Claimed effects, outcome variables and methods of measurement for health claims on foods proposed under European Community Regulation 1924/2006 in the area of oral health.

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ABSTRACT

Objective: Among the requests of authorization to apply health claims in the context of oral health proposed to the European Food Safety Authority (EFSA), the main reason for rejection is linked to the design of human intervention studies, including the inappropriate choice of outcome variables (OVs) and of their methods of measurement (MMs). The present manuscript reports the results of an investigation aimed at collecting, collating and critically analysing the information in relation to claimed effects, OVs and MMs, in the area of oral health and compliant with Regulation 1924/2006.

Methods: Claimed effects, OVs and the related MMs were collected from EFSA guidance documents and from the scientific opinions on the substantiation of health claims under Articles 13.5 and 14. The collection, collation and critical analysis of the relevant scientific literature consisted in a definition of the keywords, PubMed search strategies and in the creation of databases of references.

Results and conclusions: The critical analysis of the OVs and their MMs was performed on the basis of the literature review and was aimed at defining the appropriateness of OVs and MMs in the context each specific claimed effect.

Clinical Significance: The information provided in this document could serve to EFSA for the development of further guidance on the scientific requirements for health claims related to oral health, as well as to the stakeholders for the identification of existing and design of novel randomized controlled trials aimed at substantiating such health claims.

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Abbreviations used:

DMFS: Decayed Missing and Filled Surfaces; DMFT: Decayed Missing and Filled Teeth; EFSA: European Food Safety Authority; MM: method of measurement; OV: outcome variable; PF: Prevented Fraction; RCTs: Randomized Controlled Trials; VAS: Visual Analogue Scale; XC: mean increment in the control group; XE: mean increment in the test group.

1. INTRODUCTION

Oral health is a factor of critical importance in determining an adequate level of quality of life, as edentulism has been associated to functional limitations, psychological discomfort, physical, psychological and social disability [1]. Oral diseases can affect both teeth and periodontal tissues and can cause tooth decay and periodontitis, both of which can eventually lead to tooth loss and edentulism. The cause of these diseases is to be found in the development of a pathogenic and inflammophilic biofilm along oral tissues, which can then generate the catabolic end products necessary to the development of caries or the inflammation that sustains periodontal destruction[2]. The oral microbiologic ecosystem has been shown to be affected by several factors, both anatomical and behavioural, e.g. tooth shape and malposition, oral hygiene habits and diet. As tooth enamel and dentin are tissues that are composed of a mineralized extracellular matrix to effectively function during chewing, they are sensitive to local pH. When local pH falls below 6.2, dentin caries may ensue, and when pH falls beneath 5.5 the enamel gets damaged [3, 4]. Intake of acidic food has been associated to the onset of tooth erosions and abrasions, especially in the presence of tooth clenching or grinding. Similarly, it has long been known that the consumption of fermentable carbohydrates, such as glucose, sucrose, fructose or starch promotes caries formation, by providing substrate for dental plaque and their acidic end products, mostly lactic and acetic acid locally lower the pH and promote the formation of tissue damage [5]. This is an especially relevant issue with children and teenagers, a population subset where high sugar food and beverages are particularly popular and common and where they contribute to several further preventable diseases e.g. obesity, cardiovascular disease, hypertension and obesity-related cancers. Current recommendations suggest that sugar intake should not exceed 5% of daily energy intake [6]. Moreover, there is evidence that some food can actually reduce the incidence of caries, such as green and black tea, milk and milk products [7, 8]. Probiotics are also being actively investigated as a potential to reduce caries incidence by replacing harmful bacterial species such as

153 *Streptococcus mutans* [9]. Moreover, ascorrect and effective tooth brushing can be harder to assess in
154 children, diet control at home and at a community level (i.e. school) has been shown to be an
155 advantageous approach to reduce the incidence of caries[10]. The relation of diet with the insurgence of
156 periodontal disease is more controversial, as no food component can be singled out that can increase
157 the incidence of periodontitis [11]. However, sugar intake [12] and a high vegetable oil intake [13]
158 have been associated to an increase in gingival bleeding, and similarly low levels of vitamin C and
159 vitamin D have long been known to promote periodontitis [14, 15]. Diet can be therefore considered
160 one of the most potent factors contributing to the maintenance of adequate levels of oral health.
161 Nevertheless, the current research conducted for many nutrients has several limitations, so that their
162 importance in periodontal health still need to be fully clarified [16].
163 In this scenario, some functional foods have been the object of requests of authorization to use health
164 claims in the context of oral health, which have been proposed by stakeholders to the European Food
165 Safety Authority (EFSA), receiving both positive and negative opinions. The main concerns related to
166 the negative opinions mostly include the insufficient characterization of food/food constituent(s), the
167 lack of beneficial physiological effect of the proposed claimed effect but, most of all, the quality of the
168 studies provided for the scientific substantiation of the claims. In detail, the most critical points
169 encompass the design and the strength of the studies provided within the application, including the
170 proper choice of outcome variables (OVs) and their methods of measurement (MMs).
171 In this framework, a project has been developed with the aim of improving the quality of applications
172 provided by stakeholders to EFSA, through an appropriate choice of OVs and MMs [17].
173 Aim of the present manuscript is to gather information concerning the collection, collation and critical
174 analysis of claimed effects, OVs and MMs in the context of oral health.
175

2. MATERIALS AND METHODS: SEARCH STRATEGY

The manuscript refers to OVs and MMs collected from the relative Guidance document[18], from the applications for authorization of health claims under Articles 13.5 and 14 of Regulation 1924/2006 related to oral health [19], as well as from comments received during public consultations. The OVs and their MMs were considered only if the food/food constituent(s) was sufficiently characterized and the claimed effect, suitably defined, provided a beneficial physiological effect. Following this decision tree, 4 claimed effects with 7 OVs were evaluated under Article 13.5, whereas 2 disease risk reduction claims were selected under the Article 14.

Similarly, to the methods used in Martini et al. (2017)[17], all the MMs proposed for each OV in the scientific opinions and/or in the Guidance documents were included in the evaluation. If no methods were proposed or no proposed method was considered inappropriate, also the best or the most widely used method was included. Subsequently, individual databases of references were created on PubMed based on the keywords defined from each OV, in order to allow a specific critical analysis of the OVs and the MMs. The critical evaluation for each OV and MM was performed following a review of the literature deriving from the so obtained databases. Each OV and related MM was ranked in one of the following categories: (i) appropriate alone; (ii) appropriate only in combination with other OVs or MMs; (iii) not appropriate *per se*; (iv) not appropriate in relation to the specific claimed effect proposed by the applicant(s), (v) not appropriate alone, but useful as supportive evidence for the scientific substantiation of the claimed effect.

3. RESULTS: CITICAL EVALUATION OF OUTCOME VARIABLES AND METHODS OF MEASUREMENT

3.1. FUNCTION HEALTH CLAIMS ART 13 (5)

3.1.1. MAINTENANCE OF GUM FUNCTION

3.1.1.1. GINGIVAL INDEX

The Gingival Index, introduced by Löe and Silness in 1963, is a commonly used tool for the evaluation of the inflammatory conditions of gingival connective tissues in both children and adults[20]. Together with the Plaque Index and the Calculus Surface Index, the Gingival Index is a parameter frequently used in clinical practice for the evaluation of periodontal health status and in trials of therapeutic agents. It distinguishes between the quality and quantity of gingiva lesions, thus providing clear information both about the severity and the location of the gingiva lesions, and it is related to the four marginal areas (buccal, mesial, distal, lingual) that make up the total circumference of the gingiva, where lesions may occur. It can also be related to the interproximal gingival tissues. The Gingival Index is measured through the Gingival Index score, which associates a numeric value, ranging from 0 to 3, to gingival conditions. In detail, “0” means a normal gingiva, which is matt after drying, firm on palpation with a blunt instrument and whose color ranges from pale pink to pink; “1” is referred to a mildly inflamed gingiva which presents with slight changes in color or edema but no bleeding on probing; the score “2” means moderate inflammation, displaying a red, reddish-blue or glazy gingiva and bleeding provoked on probing; “3” is the score for severe inflammation, the considered gingiva is markedly red or reddish-blue and enlarged, with tendency to spontaneous bleeding and ulceration[21]. Therefore, the Gingival Index score includes both visual (color and contour of gums) and invasive components (bleeding). Partially erupted teeth, retained roots, teeth with periapical lesions and third molars should be not included in the measurement and there is no substitution. The scores obtained from the four areas of a tooth may be added and divided by four to give the mean Gingival Index score for the single tooth. The mean scores

may be summed up to designate the Gingival Index score for a specific group of teeth (incisors, premolars and molars). The Gingival Index score of a subject can be obtained by adding the values of each tooth and dividing by the number of teeth examined. Subjects with mild inflammation usually score from 0.1 to 1.0, those with moderate inflammation from 1.1 to 2.0, and an average score between 2.1 to 3.0 means severe gingivitis.

To evaluate the appropriateness of gingival index as outcome variable of gum function, the literature deriving from database #1 was critically evaluated (see Table 1).

The World Health Organization defines the oral health as a condition free from oral disease, pain, sores, tooth decay or loss, and other defects in the mouth [22]. Functionally, it includes the ability to bite, chew, speak, and smile. Oral health has also been seen to be associated with general health and health-related quality of life. Thus, the maintenance of gums structure leading to correct gingival functions is important at both an individual and a societal level. In this context, defined clinical parameters are therefore essential in the evaluation of the state of the gingiva, and the Gingival Index is one of the elected systems because it is efficient, quick and easy to use, with minimal instrumentation required. It accurately reflects the health state of gums. The Gingival Index is routinely assessed in both children and adults in order to obtain qualitative and quantitative information about gums status, to compare different population groups at a given time, to determine and control risk factors and to evaluate treatment efficacy. Moreover, the Gingival Index is often used as eligibility criteria for randomized controlled trials (RCTs), and depending on the requested target population it allows to discriminate between subjects, stratifying them on the basis of their gingival health status[23, 24]. Although the Gingival Index is immediate and easy to use, it is based on the association between numeric scores and visual and instrumental parameters defined by adjectives such “mild” and “severe”, whose discrimination is to be assessed by the physician, thus defying the Gingival Index score less reliable and reproducible than dichotomous systems, such as the Bleeding Index, which are based on the objective presence/absence of bleeding. Another limitation

consists in the fact that the Gingival Index is not based on a ratio scale, i.e. “2” score does not necessary means twice as much inflammation as “1” score means, thus providing less internal validity.

