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Near infrared spectroscopy as a novel non-invasive tool for the detection of lactococcosis in rainbow trout

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ABSTRACT

Lactococcosis, mainly sustained by the warm-water bacterium *Lactococcus garvieae*, is a disease associated with evident mortalities and economic lossed. Main disease signs are commonly bilateral exophthalmia, hyperpigmentation in the body, haemorrhages around the eye area, opercula, and mouth region, swollen abdomen, and anal prolapse. An effective monitoring system of fish diseases relies on rapid techniques for the timely management of infection outbreaks. We evaluated the potential of the SCiO sensor, a portable near infrared spectroscope, to assess in field the health status of rainbow trout. The study sample was composed of 20 symptomatic and 20 asymptomatic rainbow trout obtained from an aquaculture farm and submitted to molecular detection of *L. garvieae* and SCiO examination. In order to detect presence or absence of septicaemia condition, blood smears were prepared from peripheral blood. Part of the symptomatic individuals (9/20) showed septicaemia, whereas no bacteria were found in the asymptomatic individuals. PCR assay of bacterial DNA was positive in spleen and kidney of 19/20 symptomatic and in 1/20 asymptomatic individuals. Bacteriological analysis revealed *L. garvieae* in 18/20 symptomatic samples and in 2/20 asymptomatic samples. The SCiO sensor was found to be able to discriminate between healthy and sick fish [sensibility 0.95 (0.75–1.00 I.C. 95%), specificity 0.80 (0.56–0.94 I.C. 95%)]. By virtue of rapid acquisition, low cost and use on site, the SCiO was considered as a useful diagnostic tool for monitoring *L. garvieae* infection in rainbow trout.

1. Introduction

Aquaculture production has expanded rapidly and is now the world's fastest growing food-production sector, due to the increasing demand for human nutritional purpose (Naylor et al., 2021). One of the main problems affecting aquaculture sector are infectious diseases, that take a heavy toll on fish farming systems (Mishra et al., 2018), with high economic losses in aquaculture production. The annual cost due to diseases is estimated around 20% of total sector production value (Bertinetto et al., 2022a).

A major and re-emerging bacterial disease in aquaculture is lactococcosis, caused by the Gram-positive warm water pathogen *Lactococcus garvieae*. Its spread is favoured by the increase in water temperature in the Mediterranean, where it causes infection in marine and freshwater fishes, toward which rainbow trout (*Oncorhynchus mykiss*) is particularly susceptible (Meyburgh et al., 2017; Vendrell et al., 2006). Typical signs of infection are commonly anorexia, hyperpigmentation in the body, lethargy, melanosis, evident bilateral exophthalmos, and ocular extrusion (Khalil et al., 2023a; Khalil et al., 2023b; Vendrell et al., 2006). Macroscopic examination reveals extensive haemorrhaging, while necropsy discloses accumulation of ascitic fluid in the peritoneal cavity, congestion of internal organs, enlargement of the spleen (splenomegaly) and the liver, and exudate covering the brain (Khalil et al., 2023a; Meyburgh et al., 2017; Vendrell et al., 2006).

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Italy is the first fish producer of freshwater salmonids in the EU and more than 65% of the production yielded in North Italy (Pastorino et al., 2019). While there is no detailed data that estimates loss in aquaculture sector, the economic impact of lactococcosis in farmed fish is very high (Soltani et al., 2021), approximately economic losses exceed 50–80% of the total production (Eldar and Ghittino, 1999).

In the past, *L. garvieae* has been readily detectable using conventional microbiological techniques and PCR analysis, as suggested by Zlotkin et al. (1998). However, recently, *L. petauri* has emerged as a novel species closely related to *L. garvieae* (Stoppani et al., 2023), causing similar disease signs in affected organisms, as reported by Goodman et al. (2017). While *L. garvieae* has been documented as a primary pathogen in rainbow trout (Duman et al., 2020; Soltani et al., 2021), *L. petauri* has been identified as a disease-causing agent, often misidentified as *L. garvieae* due to high genetic similarity and false positive PCR reactions when commonly used primers, as suggested by Zlotkin et al. (1998), are employed (Saticioglu et al., 2023; Egger et al., 2023; Kotzamanidis et al., 2020).

