquid paraffin oil. Examples of light liquid paraffin oils for use in vaccine-adjuvants are: Drakeol®
10 6VR (Penreco), Marcol® 52 (Exxon Mobile), and Klearol® (Sonneborn).

In a preferred embodiment of the vaccine and of the use as a vaccine, further comprising an adjuvant, and wherein the adjuvant comprises an oil, all according to the invention, the vaccine is formulated as a water-in-oil emulsion.

15

A 'fish' for the invention is an aquatic organism, with fins and gills, and can be a cartilaginous- or bony fish, and can be from fresh-, brackish-, or saltwater habitats.

Preferably the fish target for the vaccines and uses of the present invention is a Tilapia.

- 20 'Tilapia' are cichlid fish, which are no longer classified in a genus of themselves, but are now classified taxonomically into several genera, the most relevant of which are the genera: Coptodon, Oreochromis, and Sarotherodon.

Therefore, embodiments of the Sa la bacterium, the descendant, or the composition, all for the use
25 according to the invention, and of the vaccine according to the invention, are characterised in that the fish is from a genus selected from: Coptodon, Oreochromis, and Sarotherodon.

In a preferred embodiment, the fish from the Coptodon, Oreochromis, or Sarotherodon genera is characterised in that it is of a species selected from: Coptodon zillii, Coptodon guineensis, Oreochromis niloticus, Oreochromis aureus, Oreochromis mossambicus, Oreochromis hornorum, and Sarotherodon
30 melanotheron; or is a hybrid of one or more of these species.

Examples of well-known hybrids of Tilapia, are crosses such as: O. aureus x O. mossambicus; O. aureus x O. niloticus; and O. niloticus x O. (urolepis) hornorum.

35

For the invention, these names of fish are to be interpreted in the same way as indicated above for the names of bacteria, namely that they are taxonomic classifications that could change in time as new insights can lead to reclassification into a new- or different taxonomic group. However, as this does not change the fish itself but only its scientific name or classification, such re-classified fish remain within the
40 scope of the invention. This also includes any subtypes, variants, crossbreeds or hybrids of these fish for the invention.

It is advantageous for the vaccination of fish to employ combination vaccines, which comprise multiple antigens from multiple pathogens. This saves time and effort which is good for the economy of operation, but also it reduces stress for the vaccinated fish as they do not need to be handled and inoculated multiple times.

Therefore in an embodiment of the vaccine and of the use as a vaccine, both according to the invention, the vaccine comprises at least one further immunogen from a fish pathogen.

The further antigens can be derived from a fish pathogen in any suitable way, for instance as a 'live' attenuated-, an inactivated-, or a subunit antigen from that micro-organism pathogenic to fish. Examples of pathogens of Tilapia are: from bacteria: *Streptococcus agalactiae* serotypes Ia (known strains), Ib, and III, *S. iniae*, and/or bacteria from the genera: *Francisella*, *Edwardsiella*, *Aeromonas*, *Vibrio*, and *Flavobacterium*, *Lactococcus*, *Aerococcus*, *Pseudomonas*, *Mycobacterium*, and *Chlamydia*; and/or from viruses: Tilapia lake virus, Tilapia larvae encephalitis virus, Iridovirus, Iridovirus-like virus, Bohle virus, infectious pancreatic necrosis virus, Parvovirus, Herpes virus, Aquabirnavirus, Betanodavirus, Ranavirus, Megalocytivirus, and Lymphocystivirus.

In a preferred embodiment of the vaccine and of the use as a vaccine, both according to the invention, the further immunogen is derived from one or more of: *S. agalactiae* Ia, *S. agalactiae* Ib, *S. agalactiae* III, and *S. iniae*.

The vaccine according to the invention can be prepared from the Sa Ia bacterium, the descendant, and/or the composition, all according to the invention, by methods as described herein, which are readily applicable by a person skilled in the art. For example, the bacteria were cultured in a medium comprising yeast extract, with 0.5 % w/v NaCl, at 32°C for at least 6 hours, with stirring, and were then harvested by centrifugation. Next the bacteria were inactivated by incubation with formalin and formulated into a water-in-oil (W/O) emulsion with a mineral oil, namely a light liquid paraffin oil.

General techniques and considerations that apply to the manufacture of vaccines under well-known standards for pharmaceutical production are described for instance in governmental directives and regulations (Pharmacopoeia, 9CFR) and in well-known handbooks, for example: "Remington: the science and practice of pharmacy" (2000, Lippincott, USA, ISBN: 683306472), and: "Veterinary vaccinology" (P. Pastoret et al. ed., 1997, Elsevier, Amsterdam, ISBN 0444819681). Commonly vaccines are prepared sterile, and are prepared using excipients of pharmaceutical quality grade.

Manufacture of vaccines will commonly include microbiological tests for sterility, and absence of extraneous agents; and may include studies in vivo or in vitro for confirming inactivation, and vaccine efficacy and -safety. After completion of the testing for quality, quantity, sterility, safety, and efficacy, the vaccine can be released for sale. All these are well-known to a skilled person.

Therefore in a further aspect the invention regards a method for the preparation of the vaccine according to the invention, the method comprising the step of admixing the Sa la bacterium, the descendant, or the composition, all according to the invention, and a pharmaceutically acceptable carrier.

- 5 In an embodiment, the method for the preparation according to the invention, is characterised in that the method comprises a step for inactivating the Sa la bacterium or the descendant, both according to the invention.

- 10 In a further aspect the invention regards the use of the Sa la bacterium, the descendant, or the composition, all according to the invention, for the manufacture of the vaccine according to the invention.

The vaccine according to the invention can be administered to a fish by different routes. In an embodiment, the vaccine according to the invention is administered by parenteral route, i.e. through the skin, e.g.: intramuscular or intraperitoneal. Alternatives are by mucosal-, immersion-, or oral route.

- 15 The preferred route of administration is intraperitoneal.

- The volume per dose of the vaccine according to the invention can be selected according to the characteristics of the specific vaccine applied, the characteristics of the target, and the intended route of application. Parenteral injection of fish is commonly done with a dose of 0.01 - 1 ml/target. Preferably the
20 dose is between 0.01 and 0.1 ml/target dose. More preferably the dose is selected from 10, 25 and 50 microliters/target dose.

- The vaccine according to the invention can be used both as a prophylactic- and as a therapeutic treatment, as it interferes with the establishment- and with the progression of Streptococcus infection in a
25 fish.

The vaccine according to the invention can serve as an effective priming vaccination, which can later be followed and amplified by a booster vaccination, with the same or with a different vaccine.