In conclusion, the Gingival Index can be used as appropriate outcome variable, better if in association with other indices of gingival status, for the scientific substantiation of health claims in the context of the maintenance of gum function.

3.1.1.1.1. VISUAL AND INSTRUMENTAL TEST

The gums health status is evaluated by an expert through the Gingival Index, which is based on the assessment of visual and invasive gingival features: the aspect, the color and the contour of gums are the primary aspects considered, together with the blood presence[25]. Gingival bleeding can either be spontaneous, thus indicating a severe ongoing gingivitis, or induced by the dentist touch. Specifically, gingival bleeding can be provoked just by touching the gingival margin with a blunt instrument; a periodontal probe with millimeter divisions is usually used at this scope, even if a triangular dental toothpick or a dental floss can also be employed. Gingival bleeding is definitely an objective, safe and reliable sign of inflammatory conditions of periodontal connective tissues but it is only one of the parameters the Gingival Index considers. Indeed, more subjective signs, such as changes in color, the presence of edema and the gums profile are also visually evaluated and therefore they only depend on the subjective opinion of the dentist[26]. Despite of its feasibility due to the easiness and the rapidity of the procedure, which have made the Gingival Index a widely used tool for the evaluation of gingival conditions, the Gingival Index is not very reliable and does not ensure a full comparability between analyses because of the great variability between measurements provided by different dentists. Nevertheless, all these aspects considered, it can be stated that the visual and instrumental test is an appropriate method for the assessment of Gingival Index.

3.1.2. REDUCTION OF DENTAL PLAQUE, ACID PRODUCTION and/or DENTAL CALCULUS

3.1.2.1. DENTAL PLAQUE

Dental plaque has been defined as the microbial community that develops on the tooth surface, embedded in an extracellular matrix of polymers of host and microbial origin. Specifically, the dental plaque is an oral biofilm, which is constituted by a heterogeneous microbial population connected by a multitude of functional and metabolic networks. The formation of dental plaque comprises an ordered sequence of events, starting from the highly specific attachment of bacteria to the host cell receptors, and then followed by a second bacterial colonization of the already formed microbial film [27, 28]. Plaque is a structured polymeric reticulum that connects the plaque/oral environment surface to the underlying tooth surface and allows molecules and bacteria to move through. This complex matrix can also accurately regulate the penetration and distribution of molecules within plaque. The carbohydrate fermentation due to the metabolic activity of the plaque microbial community leads to acids production and the subsequent impairment of oral pH, which contributes to the demineralization of tooth tissues [27, 29]. The persistence of dental plaque leads to progressive deposition of mineral layers onto its surface, which becomes harder and no longer removable by simple tooth brushing. These new mineralized deposits, named dental calculus, is in turn a suitable surface for further formation of dental plaque.

To evaluate the appropriateness of dental plaque as outcome variable of dental plaque, acid production and/or dental calculus, and as risk factor for gingivitis and for dental caries and tooth decay, the literature deriving from database #2 was critically evaluated (see Table 1).

Oral care and a correct oral hygiene are important for both oral and systemic health during life [30]and consequently, an adequate education and awareness should begin early during the infancy, so that proper oral hygiene habits are maintained throughout life. In this context, school-based intervention programs to improve oral health in children are conducted worldwide and several RCTs highlight the benefits

arising from dental plaque prevention. Dental plaque is a microbial biofilm, which forms spontaneously and it should therefore be accurately removed on a daily basis to avoid the onset of dental calculus and associated oral affections such as gingivitis or periodontitis. The removal of dental biofilm is also important because of acid production by its microbial communities, which is the main cause of progressive tooth demineralization and caries development [28]. Therefore, the dental plaque and the resulting impairment of oral pH are valid and accepted outcome variables, together with the gingival health status, which are commonly assessed to evaluate the efficacy of test formulations in the prevention or reduction of dental plaque and dental calculus formation [30]. Additionally, dental plaque is clinically evaluated to provide detailed information about the sequence of events whereby it forms, thus allowing the implementation of such procedures and devices required to prevent and reduce it [31]. In conclusion, the assessment of dental plaque presence/absence falls within the evaluation of oral hygiene in healthy adults and children, which should be routinely performed to either maintain it or to intervene whenever dental plaque occurs.

However, isolated changes in dental plaque have not generally been shown alone to reduce the risk of gingivitis as well as the risk of dental caries and the related tooth decay. In conclusion, dental plaque is an appropriate outcome variable to be used alone for the scientific substantiation of health claims focused only on the reduction of dental plaque and acid production, whereas it can be used as supportive evidence in addition to dental calculus for the scientific substantiation of health claims focused only on the reduction of dental calculus. Moreover, dental plaque may be considered an appropriate a risk factor for gingivitis and for dental caries and the related tooth decay only if changes in this factor are accompanied by evidence of reduced incidence of these diseases in humans in the context of a particular nutritional intervention (See Sections 3.2.1.2 and 3.2.2.2).

3.1.2.1.1. PLAQUE INDICES

The assessment and measurement of dental plaque provide essential information regarding an individual's oral health status and the efficacy of new treatments and products. Plaque indices are common methods used to evaluate the plaque coverage without image capture, which is instead the main feature of image analysis techniques, based on planimetric assessment of stained plaque area [30]. Planimetric dental plaque analysis expresses the plaque area as a percentage of the tooth surface covered with dental plaque. However, actually the most common basis for dental plaque scoring is the use of a numeric categorical scale, i.e. an index. A wide range of such indices are nowadays available, which have been developed over the years and some of them have undergone further modifications. All of them are based on the subjective visual evaluation of the experts and generally record dental plaque extent and thickness near the gingival margin and the coronal extension of the plaque, thus providing information on the typical pattern of progression of dental plaque accumulation. The commonest indices are those proposed by Silness and Löe, O'Leary, Navy and its modification by Rustagi, Quigley and Hein, and its modification, the Turesky index [32]. The Plaque Index was introduced by Silness and Löe in 1964 and can be properly considered the forerunner of all the indices for dental plaque measurement [33]. It consists of a numeric scale associating a score ranging from 0 to 3 to the amount of dental plaque: "0" is given when there is no plaque accumulation, "1" is given in presence of plaque film adhering to the gingival margin, which cannot be seen with the naked eye but only by using disclosing solution or probing, "2" means a visible moderate accumulation of dental plaque within the gingival pocket, on the gingival margin and/or adjacent to the tooth surface, and "3" is given when there is a heavy accumulation of dental plaque within the gingival pocket and/or on the tooth and gingival margin. This index has the advantage to be simple and therefore widely use throughout dentistry. However, considering only four scores, it conveys relatively poor discrimination capacity. O'Leary index assesses dental plaque separately on mesial, midpoint, and distal aspects of both facial and lingual surfaces for each tooth [34].

All visible plaque is scored, even if slight, and plaque scores are expressed as a percentage of the total number of potential sites. By the fact that this index has only three scores on a given tooth surface, it provides less discrimination than that given by Silness and Löe index. The Quigley and Hein index and its modification (the Turesky Index) measure the progressive coronal extension of dental plaque basing on a five scores numerical scale and considering three separate surface (mesial, distal and mid) on the tooth further divided to give a total of six areas. The Rustogi et al. modified Navy Plaque Index divides buccal and lingual surfaces into nine areas and, other than recording dental plaque on the total tooth, extends the measurement at the approximal mesial and distal tooth areas and at the marginal gingival region [35]. Despite the fact that such indices are routinely employed in dentistry trials because of the rapidity and easiness to perform, they are based on subjective evaluation thus providing low accuracy and reproducibility between studies. Nevertheless, all the previous considerations taken into account, plaque indices are appropriate methods to assess dental plaque in human intervention studies.

3.1.2.2. DENTAL CALCULUS

Dental calculus is formed from dental plaque, which is hardened by periodical deposition of mineralized layers onto its surface. The minerals constituting the dental calculus are derived from saliva and gingival crevicular fluid. This process slowly kills the microbial community of dental plaque but the new mineralized surface is in turn a suitable place for further microbial colonization and plaque deposition, processes leading to the formation of dental calculus [36, 37]. Indeed, dental calculus can be considered as mineralized dental plaque covered by a layer of non-mineralized viable bacterial plaque. The mineral proportion of dental calculus ranges from 40 to 60% and primarily consists of calcium phosphate crystals organized in four phases, namely brushite, dicalcium phosphate dihydrate, octacalcium phosphate and whitlockite [37, 38]. The organic components include mainly bacteria but also archaea like *M. oralis* and yeasts like *C. albicans.* Dental calculus can be distinguished in supragingival calculus, which is located coronally or above the gingival margin, and in subgingival calculus, located apically or below the

gingival margin in the gingival sulcus or in the periodontal pocket. Furthermore, dental calculus predominantly is formed at the buccal surface of the maxillary molars and at the lingual surface of the mandibular incisors, because of the proximity of these areas to the parotid and sublingual salivary glands [36-38]. Due to the fact that it is constituted by a high proportion of minerals, once deposited, dental calculus cannot be removed simply by tooth brushing; conversely the use of ultrasonic tools or dental hand instruments, such as a periodontal scaler, is required. Many variables have been identified to be related to the formation of dental calculus, including age, gender, ethnical background, dietary habits, location in the oral cavity, bacterial composition of dental plaque, host genetics, oral hygiene and access to professional dental care, physical disabilities, systemic diseases, tobacco smoking, drugs and medications.

To evaluate the appropriateness of dental calculus as outcome variable of dental plaque, acid production and/or dental calculus, the literature deriving from database #3 was critically evaluated (see Table 1).