Recently, distinguishing *L. petauri* from *L. garvieae* has been achieved using conventional microbiological methods, particularly sucrose fermentation, and PCR analysis, employing a novel primer pair developed by Saticioglu et al. (2023). It is noteworthy that due to their similarity in disease signs observed in rainbow trouts, differentiating these agents through morphological or pathological analysis presents some challenges. While isolating bacteria through culture methods and PCR analysis can be laborious, spectroscopic analysis has proven effective in detecting lactococcosis in diseased fish; however, it does not allow for differentiation between *L. garvieae* and *L. petauri*.

Traditional laboratory investigation techniques are often time-consuming, expensive and have limited sample flow; they also require qualified personnel to carry out the analyses, which are done on animals already with evident symptoms or who have already died. It is important to find rapid and in situ diagnostic tools, capable of diagnosing the disease in vivo, without excessive costs and easy to use.

In this context, the use of smart devices to monitor parameters in real time has opened a sustainable frontier in aquaculture (Hu et al., 2020; Vo et al., 2021; Wang et al., 2021). Among these, near infrared reflectance spectroscopy (NIR) is finding a widespread use (Ghidini et al., 2019).

NIR spectroscopy is a part of molecular vibrational spectroscopy, in which the interaction with electromagnetic radiation produces oscillations in bond lengths and angles between these bonds. It is based on the principle that each type of molecule vibrates in a certain way and that the resulting molecular vibrations interact with light to create a unique spectral profile. This allows for non-destructive investigation, with a considerable reduction in time and costs compared to traditional techniques (Ghidini et al., 2019).

The development of miniaturized, handheld NIR spectrometers has led to rapid on-site analysis and considerable improvement in practical applications for the analysis food and natural products by removing the need to transport and store laboratory samples (Bec et al., 2021; Bec et al., 2022; Kosmowski and Worku, 2018). Miniaturized NIR spectrometers, like the SCiO device, are relatively inexpensive, require minimal equipment, and are easy to use even by inexperienced personnel (Bertinetto et al., 2022b; Mayr et al., 2021). The technology found initial application in the food industry for quality control of fruit and cereals, and its potential is now being tested in other sectors (Bec et al., 2020; del Río-Celestino and Font, 2022; Cozzolino, 2021; Fu and Yiing, 2016; Kosmowski and Worku, 2018; Mulvey, 2020; Pennisi et al., 2021; Riu et al., 2020; Wiedemair and Huck, 2018; Wiedemair et al., 2019; Yakubu et al., 2020). Due to its versatility of use for monitoring critical process points (different types of information acquired quickly), it is widely applied in the agricultural and the livestock sector (Narsaiah and Shyam, 2012). Overall, it has found application in seafood authenticity and identification (Cavallini et al., 2022; Ghidini et al., 2019; Grassi et al., 2018; McVey et al., 2021; Sciuto et al., 2021), traceability, and prediction of shelf-life, though not yet for prognostic purposes in aquaculture.

To fill this gap, we evaluated the ability of NIR spectroscopy, particularly SCiO technology, to discriminate between *L. garvieae* infected and non-infected rainbow trout, by means of a portable SCiO sensor in situ. The response obtained by these readings was evaluated and compared with the outcomes of haematological, bacteriological and biomolecular analysis aiming at the proper identification of infected versus uninfected fish.

2. Materials and methods

2.1. SCiO device

The SCiO™ (Consumer Physics, Israel) is a NIR spectrometer that works at wavelengths between 740 and 1070 nm, with a declared resolution of 1 nm. The device measures 67.7 × 40.2 × 18.8 mm and weighs 35 g; its working temperature is between 4 and 35 °C, with an acquisition time of 2–5 s. The detector measures attenuated reflectance from the opaque surface of a scanned sample. It acquires data spectra via a smartphone application (SCiO Developer Toolkit) and analyses the data by means of a browser-based web application. The device is controlled by an Android/iOS (The Lab) app and communicates with a smartphone via Bluetooth connection.

2.2. Sampling

Sampling was performed in a commercial rainbow trout farm in Northern Italy during a single day in July 2022. Fish were collected from a common basin affected by a natural outbreak of lactococcosis (mean water temperature +22 °C). The sample was composed of 40 fish: 20 were macroscopically defined as symptomatic and 20 as asymptomatic. Symptoms were hyperpigmentation in the body, -bilateral exophthalmia, haemorrhages around the eye area, opercula, and mouth region, swollen abdomen, and anal prolapse (Fig. 1); furthermore, specimens display lethargic and erratic swimming. These findings were used to discriminate between symptomatic and asymptomatic individuals prior to SCiO examination.

