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RAMIRO J CASTRO BARAHONA

EFEITO DE UM PRODUTO LÁCTEO NA DESMINERALIZAÇÃO DA DENTINA RADICULAR E POTENCIAIS SOLUÇÕES PREVENTIVAS ATRAVÉS DA SUPLEMENTAÇÃO COM FLUORETO

Porto Alegre (RS), agosto de 2020.

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EFFECT OF A DAIRY PRODUCT ON ROOT DENTIN DEMINERALIZATION AND POTENTIAL PREVENTIVE SOLUTIONS THROUGH FLUORIDE SUPPLEMENTATION

CONTENTS

Abstract

The oral health condition of the older population worldwide is poor and, in most cases, severely affected by dental caries. Caries alters oral function, interfering with the masticatory process, which may compromise normal food intake. Additionally, malnutrition is common among older adults. As a way to counteract this problem, a range of dietary supplements including milk-based drinks fortified with different nutrients are commercially available. In Chile, as well as in some countries of the world, these dietary supplements are part of public government programs to improve the nutritional condition of the elderly. Dental caries has been currently defined as a sugar-dependent disease and since the majority of dietary supplements possess a high-sugar content, it is expected these products exert a negative impact on consumers' oral health. In light of the above mentioned, the aim of this thesis was to determine the cariogenicity of a Chilean milk-based drink intended for older adults on root dentin caries development and the anti-caries effect of fluoride-supplemented milk-based drink. This thesis is comprised of 4 studies: Study 1. An epidemiological study to determine the acceptability, consumption habits and caries experience of older adults who have consumed the dairy product for a prolonged period of time; Study 2. *In-vitro* determination of the cariogenic potential of the milk-based drink, using a relevant biological experimental root dentin caries biofilm model; Study 3. *In-vitro* determination of the cariogenic potential of fluoride-supplemented dairy product and Study 4. *in-situ* determination of the anti-caries effect of fluoride-supplemented milk-based drink under a high cariogenic condition. We found a statistically higher DMFT index (85.35±39.0 and 77.28±28.9; p=0.043), greater number of root caries lesions as well as higher Root Caries Index (p=0.02) in milk-based drink consumers compared to non-consumers, respectively. The milk-based drink showed higher acidogenicity and cariogenic potential against root dentin in comparison to whole of low-fat containing milk, being the cariogenicity increased by adding sucrose to the formulation. However, the supplementation of milk-based drink with 5, 10 or 20 ppmF led to a dose-response decrease on its acidogenicity, being the lowest cariogenicity found under ≥5 ppmF. *In situ* dentin demineralization under high cariogenic condition was reduced in the presence of 5 ppmF or 10 ppmF-supplemented milk-based drinks in a dose-dependent manner. The general conclusions of this research are: a**.** Daily consumption of the milk product distributed by the supplementary feeding program of the Chilean government for the elderly is related to an increased risk of developing root caries lesions; **b.** By using a root caries experimental model, milk-based drink shows a cariogenic potential for root caries development; **c.** Small amounts of fluoride (5 and 10 ppm) added to milk-based drink were able to lower its cariogenicity both *in vitro* and *in situ*.

Keywords: **root caries, fluoride, milk, fluoridated milk**

Resumo

A condição de saúde oral da população idosa em todo o mundo é precária e, na maioria dos casos, gravemente afectado pela cárie dentária. A cárie altera a função oral, interferindo com o processo mastigatório, o que pode comprometer a ingestão normal de alimentos. Além disso, a malnutrição é comum entre os idosos. Como forma de neutralizar este problema, uma gama de suplementos alimentares está comercialmente disponível, incluindo bebidas à base de leite fortificadas com diferentes nutrientes. No Chile, bem como em alguns países, estes suplementos alimentares fazem parte de programas públicos governamentais destinados a melhorar a condição nutricional dos idosos. A cárie dentária foi atualmente definida como uma doença dependente do açúcar e, dado que a maioria dos suplementos alimentares possui um elevado teor de açúcar, espera-se que estes produtos tenham um impacto negativo na saúde oral dos consumidores. Baseado nestas observações, o objetivo desta tese é determinar a cariogenicidade de uma bebida chilena à base de leite destinada a idosos no desenvolvimento de cárie radicular e o efeito anti-carie desta bebida à base de leite através adição de flúor. Esta tese é composta por 4 estudos: Estudo 1. Um estudo epidemiológico para determinar a aceitabilidade, hábitos de consumo e experiência de cárie de idosos que consumiram o produto lácteo durante um longo período de tempo; Estudo 2. Determinação *in vitro* do potencial cariogênico da bebida à base de leite, utilizando um modelo experimental *in vitro* cárie dentina radicular; Estudo 3. Determinação *in vitro* do potencial cariogênico do produto lácteo com adição de flúor e Estudo 4. Determinação *in situ* do efeito anti-cariogênico da bebida à base de leite com adição de flúor em condições de elevada cariogenicidade. Foi observado um índice CPOD estatisticamente mais elevado (85.3$\pm$39.0 e 77.28$\pm$28.9; p=0,043), um maior número de lesões das cárie radicular, bem como um índice de cárie radicular mais elevado (p=0,02) nos consumidores de bebidas à base de leite em comparação com os não consumidores, respectivamente. A bebida à base de leite apresentou maior acidogenicidade e potencial cariogênico na dentina radicular em comparação com leite desnatado com baixo teor de gordura, e a adição de sacarose à formulação aumentou a cariogenicidade do produto. No entanto, a suplementação do produto a base de leite com 5, 10 ou 20 ppmF resultou em uma diminuição diretamente proporcional da acidogenicidade sendo a cariogenicidade mais baixa encontrada abaixo de 10 ppmF. A desmineralização *in situ* da dentina em condições cariogênicas elevadas foi reduzida na presença de bebidas à base de leite com suplementação de 5 ppmF ou 10 ppmF, de forma dose-dependente. As conclusões gerais desta investigação são: a. O consumo diário do produto lácteo distribuído pelo programa de alimentação complementar do governo chileno para os idosos está relacionado com um risco acrescido de desenvolvimento de lesões de cárie radicular; b. Ao utilizar um modelo experimental de cárie radicular, a bebida à base de leite apresenta potencial cariogênico para o desenvolvimento de cárie radicular; c. Pequenas quantidades de flúor (5 e 10 ppm) adicionadas à bebida à base de leite foram capazes de diminuir a sua cariogenicidade tanto *in vitro* como *in situ*.