Dental calculus is, together with dental plaque, a recognized etiological factor in the development of periodontal disease, chronical affections characterized by the destruction of the periodontal tissues and loss of connective tissue attachment. Dental calculus and dental plaque are, in this regard, closely related by the fact that dental plaque is the substrate for building up dental calculus, which in turn provides a porous niche for microbial colonization and plaque formation on its surface [36]. Even though the effect of dental calculus is likely secondary respect to that of dental plaque on oral health, its presence should be taken into account because it is supportive to the onset of dental plaque and subsequent periodontal affections, like gingivitis and periodontitis [37]. Indeed, literature researches suggest that calculus deposition may contribute to the chronicity of the disease process because of the protection provided to dental plaque deposits from debridement, or through direct absorption of toxic substances, such as endotoxin and lipopolysaccharides. Consistently, several works demonstrate how the reduction of the amount of dental calculus, notably at specific sites like gingival margins as well as fissures and pits of

teeth, would have a favorable effect on oral health [39]. Moreover, dental calculus is a secondary outcome variable assessed, even if not alone, in intervention studies aiming to evaluate the effect of agents as well as foods in the maintenance of oral health in humans [40].

In conclusion, dental calculus is an appropriate outcome variable to be used alone for the scientific substantiation of health claims focused only on the reduction of dental calculus, whereas it can be used as supportive evidence, in addition to dental plaque, to substantiate health claims focused only on the reduction of dental plaque and acid production.

3.1.2.2.1. VOLPE-MANHOLD CALCULUS INDEX

Dental calculus detection is commonly performed through analogic visual investigations. Several indices have been proposed over the years for measuring dental calculus in order to evaluate its deposition, progression or reduction. Among all such indices, comprehending those of Greene and Vermillion, Ramfjord, Ennever, Sturzenberger and Radicke, the Volpe-Manhold Calculus Index is the most accepted index to be used in human intervention studies [41]. The Volpe-Manhold Calculus Index provides quantitative information about the amount of dental calculus in patients and also allow comparison between subjects [39, 40]. Dental calculus is assessed by measuring the height of dental calculus deposits, employing a periodontal probe graduated in millimeters, on three different planes: vertical, bisecting the center of the surface (usually the lingual surface of teeth is considered), diagonal, through the mesial-incisal or mesio-occlusal point angle of the tooth through the area of greatest calculus height and diagonal again, through the distal-incisal or distal-occlusal point angle of the tooth through the area of greatest calculus height. The measurements obtained for each tooth are totalized and by dividing the sum for the number of measurements taken, the mean height of dental calculus deposit related to a single tooth is given. Furthermore, the sum of the mean heights of each tooth divided for the number of teeth considered, provides the mean total calculus score of the subject. In RCTs evaluating the anti-calculus effect of agents or devices, the amount of dental calculus is scored at baseline and at a defined time point after the end of

the intervention program in order to obtain information on the reduction of dental calculus deposits within the intervention group in comparison to the control group. At this purpose, the reduction of dental calculus is evaluated by calculating the difference between the mean Volpe-Manhold Calculus Index score for each study group at the baseline and the Volpe-Manhold Calculus Index score measured post intervention. The reliability of the Volpe-Manhold Calculus Index in measuring dental calculus amount has been confirmed through considerable studies which found, as example, that the scores provided by the Volpe-Manhold Calculus Index is highly correlated with dental calculus dry weight, a direct quantification of dental calculus as well as with dental calculus area in square millimeters [42].

In conclusion, the Volpe-Manhold Calculus Index is an appropriate method to assess dental calculus in human intervention studies.

3.1.3. MAINTENANCE OF TOOTH MINERALIZATION

3.1.3.1. LEVEL OF EROSION / ENAMEL LOSS

Dental enamel is the outer mineralized tissue covering the tooth crown, from light yellow to grayish white in color and almost entirely composed of highly organized, tightly packed hydroxyapatite crystals that confer it hardness and strength, whereas the organic acids and water constitute less than one percent of the total volume[43]. Despite the organization of its crystals and its mineralization degree, which makes dental enamel the hardest tissue of the human body, it is highly susceptible to demineralization and erosion, due to several circumstances[44]. First, the loss of dental enamel is promoted by oral pH decrease as consequence of acids production due to the metabolic activity of members of the oral bacterial community, such as *Lactobacillus, S. mutans, Actinomyces, S.aureus* and *S. epidermidis,* exposed to dietary fermentable carbohydrates. Typically, the critical pH for tooth demineralization is around 5.5-5.7. Organic acids produced during fermentation of carbohydrates penetrate into the enamel through the aqueous phase between hydroxyapatite crystals causing the dissolution of calcium along with phosphate, leading to the erosion of tooth enamel. Saliva plays a major role in the maintenance of the equilibrium

between de- and remineralization: when saliva flow rate increases, the salivary concentration of calcium phosphate and bicarbonate also increases, thus promoting the remineralization of hydroxyapatite crystals. Moreover, the bicarbonate presence in saliva helps to reverse the falls in pH due to the consumption of carbohydrates-rich foods. Otherwise, dental enamel can be lost through a chemical process independent from bacterial involvement, e.g. due to vomiting practices in eating disorders, or due to the reflux and its frequency, the pH and the type of acid in gastroesophageal reflux disease.

To evaluate the appropriateness of dental calculus as outcome variable of level of erosion / enamel loss, the literature deriving from database #4 was critically evaluated (see Table 1).

Although dental enamel is characterized by hardness and strength provided by the highly packed structure of the hydroxyapatite crystals, it shows no regenerative properties in response to wear or tears because the ameloblasts, i.e. the cells that deposited it, are lost after dental eruption [43]. Therefore, the importance of maintaining a correct dental enamel status and the attempts of dentistry research to develop strategies to prevent enamel loss are easily understandable. Dietary habits, both of adults and children, including a high intake of food and beverages rich in fermentable carbohydrates are recognized causes of the progressive acid erosion of dental enamel, eventually leading to complete enamel loss and onset of caries. In this regard, the status of dental enamel highly reflects the erosive potential of food and beverages and its qualitative and quantitative assessment provides useful information about tooth mineralization. Frequently, changes in physical features of dental enamel, like thickening and softening are considered surrogate measures of the level of dental enamel erosion and are evaluated in *in situ* RCTs aiming either to clarify the erosive potential of aliments or to demonstrate the claimed protective action against dental enamel loss of toothpastes, mouthwashes, chewing gums and other oral care agents[44, 45]. In such studies, subjects involved usually wear a removable device holding human dental enamel, which is exposed to the normal oral and eating conditions, thus elucidating the effect of the tested products in the maintenance or impairment of tooth mineralization. It is important to consider *in situ*

studies to evaluate dental enamel erosion due to net demineralization, i.e. the loss of minerals from dental enamel occurring despite the protective, remineralizing role of saliva.

In conclusion, as the erosion level and the associated enamel loss highly reflect the mineralization status of teeth in humans, the level of erosion / enamel loss is an appropriate outcome variable to be used alone for the scientific substantiation of health claims regarding the maintenance of tooth mineralization.

3.1.3.1.1. PROFILOMETRY

Profilometry is the specific technique for the measurement of a surface's profile in order to establish its roughness. Historically, profilometry included only the use of contact profilometers, which were instruments similar in function to a phonograph, providing data on surface roughness basing on the movement of the profilometer's stylus in contact with the surface[46]. Contact profilometers are still used in measuring the level of erosion of dental enamel despite the onset of imaging technologies. The stylus, positioned vertically, is placed in contact with the surface and moved laterally for specified distance and applying specified contact force. One of the stylus extremities includes a diamond tip whose radius usually ranges from 20 and 50 µm, leading to measurement of small surface variation in height from 10 nm to 1 mm. The position of the tip in function of surface topography generates an analogue signal that is converted into a digital 2D output visualized on a display as a line profile reflecting the surface roughness. Contact profilometry has the advantage to be a direct technique meaning that no experimental models are required. Moreover, contact profilometry is easy, standardized and has high vertical and horizontal resolution, in the nanometers range. Another advantage consists in directly touching the surface, which avoids measurement errors due to the eventual presence of contaminants onto the surface. Nevertheless, some reports point out its lack of precision in measuring etching depth on dental enamel surfaces. Optical profilometry is a non-touching, imaging based technology which encompasses several methodologies, as laser triangulation, interferometry, confocal microscopy and digital holography. Vertical scanning interferometry, also known as White-Light Interferometry is the

most employed technique in dental practice for monitoring the effect of either acid erosion or conservative treatment on human dental enamel[47]. White-Light Interferometry uses a computerized optical interference microscope for the acquisition of quantitative topographic images of the enamel surface on a microscopic scale. The data obtained can be presented in the form of pseudo-color height maps, 3D images, line profiles and surface roughness and topography parameters (including Ra - average roughness; Rt - total roughness; Rku - kurtosis; Rsk - skewness; Sa - mean surface roughness; Sp - highest peak of the surface; Sv - deepest valley; Sy - total height between highest peak and deepest hole; Sz - mean of distance between the five highest peaks and five deepest holes), depending on the dedicated surface analysis software. The resolution of the White-Light Interferometry measurement is about 0.5µm in the lateral (X, Y) plane and about 1nm in the height (Z) plane. This allows micro features and large scale topographic variations to be monitored in detail. The advantages of this technique consist in the rapidity of the image acquisition, in the high reliability deriving by the fact that the instrument does not touch the sample and therefore cannot be damaged by surface wear or careless operators. Otherwise, the sensibility to surface reflectance or color may lead to experimental bias.

In conclusion, profilometry is an appropriate method to assess the level of erosion / enamel loss in human intervention studies.