- 30 The method, timing, dose and volume of the administration of the vaccine according to the invention can be adapted and optimised for the particular type of fish to be vaccinated; also taking into consideration the time and life stage when the fish could be exposed to Streptococcus infection.

- For Tilapia, the administration of the vaccine according to the invention is preferably performed as early
35 as possible, to be ahead of possible field infection, so preferably vaccinate in the setting of hatcheries or nurseries, and before transfer to facilities for the grow-out phase. With Tilapia, vaccination by injection can practically be performed on fish from a weight of about 5 grams. Commonly food is withheld the day before vaccination, and the fish are anaesthetized shortly before inoculation. All well-known in the art.

- 40 The dosing regimen for administering the vaccine according to the invention to a target fish can be in single- or in multiple doses, in a manner compatible with the formulation of the vaccine, and with the

animal husbandry particulars of the target fish in mind, and in such an amount as will be immunologically effective.

Preferably the vaccine according to the invention is given only once, i.e.: is a single shot vaccine.

5 Ideally, the regimen for the administration of the vaccine according to the invention is integrated into existing vaccination schedules of other vaccines that the target fish may require, again to reduce stress and costs. These other vaccines can be administered in a simultaneous-, concurrent-, or sequential fashion, or by so-called: 'associated use'; preferably these combinations are applied in a manner compatible with the licensed use of these vaccines.

10

It is well within the capabilities of a skilled person to optimise the vaccine according to the invention, by adapting its use or composition. For example this may involve the fine-tuning of the efficacy or the safety of the vaccine. This can be done by adapting the vaccine dose, quantity, frequency, route, by using the vaccine in another form or formulation, or by adapting the excipients of the vaccine (e.g. a stabiliser, preservative, or adjuvant).

15

The amount of antigen per animal dose of the vaccine according to the invention, can readily be determined by testing which amounts are immunologically effective, in relation to different levels of challenge infection.

20

In an embodiment the amount of inactivated *Sa la* bacteria according to the invention per ml of the vaccine according to the invention, corresponds to 1×10^6 - 1×10^{10} bacteria per ml, as present before the inactivation. Preferably the amount of *Sa la* bacteria according to the invention corresponds to 1×10^7 - 5×10^9 , 5×10^7 - 5×10^9 , or even to 1×10^8 - 1×10^9 per ml of vaccine, in this order of preference.

25

Sa bacteria can be counted using standard plate count methods on regular blood-agar plates, to determine amount in colony forming units (cfu).

At a dose volume of 50 μ l/fish, a preferred inoculum dose of the vaccine according to the invention, contains *Sa la* bacteria according to the invention corresponding to 1×10^7 - 5×10^7 /dose.

30

As described above, and as exemplified hereinafter, the vaccine according to the invention can advantageously be used to prevent or reduce infection and disease in fish from infection with *S. agalactiae la*, both of a known type and of a novel type such as disclosed herein.

35

Therefore, a further aspect of the invention relates to a method for the protection of fish against Streptococcosis, the method comprising the step of administering to said fish the vaccine according to the invention, or as obtainable by the methods or by the use, all according to the invention.

Similarly, in further aspects the invention regards:

40

- A use of the vaccine according to the invention for preventing or reducing infection by Sa bacteria, preferably by Sa la bacteria, and associated signs of disease.
- 5 - A method for preventing or reducing infection by Sa bacteria, preferably by Sa la bacteria, and associated signs of disease, the method comprising the administration of the vaccine according to the invention to fish.
- A method of vaccination of fish to prevent or reduce infection by Sa bacteria, preferably by Sa la bacteria, and associated signs of disease, the method comprising the step of inoculating said fish
10 with the vaccine according to the invention.

A further advantageous effect of the reduction of bacterial load by the vaccine according to the invention, is the prevention or reduction of shedding, and thereby the spread of Sa bacteria in the field, both vertically to offspring, and horizontally within a population, and within a geographical area. Consequently,
15 the use of the vaccine according to the invention leads to a reduction of the prevalence of Sa, preferably of Sa la.

Therefore further aspects of the invention are:

- 20 - A use of the vaccine for fish according to the invention for reducing the prevalence of Sa bacteria, preferably of Sa la bacteria, in a population or in a geographical area.
- The vaccine for fish according to the invention for reducing the prevalence of Sa bacteria, preferably of Sa la bacteria, in a population or in a geographical area.

25

The invention is described herein in various aspects and embodiments. It should be understood that any combination of these are considered to be within the scope of the invention. However merely for conciseness, not every possible combination is outlined herein in full.

30

The invention will now be further described by the following, non-limiting, examples.

Examples

Example 1: Isolation and purification of samples

- 5 In the Summer of 2021 acute disease and massive mortality outbreaks were observed in Tilapia aquaculture farms in Mexico, in fish of all sizes, and in spite of regular vaccinations against Streptococcosis. Clinical signs observed in the fish were: external pallor, corresponding to internal haemorrhages of intestines and stomach; intussusception; lethargy; and acute mortality.
- 10 Samples were taken from diseased fish from various tissues such as: brain, gill, heart, anterior kidney, liver, spleen, intestine and stomach, and used for various initial tests looking to identify the main cause of disease.

- Initial purification was performed as described in the manual by N. Buller (2014, Bacteria and Fungi from Fish and Other Aquatic Animals; A Practical Identification Manual, 2nd ed., ISBN: 978-1-84593-805-5). From individual fish, tissue samples were plated on blood agar for primary isolation, and colony identification with Gram stain. Secondary isolation was on blood agar plates. After incubation for 2
- 15 days, selected colonies were streaked on microbial agar gel columns for transportation, and these were sent to our laboratory.

- 20 Upon receipt, all bacterial samples were streaked on trypticase soy agar (TSA) agar plates. From 10 original isolates, individual colonies were picked and resuspended in TSB broth plus glycerol, and stored frozen.

The isolates from Mexico 2021 were labelled TI 2889 - TI 2898.

25

Example 2: General characterisation of the outbreak samples

Serogrouping:

- 30 The isolated bacterial samples were serotyped by latex agglutination using polyclonal antibodies specific for capsular polysaccharide serotype Ia, using a commercial kit (ImmuLex™ Streptococcus Group B type Ia; SSI Diagnostica), according to the manufacturer's instructions. All the 10 isolates from Mexico 2021 were identified as Sa-Ia.

- This was remarkable as the aquaculture farms that suffered these outbreak had been vaccinating
- 35 routinely using commercial vaccines which included Sa Ia antigens.