Palavras-chave: **cárie radicular, flúor, leite, leite fluoretado.**

Introduction

The world population is rapidly aging. In addition to the growing number of older adults worldwide, malnutrition in this age group represents an important and complex challenge (Pepersack 2009). According to the World Health Organization (WHO), the term malnutrition covers two groups of conditions that are both related to the lack of and the excess of nutrient consumption. While the excess of nutrient intake is associated to "overweight" and "obesity", the lack of nutrient consumption is associated to "undernourished" or "malnourished". According to reports from different parts of the world, malnutrition among older adults could affect up to 50% or more of the subjects belonging to this population group. The evidence indicates that an inadequate nutritional status is related to the presence of critical systemic or degenerative diseases since the nutritional status of a subject is an important modulator of the physical and physiological changes that happen over time. Specially during aging, poor nutritional status is directly associated with frailty that increases the risk of suffering geriatric syndromes, such as falls, fractures, depression, muscle fatigue, functional impairment and depressed immunity, among others (Morillas et al. 2006).

In addition to a detrimental nutritional status, the elderly population is also affected by dental caries, which is the main cause of tooth loss (Sugihara et al. 2010). Root surfaces are naturally exposed to oral environment due to aging. The exposed roots are the site where root caries start (Ekstrand et al. 2008). A very thin and sometimes non-existent layer of cement covers the dentine of the root surface. Since root dentin has a higher content of organic matrix in relation to the enamel, it is believed to be more susceptible to rapid demineralization due to the action of the acids originated by the biofilm in response to the metabolism of fermentable carbohydrates. For demineralization to occur in enamel, a pH decrease to 5.5 is necessary, meanwhile only a slight decrease in the normal local pH in dentin (under 6.7) is enough to start caries lesion (Hoppenbrouwers et al. 1987).

Dental caries negatively compromises the quality of life of people in many aspects, including functional masticatory capacity (León et al. 2014). Tooth loss may predispose to loss of functionality, which has been associated with a restriction in the variety and consistency of ingested food. Indeed, tooth loss impairs the consumption of hard foods and intense chewing, precluding the intake of foods rich in fiber, such as raw fruits and vegetables (Castrejón-Pérez and Borges-Yáñez 2014). In general, the diet of the elderly contains low amount of micronutrients (calcium, iodine, vitamin B2, zinc, phosphorus, and vitamin B12) (Rakıcıoğlu et al. 2015) but high amount in carbohydrates (Boirie et al. 2014; van Staveren and de Groot 2011); a situation that is observed in many countries around the world.

In response to this unfavorable nutritional scenario, some food supplements for elderly people are commercially available aiming to provide some additional intake of adequate nutrients. The commercial presentation of most of these nutritional supplements is a dairy drink, easy to prepare or ready to drink, and widely accepted by the consumer. Within those supplements, a dairy product intends to be consumed by elderly is available in Chile presenting the following composition: about 16 - 18% proteins; 10 - 14% fat and 50 - 60% carbohydrates. Interestingly, this dairy product contains significant amounts of sugars (20 to 36%) that may be associated to caries development. Similar products are marketed internationally, in different presentations and are accompanied by powerful advertising campaigns that support and direct their choice as safe and highly necessary supplements for a healthy diet. However, the potential health risks derived from their consumption are often omitted.

Currently and based on the available evidence, we can emphatically point out that the sugars in the diet are the determining factor for the development of dental caries (Sheiham and James 2015). According to international health-related guidelines, an adult must eat less than 10% of his daily caloric intake as free sugars (OMS 2015c). This recommendation might both avoid a potential negative effect on systemic health and also on oral health (Moynihan and Kelly 2014). It is important to mention that the worldwide leading nutritional supplements for older adults available on the market, contribute significantly to the daily caloric load of consumers, based only on their high sugar content (Popkin 2006). According to this, it is reasonable to assume that these food supplements, widely distributed without any control or prescription, could present a risk in terms of the oral health of the subjects who consumes them regularly.

It has been demonstrated that regular consumption of milk in adults and older people is positively associated with health outcomes. For example, the use of whole milk has been associated with diabetes control (Yakoob et al. 2016), normal body weight (Vanderhout et al. 2016), reduced risk of ischemic stroke (Elwood et al. 2005), lower risk of coronary disease (Steinmetz et al. 1994) and protection against some types of cancer (Prentice 2014). Although worldwide consumption of milk and dairy products is below the international recommendation for daily intake, older adults along with children are its main consumers (Singh et al. 2015).

In this context, some epidemiological studies have shown that regular milk consumption is associated with a low caries experience (Merritt et al. 2006; Petridou et al. 1996). According to different authors, some components of this drink such as: calcium, phosphate and fat would have anti-caries properties (Bowen and Pearson 1993; Harper et al. 1987; Slomiany et al. 1986). In addition, some of the proteins contained in milk, such as casein, albumin, lactoferrin and lysozyme, have demonstrated antimicrobial activity (Aimutis 2004; Johansson and Lif Holgerson

2011). In vitro studies have shown that milk has a low cariogenic potential (Bowen and Pearson 1993; Giacaman 2014; Johansson and Lif Holgerson 2011). Different authors, indicate that some components of milk, could impair the adhesion of cariogenic species to tooth surface, encourage adhesion of commensal microorganisms, decrease production of extracellular polysaccharides promote remineralization and reduce bacterial acid production (Johansson and Lif Holgerson 2011; Vacca-Smith et al. 1994). Despite these findings, further evidence, obtained from randomized clinical trials or systematic reviews, is needed to establish whether regular consumption of milk effectively provides a protective effect against dental caries.