All specimens were selected from the same tank and euthanasia was caused by an overdose of tricaine methane-sulfonate (MS-222). Both symptomatic and asymptomatic fish were weighed individually (245 ± 95 and 242 ± 71 g, respectively). The total length was also determined, i.e., 30 ± 4 and 30 ± 3 cm (symptomatic and asymptomatic fish, respectively).

2.3. SCiO calibration and data acquisition

The device was calibrated prior to analysis via insertion of the source and the detector into the outer protective case where the calibration module is embedded.

SCiO acquisitions were performed directly on site (Fig. 2). Prior to acquisition excess of water and mucus were removed from each individual. Trout were submitted to measurement three times, scanning them on a flat bench top, with the device placed over the fish. Scans were initially acquired in one specific point on the dorsal and the abdominal area (caudal to the perianal area) to account for possible variations. Data acquired by the SCiO are generated in diffuse reflectance mode, which can be affected by light-scattering spectroscopy artefacts that are not of interest for characterizing the sample under study but can interfere with correct classification. Data pre-treatment is therefore needed to reduce or eliminate these inconveniences. Standard Normal Variate (SNV) was applied for scatter correction. For each sample SCiO acquisition gives a spectrum in the full working range. Each NIR spectrum was stored in an online cloud database (Consumer Physics).

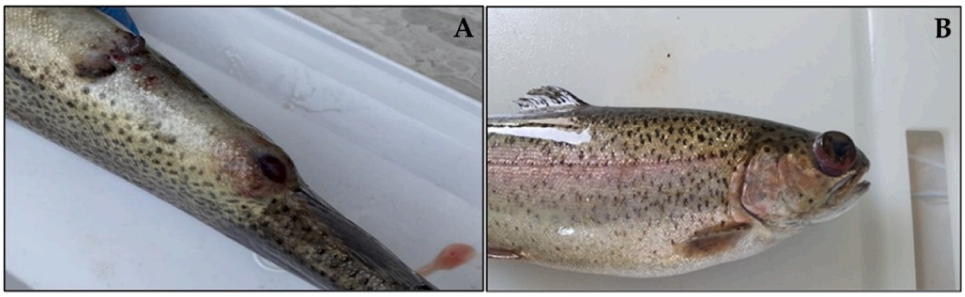

Fig. 1. Main lesions caused by lactococcosis in specimens of rainbow trout: anal prolapse (A); exophthalmia and haemorrhaging in the periorbital area (B).

Fig. 2. Adult rainbow trout analysed with the SCiO sensor over the abdominal area.

2.4. Bacterial detection in blood smears

After SCiO acquisition, blood was rapidly drawn from the caudal vein and immediately used for preparing the blood smears, which were then air-dried at room temperature, fixed with absolute methanol for 5 min (Merck Life Science, Italy) and stained with May Grunwald Giemsa, then observed under an oil immersion objective (x1000) with an optical microscope (Leica DMRB, Austria) to detect or exclude the presence of circulating bacteria attributable to *L. garvieae*.

2.5. Anatomo-pathological analysis

Individual morphometric parameters were recorded. The fish were then macroscopically examined to highlight external alterations. After dissection with sterile tools, the coelomic cavity and the internal organs were subjected to visual inspection for the evaluation of anatomopathological changes.

2.6. Bacteriological analysis

Columbia blood Agar (Liofilchem, Italy) was inoculated with kidney and spleen samples and incubated at 22 °C ± 2 °C for 72 h. Bacterial identification was carried on a VITEK MS system (bioMérieux, France) according to the manufacturer's instructions. Phenotypic identification of *L. garvieae* isolates was confirmed by a species-specific PCR, as described in paragraph 2.7 "Molecular Biology".