Haemolysis:

- TI 2893 was not found to be alpha- or beta-haemolytic after 3 days incubation at 26 °C on blood agar
- 40 plates, therefore it is: gamma haemolytic.

Sa la profiling by PCR

Two of the Mexico 2021 isolates, samples TI 2893 and TI 2898, were tested in a PCR specific for Sa la type strains, using PCR primers derived from GenBank acc. nr. : AB028896, based on Kong et al. (2005, J. Med. Micro., vol. 54, p. 1133 - 1138). Tested alongside were isolates TI 1422 and TI 1580, both are known Sa la strains, isolated from diseased Tilapia in Thailand in 2005.

In this assay, the two outbreak isolates scored identical to the results for TI 1422 and TI 1580, and thus qualify as Sa of serotype la.

10

Genetic profile:

MLST genotyping was performed as described in Jones et al. (2003, J. Clin. Microbiol., vol. 41, p. 2530 - 2536), and as improved by: Jolley et al. (2018, Wellcome Open Res., vol. 3:124). In short: using PCR primer sequences described by Jones et al., the gene-sequences for a selected number of house-keeping genes were determined: alcohol dehydrogenase (adhP), phenylalanyl tRNA synthetase (pheS), glutamine transporter protein (atr), glutamine synthetase (glnA), serine dehydratase (sdhA), glucose kinase (glcK), and transketolase (tkl). The sequences found were then compared via PubMLST, a public 'Streptococcus agalactiae typing database'. Conveniently this can be done by pasting the sequences found into one fasta sequence file, which can be compared with known sequences from Sa isolates, by uploading and comparing on the website: https://pubmlst.org/bigsub?db=pubmlst_sagalactiae_seqdef.

20

The 'allelic profile' of the TI 2893 isolate was found to match exactly with that known for Sa la strains of ST7 genotype.

25 *Biochemical profile:*

Biochemical profiling was done on the TI 2893 isolate from Mexico in 2021, using the commercial API 20™ STREP kit (bioMérieux), according to the manufacturer's instructions. The two Thailand 2005 isolates of Sa la: TI 1422 and TI 1580, were again tested alongside.

The ABI 20 Strep test results showed that isolate TI 2893 reacted for all parameters exactly as did the older Sa la isolates.

30

The new outbreak strains thus do also not differ essentially from known Sa la isolates in the sense of their basic biochemical profile.

35 *Protein profiling:*

Total cell protein, and supernatant samples were prepared from small cultures of outbreak isolate TI 2893, and for comparison also from known Sa la strain TI 1580. In short: cultures were spun down at 12,100 xg for 30 min. at 4 - 8 °C.

Secreted protein was collected and concentrated from culture medium using ammonium sulphate. The final protein pellet was resuspended in HEPES buffer, which was desalted using a commercially available desalting column.

40

The cell pellet was resuspended in PBS and OD adjusted. Cells were lysed in sample buffer and subsequently heat-treated, followed by sonication. Cell lysate was spun down to remove insoluble proteins.

Protein concentration using BCA method was measured and adjusted to 30 µg/µl for secretory protein, and to 10 µg/µl for cell lysate.

Both samples were separated by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and duplicate gels were subjected to Western blotting. The gels were stained using Coomassie blue. For the blots the first antibody used was a serum from Tilapia that had been vaccinated with commercial Sa la bacterin vaccine comprising strain TI 1422. Next, secondary mouse anti-Tilapia antibody was added to the blot, followed by tertiary anti-mouse HRP conjugated antibody.

Results are presented in Figure 1. Remarkably, it was observed that the protein band patterns of TI 2893 appeared to be identical to that of isolate TI 1580, both for the SDS-PAGE, and for the Western blot.

In conclusion:

It thus seemed that the differences between the strains of the Mexican outbreak of 2021, and known Sa la strains, are not immediately evident from standard genetic-, biochemical- and serological characterisations.

Example 3: Characterisation of serological differences

To investigate a potential serological cause behind the vaccination-breakthrough in Mexico 2021, the isolates were tested in a competitive ELISA, using antiserum from Tilapia that were vaccinated with a monovalent bacterin vaccine of Sa la isolate TI 1422, as the primary antibody for the inhibition.

Briefly: ELISA plates were coated with the TI 1422 bacterin antigen, and next the wells were blocked with casein. Subsequently, a mix of a bacterial isolate test antigens, or PBS control, and the primary antibody was added into the wells and incubated. Next, enzyme-conjugated secondary anti-Tilapia antibody was then added, followed by addition of colouring substrate. The percentage of inhibition was calculated, taking the optical density reading in control wells as 100% binding. Using this set-up, isolates having a serologic relation to the Sa la vaccine strain were expected to bind to the primary antibody, demonstrating inhibition of the control level of binding.

Results are presented in Figure 2. The relative percentage inhibition is indicated on the vertical axis. The Mexico 2021 outbreak samples are indicated on the horizontal axis, numbered TI 2889 - TI 2898. The negative controls used were bacteria of *S. iniae* (Si) and of *Tenacibaculum maritimum* (Tmar). Positive controls were bacteria from Sa la 'vaccine strain' TI 1422, and Sa la strain TI 1580.

The results show that the positive- and negative inhibition responses for the various control samples, were all as expected. For the various Mexico 2021 isolates the inhibition responses were clearly reduced to between only 2 and 15 % inhibition; much less than the inhibition of 44 - 49 % for the 'classic' Sa la isolates TI 1422 and TI 1580.

This is clear proof that the outbreak isolates were indeed much less recognised by antibodies raised against known Sa la bacteria, such as induced by commercial Sa vaccines, which explains why these vaccines could only offer little protection against the outbreaks in Mexico in 2021.

Also, in similar inhibition ELISAs, Sa la bacteria were tested that had been isolated in 2018 from diseased Tilapia from the same region of Mexico. These isolates scored more like the Thailand 2005 isolates, showing inhibition levels of 35 - 40 %. Consequently, a new type of Sa la bacteria had indeed developed in Mexico by 2021.

15

Example 4: Preparation of a seed of TI 2893

A seed was produced of isolate TI 2893, for use in various animal trials and for making the deposit at the CNCM.

TI 2893 strain was recovered from frozen glycerol stock on TSA plates and subsequently incubated at 26 - 32 °C for 3 days. Well separated colonies from TSA plates were selected for subculture to fresh TSA plates and incubated overnight. Colonies on plates were swabbed and collected in sterile saline. The preparation was inoculated into yeast extract/NaCl medium, and incubated at 26 - 32 °C. After overnight incubation 30% glycerol was added, this was filled into cryovials, and stored below -60 °C. 12 of these cryovials were sent to CNCM.

