Periodontal Condition and Subgingival Microbiota Characterization in Subjects with Down Syndrome

Maigualida Cuenca [1], María José Marín [1], Lourdes Nóvoa [2], Ana O'Connor [1], María Carmen Sánchez [1], Juan Blanco [2], Jacobo Limeres [2], Mariano Sanz [1], Pedro Diz [2,†] and David Herrera [1,*,†]

1   ETEP (Etiology and Therapy of Periodontal and Peri-implant Diseases) Research Group, University Complutense of Madrid (UCM), 28040 Madrid, Spain; maiguacu@ucm.es (M.C.); mjmarin@ucm.es (M.J.M.); aoconnor@ucm.es (A.O.); mariasan@ucm.es (M.d.C.S.); marsan@ucm.es (M.S.)
2   Medical-Surgical Dentistry Research Group (OMEQUI), Health Research Institute of Santiago de Compostela (IDIS), University of Santiago de Compostela (USC), 15782 Santiago de Compostela, Spain; lourdes.novoa@rai.usc.es (L.N.); juan.blanco@usc.es (J.B.); jacobo.limeres@usc.es (J.L.); pedro.diz@usc.es (P.D.)
*   Correspondence: davidher@ucm.es; Tel.: +34-913-941-907
†   Contributed equally.

Featured Application: Promotion of preventive actions in Down syndrome individuals is recommended, including surveillance of thyroid hormone function, improvement of oral hygiene measures and frequent evaluation of periodontal health.

Abstract: The aim was to study the subgingival microbiota in subjects with Down syndrome (DS) with different periodontal health status, using cultural and molecular microbiological methods. In this cross-sectional study, DS subjects were selected among those attending educational or occupational therapy centers in Galicia (Spain). Medical histories, intraoral and periodontal examinations and microbiological sampling were performed. Samples were processed by means of culture and quantitative polymerase chain reaction (qPCR). Microbiological data were compared, by one-way ANOVA or Kruskal-Wallis and chi-square or Fisher tests, according to their periodontal status. 124 subjects were included, 62 with a healthy periodontium, 34 with gingivitis and 28 with periodontitis. Patients with periodontitis were older ($p < 0.01$) and showed lower prevalence of hypothyroidism and levothyroxine intake ($p = 0.01$), presented significantly deeper pockets and more attachment loss ($p \leq 0.01$). Both gingivitis and periodontitis subjects showed higher levels of bleeding and dental plaque. PCR counts of *T. forsythia and* culture counts of *E. corrodens* and total anaerobic counts were significantly higher in periodontitis patients. Relevant differences were observed in the subgingival microbiota of DS patients with periodontitis, showing higher levels of anaerobic bacteria, *T. forsythia* and *E. corrodens,* when compared with periodontally healthy and gingivitis subjects. Moreover, periodontitis subjects were older, had lower frequency of hypothyroidism and higher levels of dental plaque.

Keywords: periodontitis; Down syndrome; polymerase chain reaction; microbiota; gingivitis

1. Introduction

The introduction Down syndrome (DS) is a congenital genetic disease, caused by the presence of an extra chromosome in par 21 [1], being the most common human aneuploidy among living births, with an estimated prevalence of one case per 800 births [2]. Its phenotypic expression, however, may be associated with significant genetic complexity [3]. Subjects with DS present a characteristic phenotype and intellectual disability [4], together with several systemic diseases [5], such as congenital heart disease and thyroid dysfunction, as well as a number of oral diseases [6,7]. Furthermore, there is compromise in their innate immune response [8] and DS subjects suffer more frequently from oral infections, when compared with the general population [9,10].

Early onset forms of periodontitis and late onset of caries lesions represent the most frequent oral conditions associated with DS subjects [9], which may become further aggravated by their motor disability and manual dexterity compromise, that limits their performance in oral hygiene practices [11]. Several observational studies have reported high prevalence of gingivitis and periodontitis (ranging from 58–96%) at young ages (less than 35) in DS subjects [12]. In Brazil, in a study sample of 93 DS patients aged 6–20 years, only 9% showed a healthy periodontium and 33% had periodontitis [13]; in another study including 64 DS subjects (mean age 23.8 years), 28.1% presented gingivitis and 71.9% periodontitis [14]. In The Netherlands, in a study group conformed by 182 DS subjects, 36.6% were diagnosed of periodontitis [15]. In addition, SD periodontitis patients have shown more severity, when compared with controls [16], and a higher impact in their quality of life [13]. In fact, in the latest classification of periodontal and peri-implant diseases and conditions [17], periodontitis in DS patients was included within the category "periodontitis as a manifestation of a systemic disease," in which the systemic condition has a major impact in the onset and course of periodontitis, as clearly occurs in DS subjects [18].

Furthermore, some authors have suggested a specific microbial profile associated to DS subjects with periodontitis [19,20], raising the hypothesis that a distinctive pathogenic microbiota combined with an impaired host-response [21], may result in an earlier dysbiosis and onset of periodontitis [22]. However, the evidence supporting this hypothesis is very scarce and the reported results are highly heterogeneous [19,20,23]. In light of this limited information, we have designed this observational study aimed to evaluate the subgingival microbiota of DS subjects with different periodontal health status (periodontal health, gingivitis or periodontitis), by means of cultural and molecular microbiological methods.

2. Materials and Methods

2.1. Study Population

A cross-sectional observational study was designed in a Spanish Caucasian population diagnosed of DS. This investigation followed the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) criteria for reporting [24] and was approved by the Research Ethics Committee (EC) of Santiago-Lugo, Spain (Registration Code: 2018/510). All subjects and, when appropriate, their legal guardians, confirmed in writing their agreement to participate and signed the EC-approved informed consent, once they were informed on the objectives and processes associated with this investigation.

Screening among DS subjects regularly attending seven educational or occupational therapy centers in the region of Galicia, in the North-West of Spain, was carried out between September 2017 and November 2018.

At this screening visit, information on their age, gender, presence of co-morbidities and use of current medications was collected. Study subjects were consecutively included if DS was genetically confirmed and did not have any of the following exclusion criteria: under 18 years of age, comorbidities that could influence the periodontal condition (e.g., diabetes), presence of harmful habits (e.g., smoking), having received antimicrobial therapy in the previous month (e.g., systemic antimicrobials and/or oral antiseptics), insufficient degree of collaboration for performing clinical assessment and microbiological sampling.

Upon inclusion in the study, subjects had an intraoral examination where the following periodontal variables were recorded in four sites at the six reference teeth [25] or when absent, at the adjacent tooth: plaque index (PlI) [26], probing depth (PD) (using a PCP UNC15 periodontal probe, applying a force of 20–25 g), bleeding on probing (BOP) [27], gingival recession (REC) (distance from the gingival margin to the cemento-enamel junction) and clinical attachment level (CAL). All measurements were recorded by a trained and calibrated clinical investigator (N.L.). Calibration was achieved during several sessions of repeated measurements, until the clinical investigator demonstrated an intra-class correlation coefficient (ICC) higher than 0.75 in all the periodontal variables evaluated. The intra-class correlation coefficient values for intra-examiner calibration were 0.87 (95% confidence interval = 0.75–0.98). Clinical assessments were not conducted in dental clin-

ics/chairs so, together with the specific condition of the subjects, neither full-mouth clinical evaluations or radiographical assessments were feasible.