At community level, for more than 50 years, the use of fluoride has been an effective tool for the control of dental caries. In view of the demineralization of the hard tissues of the tooth due to the production of acids by the biofilm, the presence of this ion in the microenvironment promotes the precipitation of minerals on the dental structure decreasing the demineralization and enhancing the remineralization of the dental substrate (Tenuta and Cury 2010). At the community-level, the addition of low concentrations of fluoride to drinking water was the first attempt to control dental caries development (Buzalaf et al. 2011). Initial epidemiologic studies showed that the use of fluoridated water reduced the number of caries lesions in children by as much as 60% and in permanent adult teeth nearly 35% (Peckham and Awofeso 2014). Today, studies prove water fluoridation continues to be effective in reducing tooth decay by 20–40% (Evans et al. 1995). Based on this benefit, in regions where it is difficult to implement the incorporation of fluoride into water, the consumption of fluoride-supplemented foods seems appealing. Thus, fluoridated salt, milk, sugar, bread and even cereals were proposed and used as sources of this element. Currently, evidence of the effectiveness of food fluoridation in the prevention of caries is scarce and inconclusive (Cagetti et al. 2012). Moreover, most studies have been conducted in children or adolescents.

Fluoride supplementation of milk has been a focus of interest for many years, since milk is an important part of the regular diet in children (Yeung et al. 2005). Since it was proposed (Ziegler 1953) and because of its ability to decrease the occurrence of caries lesions, milk fluoridation has been suggested for community-based caries prevention programs. Currently, clinical studies conducted in children and adolescents have shown that the regular consumption of milk supplemented with fluoride is an effective preventive measure for the onset of dental caries (Bánóczy et al. 2013). According to these reports, daily consumption of a portion of milk supplemented with fluoride concentrations ranging from 0.5 to 7 ppm determines a decrease in dmft/DMFT rates of up to 69 to 89% respectively (Bian et al. 2003; Pakhomov et al. 1995). When the implementation of these programs ends, and therefore the consumption of fluoridated milk ceases, the positive effect in terms of reducing the incidence of tooth decay disappears (Mariño et al. 2004). Along with this positive effect in the reduction of the caries experience, there is evidence supporting the fluoridation of milk as a safe and low-cost public health measure (Bánóczy et al. 2013). In fact, milk fluoridation schemes already exist in several countries like Bulgaria, Chile, China, Peru, the Russian Federation, Thailand and the United Kingdom. In contrast to this, there is no evidence about its effect in older adults.

Considering the broad consumption of milk and dairy products among older adults, fluoride supplementation of this type of food could bring an important benefit in terms of improving oral health status. Strategically, modifying a dairy product for older adults might be cost-effective with minimal risks to the subject's systemic health. Clinical and laboratory experiments, which analyze the cariogenicity or remineralizing capacity of fluoride in this food vehicle, show its high effectiveness in doses as low as 2.5 ppm (Ivancakova et al. 2003; Malinowski et al. 2012).

This thesis aims to study the potential cariogenicity of the dairy product available in Chile, as part of the Supplementary Feeding Program for the Elderly due to its high inherent sugar content. Additionally, it aims also to evaluate the anti-caries effect achieved by its supplementation with low doses of fluoride.

Research Questions

Is the Chilean dairy product, part of the Supplementary Feeding Program for the Elderly, cariogenic for root surface?

Can the supplementation of this dairy product with sodium fluoride control the demineralization of root dentin?

Objectives

Objective 1 - To determine the cariogenicity of the milk-based drink delivered by the Complementary Food Program for the Elderly (PACAM) on root dentin.

Study 1. Caries experience in elderly people consuming a milk-based drink nutritional supplement - a cross sectional study

Study 2. Cariogenicity of a milk-based drink used as dietary supplement for Older Adults, on a root caries experimental model.

Objective 2 - To assess the caries-inhibitory effect of fluoridated milk-based drink on root dentin.

Study 3. Anti-caries effect of fluoridated milk-based drink consumed by older adults on an *in vitro* root caries experimental model

Study 4. *In situ* effect of fluoridated milk-based drink on root caries development under a high cariogenic condition

Perguntas de Pesquisa

O produto lácteo chileno, parte do Programa de suplementação alimentar para idosos, é cariogênico para a superfície radicular?

A suplementação com flúoreto de sódio do produto lácteo pode controlar a desmineralização da dentina radicular?

Objetivos

Objetivo 1 - Determinar a cariogenicidade da bebida à base de leite oferecida pelo Programa de Alimentação Suplementar para Idosos (PACAM) sobre a dentina radicular.

Estudo 1. Experiência de cárie em adultos idosos que consomem uma bebida à base de leite– um estudo transversal

Estudo 2. Cariogenicidade de uma bebida à base de leite usada como suplemento dietético para adultos idosos, num modelo experimental de cárie radicular.

Objetivo 2 - Avaliar o efeito anti-cariogênico da bebida à base de leite suplementada com flúor sobre a dentina radicular.

Estudo 3. Efeito anti-caries da bebida à base de leite fluoretado, num modelo experimental in *vitro* de cárieradicular

Estudo 4. Efeito *in situ* da bebida à base de leite fluoretado no desenvolvimento de cárie radiculars sob uma condição de elevada cariogênicidade

CHAPTER 1. CARIOGENICITY OF A MILK-BASED DIETARY SUPPLEMENT DRINK FOR THE ELDERLY

Study 1. Caries experience in elderly people consuming a milk-based drink nutritional supplement, a Cross Sectional Study.

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Short-title: Caries experience and consumption of milk-based drink

Key-words: fluoride, milk, root dentin caries, epidemiology

Abstract

Objective: Malnutrition is a common condition in elderly people. WHO has recommended the use of nutritional supplements to overcome nutritional deficiencies in this age-group. These products are usually supplemented with different nutrients and a significant amount of sucrose. Since 1999, the Chilean government has implemented the Supplementary Nutrition Program for the Elderly (PACAM, for its Spanish acronym). This program consists in a monthly distribution of a milk-based drink and an instant-soup, for daily supplementation of diet. The milk-based product contains 8% sugar and low-fat milk, making it potentially cariogenic. The aim of this study was to determine whether older adults who consume PACAM products have a higher caries experience when compared to older adults not receiving the food supplement.