30

Example 5: Characterisation by PCR

Primers were designed to uniquely identify the new type of Sa la bacteria according to the invention. The primers were ordered from a contract firm, and were delivered purified and lyophilised. Primers were reconstituted in UltraPure™ water to make stocks at 100 µM, which were stored frozen.

To prepare DNA samples, the TI 2893 seed was plated on an agar plate and incubated overnight. The plate was then swabbed with 500 µl of 40 mM PBS. Of this suspension 100 µl was used for genomic DNA extraction, using the QIAamp® DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The TI 2893 genomic DNA was ultimately resuspended in AE buffer and stored.

40

PCR tests were done using a Proflex™ PCR machine (Thermo Fisher), using the default ramp rate.

A PCR reaction premix was prepared, which for each reaction included: 2.5 µl of each of the 2 primers for an assay, each at 10 µM; 19 µl of ultrapure water; and 1 µl of DNA; to a total volume of 25 µl for each

- 5 reaction. This was added to prefilled single-dose cups with lyophilized PCR beads (illustra PuReTaq™ Ready-To-Go PCR Bead; Cytiva), which were used according to the manufacturer's instructions.

Sequence comparisons were made with isolates of Sa Ia, Sa III, and negative controls were without DNA.

- 10 The cycling conditions used were largely the same, except that for the primer set of SEQ ID NOs: 3 and 4 some 'touchdown' cycles were included.

Thus, for the primer sets of SEQ ID NOs: 1 and 2 (pepN gene region); 5 and 6 (DUF3307 gene region); and 7 and 8 (yjdM gene region); the cycling conditions are as follows:

- 5 min. 95 °C
- 15 - 35 cycles of:
 - 1 min. 95 °C
 - 1 min. 58 °C
 - 2 min. 72 °C
- 10 min. 72 °C
- 20 - hold at 4 °C.

For the primer set of SEQ ID NOs: 9 and 10 (abiH gene region), the cycling conditions are as follows:

- 5 min. 95 °C
- 30 cycles of:
 - 25 • 1 min. 95 °C
 - 1 min. 63 °C
 - 2 min. 72 °C
- 10 min. 72 °C
- hold at 4 °C.
- 30

For the primer set of SEQ ID NOs: 3 and 4 (DUF1310 gene region), the cycling conditions are:

- 5 min. 94 °C
- 12 cycles of:
 - 1 min. 94 °C
 - 35 • 1 min. 66 °C for the first cycle, but reduced by 0.5 °C for each of the next 11 cycles
 - 2 min. 72 °C
- 20 cycles of:
 - 1 min. 94 °C
 - 1 min. 60 °C
 - 40 • 2 min. 72 °C
- 10 min. 72 °C

- hold at 4 °C.

Also, for the primer set of SEQ ID NOs: 11 and 12 (fbsA gene region), the cycling conditions are:

- 5 min. 95 °C
- 5 - 30 cycles of:
 - 1 min. 95 °C
 - 1 min. 63 °C
 - 1 min. 72 °C
- 10 min. 72 °C
- 10 - hold at 4 °C.

PCR products could be stored frozen until loading. Then, 10 µl from each PCR tube was loaded on a pre-cast 1 % agarose gel, and electrophoresed in the E-Gel™ system (Thermo Fisher) using standard settings, for 13 - 18 minutes depending on the size of the gel. 1 Kb Plus™ DNA ladder (Thermo Fisher)

- 15 was used as marker, according to the manufacturer's instructions.

As described above (see Table 1), these PCRs produce nucleotide fragments for the Mexico 2021 isolates (TI 2889 - 2898) that differ clearly in size from the PCR products found when using those same primers and conditions, on nucleic acid from Sa la isolates that were known before.

20

In addition, when these PCR protocols were used to test isolates from the outbreak in Honduras in 2022, isolates numbered TI 2925-2934, these produced the same PCR fragment sizes as did the Mexico 2021 isolates, and thus also qualify as Sa la bacteria according to the invention.

- 25 This also indicates that the outbreaks in Honduras in 2022 were caused by Sa la bacteria that are the same as those responsible for the Mexico 2021 outbreaks, which may have somehow contaminated the waters in other countries.

30 **Example 6: Characterisation of differences in pathology**

To characterise the level of pathogenicity of the new Mexican isolates, comparative challenge infections were done in Tilapia.

- 35 Overnight mini cultures of isolates TI 2893 and TI 1580 were prepared and quantified by measuring optical density (OD) at 660 nm using a spectrophotometer. These were used to inoculate Tilapia intraperitoneally, and after incubation, the number of mortalities were counted.

- 40 Mortality was observed already on day 1 post challenge, and reached its maximum on day 2 p.c. Signs of pathology observed were the same for the two isolates tested, thus under laboratory conditions, the overall pathology of TI 2893 (although much more acute, as shown below) showed similar clinical signs as did known Sa la isolates.

The difference in pathology was found in the number of bacteria required to cause serious disease. To test this, the number of colony forming units (cfu) inoculated for TI 2893 was set much lower than for TI 1580. The results were nevertheless impressive:

- 5 • For TI 1580, fish were inoculated i.p. with 100.000 cfu/fish, and mortality was observed for 16 of the 25 fish inoculated. That corresponds to a mortality of 64 %.
 - For TI 2893, only 25 cfu/fish were inoculated, but mortality was found for 20 of the 25 fish inoculated; that is a mortality of 80 %.
- 10 Consequently, TI 2893 seems to be at least 4000 times more pathogenic than a known pathogenic Sa la isolate.

15

Example 7: Vaccination-challenge trial with single- and combination vaccines, and cross-protection effect

7.1 INTRODUCTION

- 20 In an elaborate vaccination-challenge experiment in young Tilapia the protective effect of existing vaccines were tested in relation to a challenge infection with either known- (isolate TI 1580), or new (isolate TI 2893) Sa la bacteria. Also, the vaccines were amended by adding bacterin from the new bacteria, and the new bacteria were tested in vaccines on their own, for their (cross-)protective properties, against both types of challenges.

- 25 All vaccines used were water-in-oil (W/O) emulsions, containing formalin-inactivated Streptococcal bacteria.

7.1.1 Experimental design

- 30 The experiment was performed with a total of 330 Tilapias. The fish were equally divided into 6 groups as they came to hand. Five groups were injected intraperitoneally (i.p.) with one of the different vaccines tested:

- 'Sa1': AQUAVAQ Strep Sa1
- 'Strep-4': AQUAVAC Strep-4
- Sa1 + TI 2893 vaccine
- 35 - Strep-4 + TI 2893 vaccine, and
- TI 2893 vaccine.