Following a modification of the case definitions proposed by the Classification of Periodontal and Peri-Implant Diseases and Conditions [17], the study group was categorized as:

- Healthy periodontal condition: PD < 4 mm and BOP detected in no more than two sites (less than 10% of the total) [28],
- Gingivitis: PD < 4 mm and BOP detected in more than two sites (more than 10% of the total) [28],
- Periodontitis: PD ≥ 4 mm in, at least, one site [29].

2.2. Subgingival Biofilm Sample Collection

Subgingival samples were collected from four sites, selected by clinical criteria, namely the deepest PD with BOP per quadrant [30]. Sampling was performed after the isolation of the area by cotton rolls and after removing supragingival biofilm and/or calculus. Then, two medium-sized sterile paper tips (Maillefer, Ballaigues, Switzerland) were consecutively inserted in each site for 10 s, as subgingival as possible [30]. All eight paper points were pooled in a vial containing 1.5 mm of reduced transport fluid (RTF) [31] and sent to the Laboratory of Microbiology (Faculty of Dentistry, University Complutense of Madrid, Spain), to be processed within 24 h.

2.3. Microbiological Processing by Means of Quantitative Polymerase Chain Reaction
2.3.1. Extraction of Total Genomic DNA

Total DNA was extracted from subgingival samples using a commercial kit (MolYsis Complete 5, Molzym Gmbh & Co. KG. Bremen, Germany) following manufacturer's instructions (the protocol for bacterial DNA extraction was followed from step 6, avoiding preliminary steps). The extracted DNA was eluded in 100 μL of sterile water (Roche Diagnostic GmbH, Mannheim, Germany) and frozen at −20 °C for further analysis.

2.3.2. Polymerase Chain Reaction

Multiplex quantitative PCR (qPCR) technology was used for detecting and quantifying the bacterial DNA [32]. The sequence of the primers and probes used for *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)*, *Porphyromonas gingivalis (P. gingivalis)* and *Tannerella forsythia (T. forsythia)*, targeting against 16S rRNA gene, have been previously reported [33,34]. PCR amplification was performed in a total reaction mixture volume of 10 μL, which included 5 μL of 2×TaqMan master mixture (LC 480 Probes Master, Roche Diagnostic GmbH), optimal concentrations of primers and hydrolysis probe (300, 300 and 200 nM for *A. actinomycetemcomitans*; 300, 300 and 300 nM for *P. gingivalis* and 300, 300 and 200 nM for *T. forsythia*) and 2.5 μL of DNA from the samples. The no-template control (NTC) consisted of 2.5 μL of sterile water. Samples were subjected to an initial amplification cycle of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min in LightCycler® 480 II thermocycler (Roche Diagnostic GmbH). Each DNA sample was analyzed in duplicate.

All assays were performed using calibration curves with a linear quantitative detection range established by the slope range of 3.3–3.6 cycles/log decade, r^2 > 0.997 and an efficiency range of 1.9–2.0. Quantification was based on standard curves, which were constructed by plotting cross point cycle (Cp) values generated from qPCR against DNA extracted from serial 10-fold dilutions of purified genomic DNA from each bacterium (log of colony forming units (CFU)/mL).

2.4. Microbiological Processing by Means of Culturing

At the laboratory, aliquots of 0.1 mL from the vials were plated on two different culture media: the selective Dentaid-1 medium for the detection of *A. actinomycetemcomitans* [35] and a non-selective blood agar medium (Blood Agar Base, Oxoid, Basingstoke, England),

supplemented with hemin (5 mg/L) (Sigma, St. Louis, MO, USA), menadione (1 mg/L) (Merck, Darmstadt, Germany) and 5% of sterile horse blood (Oxoid), for detection of target periodontal pathogens (*Capnocytophaga* spp., *Campylobacter rectus (C. rectus), Eikenella corrodens (E. corrodens), Fusobacterium nucleatum (F. nucleatum), P. gingivalis, Prevotella intermedia (P. intermedia), Parvimonas micra (P. micra), T. forsythia, Actinomyces odontolyticus (A. odontolyticus)*) and for evaluating total anaerobic bacterial counts. After 2–5 days of capnophilic incubation (Dentaid-1 medium) or 7–14 days of anaerobic incubation (blood agar medium), total counts and counts of representative colonies were calculated in the most suitable plates. Suspected colonies were identified by microscopy, Gram-staining and enzyme activity (Table S1). Counts were transformed in CFU per mL of the original sample.

2.5. Microbiological Processing by Means of Next Generation Sequencing

A randomly selected group of samples, from 50 subjects, 25 with periodontitis a 25 with periodontal health, were processed by means of Illumina® Sequencing (Illumina, San Diego, CA, USA) and the results have been published in a separate article [36].

2.6. Statistical Analysis

2.6.1. Sample Size Calculation

Primary outcomes were counts and frequency of detection of each target bacterial species. A sample size calculation could not be made in light of the heterogeneity of the published microbiological results. Hence, a convenience sample was selected, being larger than those reported in previous studies: 30 periodontitis patients with DS [20], 40 [19], 67 [37] or 70 [23] subjects with DS.

2.6.2. Data Analysis

Quantitative results were expressed as CFU per mL, in median values and interquartile range (IQR) and mean values and standard deviation (SD). Secondary outcomes were other microbiological variables (proportions of target pathogens) and clinical variables of the sampled sites and Ramfjord teeth, expressed as means and SD. The unit of analysis was the patient.

To assess the normality of the distribution, Shapiro-Wilk (in group with sample sizes below 30) or Kolmogorov-Smirnov test (in group with sample sizes above 30) were performed. Differences between three groups (periodontal health, gingivitis and periodontitis) were compared by one-way ANOVA test or Kruskal-Wallis test for quantitative variables and chi-square or Fisher tests for categorical variables.

The level of statistical significance was set at $p < 0.05$. A statistical software package IBMR SPSS Statistics 25.0 (IBM Corporation, Armonk, NY, USA) was used for data analysis.

3. Results

3.1. Study Population

From the 168 subjects screened, 44 were not included due to the established exclusion criteria, so the study group was composed of 124 DS subjects (Table 1 and Table S2). After evaluating their periodontal status, their distribution was: 62 with a periodontal health (50.0%), 34 with gingivitis (27.4%) and 28 with periodontitis (22.6%).

Table 1. Demographic characteristics for each study group, with the appropriate comparisons.