Methods: A cross-sectional study was conducted with a representative sample from the Maule Region in Central Chile. The sample comprised two groups: a) PACAM consumers subjects (CS)(n=60) and b) Non-consumers of PACAM products (NCS)(n = 60). Participants received intraoral examination and coronal caries (DMFT/DMFS and SiC Index, both considering caries as cavitated lesion with dentin involvement) and root caries (RCI index; using ICDAS as diagnostic criteria) experience were recorded and scored. Additionally, a validated questionnaire was applied, to determine knowledge, acceptability and consumption habits regarding PACAM products. A 24-hour diet recall and a food frequency questionnaire were applied. Non-parametric Mann Whitney test was used to compare coronal/radicular caries experience. To explore the relationship between the caries experience with demographic and consumption variables, binary logistic regression was used. Poisson regressions were used to analyze the relationship between the presence of root caries lesions and milk-based product consumption controlled by demographic variables. A p-value<0.05 was considered significant.

Results: No differences were detected in food consumption patterns between study groups (p>0.05). CS participants had increased dairy product consumption. Higher DMFS mean value was observed in the CS group (85.35±39.0) compared with NCS (77.28±28.9), (p=0.043). The SiC index value was greater for the CS than NCS (28.89±1.8 vs 27.22±0.9; p=0.003). The multivariate analysis showed that the CS group had a higher number of root caries lesions (β=0.41; p=0.02) and higher RCI (β=7.34; p=0.02) than the non-consumer group.

Conclusion: Daily consumption of a PACAM's milk-based drink supplement seems to increase coronal and root caries risk. Further research appears needed to modify composition and raise awareness of the potential pitfalls of the program.

Introduction

The world is experiencing a rapid growth in the number and proportion of older persons. This demographic change determines an increase in the proportion of adults over 60 and over. It is expected that by 2025, more than 1.2 billion people will belong to this age group (OMS 2015a). This phenomenon poses a challenge, as it means higher demand for dental care and treatment in an already highly constrained system in most of the countries, especially in the developing world. A good nutritional status and a healthy lifestyle are crucial factors for healthy aging. Optimal nutrition has a positive effect on systemic health and well-being of older adults, delaying and reducing the risk of developing diseases, as well as maintaining functional independence (Kritchevsky 2016). However, it is common to find some kind of malnutrition in older adults (Guerrero-García et al. 2016). Dietary patterns in elderly people has been reported to be low in micronutrients (mainly calcium, iron, iodine, zinc, phosphorus and vitamins A, C, E and B), fiber and protein (Rakıcıoğlu et al. 2016) and a high percentage of carbohydrates (Boirie et al. 2014). Given limitations in the masticatory function, older people tend to select soft and highly processed foods of low nutritional value (Castrejón-Pérez and Borges-Yáñez 2014).

According to the World Health Organization (WHO), the term malnutrition covers two groups of conditions: undernutrition or deficit malnutrition, which is related to a lack of nutrients, and overnutrition, which is due to a high and unbalanced food intake (Hickson 2006). According to reports from different regions of the world, malnutrition among older adults reaches about 22% (Kaiser et al. 2010), increasing dramatically in dependent or hospitalized people, where more than 60% can be affected (Corish and Kennedy 2000). To provide adequate amounts of micronutrients, protein and calories, on a daily basis, to people who are at risk or affected by malnutrition, WHO has recommended the use of instant nutritional supplements, which are easy to prepare and consume orally (Qato et al. 2016). These products are usually fortified with proteins and fatty acids and formulated with significant amounts of sucrose. However, it is recommended that all programs involving the use of nutritional supplements, should be conducted and monitored by a trained health professional and developed according to the specific nutritional needs of the subject (Walrand 2018). Thus, different private initiatives as well as public policies have been implemented to address a lacking nutritional condition, based on the use of food supplements for the enrichment of the daily diet of the elderly. In this respect, the Chilean government has implemented since the late 1990s the "Programa de Alimentación Complementaria para el Adulto Mayor (PACAM)" (Supplementary feeding programme for the elderly (PACAM)) based on the monthly distribution of a powdered instant milk-based drink product (fat 4.5%, total carbohydrates 59.5%, sucrose 8% and 28% lactose) and an instant soup (13% protein, 11% fatty acids and 2% total sugars). This program currently benefits more than 430,000 people, representing 15.35% of the country's elderly population.

In general, subjects aged 60 and over are the main consumers of milk in the world, especially women (Singh et al. 2015). Regular milk consumption in older or middle-aged people has shown to have a positive effect on systemic health, as it is positively associated with glycemic control in diabetic patients (Yakoob et al. 2016), body weight (Vanderhout et al. 2016), reducing the risk of ischemic stroke (Elwood et al. 2005) or coronary disease (Steinmetz et al. 1994) and protects against some types of cancer (Prentice 2014). Despite the above-mentioned benefits related to regular milk consumption, an experimental study conducted in vitro by our research group have shown that the PACAM´s milk-based drink, contains potentially cariogenic nutrients such as skim milk and sucrose (Castro et al. 2018; 2019). Importantly, dental caries is the main cause of tooth loss among older adults (Sugihara et al. 2010), condition that additionally negatively affects their quality of life (León et al. 2014). The determining factor for caries is the high sugars intake in the diet (Sheiham and James 2015). In older adults, there is an increase in the incidence and prevalence of a particular type of caries lesion, root caries. These lesions affect the tooth root, typically involving the enamel-dentin junction. Multiple factors have been described in older adults that may favor the development of root caries lesions, such as decreased salivary flow, use of removable prosthetic appliances, periodontal diseases and gingival recessions, which expose root surfaces (Sugihara et al. 2010). It is important to note that because the critical pH for dentin demineralization is higher than that of coronal enamel (Peters 2010), exposed root surfaces are considered to have increased risk for caries lesions.

Given the potential caries risk posed by the consumption of dietary supplements in older persons, we aimed to evaluate whether people regularly consuming the PACAM's milk-based drink are more affected by caries than those who do not consume it.

Subjects and Methods

After obtaining Ethics approval from the University of Talca (#2014-009-RC), a cross-sectional study was conducted with the participation of older adults from the Maule Region, Chile. This region comprises 30 communes and shows poor community-based oral health index (Ministerio de Salud 2008) and also present a high proportion of rurality (INE 2015). According to the national socioeconomic characterization (CASEN 2015), the region of Maule has a large population assigned to the public social security health system (86.2%), contrasting with a low percentage of the population assigned to the private health system (5.9%). Another data that confirms the low socioeconomic level, shows that the households in the region of Maule, obtain the second lowest value of average autonomous income at a national level (CASEN 2015).