3.1.3.1.2. NANOINDENTATION

Nanoindentation is a variation of the indentation technique, the most commonly applied procedure for testing the mechanical properties of materials[45]. Specifically, nanoindentation is referred to small volume of materials and, in this context, the measurement of dental enamel features is a proper example. Recent studies have demonstrated the ability of nanoindentation to quantify the early stages of enamel softening caused by erosion *in vitro* as well as *in situ*. This technique employs a small size tip, usually made of a material, like diamond, whose hardness is known, to indent the surface of the tested material by applying a specified force. The area of the indentation in the sample is measured and the hardness of

the material is defined as the maximum load applied divided by the residual indentation area. By the fact that the indentation area is only few squared micrometers or nanometers, nanoindentation is sometimes combined with atomic-force microscopy because the latter helps to image the indentation process and permit the measurements of mechanical properties for indentation depth of less than 100 nm. Furthermore, the problems arising from the tiny dimensions of the indentation area can be overtaken by the employment of an indenter with a defined geometry, like the Berkovich tip, which has a three-sided pyramid geometry. In this case, the indentation depth is recorded during the measurement, and the related indentation area is provided using the known geometry of the tip. Then, plotting on a graphic all the parameters assessed during the penetration of the tip into the material allows the creation of the load-displacement curve, through which the hardness and the Young's modulus values can be extrapolated. Sometimes, the assessment of an area function based on the geometry of the nanoindenter tip is preferred, because it compensates for elastic load during the test. In this way, real-time nano-hardness values from a load-displacement graphic is gained. Nanoindentation has been demonstrated to be a rapid practice for evaluation of the dental enamel mechanical properties: it allows the determination of statistically significance difference in the demineralization potential of tested agents reducing tenfold the time needed for a clinical trial. Several works demonstrated that nanoindentation is an efficient and reliable tool in measuring early stages of dental enamel demineralization [45, 46].

In conclusion, nanoindentation is an appropriate method to assess the level of erosion / enamel loss in human intervention studies.

3.1.3.2. DENTAL CARIES

Dental caries, also known as tooth decay, can be properly defined as the localized destruction of susceptible dental hard tissue, i.e. enamel, cementum and dentin, due to a slow but progressive demineralization and erosion of such tissues. The disease can equally affect the crowns (coronal caries) and roots (root caries) of teeth. The dissolution of calcium phosphate mineral crystals is caused by the

metabolic activity of bacteria in dental biofilms (i.e. dental plaque), which produce acids by the fermentation of dietary carbohydrates[48]. The subsequent decrease in the surrounding pH below a critical value does not maintain tooth mineralization, thus first leading to erosion and eventually cavitations of hard tissues and then to the development of dental caries. Therefore, the onset of dental caries is always preceded by the formation and long-lasting permanence of dental plaque on the teeth surface. Dental caries is considered a chronic disease that affects people worldwide during the entire lifetime; it can already arise in early childhood as an aggressive tooth decay regarding primary teeth. Risk for caries includes physical, biological, environmental, behavioral, and lifestyle-related factors such as high number of cariogenic bacteria, inadequate salivary flow, insufficient fluoride exposure, poor oral hygiene, inappropriate methods of feeding infants, and socio-economic condition [48, 49]. Women are more predisposed than men of the same age to be affected by dental caries, and they also show a higher rate of caries development because sex- linked genetic susceptibility due to hormonal patterns[49].

To evaluate the appropriateness of dental caries as outcome variable of tooth demineralization, the literature deriving from database #5 was critically evaluated (see Table 1).

The retention of permanent teeth is of primary importance for the individual oral health status and quality of life. Dental caries is considered a pandemic disease that affects people worldwide, independently from sex and age, equally affecting toddlers and elders. Untreated caries lesions result in progressive tooth decay and loss, with heavy repercussion of the social and psyco-physical status of individuals[49]. Thus, the maintenance of oral health, specifically the prevention of dental caries development, by reducing the risk of caries lesion onset and progression is among the main purposes of researchers and clinicians[48]. Most consistent studies report assessment of caries increment as Decayed Missing and Filled Surfaces (DMFS), measured as prevented fraction (PF; PF=(XC-XE)/XC) where XC and XE are mean increment in the control group and in the test group, respectively. Therefore, the development of caries lesion can be measured as ΔDMFS, meaning the caries increment respect to the caries level at baseline, usually

552 expressed as percentage and related to a given period of time[50]. Moreover, dental caries is the primary
553 outcome in RCTs aiming to assess the role of foods, agents and oral care practices, like tooth brushing,
554 to prevent the onset of caries in the general adult population[51, 52]. In this context, dental caries
555 development, incidence as well as the net progression of tooth decay leading to caries are the most
556 commonly employed outcome variables most employed in the investigation of the effectiveness of caries-
557 preventive agents or devices. Dental caries as outcome is usually used alone, even if sometimes is
558 replaced by measurements of surrogate parameters related to dental caries, such as the degree of tooth
559 mineralization and the level of dental enamel loss and erosion. Concerning children, several RCTs have
560 been performed for the assessment of dental caries on erupting teeth into different test groups, either
561 following a prevention program or not[53, 54]. It must be noted that in this case is uncertain if results
562 obtained from the studies can be extrapolated to the adult population with permanent teeth. Nevertheless,
563 dental caries are appropriate outcome variables to be used alone for the scientific substantiation of health
564 claims related to the maintenance of tooth mineralization. Moreover, dental caries as direct measure of
565 the disease are appropriate outcome variables to be used alone for the scientific substantiation of health
566 claims related to the reduction of the risk of dental caries (See Section 3.2.2.1).

567 3.1.3.2.1. CARIES RECORDING

568 Dental caries is majorly recorded by expert visual investigation by the analysis of all visible tooth
569 surfaces[48, 55]. The examination can be performed with the employment of DMFS and Decayed
570 Missing and Filled Teeth (DMFT) indices, which allow measurement of decayed, missing and filled
571 surfaces to be assessed by completing the relative caries recording form. Usually, teeth are examined
572 after drying their surface with a cotton roll. An appropriate light source, mouth mirrors, battery-
573 illuminated dental mirrors, community periodontal index ball-end probe, community periodontal index
574 of treatment needs probes and compressed air are all tools aiding the examiner’s visual evaluation.
575 Regarding intervention programs, dental caries presence/ absence is recorded at baseline, i.e. before the

begin of the study, and then followed-up examination is taken for dental caries incidence and/or increment measurement. Usually, the evaluation comprehends different blinded examiners that ignore whether the subjects are part of the control or the intervention group, in order to not influence their response. Training and calibrating experts implied in the study is of fundamental importance for ensure consistency, uniform interpretation, understanding and application of criteria used for evaluating dental caries and conditions to be observed and recorded. Intra and inter examiner consistency can be assessed by measuring the percentage of agreement between scores, e.g. the percentage of subjects receiving the same scores between two examiners. In the context of caries, especially if the prevalence is low, a more reliable way to assess the overall agreement between examiners is the K statistic, a value correlating the actual measure of agreement with the degree of agreement, which would have occurred by chance[50]. Even if caries recording can also be practiced by using dental radiographs, mainly in cases of no visible cavities, the use of this technique is almost entirely restricted to clinical practice. Indeed, radiography is more time-consuming and expensive than visual recording approach, therefore is not suitable for RCTs involving a large number of subjects. In conclusion, caries recording by visual investigation is an appropriate method for the assessment of dental caries in human intervention studies.

3.1.3.3. pH AT TOOTH SURFACE

The pH value at tooth surface plays a fundamental role in the regulation of dental enamel status and the related tooth mineralization in humans. At neutral physiologic pH condition, dental enamel is prevented from erosion because of the saturation of calcium and phosphate ions in the surrounding oral environment. Differently, when pH falls under a critical value (pH < 5.5-5.7) calcium and phosphate are no longer sufficiently concentrated to avoid hydroxyapatite crystal dissolution, leading to tooth demineralization, the first step towards caries development. When the pH rises again, calcium and phosphate concentration also increases, mainly provided by saliva flow, thus promoting remineralization. Therefore, despite the strictly packed organization of hydroxyapatite crystals and its high mineralization

degree, which makes the dental enamel the hardest tissue of the human body, it is highly susceptible to demineralization and erosion under acid conditions. The tooth surface is physiologically covered by a microbial biofilm, which comprehends several strains of microbes, and which, at neutral pH conditions, remain stable over time. Due to the acidification of the oral micro-environment, homeostasis is impaired and there is a shift toward acid-producing and acid-tolerating species, such as *Lactobacillus*, *S. mutans*, *Actinomyces*, *S. aureus*, and *S. epidermidis*, which in turn contribute to the surrounding acidification and dental plaque development. Such plaque microbial community leads to the further drop of pH values at tooth surface through its metabolic activity comprehending the fermentation of carbohydrates contained in food and beverages[56].

To evaluate the appropriateness of pH at tooth surface as outcome variable of tooth demineralization, the literature deriving from database #6 was critically evaluated (see Table 1).

The deflection of oral pH from neutrality towards acid values is a well-known factor of teeth demineralization mainly due to the dietary intake of acidogenic food and beverage[57]. Therefore, the aim of investigations is to compare the effect of different types of food or beverages on the pH at various tooth surfaces during and after ingestion in healthy subjects without enamel erosion. The tooth surfaces where pH is usually recorded include the palatal surface of the upper central incisors and premolars and the facial surface of the incisors. Regarding drinks, pH measuring at tooth surface has led to the discovery that the drinking method, as well as the type of drink, strongly affects tooth-surface pH and thereby the risk for tooth demineralization. The studies found in literature provide evidences about the strong association between the drop of pH at tooth surface due to surrounding acidification and the erosion of dental enamel leading to tooth demineralization. Nevertheless, the response to acid challenge, in terms of the time taken for subjects to normal pre-drinking pH levels, often shows considerable individual variations and further investigation to clarify these aspects should be taken into account[58].

In conclusion, pH at tooth surface is not an appropriate outcome variable to be used alone for the scientific substantiation of health claims in the context of maintenance of tooth mineralization. However, it can be used as supportive of a mechanism through which the food/constituent could exert the claimed effect, in addition to parallel measurement of other surrogate parameters of tooth mineralization (e.g. dental caries and level of enamel erosion), for the scientific substantiation of such health claims.