	Periodontal Health (H) (n = 62)						Gingivitis (G) (n = 34)						Periodontitis (p) (n = 28)						ANOVA p Values			
Age	mean	SD	min	max			mean	SD	min	max			mean	SD	min	max			all	H-G	G-P	H-P
age	21.4	7.5	6	40			22.6	6.0	11	34			27.9	7.2	10	42			<0.01 *	1.00	0.01 *	<0.01 *
Gender	female	male		%female	%male		female	male		%female	%male		female	male		%female	%male		all	H-G	G-P	H-P
gender	27	35		43.5%	56.5%		18	15		54.5%	45.5%		10	18		35.7%	64.3%		0.33	0.92	0.42	1.45
Systemic Conditions	none	one	≥2	%none	%one	% ≥ 2	none	one	≥2	%none	%one	%(≥2)	none	one	≥2	%none	%one	% ≥ 2	all	H-G	G-P	H-P
n systemic conditions	31	18	13	50.0%	29.0%	21.0%	13	15	6	38.2%	44.1%	17.7%	17	9	2	60.7%	32.1%	7.1%	0.52	1.51	0.53	1.48
Specific Conditions	n (no)	n (yes)		% (no)	% (yes)		n (no)	n (yes)		% (no)	% (yes)		n (no)	n (yes)		% (no)	% (yes)		all	H-G	G-P	H-P
cardiovascular diseases	62	0		100.0%	0.0%		34	0		100.0%	0.0%		27	1		96.4%	3.6%		0.22		1.35	0.93
cardiopathies	55	7		88.7%	11.3%		30	4		88.2%	11.8%		23	5		82.1%	17.9%		0.69	3.00	2.16	1.51
hypothyroidism	36	26		58.1%	41.9%		19	15		55.9%	44.1%		25	3		89.3%	10.7%		0.01 *	2.51	0.01 *	0.01 *
levothyroxine intake	37	25		59.7%	40.3%		20	14		58.8%	41.2%		25	3		89.3%	10.7%		0.01 *	2.81	0.02 *	0.01 *

SD, standard deviation; min, minimum; max, maximum; fem, female.* Statistically significant differences.

Differences in age among groups were statistically significant ($p < 0.01$), since patients with periodontitis (mean age 27.9 years) were significantly older than those with periodontal health (mean age 21.4 years; $p = 0.01$) or with gingivitis (mean age 22.6 years; $p < 0.01$). No significant differences were detected for gender, number of co-morbidities and presence of cardiovascular conditions.

Conversely, statistically significant differences among groups ($p = 0.01$) were detected for presence of hypothyroidism and intake of supplemental thyroxine medication (levothyroxine). Hypothyroidism was significantly less prevalent in periodontitis subjects (10.7%), when compared with periodontally healthy (41.9%; $p = 0.01$) and gingivitis subjects (40.3%; $p = 0.01$).

3.2. Clinical Outcome Variables

PD showed significant differences among groups for both, sampling sites and Ramfjord index teeth ($p < 0.01$), with deeper pockets in periodontitis patients (3.28 mm and 2.76 mm, respectively), than in healthy subjects (2.03 and 2.09, respectively; $p < 0.01$) and gingivitis patients (2.20 and 2.19, respectively; $p < 0.01$). Similar differences were observed for proximal PD at Ramfjord teeth and for CAL. BOP also showed significant differences among groups, for both sampling sites and Ramfjord teeth ($p < 0.01$) but differences corresponded to significant higher values in gingivitis (38.2% and 29.1%, respectively; $p < 0.01$) and periodontitis patients (50.9% and 35.8%, respectively; $p < 0.01$), when compared with healthy subjects (7.8% and 5.7%, respectively). Similar results were observed for PlI, with lower values in the periodontal health group and significantly higher levels in gingivitis and periodontitis patients, with no differences between them (Table 2 and Table S3).

3.3. Subgingival Microbiota as Evaluated by Means of qPCR

Differences in PCR counts were significantly different among groups ($p = 0.01$), with significantly higher counts in periodontitis patients, when compared with the periodontal health ($p = 0.01$) or gingivitis ($p = 0.02$) groups (Table 3 and Table S4).

Frequency of detection and counts of *A. actinomycetemcomitans* were low in all groups, although with increasing frequencies and counts in periodontitis subjects but differences were not statistically significant. *P. gingivalis*, also showed increasing frequencies and counts in periodontitis but, again, differences were not statistically significant. *T. forsythia*, showed high frequencies of detection (prevalence) in all groups (67.9% in periodontitis, 47.0% in gingivitis and 48.8% in periodontal health), with no statistically significant differences among groups, while PCR counts demonstrated significant differences ($p = 0.01$), with higher percentages in periodontitis when compared with gingivitis ($p = 0.02$) or periodontally healthy ($p = 0.01$) subjects.

3.4. Subgingival Microbiota as Evaluated by Means of Culture

Total anaerobic counts were significantly different among groups ($p < 0.01$), with significantly higher counts in periodontitis, as compared with periodontal health ($p < 0.01$).

Only four target bacterial species (*P. gingivalis, P. intermedia, T. forsythia* and *E. corrodens*) showed an overall increase in frequency of detection, counts and proportions, as the periodontal status worsened. *P. micra* and *A. odontolyticus* demonstrated higher counts, proportions and frequencies in healthy subjects. *C. rectus* and *Capnocytophaga* spp. presented the highest counts, proportions and frequencies in gingivitis patients. Finally, *A. actinomycetemcomitans* was not detected with culture methods.

Statistically significant differences among groups were only detected for *E. corrodens* ($p = 0.01$), corresponding to higher frequencies of detection ($p = 0.01$), proportions ($p = 0.01$) and counts ($p = 0.01$) in periodontitis patients, when compared with periodontal health subjects (Tables 4 and 5, Tables S5 and S6).

Table 2. Periodontal clinical outcomes, expressed as medians and interquartile ranks (IQR), for each study group, with the appropriate comparisons.

	PERIODONTAL HEALTH (H) (n = 62)				GINGIVITIS (G) (n = 34)				PERIODONTITIS (P) (n = 28)				*Kruskal-Wallis P Values*							
	Sampling Sites		Ramfjord Teeth		Sampling Sites		Ramfjord Teeth		Sampling Sites		Ramfjord Teeth		Sampling Sites				Ramfjord Teeth			
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	All	H-G	G-P	H-P	all	H-G	G-P	H-P
PD (mm)	2.00	0.25	2.04	0.17	2.25	0.50	2.10	0.26	3.25	0.75	2.68	0.63	<0.01 *	0.06	<0.01 *	<0.01 *	<0.01 *	0.25	<0.01 *	<0.01 *
proximal PD (mm)			2.08	0.25			2.25	0.33			3.00	0.65					<0.01 *	0.07	<0.01 *	<0.01 *
gingival REC (mm)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.45				0.42			
CAL (mm)	2.00	0.25	1.88	0.42	2.25	0.50	1.67	0.47	3.25	0.75	2.81	0.63	<0.01 *	0.25	<0.01 *	<0.01 *	<0.01 *	0.15	<0.01 *	<0.01 *
BOP	0.0%	0.0%	4.2%	8.3%	25.0%	25.0%	25.0%	21.1%	50.0%	50.0%	35.4%	29.2%	<0.01 *	<0.01 *	0.51	<0.01 *	<0.01 *	0.00	1.00	<0.01 *
plaque index (PlI)	0.5%	0.5%	45.8%	45.8%	75.0%	50.0%	81.3%	43.8%	75.0%	43.8%	77.5%	41.7%	<0.01 *	0.01 *	1.00	<0.01 *	<0.01 *	<0.01 *	1.00	<0.01 *
proximal PlI			58.3%	66.7%			85.4%	35.4%			83.3%	33.3%					<0.01 *	<0.01 *	1.00	<0.01 *

PD, probing depth; REC, recession; CAL: clinical attachment level; BOP, bleeding on probing. * Statistically significant differences.