Sample size calculation: The sample size was calculated based on the data that older adults with a good nutritional status have less root surfaces areas affected by caries, compared to those with an inadequate nutritional status (Yoshihara et al. 2007). According to the Yoshihara et al. (2007) study, among subjects with good nutritional status and those with poor nutritional status, there was a mean difference of one tooth surface affected by root caries and a common standard deviation of 1.9. An alpha risk of 0.05 and a beta error of 0.2 were accepted in a bilateral contrast. The calculation resulted in a number of 114 subjects needed, divided in two groups of equal number (57 subjects each). To avoid any data loss during processing, a total sample of 120 subjects, divided into two groups of 60 individuals each, was recruited. Sample size calculation was estimated with the software GRANMO v.7.12.

A total of 10 communes of the Maule Region were randomly selected (4 urban and 6 rural, proportional to the demographic distribution of the Maule region). The primary sampling units in each commune corresponded to the Family Health Centers (CESFAM). The CESFAM with the highest population coverage within their territory was selected. In the second stage, individuals (secondary sampling units) over 60 years old who were registered in the CESFAMs were selected through a systematic random selection procedure. The information related to demographic data (age, sex and place of residence) were obtained from the clinical records, as well as the subject's membership into PACAM. The groups were categorized in: a) subjects who consumed the milk-based drink product for at least two years (CS) (n=60) and b) subjects non-consumers of the dairy product (NCS) (n=60). In both groups, subjects had to have at least 5 teeth.

Diet assessment: A previously trained interviewer applied a guided questionnaire (Contreras 2011), for subjects in both groups. This questionnaire determined the acceptability and consumption habits related to the products supplied by PACAM (i.e., added sugar and volume consumed). To evaluate each subject's diet patterns, a "24-hour diet recall" and a "food frequency questionnaire" were applied (Ministerio-de-Salud-Chile. 2014). For both surveys, simple portion/size explanations were used to guide the respondents' answers. Participants were interviewed personally, and their responses were recorded. The portion/size were transformed in grams per day (gr/day) using a table of home measurements and their equivalent value (Ministerio-de-Salud-Chile. 2014). Subsequently, the gr/day of each food group was analyzed and calculated (i.e., Dairy, Meat, Oils, Candies, Sugared dough, Sugared beverages and Sweeteners).

Carious lesions assessment: An experienced examiner previously trained and calibrated in the diagnosis of caries lesions (kappa index inter examiner: 0.69, intra examiner: 0.78), performed clinical examination. Each examination was carried out in a dental box-office, with the aid of artificial light and examination kits. After cleaning and drying the dental surfaces, for root lesions, ICDAS criteria was used (Ismail et al. 2007). Teeth with root surfaces with caries lesions, restored and exposed/healthy, were recorded (Shivakumar et al. 2009). The criterion for considering a coronal caries lesion for the "carious" component of DMFT/S was a cavitated lesion with dentin involvement (World-Health-Organization 2013).

Statistical analysis:

The prevalence and caries experience were analyzed. For coronal caries lesions, DMFT/S was calculated (sum of "decayed", "fillings" and "missing" in relation to **T**ooth/**S**urface). Additionally, SiC (Significant caries index) (Bratthall 2000) and root caries index (RCI%) (Katz 1980), were calculated. Sociodemographic characteristics were compared by study groups using Chi-squared tests and Mann-Whitney.

Data collected were analyzed according to measures of dispersion and central tendency. Normality tests were performed using the Kolmogorov-Smirnov Test. Because data were not normally distributed, the non-parametric U-Mann Whitney test was used to compare the diet consumption patterns and coronal/root caries experience among the study variables (i.e., demographics and PACAM). The possible relationship between the coronal caries experience and variables such as sex, area of residence, consumption of the milk-based drink product, volume of dairy product consumed and addition of sugar to the milk-based drink supplied by PACAM was determined through a binary logistic regression. For binary logistic regression, coronal caries experience (DMFS) of the CS was dichotomized in relation to a SiC value of 63. This value (cut-off point) corresponded to the minimum DMFS value of one third of the study group (n: 120) with the highest caries score. Poisson regressions were used to analyze the relationship between the presence of root caries lesions (number of root caries lesions and RCI%) and consumption of milk-based drink controlled by demographic variables. Significant differences were considered if $p<0.05$. Data processing and manipulation was performed using the statistical program SPSS version 25.0 (IBM, NY, USA).

Results

Information is shown for both study groups by sex, age and geographical distribution in Table 1. There were no statistically significant differences in the study groups regarding age and residency ($p>0.05$). The CS participants were older than NCS, with a mean age of 73.45(1.8) and 70.21(3.6), respectively ($p< 0.001$)

Table 1. Distribution and characterization of both groups. Consumers of the milk-based drink product (CS) and non-consumers of the milk-based drink product (NCS) according to sex and geographical distribution (n(%)) and age (n(SD)),

	CS (n=60)	NCS (n=60)	Test
Sex			
Men n(%)	25 (41.4)	26 (42.8)	
Female n(%)	35 (58.6)	34 (57.1)	X^2= 0.03, p = 0.85.
Age mean (SD)	73.45 (1.8)	70.21 (3.6)	MW = 8.73, p <0.001.
Geographical distribution			
Urban n(%)	44 (72.8)	40 (65.7)	X^2= 0.63, p = 0.43.
Rural n(%)	16 (27.2)	20 (34.3)	

The daily portions/size of different food groups consumed by the subjects demonstrated no differences between the study groups, with the exception of dairy products. The CS group showed a higher daily intake of milk ($p<0.05$).

Table 2. Dietary assessment between consumers (CS) and non-consumers (NCS) of the milk-based drink product. Average quantity (SD) in g/day or cc/day of some foods consumed daily.