3.1.3.3.1. MICROELECTRODE METHOD

Over the last century several methods have been developed to measure plaque pH and the pioneer has been the fine touch or probing electrode created by Stephan using antimony in 1938[59]. It was used to monitor the effect on plaque pH of rinsing with various concentrations of glucose at different sites of the mouth, including the labial surface of both the upper and the lower anterior teeth. By the fact that this technique has been seen to give inconsistent results in terms of pH measurement accuracy when placed in biological materials, the touch antimony electrode has been then modified and improved by others, giving rise to glass electrode by Clement in 1949, which instead gave higher performances in comparison to those provided by the antimony microelectrodes and offered the greatest possibility of ultimate accuracy for in vivo plaque pH measurement. The main disadvantage of microglass electrodes was fragility. Furthermore, several authors report the use of various type of antimony- and glass- based modified electrodes, which were more or less accurate for the measurement of plaque pH at different intraoral sites and the related potential cariogenicity of foods. Despite the antimony microelectrode is sometimes still used, it is nowadays considered outdated and it has been replaced by the Beetrode iridium-iridium oxide touch microelectrode, which is particularly suitable for the intraoral use because of the small size, the versatility and the quick response time, even if the problem of fragility remains. Therefore, it is widely used to detect *in situ* plaque pH changes, in RCTs that aim to clarify the effect of the intake of certain food on oral pH variation[58]. Usually the microelectrodes are connected to a display unit. First of all, the extremity of new and sterile pH sensors is submersed in distilled water for several

hours prior to use. Then, the microelectrodes are stored in a neutral reference buffer (pH = 7), in order to calibrate the instrument before the plaque pH assessment. Once the electrode grounding device is placed sublingually, the tip of the microelectrodes is placed in contact with the dental plaque biofilm and held still until the reading on the display unit has stabilized and data is recorded. Between each consecutive reading it is important to rinse the microelectrodes in distilled deionized water in order to avoid cross-contaminated measures. Collected data are reported on the device display, which often show them as a curve reporting the pH trend related to the time course. Through parametric and non-parametric statistical analysis, difference plaque pH values (e.g. the mean, the minimum, the mean minimum plaque pH) between the groups treated with different study conditions and at different time intervals are assessed.

In conclusion, the low plaque pH derived from acid production by plaque bacteria is useful to the process of caries risk assessment, and microelectrode are reliable and accurate devices to monitor plaque pH variations both clinically and in RCTs evaluating the cariogenicity of fermentable carbohydrates. Therefore, the microelectrode method is an appropriate method to assess pH at tooth surface and plaque pH in human intervention studies.

3.1.3.4. PLAQUE pH

Dental plaque has been defined as the microbial community that develops on the tooth surface, embedded in an extracellular matrix of polymers of host and microbial origin. Specifically, the dental plaque is an oral biofilm, physiologically present in healthy individuals, providing protection against invading microbes. Dental plaque is constituted by a heterogeneous microbial population, which is connected by a multitude of functional and metabolic networks and remains relatively stable over time (microbial homeostasis)[29, 60]. The carbohydrates fermentation due to the metabolic activity of the plaque microbial community leads to acid production and the subsequent impairment of plaque pH, which contributes to enamel loss and the demineralization of tooth tissues[61]. Indeed, under acid conditions, homeostasis is impaired, and there is a shift toward acid-producing and acid-tolerating species, such as

Lactobacillus, *S. mutans*, *Actinomyces, S. aureus*, and *S. epidermidis*, that in turn contribute to the surrounding acidification. Organic acids produced during fermentation of carbohydrates penetrate into the enamel through the aqueous phase between hydroxyapatite crystals causing the dissolution of calcium along with phosphate, leading to demineralization of tooth enamel. Saliva plays a major role in the maintenance of the equilibrium between de- and remineralization: when saliva flow rate increases, the salivary concentration of calcium phosphate and bicarbonate also increases, thus promoting the remineralization of hydroxyapatite crystals. Moreover, the bicarbonate in saliva helps to reverse the falls in plaque pH due to the consumption of carbohydrates-rich food.

To evaluate the appropriateness of plaque pH as outcome variable of tooth demineralization, the literature deriving from database #2 was critically evaluated (see Table 1).

Plaque pH values are related to the production of organic and inorganic acids, resulting from carbohydrates fermentation due to the metabolic activity of plaque microbial community [60, 61]. By the fact that an acid surrounding has detrimental consequences on tooth mineralization, the plaque pH is considered by experts as an established measure of the potential tooth demineralization [62]. Randomized controlled plaque pH studies frequently assess plaque pH variation after the ingestion or use of specific products or aliments in order to elucidate their role in the maintenance of the correct de- and remineralization equilibrium, by acting on pH stability. In this regard, often assessed outcome variables are the minimum, the mean and the mean minimum plaque pH, thanks to which is possible, when assessed at different times, to build up a graphic curve, to calculate the relative Area Under the Curve and then to monitor plaque pH changes over time, finally providing a quantitative expression about the impact of the aliment on the maintenance of tooth mineralization[62]. Clinically speaking, a pH value of 5,5 is considered the critical threshold for tooth mineralization, below which dissolution of tooth enamel may occur[60, 61]. Nevertheless, it is worth remembering that the direct evaluation of tooth

mineralization should always be provided, through the assessment of specific surrogate parameters of tooth mineralization status, e.g. the level of dental enamel erosion and loss.

In conclusion, plaque pH is an appropriate outcome variable for the scientific substantiation of health claims in the context of maintenance of tooth mineralization, only in combination with other direct measurements of tooth mineralization, such dental caries and the level of enamel erosion.

3.1.3.4.1. MICROELECTRODE METHOD

See Section 3.1.3.3.1

3.1.3.4.2. INTRA-ORALLY MOUNTED ELECTRODES

The cariogenic potential of a dietary product is usually evaluated by measuring plaque pH in vivo during and some minutes after consumption of the product using intra-orally mounted pH electrodes, also known as indwelling electrodes. The first indwelling electrodes were miniature transmitters including a power supply, glass and references electrodes which were mounted on removable partial dentures and used for telemetering the interdental plaque pH in adults, via radiotelemetry. Then, the so called “Zurich” system has been developed by Graf and Muhlemann, which was based on a natural, hollow tooth placed into a cobalt-chromium mandibular dental prosthesis. An artificial contact point was then created between the hollow tooth and the adjacent natural abutment tooth through a glass microelectrode inserted into the tooth. Plaque pH values by radiofrequency were then collected from subjects first retrained to oral hygiene for several days in order to accumulate plaque on the device and then subjected to various carbohydrates challenges. Further, several researchers improved the technology underlying those forerunner electrodes, giving rise to various types of modified system: as example, a portable telemeter with the advantage of plaque pH reading while allowing subjects to conduct their normal daily activities, or the Hydrogen Ion Sensitive Field Effect Transistor, devised by Esashi and Matsuo, which conveyed wire telemetry measurement with low resistance causing few insulation problems [63]. Other than plaque pH measurement, Hydrogen Ion Sensitive Field Effect Transistor was also used to investigate the

718 microbiology of the plaque growing on it. The intraorally mounted electrodes (i.e. the indwelling
719 electrodes) have been, and still are, very useful and accurate tools in the evaluation of food acidogenicity,
720 the effect of food additives on plaque pH and the evaluation of chewing gum on plaque pH, among many
721 other applications. This technique continues to be popular and has been subject to further developments
722 for denture applications[59]. Despite the advantages, there are few centers worldwide that have
723 indwelling plaque pH telemetry facilities, perhaps due to the costs and the high technical skills required.
724 In conclusion, the intra-orally mounted pH electrodes are appropriate methods to assess plaque pH in
725 human intervention studies.

726 **3.1.4. REDUCTION OF ORAL DRYNESS**

727 3.1.4.1. SALIVA FLOW

728 Saliva is a clear, slightly acidic exocrine oral fluid, composed of more than 99% of water, while the
729 remaining 1% consists of proteins, electrolytes, such as sodium and potassium and nitrogenous products,
730 including urea and ammonia[64]. The whole saliva is composed by the mucoserous exocrine fluid
731 secreted from the major (parotid, submandibular and sublingual) and minor salivary glands, and by an
732 exudate, the gingival crevicular fluid, containing oral bacteria, their metabolic products and food debris.
733 Saliva flow by minor salivary glands is continuously supplied during the day and night, whereas the
734 major salivary glands only secrete saliva in response to mechanical stimuli associated with lips and
735 tongue movement as well as responding to mucosal dryness to protect and lubricate the oral cavity. Such
736 salivation is known as unstimulated saliva flow even if it depends on nervous stimulation and it is mainly
737 secreted by submandibular and sublingual glands (submandibular/sublingual saliva) while an inferior
738 quote derives from parotid and minor glands activity. Otherwise, stimulated saliva flow refers to the
739 increase in salivation in response to taste, smell, visual and mechanical stimuli occurring at mealtime and
740 the parotid glands contribute for more than a half of total salivary secretions[64]. Despite previously
741 reported, conflicting literature findings, it is nowadays acknowledged the unstimulated and stimulated

whole and submandibular/sublingual saliva flow rates decrease with ageing in a proportional way between sexes; conversely, parotid and minor gland saliva flow rates do not seem to be affected by age, not being significantly lower in elder people respect to young adults[64, 65]. Moreover, saliva flow and electrolytes concentration are known to vary with circadian rhythms. The volume and the electrolyte composition of saliva are not only influenced by the moment of the day but also by hormonal regulation, like the pregnancy related hormonal changes which has been seen to increase salivation in women. In general, under healthy condition, adults approximately produce 500-1500 ml saliva per day or, differently speaking, up to 6 ml per minute[66].

To evaluate the appropriateness of saliva flow as outcome variable of oral dryness, the literature deriving from database #7 was critically evaluated (see Table 1).

Saliva plays a relevant and irreplaceable role in the maintenance of oral homeostasis. Indeed, other than being required for the modulation of pH levels in order to avoid tooth demineralization and erosion and for the protection of hard and soft tissues against bacteria, virus and fungi, an adequate saliva secretion is indispensable to maintain the proper moistening and lubrication of the oral tissues, to favor swallowing and speaking and to protect the oral cavity from oral dryness and ulceration[66]. Therefore, the maintenance of the correct salivary flow is a prerequisite to avoid oral dryness in healthy individuals with a favorable effect on their health related quality of life[65]. Literature searching provides numerous works, in particular RCTs and clinical studies aiming to clarify and compare the effect of different agents as stimulating for salivation in healthy subjects[67]. Depending on the aim of the study, unstimulated or stimulated whole saliva flow are taken into account. Moreover, even if saliva flow is mainly considered as a primary outcome, it is sometimes evaluated together with salivary secretion under mechanical stimulation in order to assess salivary gland function. Furthermore, despite the fact that saliva flow is considered as the main parameter to obtain objective information on mouth conditions, specifically dryness or moisture, great relevance is also given to self-perceived oral dryness in diagnosis, clinical

766 studies, as well as human intervention studies aiming to evaluate the effect of a food, its components and
767 agents on the reduction of dryness.
768 In conclusion, saliva flow is an appropriate outcome variable for the scientific substantiation of health
769 claims related to the reduction of oral dryness, better if used in combination with self-perceived oral
770 dryness.