The 6th group was injected with standard vaccine dilution buffer (SVDB) to serve as unvaccinated control. SVDB is a phosphate-buffered physiological salt solution.

- 40 Three weeks post-vaccination, fish from each group were challenged with either isolate TI 2893 (Sa la, Mexico 2021), or with isolate TI 1580 (Sa la, Thailand 2005). Post-challenge observations (mortality and

clinical signs) were done for 14 days post challenge, during which period dead and moribund fish were collected daily and samples of internal organs were plated on non-selective media to determine presence of the challenge bacterium.

NB: As *S. agalactiae* is a potential zoonosis for humans and other mammals, appropriate health- and bio-safety measures were required throughout the experiment.

7.2 MATERIALS AND METHODS

7.2.1 Vaccines

AQUAVAC® Strep Sa1 (MSD Animal Health) (herein: Sa1), as described herein, is a commercial bivalent vaccine, of formalin inactivated *Sa* bacteria from *Sa* Ia isolate TI 1422, and from *Sa* III isolate TI 1428; formulated with Montanide™ ISA 763A VG.

AQUAVAC® Strep-4 (MSD Animal Health) (herein: Strep-4), is a commercial quadrivalent vaccine, of formalin inactivated *Streptococci*: *Sa* Ia isolate TI 1422, *Sa* Ib isolate 513, *Sa* III isolate TI 1428, and *S. iniae* isolate SB430; formulated in light liquid paraffin oil.

'TI 2893 vaccine' is a W/O emulsion of the new bacterium according to the invention, formalin inactivated, and formulated in light liquid paraffin oil.

In short: the TI 2893 isolate was revived, plated, swabbed, taken up into a suspension, and cultured in yeast extract/NaCl medium as described above. Next the culture was then inactivated with 0.5 % v/v formalin overnight at 26 - 32 °C, and was then stored at 4 °C, until the vaccine was formulated.

For emulsification, the formalin-killed bacterial culture TI 2893 was mixed with the paraffin oil in a 45 % water : 55 % oil ratio, with Span™ and Tween80™ as surfactants. Next this composition was emulsified using an IKA T25 Ultrathorax at 11,000 rpm for a total of 3 minutes; the water-phase droplet size was checked by microscopy. 25 ml of the vaccine was then filled in each of 4 100 ml glass bottles, and stored at 4 °C until use. The amount of bacteria in the TI 2893 vaccine corresponded to 5×10^8 cells/ml vaccine.

The double vaccinations, Sa1 + TI 2893, and Strep-4 + TI 2893, were given as consecutive doses by i.p. injection.

7.2.2 Challenge materials

TI 2893 seed was available in frozen storage at -60 °C in 1.5 ml vials, at: 6.5×10^9 cfu/ml.

TI 1580 seed was available at -60 °C in 1 ml vials, at: 5.2×10^8 cfu/ml.

For TI 1580, 1 ml of seed was inoculated into 100 ml of yeast extract/NaCl medium, and incubated at 26 – 32 °C with agitation overnight.

For TI 2893, the stock was revived, plated, swabbed, taken up into a suspension, and cultured in yeast
5 extract/NaCl medium as described above.

Both end cultures will then be separately diluted with 0.9 % w/v NaCl for use in the challenge. To control the challenge amount, each challenge suspension was serially diluted and spread-plated in duplo on TSA plates to determine the viable count of each challenge dose. The viable count was determined after
10 challenge suspension inoculation.

7.2.3 Test animal

Tilapia (*Oreochromis spec.*) were used, as hatchery produced fingerlings, from: Temasek Life Sciences
15 Laboratory, Singapore. Average weight at arrival was 2 g/fish. Fish were acclimatised in a quarantine tank, and were on average 10 g/fish at the start of the experiment.

Only healthy fish were used, at 330 in total.

The fish were not marked individually, but the different groups were kept in separate labelled tanks.

20

7.2.4 Water conditions

- salinity was at 2 parts per thousand after vaccination, and was freshwater after challenge.
- temperature was 28 °C ± 2 °C after vaccination, and 30 °C ± 2 °C after challenge.
- tank size was 500 L after vaccination, and 70 L after challenge.

25

Water conditions were monitored daily: O₂, NH₄, NO₂, and NO₃ concentrations; salinity; water temperature; and pH.

30 7.2.5 Feeding

After vaccination, fish were fed at 2-4 % of their body weight per day, which was adjusted weekly for each group. After challenge the fish were fed ad libitum.

However, the fish were starved for at least 12 hours prior to manipulations such as transfer to tanks and weighing. Also, the fish were starved for at least 48h prior to vaccination and challenge.

35

7.2.5 Treatment

Vaccination

When the fish reached an average weight of 10 grams, they were taken from the quarantine tank and
40 divided equally and randomly into 6 groups.

Fish were anaesthetized with AQUI-S®. Vaccination was then performed individually by i.p. injection, about half-way between the base and the tip of the pelvic fin.

The vaccines 'Sa1' and TI 2893, were administered i.p. as 50 µl dose; the Strep-4 vaccine was given as 100 µl dose. The mock-vaccination with buffer was given i.p. as a 150 µl dose.

- 5 The combined vaccinations: Sa1 + TI 2893, and Strep-4 + TI 2893, were each given as two subsequent doses: as 50 and 50 µl; and as 100 and 50 µl doses, respectively.

Challenge

- 10 Challenge was performed at 3 weeks post-vaccination; the fish were then at an average weight of 15.5 gram. First the fish were starved for at least 48 hours prior to the challenge, to ensure complete emptying of the gastro-intestinal tract and thereby preventing injury to the internal organs as a result of the injection. Then the fish were anaesthetized. The challenge inoculation was given i.p. as 100 µl dose of either the TI 1580 or the TI 2893 bacteria.

- 15 The challenge compositions used were:

- TI 2893: 253 cfu/ml
- TI 1580: 1×10^6 cfu/ml

Immediately after inoculation, the fish were transferred to 12 separate challenge holding tanks.

20

Monitoring

- Fish were monitored after vaccination and after challenge infection for any direct effects from the treatment of the sedation. Later the fish were observed at least once a day. Post-challenge monitoring was for 14 days. Typical clinical signs included erratic swimming (spiralling or spinning) and
- 25 exophthalmia. Dead fish were subjected to section and examination of the internal organs. For bacteriological sampling, the brain and/or internal organs were sampled and plated on TSA plates. The plates were incubated at 26 - 32 °C for 1 - 3 days before scoring them.