Table 3. Microbiological findings (quantitative polymerase chain reaction, qPCR), with PCR counts expressed as means and standard deviations (SD) or as medians and interquartile ranks (IQR) and frequencies of detection as percentages, for the complete study population and for each study group, with the appropriate comparisons.

All Subjects (*n* = 124)				
qPCR Counts–Mean & SD	**Mean**	**SD**	**_n_**	**(+)**	**prev.**
A. actinomycetemcomitans	6857	41,713	124	11	8.87%
p. gingivalis	64,064	458,060	124	15	12.10%
T. forsythia	342,644	1,755,259	124	65	52.42%

	Periodontal Health (H) (*n* = 62)			Gingivitis (G) (*n* = 34)			Periodontitis (*p*) (*n* = 28)		
qPCR counts–mean & SD	**mean**	**SD**	**_n_**	**mean**	**SD**	**_n_**	**mean**	**SD**	**_n_**
A. actinomycetemcomitans	406	2077	62	5601	22,125	34	22,668	83,438	24
p. gingivalis	5591	35,883	62	6396	33,297	34	263,564	947,768	23
T. forsythia	88,497	249,908	62	45,934	99,763	34	1,265,688	3,569,224	9

	Periodontal Health (H) (*n* = 62)					Gingivitis (G) (*n* = 34)					Periodontitis (*p*) (*n* = 28)					Counts (Kruskal-Wallis *p* value)				Frequency of Detection (*p* value)			
qPCR counts–median & IQR	**median**	**IQR**	**_n_**	**(+)**	**prev.**	**median**	**IQR**	**_n_**	**(+)**	**prev.**	**median**	**IQR**	**_n_**	**(+)**	**prev.**	**all**	**H-G**	**G-*p***	**H-P**	**all**	**H-G**	**G-P**	**H-P**
A. actinomycetemcomitans	0	0	62	4	6.45%	0	0	34	3	8.82%	0	0	24	4	14.29%	0.41				0.51	2.09	2.07	0.75
P. gingivalis	0	0	62	6	9.68%	0	0	34	4	11.76%	0	0	23	5	17.86%	0.41				0.56	2.22	2.16	0.92
T. forsythia	0	15,625	62	30	48.39%	0	17,875	34	16	47.06%	22,350	1,372,000	9	19	67.86%	0.01 *	1.00	0.02 *	0.01 *	0.18	2.70	0.30	0.26

n, number of samples; (+), samples with pathogen detection; prev., frequency of detection of the target pathogen; A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans; P. gingivalis: Porphyromonas gingivalis; T. forsythia: Tannerella forsythia.* Statistically significant differences.

Table 4. Microbiological findings (culture), with counts (colony forming units per mL) expressed as medians and interquartile ranks (IQR) and frequencies of detection expressed as percentage, for each study group, with the appropriate comparisons.

Culture Counts	Periodontal Health (H) (n = 62)					Gingivitis (G) (n = 34)					Periodontitis (P) (n = 28)					Counts (p Values) ^		Frequency of Detection (p Value)		
	Median	IQR	n	(+)	Prev.	Median	IQR	n	(+)	Prev.	Median	IQR	n	(+)	prev.	all	all	H-G	G-P	H-P
Total anaerobic bacteria	1,435,000	1,617,500				1,735,000	2,402,500				4,065,000	4,957,500				<0.01 *				
A. actinomycetemcomitans	0	0	62	0	0.0%	0	0	34	0	0.0%	0	0	28	0	0.0%					
P. gingivalis	0	0	62	11	17.7%	0	0	34	7	20.6%	0	7505	28	8	28.6%	0.37	0.50	2.20	1.40	0.73
P. intermedia	0	625	62	22	35.5%	0	1250	34	14	41.2%	600	9000	28	15	53.6%	0.13	0.27	1.74	0.99	0.32
T. forsythia	580,645	0	62	1	1.6%	0	60,000	34	3	8.8%	0	0	28	2	7.1%	0.23	0.21	0.38	3.00	0.68
P. micra	0	0	62	4	6.5%	0	0	34	2	5.9%	0	0	28	0	0.0%	0.40	0.20	3.00	1.49	0.92
F. nucleatum	20,000	33,250	62	60	96.8%	20,000	68,125	34	29	85.3%	20,000	27,750	28	27	96.4%	0.99	0.10	0.28	0.63	3.00
C. rectus	0	0	62	3	4.8%	0	0	34	4	11.8%	0	0	28	2	7.1%	0.47	0.48	0.72	2.04	1.93
E. corrodens	0	0	62	8	12.9%	0	0	34	6	17.6%	0	10,000	28	11	39.3%	0.01 *	0.01 *	1.67	0.17	0.01 *
Capnocytophaga spp.	0	0	62	6	9.7%	0	0	34	7	20.6%	0	0	28	3	10.7%	0.25	0.32	0.63	1.47	3.00
A. odontolyticus	0	0	62	5	8.1%	0	0	34	1	2.9%	0	0	28	1	3.6%	0.54	0.49	1.25	1.47	1.98

^ Counts (Kruskal-Wallis *p* value). For comparison between groups, they were available just for anaerobic counts (H-G, 0.25; G-P, 0.43; H-P, 0.00 *) and *E. corrodens* (H-G, 1.00; G-P, 0.11; H-P, 0.01 *). * Statistically significant differences. n, number of samples; (+), samples with pathogen detection; prev., frequency of detection of the target pathogen; *A. actinomycetemcomitans: Aggregatibacter actinomycetemcomitans; P gingivalis: Porphyromonas gingivalis; T. forsythia: Tannerella forsythia; P. micra: Parvimonas micra; F. nucleatum: Fusobacterium nucleatum; C. rectus: Campylobacter rectus; E. corrodens: Eikenella corrodens; A. odontolyticus: Actinomyces odontolyticus.*

Table 5. Microbiological findings (culture), with proportions of total anaerobic microbiota in percentage, expressed as medians and interquartile ranks (IQR), for each study group, with the appropriate comparisons.

	Periodontal Health (H) ($n = 62$)		Gingivitis (G) ($n = 34$)		Periodontitis (P) ($n = 28$)		p Value ^
Proportions	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**All**
A. actinomycetemcomitans	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	
P. gingivalis	0.00%	0.00%	0.00%	0.00%	0.00%	0.58%	0.37
P. intermedia	0.00%	0.05%	0.00%	0.06%	0.01%	0.41%	0.24
T. forsythia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24
P. micra	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.40
F. nucleatum	1.29%	2.77%	0.91%	3.31%	0.59%	1.76%	0.21
C. rectus	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.48
E. corrodens	0.00%	0.00%	0.00%	0.00%	0.00%	0.28%	0.01 *
Capnocytophaga spp.	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.26
A. odontolyticus	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.51

^ Proportions of total anaerobic microbiota (Kruskal-Wallis p value); for comparison between groups, they were available just for *E. corrodens* (H-G, 1.00; G-P, 0.13; H-P, 0.01 *). * Statistically significant differences. *A. actinomycetemcomitans*: *Aggregatibacter actinomycetemcomitans*; *P. gingivalis*: *Porphyromonas gingivalis*; *T. forsythia*: *Tannerella forsythia*; *P. micra*: *Parvimonas micra*; *F. nucletum*: *Fusobacterium nucleatum*; *C. rectus*: *Campylobacter rectus*; *E. corrodens*: *Eikenella corrodens*; *A. odontolyticus*: *Actinomyces odontolyticus*.