771 3.1.4.1.1. *IN VIVO* SALIVA COLLECTION AND MEASUREMENT

772 The accurate measurement of saliva flow has been seen to be important in several clinical, experimental
773 and diagnostic studies. In particular, RCTs usually take into account and provide measurements of whole
774 saliva flow, because its impairment is the prime responsible of oral dryness in healthy population.
775 Sometimes, the stimulated saliva flow is considered in studies aiming to clarify the effect of agents as
776 stimulating of salivation[67]. Saliva flow is normally measured as a volume expressed in ml and can be
777 collected through different methods, both under unstimulated and stimulated conditions. Standardization
778 of the collecting method is crucial because of the significant variation of the saliva flow rate among
779 individuals and also in the same individual under different conditions. Whatever is the chosen
780 methodology, subjects are previously instructed to thoroughly rinse their mouth, usually with deionized
781 water and to empty it from saliva just before the collection. Then, they are seated comfortably for five
782 minutes, minimizing the orofacial movements, with open eyes and their head tilted slightly forward. Five
783 minutes are considered an appropriate time period to collect saliva samples, regardless of the method.
784 The most commonly used methods to collect whole unstimulated saliva are four: the draining, spitting,
785 suction and swab or absorbent method. According to the draining technique, subjects expectorate saliva
786 after the collecting period, into a previously weighed, graduated tube, through a funnel. The spitting
787 method is similar to the draining method, except for the fact that saliva is accumulated at the floor of the
788 mouth and collected every minute. Referring to the suction method, saliva is continuously collected with
789 an aspirator or a saliva ejector from the floor of the mouth into a graduated tube. Lastly, the swab or

absorbent method consists in collecting saliva by a swab, cotton or gauze sponge of known weight placed at the orifices of the major glands and, at the end of the defined period, in weighing the collecting material again. Direct comparison of these methodologies shows that the suction and the swab methods are affected by variability and some degree of stimulation and therefore these are not the most suitable techniques when unstimulated saliva sample is needed. Moreover, the swab method has been found to be the least reliable. Differently, the draining and spitting methods provide similar results and are both reproducible and reliable. The spitting method is also widely used for stimulated whole saliva collection. Salivation can be easily stimulated with paraffin wax, rubber bands, gum base, and citric acid. Devices usually employed for collecting saliva after stimulation include the Lashley cup or a modified Carlson Crittenden device, which is an easy and reliable device to collect saliva from parotid glands, consisting of a plastic or metal cup with an inner and outer chamber, the former connected to plastic tube that carries saliva to the collection vessel, the latter attached to a suction-inducing device. Cannulating the Wharton's duct is necessary to collect saliva from supramandibular gland, but caution should be made because the polietylene tube employed risks to damage the thin wall of the duct. The so called "segregators" allow the simultaneous collection from submandibular and sublingual saliva through a system of tubes and chambers. In order to collect mixed submandibular and sublingual saliva, the method introduced by Fox and colleagues in 1985 can be used[68]; it consists in collecting saliva from the floor of the mouth by gently aspirating with a micropipette after blocking Stensen's duct and isolating Wharton's duct. Moreover, by the use of a pipette or absorbent filter paper or strip, minor gland secretion can be collected from the oral mucosa, lips or palate, depending on the necessity, and the quantification of the sample can be performed with Periotron, an instrument which measures small volumes of fluids. Another apparatus for the collection of submandibular and sublingual saliva has been developed by Wolff and colleagues in 1997, which has been demonstrated to be reliable, easy to use, safe and comfort for the patients because no cannulation of the ducts is required: it consists of collecting tubing, a buffering chamber, a storing

tube, and a suction device[69]. In conclusion, *in vivo* measurements are appropriate methods to collect and measure saliva flow in human intervention studies.

3.1.4.2. SELF-PERCEIVED ORAL DRYNESS

Oral dryness, also known as xerostomia, is a condition mainly due to changes in biochemical composition of saliva and reduced saliva flow, with detrimental consequences on the lubrication and moistening of oral tissues. Xerostomia implicates self-perceived oral dryness, sequentially leading to oral discomfort, difficulty in speaking and swallowing[70]. One of the main causes of reduced salivation is drug consumption, mainly antidepressant, but also diuretic, Angiotensin Converting Enzyme inhibitors, oral hypoglicemiants, acetylsalicylic acid, iron supplement and drugs used for cardiovascular disease and hypertension. In addition, drugs considered to be xerostomizing are those used for the treatment of diabetes, obesity, epilepsy, Parkinson's disease, diarrhea, asthma and urinary incontinence. By the fact that older people are more likely to take medicines, they are majorly predisposed to be affected by xerostomia and it is further well-recognized that self-perceived oral dryness is mostly experienced by women, regardless of age. The literature reports a higher probability to suffer from oral dryness at night, probably because of the habit of mouth-breathing. Lifestyle behaviors, like smoking cigarettes or chewing tobacco, also negatively affect the perception of dryness. It has been reported that salivation is impaired in Sjögren's and Alzeheimer's syndromes, HIV/AIDS, diabetes, anemia, hypertension and rheumatoid arthritis[70]. Lastly, damages at nerves controlling salivary glands, often due to radiation exposure during chemotherapy in case of neck or head cancer[71], as well as dehydration caused by fever, vomiting, excessive sweating, burning mouth, blood loss and diarrhea represent other eventualities responsible of self-perceived oral dryness.

To evaluate the appropriateness of self-perceived oral dryness as outcome variable of oral dryness, the literature deriving from database #8 was critically evaluated (see Table 1).

Self-perceived oral dryness, or xerostomia, refers to the subjective sensation of dry mouth[72]. It has been reported that the unstimulated whole salivary flow rate is more strongly correlated with xerostomia than the stimulated whole salivary rate[73]. Additionally, some patients who complain of xerostomia do not exhibit a decrease in the flow rate of whole saliva, leading to the hypothesis that minor salivary gland secretion are involved, when impaired, in inducing xerostomia. The recognized threshold under which xerostomia can be properly defined is 0.2 ml per minute, referring to the unstimulated saliva flow rate. Oral dryness has a significant effect primarily on patient's oral health. Indeed, it reduces salivary buffering capacity and decreases the level of salivary protective proteins, thus increasing the risk of caries, dental plaque and gingivitis. Moreover, the overall health, quality of life and well-being are also affected as it is demonstrated by patients complaining about speech and swallowing difficulties, changes in taste sensation and decrease in dietary intake[71, 72]. Clinician's evaluation does not always agree with patient's assessment; therefore, great relevance is given to self-perceived oral dryness in diagnosis, clinical studies, as well as human intervention studies aiming to evaluate the effect of a food, its components and agents on the reduction of dryness. By the fact that xerostomia can be derived by other medical conditions, it is necessary that the population involved in RCT's be healthy, in order to not void the results[73].

In conclusion, self-perceived oral dryness is an appropriate outcome variable better if used in combination with the objective assessment of saliva flow for the scientific substantiation of health claims related to the reduction of oral dryness.

3.1.4.2.1. VALIDATED QUESTIONNAIRES

The subjective sensation of oral dryness is commonly assessed by questionnaires based on focused questions in order to increase reproducibility, obtain standardized results and reduce misinterpretation. After an accurate literature search, the Bluestone Mouthfeel Questionnaire has been found to be the most widely used questionnaire to be administered to subjects to evaluate their perception of dry mouth and

general “mouthfeel”[74]. Commonly, the Bluestone Mouthfeel Questionnaire is administered to subjects immediately after the unstimulated salivary flow rate measurement and their perception of oral dryness is recorded by using eleven items assessed on a Visual Analogue Scale (VAS), flanked by “not at all” at one extremity and “strongly agree”, or similar statement, at the other. The subjects are asked to mark a vertical line on the VAS in order to describe their mouth conditions and dryness sensation. Then, the marks are converted into the corresponding millimeters on the scale of 0 to 100 mm[73]. The Bluestone Mouthfeel Questionnaire is appreciated for its accuracy in differentiate subjects complaining about dry mouth compared to those without dry mouth. The Bluestone Mouthfeel Questionnaire also provides test-retest reliability and highly correlates with whole unstimulated saliva flow rate, often simultaneously collected and measured (in millimeter per minute) to provide adjunctive information about oral dryness condition. Several modifications of the original questionnaire have been developed according to different languages and/or necessities, thus frequently including also questions about self-perceived bad breath or smoking habits. In conclusion, validated questionnaires, particularly the Bluestone Mouthfeel Questionnaire, are appropriate methods to measure self-perceived oral dryness in human intervention studies.

3.2. RISK REDUCTION CLAIMS Art 14(a)

3.2.1. GINGIVITIS

Gingivitis is a non-destructive, reversible periodontal disease, consisting in the inflammation of interdental and marginal gingival tissue without loss of the underlying, supportive connective tissue. Various types of gingivitis exist and, according to the World Workshop in Clinical Periodontics of 1999, they can be categorized into two main groups, each further divided into subgroups: plaque-induced gingivitis and non-plaque-induced gingivitis, the latter including, e.g. gingivitis of fungal, viral or genetic origins. The interest of clinicians for gingivitis began to grow since the early 60’s when a series of epidemiologic studies were carried out worldwide to assess the prevalence and severity of this disease.