2.7. Molecular biology

PCR was used to determine the positivity to *L. garvieae* in the kidney and the spleen, which are the target organs of infection. Tissue samples were aseptically collected and stored at −20 °C. DNA extraction from tissue samples (25 mg) was performed using a ReliaPrep gDNA Tissue Miniprep System kit (Promega, USA). Amplification was performed as reported by Zlotkin et al. (1998). Amplicons were run inside an electrophoretic cell on 2% Gelgreen stained agarose gel and then visualized under UV exposure by a transilluminator. A 50–2000 Kb ladder was used as a molecular marker. The amplicons were purified with an ExtractMe DNA Clean-up and Gel-out kit (Blirt, Poland) according to the manufacturer's instructions. The purified PCR products were bidirectionally sequenced using Big Dye 1.1 (ThermoFisher Scientific, USA) and the same primers as in PCR amplification. Cycle sequencing products were purified Dye Ex 2.0 spin kit (Qiagen) and sequenced on an ABI3130xl genetic analyser (ThermoFisher Scientific). Contig assembly of forward and reverse DNA sequences for each isolate was performed using Lasergen software package (DNASTAR, USA). Sequences were compared from the GenBank database using the Basic Local Alignment Search Tool (BLAST) search algorithm.

In order to discriminate between *L. garvieae* and *L. petauri*, a further PCR analysis was done, according to the protocol of Stoppani et al. (2023).

2.8. Data analysis

A 2 × 2 contingency table was created to determine sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the SCiO device. Statistics are reported as absolute values with their 95% confidence interval (95% CI); the α value was set at 0.05. Principal Component Analysis (PCA) was performed to check for differences in SCiO spectra between sick and healthy individuals. Data analysis was performed using GraphPad Prism (version 9.4.1.; GraphPad Software, Inc.).

3. Results and discussion

3.1. SCiO acquisition

SCiO acquisitions provided more reliable results in the proximity of the abdominal area, while the animal's pigmentation in the dorsal area did not allow the differentiation of spectra. Fig. 3 illustrates the SCiO spectra of all the analysed individuals, with yellow lines referring to

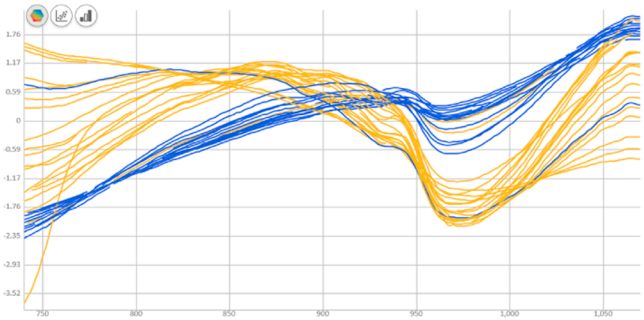

Fig. 3. Trend of SCiO spectra recorded in healthy (yellow lines) and sick (blue lines) rainbow trout (blue line). Wavelength (nm) is reported on the abscissa and reflectance (Ra) on the ordinate axis.

healthy samples and blue lines to sick rainbow trout. Fig. 4 shows the outcome of Principal Component analysis (PCA), in which a clear separation between the categories investigated was observed.

3.2. Molecular biology, bacteriological analysis and bacterial detection in blood smear

The results of PCR-based detection according the protocol of Stoppani et al. (2023) revealed positivity only for *L. garvieae*.

The results of PCR-based detection of *Lactococcus garvieae* DNA, the classical bacteriological diagnosis, and the bacteria detection in peripheral blood smears (septicaemia condition) are illustrated for each individual (Table 1).

SCiO had a sensibility of 0.95 (0.75–1.00 C.I. 95%) and a specificity of 0.80 (0.56–0.94 C.I. 95%). The positive predictive value was 0.83 (0.66–92.00 C.I. 95%), while the negative predictive value was 0.94 (0.70–0.99 C.I. 95%). Test accuracy was 0.88 (0.73–0.96 C.I. 95%).

For the individuals with macroscopically detectable symptoms, the NIR spectra data were in line with the results of molecular biology findings in all samples, except for samples 4/S and 16/S (10%). Sample 16/S was actually a healthy (uninfected) individual, although classified as symptomatic by macroscopic examination, because both classical bacteriology and PCR excluded the presence of *L. garvieae*.

Sample 4 S was classified as sick based on-SCiO data, while PCR and bacteriology gave a negative result: sequencing analysis revealed the presence of *Yersinia ruckeri DNA*, a pathogen causing symptoms partially similar to those reported for lactococcosis in rainbow trout (Kumar et al., 2015).