4. Discussion

In the present study, a large cohort of 124 subjects with DS showed significantly higher PCR counts of *T. forsythia,* culture counts of *E. corrodens* and total anaerobic counts, when comparing periodontitis with periodontal health or gingivitis. Subjects with periodontitis were also significantly older and the prevalence of hypothyroidism and levothyroxine intake significantly lower, as compared with the periodontal health and gingivitis groups.

Studies that have previously assessed the subgingival microbiota in DS subjects, have included a limited number of subjects and applied culture-independent techniques [19,20,23,37]. In the present study, a combination of culture and molecular techniques were selected, since they can be considered as complementary methodologies [32,38]. Using these techniques, higher counts and frequencies of detection of relevant periodontal pathogens, such as *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *T. forsythia* and *E. corrodens*, were identified in periodontitis, when compared with gingivitis and periodontally healthy DS subjects, as it has been previously reported in periodontitis patients without DS [39]. These quantitative and qualitative differences in the subgingival microbiota of periodontitis subjects may, in part, explain the early onset and progression of periodontitis in DS subjects, as it has been highlighted in previous studies [20,37]. However, in the present study, statistically significant differences among groups were only observed for culture counts of *E. corrodens* and total anaerobic counts, between periodontal health and periodontitis patients, and for qPCR counts of *T. forsythia,* when comparing periodontitis with periodontal health or gingivitis patients. This microbiological profile is also in agreement with the results obtained using Next Generation Sequencing (NGS), reported in a subset of this study population, which showed significantly higher levels of the genera *Tannerella*, *Porphyromonas* and *Aggregatibacter* in periodontitis, as compared with the periodontal health group [36].

In the present study, when using qPCR, *T. forsythia* was the most prevalent bacterial species in periodontitis patients (67.9%) but also in the other two groups. A similar trend has been reported in previous studies: Martinez-Martinez et al. [20] reported that *T. forsythia* was the most prevalent species in periodontitis DS patients (95.5%), being significantly higher when compared with periodontal health; Amano et al. [37] reported a high frequency of detection for *T. forsythia* (89.7%) in periodontitis DS patients, also significantly higher than in gingivitis patients; and *T. forsythia* was the only bacterial species with significantly higher presence and counts in DS subjects, when compared with healthy controls and individuals with cerebral palsy [23].

For *A. actinomycetemcomitans,* low frequencies of detection in the three study groups were reported in the present study. Much higher frequencies were detected in previous studies [20,37]. These differences could be explained by geographical variability, since a low prevalence of this bacterium has been reported in Spain [40,41] or due to technical aspects, such as the use of different probe-primers in the PCR technique or to differences in the patient selection process.

Higher counts and frequencies, for *T. forsythia* and *A. actinomycetemcomitans*, were reported when using qPCR, compared with culture. Similar results have been found in previous comparative studies [42]. These differences could be explained by the ability of qPCR to detect DNA of both viable and non-viable bacteria and to the lower detection limits of qPCR [32,38]. In addition, detection for *T. forsythia* by culturing is challenging [43,44], resulting in higher sensitivity and lower specificity when comparing qPCR technique *versus* culture [32,43,45]. However, the overall microbial tendency of an increase in prevalence for the three main target bacterial species from periodontal health to gingivitis and to periodontitis, was similar irrespective of the applied microbiological diagnostic technique.

The use of different microbiological technologies makes difficult to interpret the results from different studies, although common trends can be identified. In the present study, in which two complementary techniques were used, qPCR yielded higher counts for the three main target bacterial species, and higher prevalence for *T. forsythia* and *A. actinomycetemcomitans*, than culturing techniques, but the same tendency was observed with both methods, that is, an increase in prevalence/counts for the three main target bacterial species from health to gingivitis and to periodontitis. Both techniques are restricted in terms of target bacterial species, in this case three for qPCR and ten for culture. Other techniques may have a wider target such a checkerboard DNA-DNA hybridization: in the study by Sakellari et al. [23], 14 species were targeted and *T. forsythia* was identified as a relevant pathogen in all age cohorts, while *P. gingivalis*, *C. rectus*, *P. intermedia* and *A. actinomycetemcomitans* were relevant (among others) in the older cohorts; conversely, in the study by Khocht et al. [19], 40 species were targeted and *Treponema socrankii* showed significantly higher levels in DS subjects with periodontitis, as compared to individuals without periodontitis. These restrictions in the number of target species can be solved today using Next Generation Sequencing approaches, as shown in a publication based on subset of samples of the present study, but only comparing periodontitis and healthy periodontal subjects [36]: significant differences were observed at different taxonomical levels, with periodontitis patients demonstrating not only higher levels of periodontal pathogens, including *Tannerella*, *Treponema*, *Porphyromonas* and *Aggregatibacter* but also of new putative pathogens, such as species of *Peptostreptococcus*, *Filifactor*, *Fretibacterium* and *Desulfobulbus*; subjects with periodontal health showed more frequently species of *Veillonella*, *Neisseria*, *Gamella* and *Granulicatella*.

Different case definitions have been used to assess the periodontal status of DS subjects. For example, López-Pérez et al. [16] defined four categories as sound (CAL, 0 to 1 mm), mild periodontitis (CAL, 2 to 3 mm), moderate periodontitis (CAL, 4 to 5 mm) and severe periodontitis (CAL, 6 mm or more); while Khocht et al. [19] defined periodontitis as 5% or more teeth with CAL of 5 mm or more. In the present study, a more solid reference, the one proposed by the 2018 Classification of Periodontal and Peri-Implant Diseases and Conditions [17,28,29], was applied, with some modifications for the optimal use of the registered variables. The significant differences in PD, CAL and BOP among the studied categories were expected, since those variables were part of the case definition. For PlI, closely associated with BOP, it should be highlighted the large magnitude of the differences between periodontal health (around 45%), when compared with gingivitis and periodontitis (over 70%). The influence of this finding in the overall results deserve a careful analysis and the influence of a poor plaque control in the onset of gingivitis and periodontitis should be considered. In light of the possible impact of the limited skills in supragingival biofilm control in DS individuals, this factor should be taken into consideration [11].

In the present study, patients with periodontitis presented a significantly lower prevalence of hypothyroidism (10%) and the use of substitute medication (levothyroxine), compared with periodontal health and gingivitis subjects (42–44%). Hypothyroidism is the most common pathological hormone deficiency and, among the known risk factors for developing hypothyroidism, DS has been listed, usually as an autoimmune thyroiditis [46], which is consistent with the higher prevalence of autoimmune diseases in DS. Thus, the estimated prevalence of thyroid disorders in DS subjects reaches 40% in some series [47] and thanks to systematic screening, usually hypothyroidism is detected in subclinical stages in DS individuals [48], being subclinical hypothyroidism the most common detected thyroid abnormality in DS subjects [49]. Since the rate of conversion to overt hypothyroidism has been reported to be low in follow up studies [50], treatment of subclinical hypothyroidism is only advised by most authors in case of conversion to overt hypothyroidism [51].