- 30 7.2.6 Evaluation of results

Cumulative mortality

Mortality after challenge was expressed and compared as percentage of cumulative mortality.

This is calculated as follows:

- 35 % cumulative mortality = (total number of fish died / total number of fish in a group) x 100 %

Vaccine efficacy

- The efficacy of the vaccines to protect fish against the challenges was determined as the Relative Percentage Survival (RPS). The RPS values for the vaccine groups will be calculated using the following
- 40 formula:

$$RPS = \left\{ 1 - \left(\frac{\% \text{ mortality in vaccinates}}{\% \text{ mortality in controls}} \right) \right\} \times 100$$

5 This is also described in WO 2011/048041, pages 16-17.

7.3 RESULTS

10

The experiment went well in that no unexpected events intervened causing disruption or unexpected infection and mortalities. Also the vaccination- and challenge treatments caused no problems of their own, and the challenge infections had the expected effects on the non-vaccinated fish.

15 7.3.1 Mortality

The cumulative mortality results are presented in Figures 3 and 4, respectively for the challenges with the TI 1580 or the TI 2893 isolates.

20 The % cumulative mortality observed, is presented on the vertical axis, as function of the days post challenge, which are indicated on the horizontal axis. The different test groups are indicated by different signs, as indicated in the legend.

What can readily be observed is that isolate TI 1580 caused a lesser mortality in the nonvaccinated fish than did isolate TI 2893: 64 vs 80 %. This even through the challenge dose for TI 2893 was 4000 times lower. This confirms the results observed in the experiment disclosed in Example 6 above.

25

Mortality caused by TI 1580 challenge was drastically reduced by all types of vaccination applied, also by the TI 2893 vaccine. Mortality from the TI 2893 challenge infection was not so well reduced by the classic Sa vaccines Sa1 and Strep-4. This confirmed the observations made during the Mexican outbreak of 2021.

30 When TI 2893 vaccine was applied, challenge-mortality was nearly prevented, both when the TI 2893 vaccine was used on its own, and -even more effectively- when it was combined with one of the classic vaccines.

Similar effects as for mortality, were also seen in the vaccination results.

35

7.3.1 Vaccination efficacy

Table 2: Results of the vaccinations for the two types of challenges given

Challenge	TI 1580	TI 2893
Vaccine	% RPS	% RPS
Sa1	81	20
Strep-4	100	50
Sa1+ TI 2893	81	85
Strep-4 + TI 2893	94	85
TI 2893	81	95
Control	--	--

Table 2 presents the efficacy of the various vaccines administered and tested against the two types of challenge infections. The vaccination effect is presented in % RPS

The 'classic' vaccines Sa1 and Strep-4 protected well against TI 1580 challenge, with RPS values at 81 and 100 %, respectively. For those two vaccines the protection against TI 2893 challenge was however much less, at 20 and 50 % RPS, respectively.

The TI 2893 vaccine, even as a monovalent vaccine, protected well, both against a homologous challenge (TI 2893), and against a heterologous challenge (TI 1580), at: 95 and 81 % RPS, respectively. When combined with the classic Sa la vaccines, TI 2893 vaccine was able to bring protection against TI 2893 up to very good levels, from 20 - 50 % up to: 85 % RPS. Also, adding the TI 2893 vaccine to the Sa1 and Strep-4 vaccines did not really affect protection against the TI 1580 challenge.

NB: In this system of efficacy assessment by % RPS, the mortality in the negative control group is set as the basis for the calculation of RPS value of the other groups.

7.4 CONCLUSIONS

Vaccines against known Sa la isolates were found to be little effective against a challenge infection with a new Sa la bacterium according to the invention, isolate TI 2893. This reflected the serious outbreaks of disease observed in Tilapia aquaculture farms in Mexico in summer of 2021.

Advantageously however it was found that a vaccine prepared from this very pathogenic isolate, could effectively protect against mortality caused by that same TI 2893 isolate, but also against mortality induced by challenge with a known Sa la isolate (TI 1580).

Also, the TI 2893 vaccine could be added to the existing Sa vaccines, to supplement those with protective effect against TI 2893.

The present invention thus allows to make and use of vaccines for fish that protect well against known Sa la bacteria and against the new outbreak type Sa la bacteria according to the invention.

Example 8: Genome sequence analysis

Sample preparation:

5 The isolate TI 2893 (corresponding to the sample as deposited) was cultured in 50 ml volume as described above. The cell pellet was harvested, frozen at -20 °C, and sent to a CRO (BaseClear, Leiden, The Netherlands) for DNA sequencing. The cells were lysed mechanically using ZR BashingBead™ Lysis tubes, and vortexing for 5 minutes. Next, genomic DNA was extracted using the ZymoBIOMICS™ DNA miniprep kit according to the manufacturer's instructions. The extracted genomic DNA was quantified and normalized based on a measurement using the Qubit™ Broad Range kit.

10

DNA sequencing

Long-read sequencing was performed using Oxford Nanopore™ Technologies as follows: library preparation was performed with 1D ligation, and sequence reads were generated using the Oxford Nanopore GridION™ system with a R9.4.1 flowcell. Basecalling was performed using Guppy™ v. 5.0.13.

15 The ONT sequencing statistics were as presented in Table 2.

Table 2: ONT sequencing statistics

Sample	Total nr of reads	Mapped nr of reads	% reads used	Yield in Mbp	Read n50	Mean nt	Mean qscore	Average coverage
TI 2893	669682	662032	98,86%	5892	17959	8798	12.32	2724 x

20 For short read sequencing, the library preparation was done using the Illumina Nextera™ XT DNA Library prep kit. Sequencing of the libraries was then performed on a Novaseq™ 6000 system with an Illumina PE150 kit. Paired-end sequencing resulted in FASTQ read sequence files using bcl2fastq v. 2.20 (Illumina). Initial quality assessment was based on data passing the Illumina Chastity™ filtering. Subsequently, reads containing PhiX control signal were removed using a filtering protocol (developed by

25 the CRO). In addition, reads containing (partial) adapters were clipped up to a minimum read length of 50 bp. The second quality assessment was based on the remaining reads using the FASTQC quality control tool v. 0.11.8. The Illumina sequencing statistics are provided in Table 3.