The possibility of SCiO error in the detection of infection was higher for the asymptomatic individuals. Sample 12/AS resulted as false negative for SCiO, while samples 2/AS, 14/AS and 19/AS resulted as false positives. In detail, sample 19/AS displayed skin darkening around the perianal region, which could have influenced the data acquisition. Sample 4/AS is an example of the prognostic ability of the SCiO: despite the absence of evident symptoms, the individual resulted sick by analysis with the SCiO device and by PCR.

There are various hypotheses for the mechanism by which the device proposes differential spectra between sick and healthy individuals, but the most plausible one is the fatty acids profile of analysed tissues. Fatty acids and lipids present in the skin, sub-cutaneous tissues and in visceral fat may produce different curves of reflected light (Trbovic et al., 2012). Since reduced feed intake is one of the early symptoms of lactococcosis, the SCiO sensor might detect changes in abdominal fat composition consequent to feed deprivation and fatty acids mobilization. At a wavelength of 940 nm, the graphs display a spectral peak characteristic of infected individuals, which could correspond to the presence of CH groups or OH groups at the terminal group of fatty acids (Mulvey, 2020; Wiedemair et al., 2019). We attempted to find a correlation between the SCiO data and the weight of individuals to determine whether anorexia potentially affects the data recorded by the SCiO but there was no evidence of this link, most likely due to the rapid evolution of symptoms, unlike loss of weight generally takes longer to be evident.

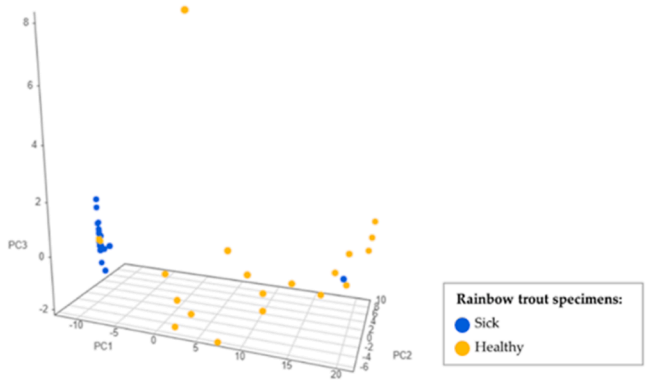

Fig. 4. Principal component analysis (PCA) information about adult rainbow trout specimens (healthy and sick) described by multiple inter-correlated quantitative variables.

Moreover, it is known that lactococcosis induces severe inflammatory and haemorrhagic lesions in the intestine and in the surrounding abdominal fat (Shanin et al., 2021), which could be detected by the SCiO device as alterations of biochemical properties in affected tissues. A future area worthy of investigation is the "reading" capacity of the device to penetrate tissues and organs (Sun et al., 2020), as well as the expression in the visceral fat under investigation of chemical mediators belonging to the adipokines group.

This varied class of molecules is secreted by the adipocytes and immune cells colonising the adipose tissue, and is involved in the modulation of nutrient metabolism and in endocrine/immune functions (Pignatelli et al., 2014; Veenstra et al., 2018; Weidinger et al., 2018). The literature on the salmonid immune system recently described the presence of a "bursa-like organ" in the proximity of the anal area (Bjørgen and Koppang, 2021; Loken et al., 2020). This newly identified organ is colonised by T lymphocytes and is probably highly exposed to both enteric and environmental antigens. Its function and immune reactivity could be properly investigated also in rainbow trout affected by *L. garvieae* infection, to determine whether the SCiO detection spectres are potentially influenced by its physiological variations.

The sensitivity of data acquisition is important aiming at the use of the SCiO device in field applications and shows whether the device is able to correctly identify healthy status.

The detection of the lowest possible number of false negatives is essential to prevent outbreaks, i.e., individuals with normal values but actually carrying an infection. Furthermore, early infected fish, still asymptomatic in farmed stocks set the conditions for an outbreak to spread quickly. For this reason a diagnostic tool should easy to use and reliable. Even a few infected fish albeit apparently asymptomatic, can be sufficient to trigger a new outbreak, in which the related mortality can occur within days. The periodical use of the SCiO device could help farmers to detect a potential problem in its early stages and avert economic loss. Early diagnosis has multiple management implications for farmers (Kain, 2022). There are compounds (immunostimulant or natural antimicrobials) that can be used as prevention or early treatment of infectious diseases in aquaculture, and they can be provided within functional feeds as soon as a biological risk is detected (Kain, 2022).