Since the endocrine system can modulate the immune system in a bidirectional manner [52] and, in a recent scoping review, on 29 selected articles, a positive relationship between hypothyroidism and periodontitis was found [53], thus the findings of the present study are totally unexpected. One possible explanation is that periodontitis patients are actually suffering subclinical hypothyroidism and/or they have not yet been properly diagnosed [54], and, this were true, the surveillance of hypothyroid hormone function can be further supported in DS individuals. However, only speculations can be made at this point, since the previous hypothesis cannot be tested now.

In the present study, patients with periodontitis were significantly older that those in periodontal health or gingivitis groups. This finding agrees with previous reports, that have pointed out that the prevalence of periodontal diseases in DS, as it occurs in the general population, increases with age [14,15]. It is also relevant to highlight that the prevalence of periodontitis in DS individual is relatively high at a young age, ranging 58–96% in subjects younger that 35 years old [12]. As for other periodontitis as a manifestation of systemic diseases, periodontitis in DS individual presents more frequently with earlier onset, faster and generalized and more severe progression, as compared with individuals without a systemic immune compromised [16,23]. A longitudinal study has also shown that periodontal pathogens can be frequently detected in DS subjects with periodontitis, for up to 6 months, despite periodontal treatment and frequent supportive periodontal care, suggesting a higher risk of progression and giving more relevance to supragingival biofilm control as performed by the subject [55]. The importance of poor oral hygiene has been also emphasized in a recent study, suggesting a larger impact of a poor oral hygiene and an impaired immune response in the development of periodontal diseases in DS subjects [56]. However, the possible relevance of specific periodontal pathogens should not be discarded, since a recent 24-month study has even reported an association of high counts of *P. gingivalis* with risk of disease progression [57], although the study population did not include DS patients.

The present study has clinically and microbiologically evaluated a large group of DS individuals. However, some limitations have to be acknowledged, being the main limitation that periodontal diagnosis was based on a partial-mouth clinical evaluation (Ramfjord teeth), with no radiographical evaluation, which may have underestimated disease levels. This approach was selected to ease the evaluation of the special patient population included in the study and knowing that partial-mouth recording systems may present adequate degrees of accuracy [58]. Additional limitations can also be listed: (i) in a cross-sectional design, causal associations cannot be established [59]; (ii) some confounding factors may have not been properly controlled, such as diet, life-style and habits, care givers support in oral hygiene; and (iii) differences in age and thyroid disfunction may have impacted both clinical and microbiological findings.

5. Conclusions

In the present study, DS patients with periodontitis were characterized, as compared with those with periodontal health or gingivitis, by an older age, a lower frequency of thy-

roid dysfunction, deeper periodontal pockets and more attachment loss, higher prevalence of bleeding on probing and dental plaque accumulation and higher levels of anaerobic bacterial counts, *T. forsythia* and *E. corrodens*. It is, therefore, important, to promote actions for DS individuals, including surveillance of thyroid hormone function, improvement of oral hygiene measures and frequent evaluation of periodontal health, in order to make possible an early detection of disease (i.e., gingivitis) and, thus, providing adequate treatment or help in maintaining periodontal health, when it is still preserved.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-341 7/11/2/778/s1, Supplementary Table S1. Presumptive identification of bacterial species in culture. Supplementary Table S2. Demographic characteristics for the complete study population. Supplementary Table S3. Periodontal clinical outcomes, expressed as means and standard deviations (SD), for the complete study population and for each study group. Supplementary Table S4. Microbiological findings (counts and frequencies of detection), as evaluated by means of quantitative polymerase chain reaction, expressed as means and standard deviations (SD), for the complete study population and for each study group. Supplementary Table S5. Microbiological findings, as evaluated by means of culture, with counts expressed as means and standard deviations (SD) and frequencies of detection expressed as percentage, for the complete study population and for each study group. Supplementary Table S6. Microbiological findings (proportions), as evaluated by means of culture, expressed as means and standard deviations (SD), for the complete study population and for each study group.

Author Contributions: M.C., M.J.M., A.O., M.C.S., contributed to data acquisition (microbiological analyses), statistical analyses and critically revised the manuscript; L.N., contributed to conception, data acquisition (clinical evaluations) and critically revised the manuscript; J.B., J.L., critically revised the manuscript; M.S., D.H., P.D., contributed to conception, data analysis and interpretation, critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki of the World Medical Association (2008) and approved by the Research Ethics Committee of Santiago-Lugo, Spain (registration code: 2018/510).

Informed Consent Statement: Informed consent was obtained from all subjects involved (and their legal guardians, where appropriate) in the study.

Data Availability Statement: Data available on request due to restrictions. The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethical issues.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lejeune, J.; Gautier, M.; Turpin, R. Study of somatic chromosomes from 9 mongoloid children. *C. R. Hebd. Seances Acad. Sci.* **1959**, *248*, 1721–1722. [PubMed]
2. Shin, M.; Besser, L.M.; Kucik, J.E.; Lu, C.; Siffel, C.; Correa, A.; Congenital Anomaly Multistate, P.; Survival, C. Prevalence of Down syndrome among children and adolescents in 10 regions of the United States. *Pediatrics* **2009**, *124*, 1565–1571. [CrossRef] [PubMed]
3. Asim, A.; Kumar, A.; Muthuswamy, S.; Jain, S.; Agarwal, S. Down syndrome: An insight of the disease. *J. Biomed. Sci.* **2015**, *22*, 41. [CrossRef] [PubMed]
4. Abanto, J.; Ciamponi, A.L.; Francischini, E.; Murakami, C.; de Rezende, N.P.; Gallottini, M. Medical problems and oral care of patients with Down syndrome: A literature review. *Spec. Care Dent.* **2011**, *31*, 197–203. [CrossRef] [PubMed]
5. Lagan, N.; Huggard, D.; Mc Grane, F.; Leahy, T.R.; Franklin, O.; Roche, E.; Webb, D.; O' Marcaigh, A.; Cox, D.; El-Khuffash, A.; et al. Multiorgan involvement and management in children with Down syndrome. *Acta Paediatr.* **2020**. [CrossRef]
6. Mubayrik, A.B. The Dental Needs and Treatment of Patients with Down Syndrome. *Dent. Clin. N. Am.* **2016**, *60*, 613–626. [CrossRef]
7. Scalioni, F.A.R.; Carrada, C.F.; Martins, C.C.; Ribeiro, R.A.; Paiva, S.M. Periodontal disease in patients with Down syndrome: A systematic review. *J. Am. Dent. Assoc.* **2018**, *149*, 628–639.e611. [CrossRef]

8.   Bloemers, B.L.; van Bleek, G.M.; Kimpen, J.L.; Bont, L. Distinct abnormalities in the innate immune system of children with Down syndrome. *J. Pediatr.* **2010**, *156*, 804–809. [CrossRef]

9.   Hennequin, M.; Allison, P.J.; Veyrune, J.L. Prevalence of oral health problems in a group of individuals with Down syndrome in France. *Dev. Med. Child. Neurol.* **2000**, *42*, 691–698. [CrossRef]