Based on these studies, two main concepts have been formulated: first of all, a positive association has been demonstrated between the level of oral hygiene and the presence and increasing severity of gingivitis; secondly, gingivitis has been recognized as an early form of periodontitis, which, in time and in absence of treatment, spontaneously progresses without remission to periodontitis. The primary etiology of gingivitis is the attachment and growth of microbial species on teeth surfaces at or near gingival margins, thus forming dental plaque. The deposition of the bacterial biofilm extending below the gum line often results in inflammation, thus promoting the onset of the most common form of gingivitis, namely the plaque-induced gingivitis. The microbial species of dental plaque that are mainly involved in the pathophysiology of gingivitis are Gram negative bacteria like *P. gingivalis*, spirochetes like *T. denticola* and fungal species like *H. actinomycetemcomitans*. These microorganisms produce degradative enzymes and toxins, such as lipopolysaccharide or lipoteichoic acid, which promote an inflammatory response in the gum tissue. The overgrowth and override of these periodontopathic bacteria as compared to other microbial species in dental plaque can be explained, according to the "ecological plaque hypothesis", by the rise of local pH above the normal neutral value due to the increased secretion of the gingival crevicular fluid in response to inflammation.

Gingivitis onset and progression can be easily avoided by the accurate, daily removal of dental plaque, while a lack of oral hygiene is a precondition for the accumulation of dental plaque and the development of gingivitis. Furthermore, if not treated, gingivitis may become chronic and periodontal tissues are not only inflamed but eventually damaged, resulting in a severe pathological condition known as periodontitis. The progression of the gingivitis to periodontitis is an unpredictable event, being based on individual predisposition, local, systemic and exogenous factors.

Gingivitis, especially at the beginning, is mostly asymptomatic, so that many people are not aware of suffering from it, besides occasional bleeding upon tooth brushing. Nevertheless, among the main signs,

which are associated to inflammation, are: redness, tenderness and swelling of gums, presence of pus, pain when chewing or touching and persistent foul-smelling breath.

Other than poor oral hygiene levels, numerous other factors have been recognized to predispose to gingivitis, including chewing or smoking tobacco, tooth crowding, poorly fitted dental appliances, pregnancy, genetic factors, psychosocial stress, certain diseases, like diabetes and immunosuppression conditions. Moreover, the use of oral contraceptives, steroids, anticonvulsants, calcium channel blockers and chemotherapeutic agents is likely to play a role in increasing the predisposition to gingivitis.

Gingivitis is found already in early childhood; its prevalence and severity rise during adolescence and then tend to stabilize in older age groups. Gingivitis differs in children and adults; indeed, clinical signs of gingivitis rarely appear as dental plaque accumulation and the inflammatory infiltrate mainly consists of T lymphocytes in children while, in adults, the T lymphocyte infiltrate is promptly replaced by B cells and plasma cells as the clinical conditions worsen.

The American Dental Association has identified gingivitis and periodontitis as major causes of tooth loss and decay in adults, sustained by the fact that, as example, 82% of adults in the USA have gingivitis affecting one or more sites. Thus, the maintenance of gums structure leading to the correct gingival functions is important at both an individual and a societal level. In the recent years, the interest on the study of gingivitis in children has increased because it provides an appropriate model illustrating the lifetime impact of gingival and periodontal infections occurring in childhood on future oral and systemic health.

3.2.1.1. *S. mutans*

S.mutans is a facultative anaerobic, spherical Gram positive bacterium that was firstly isolated from human carious lesions by Clark in 1924. *S. mutans* exclusively colonized the oral cavity environment, where it plays a fundament role in the development of dental plaque, the oral biofilm recognized as prerequisite for gingivitis, tooth decay and dental caries onset. The colonization of the oral cavity,

especially the tooth surface, and the formation of microbial biofilm by *S. mutans* are derived by its ability to adhere to solid surfaces, to survive in an acidic environment and to specifically interact with other microorganisms colonizing this oral ecosystem. The attachment of *S. mutans* to the tooth surface implies the adhesion to a previously formed pellicle of salivary origin and involves specific interaction between pellicle components and *S. mutans* surface receptors as dextran-based polysaccharide. Further extracellular polysaccharides synthesized by *S. mutans* are glucans and fructans, which derived from sucrose and are considered to be critically important, especially glucans, in the dental plaque formation and hence in the pathogenesis of dental caries because of their insolubility to water and the ability to promote adhesion when synthesized de novo on tooth surfaces [75]. Moreover, the maturation of dental plaque is known to be mediated by the synthesis of glucans by *S. mutans*. *S. mutans* is commonly found, other than on the tooth surface, on the gingival margin and within the periodontal pockets, whose low oxygen tension conditions favors the growth of the microaerophilic species. The accumulation of dental plaque in these areas triggers an inflammatory reaction of soft tissue, often leading to the onset of gingivitis. *S. mutans* is an acidogenic microorganism, mainly producing lactate as result of the fermentation of dietary carbohydrates. This metabolic process further sustains the lowering of pH level at tooth surface and within the plaque biofilm, causing the erosion of the dental enamel, through the demineralization of hydroxyapatite crystals. The progressive impairment of tooth mineralization under acid conditions is the precondition for the formation of tooth cavitations and caries lesions.

To evaluate the appropriateness of *S. mutans* as risk factor for gingivitis, the literature deriving from database #9 was critically evaluated (see Table 1).

Processes in the oral cavity related to human disease are predominately driven by reactions occurring within the complex microbial biofilm communities. Indeed, clinical observation in humans demonstrate that dental plaque formation is a prerequisite for both dental caries and periodontal diseases development. Despite the fact that *S. mutans* is one of the main cariogenic factor of dental caries[76], its prevalence in

the dental plaque has no clear relationship to gingival inflammation. Indeed, as literature works state, it is still uncertain whether the colonization of *S. mutans* within dental plaque is related or not to gingivitis. The results provided by several works indicate that gingivitis does not appear to be associated with the proportion or the percentage of *S. mutans* in dental plaque, even if it plays a considerable role in the early formation of dental plaque biofilm. Differently, the microbial species mostly associated to the pathophysiology of gingivitis and other periodontal diseases are Gram negative bacteria, like *P. gingivalis*, spirochetes such as *T. denticola* and fungal species like *H. actitiomycetemcomitans*[77]. Therefore, the control and the reduction of *S. mutans* infections in dental plaque is recognized to be important only for the treatment of dental caries but not for the reduction of the risk of gingivitis. In other words, isolated changes in colonization of *S. mutans* have not generally been shown alone to reduce the risk of gingivitis. In conclusion, colonization of *S. mutans* may be considered an appropriate risk factor for gingivitis only if changes in this factor are accompanied by evidence of reduced incidence of these diseases in humans in the context of a particular nutritional intervention.

3.2.1.1.1. MICROBIOLOGICAL ANALYSIS

The diagnosis of periodontal disease is based on the identification of such microorganisms with a related pathological meaning. In this context several methodologies have been developed and adjusted over the year, comprehending enzymatic methods, microspectrometry and microscopic examinations of plaque and salivary samples. Differently, in RCTs, the purpose is to correlate the levels of the species under examination, i.e. *S. mutans,* with the state and/or the course of disease[78]. In this way, information can be obtained on the action of a specific agent in either preventing or reducing it, through its effect on *S. mutans.* The common practice envisages that *S. mutans* colonies are identified by microbiological and eventually morphological and biochemical procedures, then counted and often expressed as percentage. Specifically, salivary or dental plaque samples are collected from recruited subjects and spread over a selective culture medium for *S. mutans* (e.g. Mitis Salivarius bacitracin, Mitis Salivarus agar medium

supplemented with specific concentrations of bacitracin and sucrose, depending on the followed experimental protocol). After incubating for 48 hours at 37 °C under microaerophilic conditions, *S. mutans* appears on the culture plate as small, rough, raised and adherent colonies. The eventual atypical colonies are further investigated with biochemical analysis, like the mannitol and sorbitol test. The colonies so identified are quantified by counting with an analogic or electronic colony counter and the count is expressed as Colony-Forming Unit/mL of diluted plaque or salivary samples. The final comparison between counts obtained at baseline and at defined time points allows investigators to determine what salivary or dental plaque levels of *S. mutans* are associated with the considered pathology[78, 79]. Despite the described methodology is time consuming and its reliability strictly depends on the precision and accuracy of the investigator, it is still the more widely accepted and employed technique for measuring the concentration of *S. mutans* in human sample.

In conclusion, microbiological analysis is an appropriate method for the assessment of the colonization by *S. mutans* in human intervention studies.

3.2.1.2. DENTAL PLAQUE

See Section 3.1.2.1.

3.2.1.2.1. PLAQUE INDICES

See Section 3.1.2.1.1.

3.2.1.3. PLAQUE pH

As already mentioned (Section 3.1.3.4), dental plaque is an oral biofilm, defined as a heterogeneous microbial community that develops on the tooth surface, embedded in an extracellular matrix of polymers of host and microbial origin. The dental plaque formation encompasses an ordered sequence of events, starting from the highly specific attachment of bacteria to the host cell receptors, and then followed by a second bacterial colonization of the already formed microbial film[60]. Plaque architecture is a structured polymeric reticulum that connects the plaque/oral environment surface to the underlying tooth surface

and allows molecules and bacteria to move throughout the plaque. This complex matrix can also accurately regulate the penetration and distribution of molecules within the plaque. Carbohydrates, such as starch and sucrose, are easily metabolized by the plaque microbial community, mostly Streptococci like *S. mutans* and *S. sobrinus*, to acids[80]. A persistent acidic environment within the biofilm results in demineralization of tooth enamel and, in the long run, this process leads to cavitation and caries development[60, 61], especially among children due to their high dietary intake of food containing high concentration of fermentable carbohydrates (e.g sweets, biscuits, snacks, sweet drinks)[80]. Regarding gingivitis, the overgrowth and override of periodontopatic microorganisms, such as *P. gingivalis, T. denticola* and *H. actinomycetemcomitans* respect to the other microbial species in dental plaque can be explained, according to the "ecological plaque hypothesis", by the rise of local pH above the normal neutral value due to the increased secretion of the gingival crevicular fluid in response to inflammation. The persistent presence of bacterial biofilm at gingival margins often results in inflammation of the periodontal tissues, thus favoring the onset of plaque-induced gingivitis[28].

To evaluate the appropriateness of dental plaque as risk factor for gingivitis, the literature deriving from database #2 was critically evaluated (see Table 1).