4. Conclusions

The study findings demonstrate promising performances of this NIR spectroscope in discriminating between healthy and infected fish. Rapid data acquisition, low cost and on-site are the particular advantages related to its use: application in the field and potential employ as a diagnostic tool for monitoring disease in farmed fish. A future area of focus will be the study of asymptomatic animals and the determination of the SCiO ability to correctly identify the disease in its prodromal stage.

Institutional review board statement

The study was conducted in accordance with the criteria approved by an ethical committee (n. 03/2022 OPBA UNIUD). Animal handling was performed in conformity with European/Italian guidelines on animal welfare (L.D.No.26/2014, implementation of the European Directive 2010/63/EU).

CRediT authorship contribution statement

Maganza Alessandra: Formal analysis. **Fariano Lucio:** Writing – original draft, Writing – review & editing. **Montemurro Vittoria:**

Table 1
Results of SCiO acquisition compared to bacteriology, PCR and blood smear analysis.

Sample	ID/signs disease	Bacteriological analysis		DNA detection by PCR		Bacteria in blood smear	SCiO profile
		Kidney	Spleen	Kidney	Spleen		
1	1/S	*L. garvieae*	n.d.	+	+	-	sick
2	2/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
3	3/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
4	4/S	n.d.	n.d.	-	-	-	sick
5	5/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
6	6/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
7	7/S	*L. garvieae*	n.d.	+	+	-	sick
8	8/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
9	9/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
10	10/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
11	11/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
12	12/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
13	13/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
14	14/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
15	15/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
16	16/S	n.d.	n.d.	-	-	-	healthy
17	17/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
18	18/S	*L. garvieae*	*L. garvieae*	+	+	-	sick
19	19/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
20	20/S	*L. garvieae*	*L. garvieae*	+	+	+	sick
21	1/AS	n.d.	n.d.	-	-	-	healthy
22	2/AS	n.d.	n.d.	-	-	-	sick
23	3/AS	n.d.	n.d.	-	-	-	healthy
24	4/AS	*L. garvieae*	n.d.	+	+	-	sick
25	5/AS	n.d.	n.d.	-	-	-	healthy
26	6/AS	n.d.	n.d.	-	-	-	healthy
27	7/AS	n.d.	n.d.	-	-	-	healthy
28	8/AS	n.d.	n.d.	-	-	-	healthy
29	9/AS	n.d.	n.d.	-	-	-	healthy
30	10/AS	n.d.	n.d.	-	-	-	healthy
31	11/AS	n.d.	n.d.	-	-	-	healthy
32	12/AS	n.d.	*L. garvieae*	+	+	-	healthy
33	13/AS	n.d.	n.d.	-	-	-	healthy
34	14/AS	n.d.	n.d.	-	-	-	sick
35	15/AS	n.d.	n.d.	-	-	-	healthy
36	16/AS	n.d.	n.d.	-	-	-	healthy
37	17/AS	n.d.	n.d.	-	-	-	healthy
38	18/AS	n.d.	n.d.	-	-	-	healthy
39	19/AS	n.d.	n.d.	-	-	-	sick
40	20/AS	n.d.	n.d.	-	-	-	healthy

S = Symptomatic, **AS** = Asymptomatic; **n.d.** = not detected.

Formal analysis. **Sciuto Simona:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Bozzetta Elena:** Supervision. **Volpatti Donatella:** Conceptualization, Investigation, Methodology, Writing – original draft. **Acutis Pier Luigi:** Conceptualization, Supervision. **Esposito Giuseppe:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Colussi Silvia:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. **Pastorino Paolo:** Data curation, Formal analysis, Funding acquisition, Methodology, Software, Writing – original draft, Writing – review & editing. **Khalil Sarker Mohammed Ibrahim:** Formal analysis, Writing – original draft. **Stoppani Nadia:** Formal analysis. **Esposito Giovanna:** Methodology. **Prearo Marino:** Funding acquisition, Writing – original draft. **Gabetti Alice:** Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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