10. Moreira, M.J.; Schwertner, C.; Jardim, J.J.; Hashizume, L.N. Dental caries in individuals with Down syndrome: A systematic review. *Int. J. Paediatr. Dent.* **2016**, *26*, 3–12. [CrossRef]

11. Krishnan, C.S.; Kumari, B.N.; Sivakumar, G.; Iyer, S.P.; Ganesh, P.R. Evaluation of oral hygiene status and periodontal health in Down's syndrome subjects in comparison with normal healthy individuals. *J. Indian Acad. Dent. Spec. Res.* **2014**, *1*, 47–49. [CrossRef]

12. Morgan, J. Why is periodontal disease more prevalent and more severe in people with Down syndrome? *Spec. Care Dent.* **2007**, *27*, 196–201. [CrossRef] [PubMed]

13. Amaral Loureiro, A.C.; Oliveira Costa, F.; Eustaquio da Costa, J. The impact of periodontal disease on the quality of life of individuals with Down syndrome. *Downs Syndr. Res. Pract.* **2007**, *12*, 50–54. [CrossRef] [PubMed]

14. Nuernberg, M.A.A.; Ivanaga, C.A.; Haas, A.N.; Aranega, A.M.; Casarin, R.C.V.; Caminaga, R.M.S.; Garcia, V.G.; Theodoro, L.H. Periodontal status of individuals with Down syndrome: Sociodemographic, behavioural and family perception influence. *J. Intellect. Disabil. Res.* **2019**, *63*, 1181–1192. [CrossRef]

15. van de Wiel, B.; van Loon, M.; Reuland, W.; Bruers, J. Periodontal disease in Down's syndrome patients. A retrospective study. *Spec. Care Dent.* **2018**, *38*, 299–306. [CrossRef]

16. Lopez-Perez, R.; Borges-Yanez, S.A.; Jimenez-Garcia, G.; Maupome, G. Oral hygiene, gingivitis, and periodontitis in persons with Down syndrome. *Spec. Care Dent.* **2002**, *22*, 214–220. [CrossRef]

17. Caton, J.G.; Armitage, G.; Berglundh, T.; Chapple, I.L.C.; Jepsen, S.; Kornman, K.S.; Mealey, B.L.; Papapanou, P.N.; Sanz, M.; Tonetti, M.S. A new classification scheme for periodontal and peri-implant diseases and conditions-Introduction and key changes from the 1999 classification. *J. Clin. Periodontol.* **2018**, *45* (Suppl. 20), S1–S8. [CrossRef]

18. Jepsen, S.; Caton, J.G.; Albandar, J.M.; Bissada, N.F.; Bouchard, P.; Cortellini, P.; Demirel, K.; de Sanctis, M.; Ercoli, C.; Fan, J.; et al. Periodontal manifestations of systemic diseases and developmental and acquired conditions: Consensus report of workgroup 3 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J. Clin. Periodontol.* **2018**, *45* (Suppl. 20), S219–S229. [CrossRef]

19. Khocht, A.; Yaskell, T.; Janal, M.; Turner, B.F.; Rams, T.E.; Haffajee, A.D.; Socransky, S.S. Subgingival microbiota in adult Down syndrome periodontitis. *J. Periodontal Res.* **2012**, *47*, 500–507. [CrossRef]

20. Martinez-Martinez, R.E.; Loyola-Rodriguez, J.P.; Bonilla-Garro, S.E.; Patino-Marin, N.; Haubek, D.; Amano, A.; Poulsen, K. Characterization of periodontal biofilm in Down syndrome patients: A comparative study. *J. Clin. Pediatr. Dent.* **2013**, *37*, 289–295. [CrossRef]

21. Khocht, A.; Russell, B.; Cannon, J.G.; Turner, B.; Janal, M. Oxidative burst intensity of peripheral phagocytic cells and periodontitis in Down syndrome. *J. Periodontal Res.* **2014**, *49*, 29–35. [CrossRef] [PubMed]

22. Hajishengallis, G.; Lamont, R.J. Beyond the red complex and into more complexity: The polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol. Oral Microbiol.* **2012**, *27*, 409–419. [CrossRef] [PubMed]

23. Sakellari, D.; Arapostathis, K.N.; Konstantinidis, A. Periodontal conditions and subgingival microflora in Down syndrome patients. A case-control study. *J. Clin. Periodontol.* **2005**, *32*, 684–690. [CrossRef] [PubMed]

24. von Elm, E.; Altman, D.G.; Egger, M.; Pocock, S.J.; Gotzsche, P.C.; Vandenbroucke, J.P.; Initiative, S. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: Guidelines for reporting observational studies. *J. Clin. Epidemiol.* **2008**, *61*, 344–349. [CrossRef] [PubMed]

25. Ramfjord, S.P. Indices for Prevalence and Incidence of Periodontal Disease. *J. Periodontol.* **1959**, *30*, 51–59. [CrossRef]

26. O'Leary, T.J.; Drake, R.B.; Naylor, J.E. The plaque control record. *J. Periodontol.* **1972**, *43*, 38. [CrossRef] [PubMed]

27. Ainamo, J.; Bay, I. Problems and proposals for recording gingivitis and plaque. *Int. Dent. J.* **1975**, *25*, 229–235.

28. Chapple, I.L.C.; Mealey, B.L.; Van Dyke, T.E.; Bartold, P.M.; Dommisch, H.; Eickholz, P.; Geisinger, M.L.; Genco, R.J.; Glogauer, M.; Goldstein, M.; et al. Periodontal health and gingival diseases and conditions on an intact and a reduced periodontium: Consensus report of workgroup 1 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J. Clin. Periodontol.* **2018**, *45* (Suppl. 20), S68–S77. [CrossRef]

29. Tonetti, M.S.; Greenwell, H.; Kornman, K.S. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *J. Clin. Periodontol.* **2018**, *45* (Suppl. 20), S149–S161. [CrossRef]

30. Mombelli, A.; McNabb, H.; Lang, N.P. Black-pigmenting gram-negative bacteria in periodontal disease. II. Screening strategies for detection of P. gingivalis. *J. Periodontal Res.* **1991**, *26*, 308–313. [CrossRef]

31. Syed, S.A.; Loesche, W.J. Survival of human dental plaque flora in various transport media. *Appl. Microbiol.* **1972**, *24*, 638–644. [CrossRef] [PubMed]

32. Marin, M.J.; Ambrosio, N.; O'Connor, A.; Herrera, D.; Sanz, M.; Figuero, E. Validation of a multiplex qPCR assay for detection and quantification of Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythia in subgingival plaque samples. A comparison with anaerobic culture. *Arch. Oral Biol.* **2019**, *102*, 199–204. [CrossRef] [PubMed]

33. Boutaga, K.; van Winkelhoff, A.J.; Vandenbroucke-Grauls, C.M.; Savelkoul, P.H. Comparison of real-time PCR and culture for detection of Porphyromonas gingivalis in subgingival plaque samples. *J. Clin. Microbiol.* **2003**, *41*, 4950–4954. [CrossRef] [PubMed]

34. Boutaga, K.; van Winkelhoff, A.J.; Vandenbroucke-Grauls, C.M.; Savelkoul, P.H. Periodontal pathogens: A quantitative comparison of anaerobic culture and real-time PCR. *FEMS Immunol. Med. Microbiol.* **2005**, *45*, 191–199. [CrossRef] [PubMed]