As already mentioned, the WHO defines oral health as a condition free from oral disease, pain, sores, tooth decay or loss, and other defects in the mouth. Functionally, it includes the ability to bite, chew, speak, and smile. Oral health has also been proven to be associated with general health and health-related quality of life. Thus, oral health is important both at individual and socially level and the prompt identification of conditions potentially detrimental to oral well-being is equally relevant, in order to timely intervene, thus avoiding the eventual onset of oral diseases. In this context, gingivitis is a common reversible gums inflammation, mainly caused by plaque acid accumulation at the gingival margin[81]. Despite the rise of gingival crevicular fluid pH values above the neutrality plays a fundamental role in the onset of gingivitis because it triggers the growth of periodontophatic microbial species of plaque[28],

the same cannot be said for plaque pH. Indeed, changes in plaque pH levels have not been found to be related to risk of gingivitis. Conversely, a great contribution in the pathophysiology of gingivitis is made by the accumulation of dental plaque on interdental and marginal gingivae and by the replacement of Gram-positive bacteria by the Gram-negative periodontopatic population[81]. It is therefore easily understandable how neutralization of plaque acid could prevent gingival inflammation and reduce caries incidence, thus providing a beneficial effect for the general oral health[28]. The presence of dental plaque at gingival margin is only one among several outcome variables (e.g. gingival color and contour and histological changes) assessed in clinical studies to discriminate between the presence/absence of inflamed conditions of gums. However, changes in plaque pH have not generally been shown to reduce the risk of gingivitis. Therefore, plaque pH may be considered an appropriate risk factor for gingivitis only if changes in this factor are accompanied by evidence of reduced incidence of these diseases in humans in the context of a particular nutritional intervention.

3.2.1.3.1. MICROELECTRODE METHOD

See Section 3.1.3.4.1.

3.2.1.3.2 INTRA-ORALLY MOUNTED ELECTRODES

See Section 3.1.3.4.2.

3.2.2. DENTAL CARIES AND TOOTH DECAY

Dental caries, also known as tooth decay, can be properly defined as the localized destruction of susceptible dental hard tissue, i.e. enamel, cementum and dentin, due to a slow but progressive demineralization and erosion of such tissues. The disease can equally affect the crowns (coronal caries) and roots (root caries) of teeth. If not treated, dental caries can lead to complications, including infection, inflammation and abscess formation in the tissue around the tooth, up to the eventual tooth loss. Despite the main symptoms are pain and difficulty with eating, the initial caries lesion can be asymptomatic, so that the individual might not be aware to be affected by it. Only a white spot lesion is at that time evident,

which can further become brown as the demineralizing process continues. Even if, at the beginning, the lesion is reversible, once a cavity in the dental structure is formed, the lost portion of tooth cannot be regenerated. The dissolution of calcium phosphate mineral crystals is caused by the metabolic activity of bacteria in dental biofilms (i.e. dental plaque) which produce acids by the fermentation of dietary carbohydrates. The subsequent decrease in the surrounding pH below a critical value does not maintain tooth mineralization, thus first leading to erosion and eventually cavitations of hard tissues and then to the development of dental caries. Therefore, the onset of dental caries is always preceded by the formation and long-lasting permanence of dental plaque on the teeth surface. The lowering of plaque pH also causes a shift within the bacterial community of the plaque, favoring the overgrowth and override of the cariogenic species, most prominently *S. mutans*, *S. sobrinus* and *Lactobacillus* species. Dental caries is considered a chronic disease that affects people worldwide during the entire lifetime; it has been estimated that up to 36% of the world population suffers from caries, with a major incidence among the developed world due to the great consumption of simple sugar rich food. Dental caries can already arise in early childhood as an aggressive tooth decay regarding primary teeth. Risk for caries includes physical, biological, environmental, behavioral, and lifestyle-related factors such as high number of cariogenic bacteria, inadequate salivary flow, insufficient fluoride exposure, poor oral hygiene, inappropriate methods of feeding infants, and socioeconomical situation. Women are more predisposed than men of the same age to be affected by dental caries, and they also show a higher rate of caries development because sex- linked genetic susceptibility due to hormonal patterns.

3.2.2.1. DENTAL CARIES

See Section 3.1.3.2.

3.2.2.1.1. CARIES RECORDING

See Section 3.1.3.2.1.

3.2.2.2. DENTAL PLAQUE

See Section 3.1.2.1.

3.2.2.2.1. PLAQUE INDICES

See Section 3.1.2.1.1.

3.2.2.3. *S. mutans*

S.*mutans* has been already described as a risk factor of gingivitis (See Section 3.2.1.1).

To evaluate the appropriateness of *S. mutans* as risk factor for dental caries and tooth decay, the literature deriving from database #9 was critically evaluated (see Table 1).

As mentioned in Section 3.2.1.1, processes in the oral cavity related to human disease are mainly driven by reactions occurring within the complex microbial biofilm and it has been shown that dental plaque formation is a prerequisite for both dental caries and periodontal diseases development. Dental caries is a multifactorial disease encompassing host, agent and environmental factors, which are strictly intertwined. Therefore, strategies for reducing the risk of dental caries should focus on disrupting the interaction between all the risk factors that are thought to be implicated in dental caries. Nevertheless, because *S. mutans* has been recognized as one of the main cariogenic factors[76], the presence (expressed as proportion or percentage) of *S. mutans* colonies is often evaluated alone as outcome variable related to dental caries presence/absence in RCTs assessing the effect of antimicrobial agents on the reduction of dental caries development[78]. Conversely, some studies suggest that the high presence of *S. mutans* in dental plaque is not sufficient to justify the onset of carious lesions, but rather multiple cariogenic species, such as *S. mutans*, *S. mitis*, *Rothia*, *Actynomices*, *Lactobacillus*, *Bifidobacterium* and even fungal species like *Candida* could account for dental plaque to become cariogenic[82]. In conclusion, it can be stated that isolated changes in colonization of *S. mutans* have not generally been shown alone to reduce the risk of dental caries. Therefore, colonization of *S. mutans* may be considered an appropriate risk

factor for dental caries only if changes in this factor are accompanied by evidence of reduced incidence of these diseases in humans in the context of a particular nutritional intervention.

3.2.2.3.1. MICROBIOLOGICAL ANALYSIS

See Section 3.2.1.1.1.

3.2.2.4. PLAQUE pH

Plaque pH has been already described as outcome of tooth demineralization (See Section 3.1.3.4) and as risk factor of gingivitis (See Section 3.2.1.3).

To evaluate the appropriateness of plaque pH as risk factor for dental caries and tooth decay, the literature deriving from database #2 was critically evaluated (see Table 1).

As previously mentioned, dental plaque is characterized by acid pH, due to bacterial carbohydrates fermentation, which dissolves tooth enamel, provoking cavitation and dental caries. Therefore, neutralization of plaque acid could prevent gingival inflammation and reduce caries incidence. Relating to caries, it must be said that the acidification of the environment surrounding teeth due to plaque acid is the necessary condition for the eventual subsequent caries development. Therefore, the plaque acid is often assessed in several literature RCTs evaluating the cause and effect relationship between foods intake or devices use and the incidence of caries [61, 62]. However, isolated changes in plaque pH have not generally been shown alone to reduce the risk of dental caries and the related tooth decay. Therefore, plaque pH may be considered an appropriate risk factor for dental caries and the related tooth decay only if changes in this factor are accompanied by evidence of reduced incidence of these diseases in humans in the context of a particular nutritional intervention.

3.2.2.4.1. MICROELECTRODE METHOD

See Section 3.1.3.4.1.

3.2.2.4.2. INTRA-ORALLY MOUNTED ELECTRODES

See Section 3.1.3.4.2.

4. CONCLUSIONS

The present paper provides information related to the collection, collation and critical analysis of claimed effects, OVs and MMs that have been proposed so far in the context of oral health, compliant with the European Regulation. This critical analysis could represent a useful tool for applicants during the design or selection (or design) of human intervention studies aimed to substantiate health claims related to oral health. Moreover, information could serve as basis for EFSA to develop further guidance to applicants in the preparation of new applications for authorization of health claims in the context of oral health. Nevertheless, it is worth repeating that many other issues, such adequate sample size, study design and adequate statistical analysis, are decisive for receiving a positive opinion from EFSA.

CONFLICT OF INTERESTS

None.

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Tab.1 Strategies used for retrieving the literature pertinent with outcome variables and methods of measurement related to oral health.

DB Number	Syntax	Total articles	Narrative reviews	Systematic reviews/ metanalyses	Validation studies	Outcome variables/Risk factors
1	"periodontal index"[mesh] AND "english"[language] AND "humans"[mesh]	6370	201	75	43	gingival index
2	"dental plaque"[mesh] AND "english"[language] AND "humans"[mesh]	10975	1091	165	35	dental plaque plaque pH
3	"dental calculus"[mesh] AND "english"[language] AND "humans"[mesh]	1768	121	13	6	dental calculus
4	("tooth demineralization"[mesh] OR "tooth erosion"[mesh] OR "dental enamel"[mesh] OR "enamel loss"[title/abstract]) AND "english"[language] AND "humans"[mesh]	35658	3219	645	212	level of erosion /enamel loss
5	"dental caries"[mesh] OR ("tooth"[mesh] AND "decay"[title/abstract]) AND "english"[language] AND "humans"[mesh]	25444	2532	578	168	dental caries
6	"hydrogen-ion concentration"[mesh] AND ("tooth"[mesh] OR "dentition"[mesh] OR "mouth"[mesh] OR "oral"[title/abstract]) AND "english"[language] AND "humans"[mesh]	3921	243	14	19	pH at tooth surface

7	(("saliva"[mesh] AND "flow"[title/abstract]) OR "salivation"[mesh]) AND "english"[language] AND "humans"[mesh]	3571	264	27	26	saliva flow
8	"xerostomia"[mesh] AND "english"[language] AND "humans"[mesh]	19496	1619	143	46	self-perceived oral dryness
9	"streptococcus mutans"[mesh] AND "english"[language] AND "humans"[mesh]	4053	226	18	7	S.mutans

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