	CS mean (SD)	NCS mean (SD)	P-VALUE
Dairy (cc/day)	392.18 (208.35)	204.16 (212.04)	**0.0001***
Meat (gr/day)	103.16 (43.09)	91.38 (42.38)	0.241
Oils (gr/day)	36.35 (31.47)	50.86 (65.5)	0.97
Candies (gr/day)	18.34 (21.9)	24.4 (25.68)	0.312
Sugared masses (gr/day)	7.81 (16.43)	4.21 (8.47)	0.675
Sugary drinks (cc/day)	220.784 (249.49)	434.248 (325.96)	0.092
Sweeteners (gr/day)	0.083 (0.096)	0.068 (0.092)	0.529

Bold values indicate statistical difference between CS and NCS

Most CS (68%) drank more than one cup (200 cc) of the milk-based drink product per day and also sweetened the product with sugar or some non-caloric sweetener (70%). Most subjects (64%) reported very low soup intake (1 dish or less per day) or no soup consumption at all (Table 3).

Table 3. Consumption habits (quantity/day) of products supplied by the PACAM in the CS group (n = 60)

Milk-based drink product consumption n(%)	
≤1 cup	19 (32%)
>1 and ≤2 cups	33 (55%)
> 2 cups	8 (13%)

Addition of sweetener to the dairy product n(%)	
Sugar	14 (23%)
Sweetener	28 (47%)
No addition	18 (30%)

Consumption of instant Soup n(%)	
> 1 plate	22 (36%)
≤ 1 plate	37 (61%)
No consumption	1 (3%)

Coronal Caries Experience

Table 4 shows a greater number of decayed teeth in men than in women (6.36 v/s 5.09; p=0.018). Also, greater number of filled teeth (2.19 v/s 0.98; p=0.006) and filled surfaces (3.31 v/s 1.49; p=0.006) were found in subjects from urban areas compared to rural areas. However, no difference in DMFT/S was observed among those areas. A higher DMFS value was reported in CS compared to NCS (85.35 v/s 77.28; p=0.043).

Table 4. Mean and standard deviation (SD) of coronal caries experience, according to predictor variables (sex, place of residence and affiliation to the supplementary feeding program PACAM).

		Coronal Caries - Tooth				Coronal Caries - Surface			
		D	F	M	DMFT	D	F	M	DMFS
Variables		**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
		5.57 (3.2)	1.76 (2.8)	14.36 (7.8)	21.6 (6.9)	8.78 (6.1)	2.66 (4.2)	69.88 (36.1)	81.32 (34.4)
Sex	Women (n = 75)	5.09 (3.2)	2.31 (3.3)	15.20 (7.3)	22.60 (5.9)	8.45 (6.5)	3.43 (5.0)	72.99 (32.0)	84.87 (29.2)
	Men (n = 45)	6.36 (3.0)	0.84 (1.3)	12.96 (8.5)	22.16 (8.2)	9.33 (5.3)	1.38 (2.1)	64.69 (42.0)	75.40 (41.4)
		p= 0.018	p= 0.059	p= 0.138	p= 0.209	p= 0.123	p= 0.064	p= 0.342	p= 0.453
Place of residence	Rural (n = 43)	5.56 (3.2)	0.98 (1.8)	14.63 (8.3)	21.16 (7.6)	8.12 (4.6)	1.49 (2.8)	70.60 (38.1)	80.2 (36.8)
	Urban (n = 77)	5.57 (3.2)	2.19 (3.1)	14.21 (7.6)	21.97 (6.5)	9.16 (6.7)	3.31 (4.7)	69.47 (35.2)	81.94 (33.2)
		p= 0.685	**p= 0.006**	p= 0.673	p= 0.792	p= 0.568	**p= 0.006**	p= 0.816	p=0.887
Program	NCS (n = 60)	5.87 (3.0)	2.03 (2.8)	13.73 (6.6)	21.63 (5.4)	8.55 (4.3)	3.12 (4.7)	65.62 (31.4)	77.28 (28.9)
	CS (n = 60)	5.27 (3.3)	1.48 (2.7)	14.98 (8.9)	21.73 (8.2)	9.02 (7.4)	2.20 (3.7)	74.13 (40.2)	85.35 (39.0)
		p= 0.369	p= 0.098	p= 0.259	p= 0.207	p= 0.748	p= 0.158	p= 0.097	**p= 0.043**

* Bold values indicate differences in average per group according to Mann Whitney test, p < 0.05.

Binary regression shows that subjects living in urban areas and those who consumed more than one cup of milk-based drink product per day had a greater probability of being more severely affected by coronal caries (OR 4.84 and 4.24, respectively) (Table 5).

Table 5. Association between predictor variables (sex, place of residence, consumption of milk-based drink product, volume of milk-based product consumed, addition of sugar to the milk-based product) and experience of coronal caries (DMFS), dichotomized according to the SiC index, of the consumer subjects (CS) (n=60).

	Low DMFS* N (%) 15 (25%)	High DMFS* N (%) 45 (75%)	Unadjusted OR	95% CI	Unadjusted P-value	Adjusted OR	95% CI	Adjusted P-value
Sex								
Women	7 (46.7%)	30 (66.7)						
Men	8 (53.3%)	15 (33.3)	0.44	0.13 - 1.44	1.17	0.52	0.14 - 1.92	0.33
Place of residence								
Rural	8 (53.3%)	11 (24.4%)						
Urban	7 (46.7%)	34 (75.6%)	3.53	1.04 - 11.98	**0.04**	4.84	1.18 - 19.78	**0.03**
Milk-based drink consumption								
Under than 200 mL/day	7 (46.7%)	10 (22.2%)						
Over 200 mL/day	8 (53.3%)	35 (77.8%)	3.06	0.89 - 10.52	0.07	4.24	1.03 - 17.42	**0.04**
Addition of sugar to dairy product								
do not add	12 (80%)	34 (75.6%)						
Adding	3 (20%)	11 (24.4%)	1.29	0.31 - 5.44	0.72	1.71	0.37 - 7.93	0.49

Significant regression model. $\chi2(4)= 10.20$; $p=0.04$, Nagelkerke 23%. Bold values indicate statistical differences.

* Low DMFS = low SiC value (<63) and high DMFS = value greater or equal to SiC (≥63)

Root Caries Lesions

When comparing data between both groups regarding root caries experience (number of root caries lesions and root caries index), no differences were observed (Table 6).

Table 6. Distribution and comparison of root caries lesions (RCLs, mean; SD) and root caries index [RCI%, mean; SD] by sex, place of residence and milk-based drink product consumption.