35. Alsina, M.; Olle, E.; Frias, J. Improved, low-cost selective culture medium for Actinobacillus actinomycetemcomitans. *J. Clin. Microbiol.* **2001**, *39*, 509–513. [CrossRef] [PubMed]

36. Novoa, L.; Sanchez, M.D.C.; Blanco, J.; Limeres, J.; Cuenca, M.; Marin, M.J.; Sanz, M.; Herrera, D.; Diz, P. The Subgingival Microbiome in Patients with Down Syndrome and Periodontitis. *J. Clin. Med.* **2020**, *9*, 2482. [CrossRef]

37. Amano, A.; Kishima, T.; Akiyama, S.; Nakagawa, I.; Hamada, S.; Morisaki, I. Relationship of periodontopathic bacteria with early-onset periodontitis in Down's syndrome. *J. Periodontol.* **2001**, *72*, 368–373. [CrossRef]

38. Marin, M.J.; Ambrosio, N.; Herrera, D.; Sanz, M.; Figuero, E. Validation of a multiplex qPCR assay for the identification and quantification of Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis: In vitro and subgingival plaque samples. *Arch. Oral Biol.* **2018**, *88*, 47–53. [CrossRef]

39. Ambrosio, N.; Marin, M.J.; Laguna, E.; Herrera, D.; Sanz, M.; Figuero, E. Detection and quantification of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans in bacteremia induced by interdental brushing in periodontally healthy and periodontitis patients. *Arch. Oral Biol.* **2019**, *98*, 213–219. [CrossRef]

40. Herrera, D.; Contreras, A.; Gamonal, J.; Oteo, A.; Jaramillo, A.; Silva, N.; Sanz, M.; Botero, J.E.; Leon, R. Subgingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. *J. Clin. Periodontol.* **2008**, *35*, 106–113. [CrossRef]

41. Minguez, M.; Pousa, X.; Herrera, D.; Blasi, A.; Sanchez, M.C.; Leon, R.; Sanz, M. Characterization and serotype distribution of Aggregatibacter actinomycetemcomitans isolated from a population of periodontitis patients in Spain. *Arch. Oral Biol.* **2014**, *59*, 1359–1367. [CrossRef]

42. Lau, L.; Sanz, M.; Herrera, D.; Morillo, J.M.; Martin, C.; Silva, A. Quantitative real-time polymerase chain reaction versus culture: A comparison between two methods for the detection and quantification of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis in subgingival plaque samples. *J. Clin. Periodontol.* **2004**, *31*, 1061–1069. [CrossRef]

43. Bankur, P.K.; Nayak, A.; Bhat, K.; Bankur, R.; Naik, R.; Rajpoot, N. Comparison of culture and polymerase chain reaction techniques in the identification of Tannerella forsythia in periodontal health and disease, an in vitro study. *J. Indian Soc. Periodontol.* **2014**, *18*, 155–160. [CrossRef] [PubMed]

44. Paster, B.J.; Boches, S.K.; Galvin, J.L.; Ericson, R.E.; Lau, C.N.; Levanos, V.A.; Sahasrabudhe, A.; Dewhirst, F.E. Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **2001**, *183*, 3770–3783. [CrossRef] [PubMed]

45. Kotsilkov, K.; Popova, C.; Boyanova, L.; Setchanova, L. Comparison of culture method and real-time PCR for detection of putative periodontopathogenic bacteria in deep periodontal pockets. *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 1–7. [CrossRef]

46. Roberts, C.G.; Ladenson, P.W. Hypothyroidism. *Lancet* **2004**, *363*, 793–803. [CrossRef]

47. Amr, N.H. Thyroid Disorders in Subjects with Down Syndrome: An Update. *Acta Biomed.* **2018**, *89*, 132–139. [CrossRef]

48. Karlsson, B.; Gustafsson, J.; Hedov, G.; Ivarsson, S.A.; Anneren, G. Thyroid dysfunction in Down's syndrome: Relation to age and thyroid autoimmunity. *Arch. Dis. Child.* **1998**, *79*, 242–245. [CrossRef]

49. King, K.; O'Gorman, C.; Gallagher, S. Thyroid dysfunction in children with Down syndrome: A literature review. *Ir. J. Med. Sci.* **2014**, *183*, 1–6. [CrossRef]

50. Prasher, V.; Ninan, S.; Haque, S. Fifteen-year follow-up of thyroid status in adults with Down syndrome. *J. Intellect. Disabil. Res.* **2011**, *55*, 392–396. [CrossRef]

51. Guaraldi, F.; Rossetto Giaccherino, R.; Lanfranco, F.; Motta, G.; Gori, D.; Arvat, E.; Ghigo, E.; Giordano, R. Endocrine Autoimmunity in Down's Syndrome. *Front. Horm. Res.* **2017**, *48*, 133–146. [CrossRef] [PubMed]

52. Klein, J.R. The immune system as a regulator of thyroid hormone activity. *Exp. Biol. Med.* **2006**, *231*, 229–236. [CrossRef] [PubMed]

53. Aldulaijan, H.A.; Cohen, R.E.; Stellrecht, E.M.; Levine, M.J.; Yerke, L.M. Relationship between hypothyroidism and periodontitis: A scoping review. *Clin. Exp. Dent. Res.* **2020**, *6*, 147–157. [CrossRef] [PubMed]

54. Pinto, A.; Glick, M. Management of patients with thyroid disease: Oral health considerations. *J. Am. Dent. Assoc.* **2002**, *133*, 849–858. [CrossRef] [PubMed]

55. Sakellari, D.; Belibasakis, G.; Chadjipadelis, T.; Arapostathis, K.; Konstantinidis, A. Supragingival and subgingival microbiota of adult patients with Down's syndrome. Changes after periodontal treatment. *Oral Microbiol. Immunol.* **2001**, *16*, 376–382. [CrossRef]

56. Kosaka, M.; Senpuku, H.; Hagiwara, A.; Nomura, Y.; Hanada, N. Oral infection, periodontal disease and cytokine production in adults with Down syndrome. *Med. Res. Arch.* **2017**, *5*. Available online: https://esmed.org/MRA/mra/article/view/960 (accessed on 14 January 2021). [CrossRef]

57. Kakuta, E.; Nomura, Y.; Morozumi, T.; Nakagawa, T.; Nakamura, T.; Noguchi, K.; Yoshimura, A.; Hara, Y.; Fujise, O.; Nishimura, F.; et al. Assessing the progression of chronic periodontitis using subgingival pathogen levels: A 24-month prospective multicenter cohort study. *BMC Oral Health* **2017**, *17*, 46. [CrossRef]

58. Fernandez, E.; STROBE Group. Observational studies in epidemiology (STROBE). *Med. Clin.* **2005**, *125* (Suppl. 1), 43–48. [CrossRef]

59. Relvas, M.; Tomas, I.; Salazar, F.; Velazco, C.; Blanco, J.; Diz, P. Reliability of partial-mouth recording systems to determine periodontal status: A pilot study in an adult Portuguese population. *J. Periodontol.* **2014**, *85*, e188–e197. [CrossRef]