Additionally to the genome, a plasmid of 4441 bp was assembled using the plasmidSPAdes script of

30 SPAdes v. 3.15.3 (Antipov et al., 2016, Bioinformatics, vol. 32, p. 3380 - 3387) on the complete Illumina read set.

Table 3: Illumina sequencing statistics

Sample name	Number of reads	Mapped nr of reads	% mapped reads	Yield in Mbp	Average quality score	Coverage
TI 2893	3582406	3274752	91,41	530	35,03	231 x
TI 2893 plasmid		258857	7,23			8023 x

Genome assembly and annotation

- 5 The ONT reads obtained, were used with Flye 2.9 (M. Kolmogorov et al., 2019, Nature Biotechnology, vol. 540, p. 540-546) to produce the draft genome assembly, followed by a polishing step using Medaka v. 1.4.3. A subsequent polishing step using the Illumina paired reads (Pilon, v. 1.23) resulted in the final version of the genome assembly. The assembly statistics are provided in Table 4.

10 Table 4: Genome assembly statistics

TI 2893	Nr scaffolds	Maximum scaffold size	GC %
chromosome	1	2113287	35,7
plasmid	1	4441	37,8

Subsequently, prokaryotic genome annotation was performed on the assembled contig using an annotation pipeline based on Prokka (T. Seemann, 2014, Bioinformatics, vol. 30, p. 2068-2069). The

- 15 pipeline included the programs:

- Prokaryote gene prediction, by Prodigal (Hyatt et al., 2010, Bioinformatics, vol. 11, p. 119) v. 2.6.3,
- rRNA detection, using BARRNAP (Seemann, 2014, supra) v. 0.8,
- tRNA prediction, by Aragorn (Laslett et al., 2004, Nucleic Acids Res., vol. 32, p. 11-16) v. 1.2.38, and
- pCDS physico-chemical properties (BaseClear, Leiden, the Netherlands).

20

And by using the public databases:

- Uniprot-Swissprot, v. 2019_08; and
- Rfam (Kalvari et al., 2017, Nucleic Acids Res., vol. 49, D1), v. 14.1.

- 25 Proteins were inferred by homology using:

- EC number, from UniProt BLAST best hit (Apweiler et al., 2004, Nucleic Acids Res., vol. 32, D115-9; Pundir et al., 2016, doi.org/10.1002/0471250953.bi0129s53);
- Signal peptide, and: Cellular localization, both from SignalP (Petersen et al., 2011, Nat. Methods, vol. 8, p. 785-786) v. 4.1;
- 30 - CAZY number, and: Function annotation, both from UniProt BLAST best hit (Apweiler et al., 2004; Pundir et al., 2016; both supra); and
- Conserved domains, by HMMER-3 (Eddy et al., 2011, Nucleic Acids Res., vol. 39, W29-37).

As is well-known in the art, proteins are 'inferred by homology' when their existence is probable because clear orthologs exist in closely related species.

5

Genome analysis

SEQ ID NO: 13 presents the full 2.1 Mb of the genomic DNA sequence from the bacterium deposited as isolate CNCM I-5929. The sequence is presented in such a way that nt number 1 is the first nucleotide of the dnaA gene. Several other annotated genes are indicated herein by a nt number indicating their region of location in the genome as presented in SEQ ID NO: 13.

10

Based on the genomic sequence of the Sa la bacteria according to the invention, several genetic determinants were analyzed, such as: 16S rRNA genes; genes for multilocus sequence typing; mobile genetic elements; pro-phage typing; and molecular serotyping. Results have been described

15

hereinbefore. Other relevant features of the genome organization found in SEQ ID NO: 13, are:

- Six rRNA cluster regions are located between nt 15400 and 440300.
- MLST sequence typing was done based on the sequence of 7 housekeeping genes: adhP (around nt 72430); tkt (around nt 298000); glcK (around nt 516380); atr (around nt 539580); pheS (around nt 923670); glnA (around nt 1760150); and sdhA (around nt 2082200).
- The cpsla gene cluster is located in the region of nt 1194000 - 1213000.
- A CRISPR array is located around nt 964400.
- In the genome of isolate TI 2893, no pro-phage was detected.

20

Marker genes unique to the highly virulent Sa la bacteria according to the invention, were identified by comparing their genomic sequence to that of known Sa la isolates, using OrthoVenn analysis (Xu et al., 2019, Nucleic Acids Res., vol. 47, W52-W58); and by using LastZ alignment v. 1.02.00 (Geneious™ Prime suite, v. 2022.1.1). As described in detail hereinbefore, selective PCR primer sets were developed to identify the genetic markers specific for an Sa la bacterium according to the invention.

25

A further example of such analyses is the comparison with the genome sequence of an Sa la that was isolated in 2009 in China from a Tilapia without clear symptoms of disease: isolate TFJ0901, of which the sequence is disclosed in GenBank acc. nr. NZ_CP034315. Both TI 2893 and TFJ0901 are Sa la, are of sequence type 7, do not encode a pro-phage, and in regard to the integrated mobile genetic elements they contain, both have: 4 copies of nr. IS 1161; 1 copy of nr. IS 1501; and 6 copies of nr. IS 702.

30

However, TI 2893 comprises 5 copies of nr. IS 6110 and 5 copies of nr. IS 630, whereas TFJ0901 has no copies of either of IS 6110 or IS 630. As the insertion of such mobile elements may affect the expression or regulation of genes, they may be linked to changes in the bacteria's replication, virulence, or pathogenicity.

35

Specifically: in TI 2893 four of the marker genes identified for the invention, comprise a mobile genetic element:

40

- The yjdM gene, around nt position 991000 on the genome, contains an IS 630 element,
- The DUF3307 gene, around position 1028000, contains at its 5' side, an IS 6110 element,
- Similarly, the abiH gene, around position 1274000, contains at its 5' side, an IS 6110 element, and
- The DUF1310 gene, around position 1935000, contains an IS 6110 element.

5

All these mobile genetic elements are known in the art:

- IS 1161 is an insertion element encoding a putative transposase, similar to aa sequence UniProtKB: P37245.
- IS 1501 is an insertion element encoding an uncharacterized 19.7 kDa protein, similar to aa
10 sequence UniProtKB: P60046.
- IS 702 is an insertion element encoding a putative transposase of 128 aa, similar to aa sequence UniProtKB: Q00462, and a putative transposase of 130 aa, similar to aa sequence UniProtKB: Q00462.
- IS 630 is an insertion element containing a hypothetical gene encoding 169 aa, and an
15 uncharacterized 39 kDa protein, similar to aa sequence: UniProtKB: P16943.
- IS 6110 is an insertion element containing a Rv0795 gene encoding an uncharacterized 12kDa protein, similar to aa sequence UniProtKB: P9WKH5. IS 6110 is often associated with IS 904, encoding a transposase, similar to aa sequence UniProtKB: P35878.