		Root Caries	
Variables		**Number ofRCLs Mean (SD)**	**RCI Mean (SD)**
		1.19 (1.1)	18.55 (17.8)
Sex	Women (n = 75)	1.14 (1.0)	17.59 (15.3)
	Men (n = 45)	1.27 (1.3)	20.15 (21.5)
		p= 0.950	p= 0.867
Place of residence	Rural (n = 43)	0.90 (0.8)	14.4 (14.7)
	Urban (n = 77)	1.35 (1.2)	20.86 (19.0)
		p= 0.070	p= 0.064
Program	NCS (n = 60)	0.93 (0.7)	14.6 (12.2)
	CS (n = 60)	1.4 (1.3)	22.5 (21.4)
		p= 0.077	p= 0.063

* differences in mean per group according to Mann Whitney test, $p < 0.05$.

According to a multivariate analysis, non-consumers of the milk-based product (β= -0.41, p= 0.02), are less likely to have root surfaces affected by caries (Table 7.A). Additionally, CS show higher RCI, compared to non-consumers (β=-0.17, p=0.02) (Table 7.B).

Table 7. Poisson regression for number of root caries lesions (A) and RCI (B) (n=120) adjusted by predictor variables (sex, place of residence and consumption of milk-based drink product).

A*. Variables	Beta	Standard error	p-value	B**. Variables	Beta	Standard error	p-value
Constant	0.55	0.16	<0.001	Constant	3.05	0.07	<0.001
Sex (woman)	-0.13	1.17	0.45	Sex (woman)	-0.26	0.07	<0.001
Residence (Rural)	-0.38	0.19	0.05	Residence (Rural)	-0.44	0.07	<0.001
Program (NCS)	-0.41	0.17	0.02	Program (NCS)	-0.17	0.07	0.02

*Significant model *xi* (3) = 11,21; p = 0,01.
NCS, subjects non consumer.

**Significant model *xi* (3) = 56,27; p < 0,001.
NCS, subjects non consumer.

Discussion

The results of this investigation show that a milk-based drink product, with added sugars (8%) is cariogenic and could be a risk factor for both coronal and root caries in people who consume it. These epidemiological findings are consistent with our *in vitro* experimental results (Castro et al. 2019).

Milk is a widely consumed food throughout the world and its intake increases with age. The average amount of milk ingested daily by an adult is 0.57 portions per day (114 cc), while for women over 60, this amount can reach 0.68 portions per day (136 cc) (Singh et al. 2015). In general, a higher milk consumption is observed in developed countries compared to poor or tropical countries (Singh et al. 2015). Data from Latin America indicate that the region shows a moderate consumption of this food with an average amount of 30-150 kg per capita/year (FAO 2013). According to our data, regular milk consumption of the subjects consuming the dairy product would reach 18.25 kg/year (on average 50g of product/02 cups per day), which is less than the average in the Latin American region. As the beneficiaries of PACAM are from low socio-economic status and the program increases the consumption of the milk product , it is clear that this program is necessary and makes a real contribution to supplementing the daily diet of consumers, so the discussion on its potential caries risk must be addressed and it is of relevance.

Although the higher rurality of the Maule region, and this condition was reflected in the studied sample, no significant differences between program users (CS) and non-users (NCS) and the residence location was detected. It should be noted that in rural areas, access to health services is limited compared to urban areas. Distance to provider centers might explain the differences in access. This difference in access to health services may explain why, although subjects in the rural and urban areas have the same caries experience, subjects in the rural area have less filled teeth. Yet, rural communities did have access to the food supplement and no differences were found in consumption between residence groups. In general, PACAM has good acceptance among older adults. In fact, among NCS, the main reason for this condition was that they did not meet the requirements to be part of the program. To be eligible, older people have to be in treatment or active control in some public health center. Despite the scarce evidence available on the benefits of the program, it has been demonstrated that the regular consumption of food supplements provided by the program, cause a significant increase in the daily intake of energy and some micronutrients such as vitamin B12, C, E and zinc (Masi and Atalah 2008)

In Chile, older people have a high caries prevalence (Mariño et al. 2015), and this situation could be aggravated by the consumption of the milk product supplied by PACAM. When comparing caries experience among the study groups, program users had more coronal caries lesions, with higher DMFS and SiC indexes compared to non-users. Additionally, program users showed a greater experience of root caries, with higher number of root caries lesions and a higher RCI. It is important to note that no difference in eating habits (diet surveys) was observed between program users and non-users, except for the consumption of the dairy product that was higher in the program users. Importantly, most users reported to consume more than one cup of dairy product per day, which means additional sugars intake that may explain the differences found.

It has been stated that there would be a possible protective role against dental caries associated with regular milk consumption, but the evidence is not clear. As reported by some researchers, milk proteins such as casein and lactoferrin (Johansson and Lif Holgerson 2011), whey proteins, fat content (Shetty et al. 2011), and the high concentration of calcium and phosphorus (Grenby et al. 2001), would be important factors that would alter the adhesion of cariogenic bacteria, interfering with dental biofilm development, or promoting remineralization (Merritt et al. 2006). Our studies have shown, meanwhile, that milk does not decrease the demineralization of enamel and dentin subjected to a cariogenic challenge through frequent exposure to sucrose (Giacaman et al. 2012). On the other hand, lipids contained in whole milk (3.1% fat content) could modulate the effect of lactose, as well as the presence of sucrose, presenting lower cariogenicity (Giacaman 2014). This could be explained by a potential protective mechanism of fatty acids that has been described both *in vitro* (Giacaman et al. 2015), as *in vivo* (Giacaman et al. 2016). These molecules are not metabolized by bacteria (Schuster et al. 1980), they may present antimicrobial activity being able to inhibit glucose transport through bacterial cell membrane and inhibit cell adhesion (Williams et al. 1982). As the fat content of the milk decreases (semi-skimmed or skimmed milk) or when sugar is added, milk becomes highly cariogenic in both enamel and root dentin (Giacaman 2014), comparable to 10% sucrose solutions.