- 20 SEQ ID NO: 14 presents the full DNA sequence of the plasmid found in Sa la bacteria according to the invention: isolate TI 2893, deposited as: CNCM I-5929.

The plasmid that was detected in TI 2893 was also found in some known Sa la isolates obtained previously from several other countries, and which induced only 'normal' Sa la pathology in Tilapia.

- 25 Nevertheless, the conservation of this plasmid in the bacterium according to the invention marks that it is evidently important for the bacterium.

Legend to the figures

Figure 1

Photographs of Coomassie-stained SDS-Page gel (panel A), and of Western blot stained with a first antibody from fish vaccinated with a Sa Ia bacterin vaccine (panel B). Details are in Example 2.

In both panels: lane 1: Molecular weight marker - Mws are indicated at the left side; lane 2: whole cell protein lysate of isolate TI 1580 (Thailand 2005); lane 3: whole cell protein sample of isolate TI 2893 from Mexico 2021 outbreak; lane 4: supernatant of TI 1580 mini culture; lane 5: supernatant of TI 2893 mini culture.

Figure 2

Graphical representation of the results of an inhibition-type ELISA on the ten Mexico 2021 isolates, using as primary- and inhibition antibody, a Tilapia serum raised against an Sa Ia bacterin vaccine. Details are in Example 3.

On the vertical axis is indicated the level of inhibition relative to no antigen controls. On the horizontal axis are the various isolates tested: numbers TI 2889 - TI 2898 are the ten isolates from the Mexico 2021 outbreak; 'vaccine strain' is Sa Ia TI 1422; TI 1580 is another known Sa Ia isolate; 'Si' and 'Tmar' are the negative control antigens from respectively: *S. iniae* (Si) and *Tenacibaculum maritimum* bacteria. The indication 'ND' is for: not detectable.

Figures 3 and 4

Graphical representation of the % cumulative mortality as observed in the vaccination-challenge experiment as disclosed in Example 7. Figure 3: challenge with the 'classic' Sa Ia isolate TI 1580; Figure 4: challenge with the new Sa Ia bacterium according to the invention: TI 2893.

Claims

1. *Streptococcus agalactiae* bacterium of serotype Ia (Sa Ia) having the characterising features of the bacterium as deposited at the Collection Nationale de Cultures de Microorganismes (CNCM), Institut Pasteur, Paris, France, under accession number: CNCM I-5929.
2. The Sa Ia bacterium according to claim 1, characterised in that in a polymerase chain reaction (PCR) with genetic material from said bacterium, a nucleotide (nt) fragment of a specific size is produced, when using a specific primer set, as follows:
 - a fragment of 500 nt, with the primer set of SEQ ID NO: 1 and SEQ ID NO: 2; or
 - a fragment of 1672 nt, with the primer set of SEQ ID NO: 3 and SEQ ID NO: 4; or
 - a fragment of 1772 nt, with the primer set of SEQ ID NO: 5 and SEQ ID NO: 6; or
 - a fragment of 1912 nt, with the primer set of SEQ ID NO: 7 and SEQ ID NO: 8; or
 - a fragment of 2016 nt, with the primer set of SEQ ID NO: 9 and SEQ ID NO: 10; or
 - a fragment of 876 nt, with the primer set of SEQ ID NO: 11 and SEQ ID NO: 12.
3. The Sa Ia bacterium according to claims 1 or 2, characterised in that in a PCR with genetic material from said bacterium, a nt fragment is produced of the sizes as defined in claim 2, with two or more of the primer sets as defined in claim 2.
4. The Sa Ia bacterium according to any one of claims 1 - 3, characterised in that in a PCR with genetic material from said bacterium, a nt fragment is produced of the sizes as defined in claim 2, with all six of the primer sets as defined in claim 2.
5. The Sa Ia bacterium according to any one of claims 1 - 4, characterised in that the bacterium is the bacterium as deposited under accession number: CNCM I-5929, or is a descendant from said deposited bacterium.
6. The Sa Ia bacterium according to any one of claims 1 - 5, or the descendant according to claim 5, characterised in that the bacterium has a genome having 90 % nucleotide sequence identity with the sequence of SEQ ID NO: 13.
7. Composition comprising a preparation of the Sa Ia bacterium or of the descendant, both according to any one of claims 1 - 6.
8. The composition according to claim 7, characterised in that the preparation comprises the Sa Ia bacterium or the descendant, both according to any one of claims 1 - 6, in inactivated form.
9. Diagnostic test kit, characterised in that said kit comprises a container comprising the Sa Ia bacterium or the descendant, both according to any one of claims 1 - 6, and/or comprising the composition according to claims 7 or 8.

10. The Sa la bacterium or the descendant, both according to any one of claims 1 - 6, and/or the composition according to claims 7 or 8, for use as a vaccine for fish against Streptococcosis.
11. Vaccine for fish against Streptococcosis, characterised in that said vaccine comprises the Sa la bacterium or the descendant, both according to any one of claims 1 - 6, and/or the composition according to claims 7 or 8, and a pharmaceutically acceptable carrier.
12. The vaccine according to claim 11, further comprising an adjuvant.
13. The Sa la bacterium, the descendant, or the composition, all for the use according to claim 10, or the vaccine according to claims 11 or 12, characterised in that the fish is from a genus selected from: Coptodon, Oreochromis, and Sarotherodon.
14. The Sa la bacterium, the descendant, or the composition, for the use, or the vaccine, all according to claim 13, characterised in that the fish is of a species selected from: Coptodon zillii, Coptodon guineensis, Oreochromis niloticus, Oreochromis aureus, Oreochromis mossambicus, Oreochromis hornorum, and Sarotherodon melanotheron; or is a hybrid of one or more of these species.
15. Method for the preparation of the vaccine according to claims 11 or 12, the method comprising the step of admixing the Sa la bacterium or the descendant, both according to any one of claims 1 - 6, and/or admixing the composition according to claims 7 or 8, and a pharmaceutically acceptable carrier.
16. The method according to claim 15, characterised in that the method comprises a step for inactivating the Sa la bacterium or the descendant, both according to any one of claims 1 - 6.
17. Use of the Sa la bacterium or the descendant, both according to any one of claims 1 - 6, and/or of the composition according to claims 7 or 8, for the manufacture of the vaccine according to claims 11 or 12.
18. Method for the protection of fish against Streptococcosis, the method comprising the step of administering to said fish the vaccine according to claims 11 or 12, or the vaccine as obtainable by the methods according to claims 15 or 16, or as obtainable by the use according to claim 17.