In general, non-specialized dietary supplements for older adults, like Enprocal®,Ensure®, Fortisip® and Años Dorados® in Chile (the brand used for the PACAM product), whose most common commercial presentation is a milk-based drink, are usually fortified with protein and fatty acids, similar to those found in milk, but have a significant amount of sucrose in their formulation (between 8 and 50%), which exceeds any caries-decreasing effect, as described before. Considering that the PACAM dairy dietary supplement contains in its composition a low percentage of fat, plus 8% of sucrose and is consumed more than once a day (on average 392.18 cc/day), it would be expected that this product represents a risk in terms of consumers' oral health. Our results seem to confirm this hypothesis.

Although there are many modulating factors that can affect the natural course of caries disease and determine whether new lesions can appear, the situation of older adults should be considered as a particular case. Indeed, to the higher sugars consumption in the diet, the typical salivary flow reduction due to glandular atrophy or polypharmacy, root surfaces exposure and the wearing of prosthetic devices, may represent added caries risk (Sugihara et al. 2010). Although it has been demonstrated the contribution of nutritional supplementation to the general health, it is important to consider that the incorporation to the daily diet of dairy products with sugars added, may represent a risk for oral health.

Within the limitations of this study, the lack of information regarding the periodontal condition of the subjects (periodontitis or gingivitis) is indicated, as well as a determination of the plaque index, an important factor since it is directly related to the amount of biofilm present on the teeth.

We can conclude that when considered as a whole, the results of this study suggest that daily consumption of the PACAM nutritional supplement for older adults, which contains sucrose in its composition, increases the risk of presenting caries lesions. The results suggest the need to review the formulation, in terms of components, of the products supplied by supplementary feeding programs. Milk reformulation, eliminating the elements that may represent a risk to oral health may be an alternative to preserve its nutritional benefits, avoiding potential oral health damage. In addition, it would be beneficial to incorporate or adjust the content of those nutrients that can play a protective role against cariogenicity, such as proteins (Diamanti et al. 2011), fatty acids (Huang et al. 2011) or the addition of fluorides.

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Study 2. Cariogenicity of a Milk-based Drink used as a Dietary Supplement for Older Adults using a Root Caries Experimental Model.

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Short-title: Cariogenicity of Dietary supplement on a root caries model

Key-words: Milk, sucrose, dentin, demineralization, *Streptococcus mutans*

Declaration of interests

The authors declare no conflict of interest.

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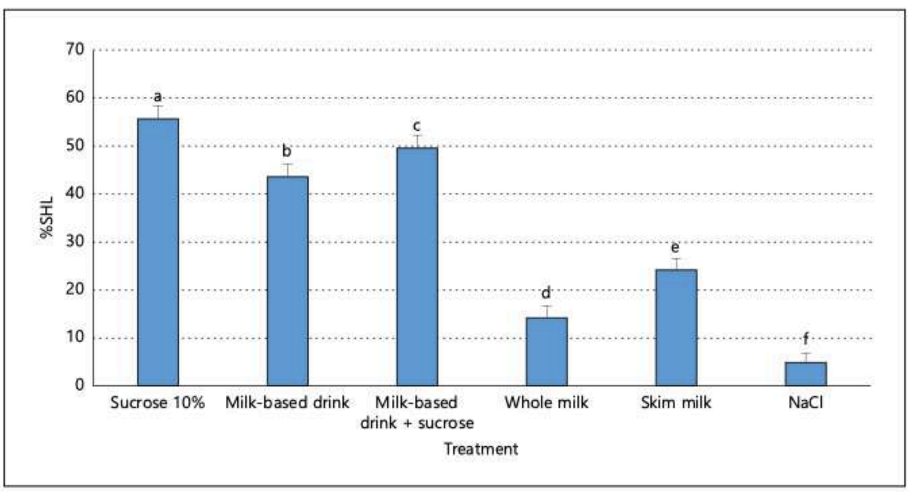

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Cariogenicity of a Milk-Based Drink Used as a Dietary Supplement for Older Adults Using a Root Caries Experimental Model

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Keywords

Milk · Sucrose · Dentin · Demineralization · *Streptococcus mutans*

Abstract

The aim of this study was to evaluate the cariogenicity of a milk-based drink intended for older adults that was used as part of a governmental initiative in Chile to improve their nutritional conditions. This drink contains a high concentration of sugars, which can contribute to root caries development. To test this hypothesis, an experimental biofilm/caries model was used. Dentin slabs were used to grow biofilms of *Streptococcus mutans* UA159. Slabs/biofilms were exposed 3× per day to bovine milk with different fat content, the milk-based drink, and the milk-based drink supplemented with 10 g of sucrose added per serving. Slabs exposed to 10% sucrose or 0.9% NaCl were used as positive and negative controls, respectively. Biofilms were analyzed for bacterial counts and acidogenicity. Dentin demineralization was estimated by the loss of surface microhardness and integrated mineral loss. Results were compared by analysis of variance and Tukey's test. The milk-based drink showed higher acidogenicity than milk with its entire (whole) or reduced total fat content (skim). The milk-based drink supplemented with sucrose had similar acidogenicity as the 10% sucrose positive control ($p = 0.506$). Whole milk exposure elicited lower bacterial counts than the positive control, the milk-based drink, and the milk-based drink supplemented with sucrose ($p = 0.002$; 0.006 and 0.014 respectively). Although skim milk induced higher demineralization than whole milk, both milk types produced lower demineralization than the milk-based drink. Regarding integrated mineral loss, demineralization induced by the milk-based drink and the milk-based drink supplemented with sucrose was similar to that induced by the positive control and skim milk ($p > 0.05$). Sugar-containing milk-based drinks used as dietary supplements for older adults may be highly cariogenic and could represent a potential risk for root caries.

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Introduction

The world is rapidly aging [Beard et al., 2016]. The oral health condition of the older population is typically poor and, in most cases, severely affected by dental caries [Liu et al., 2013; Mariño and Giacaman, 2014]. Caries alters oral function, interfering with the masticatory process, which may compromise the nutritional condition [Castrejón-Pérez and Borges-Yáñez, 2014]. Caries-induced tooth damage is the main causative factor for tooth loss in the elderly [Sugihara et al., 2010]. Besides the oral consequences of caries, the disease also negatively influences the quality of life [León et al., 2014].

On the other hand, malnutrition is often found in the elderly [Guerrero-García et al., 2016; Volkert, 2013]. Dietary patterns of older adults usually contain low amounts of macro and micronutrients (calcium, iodine, vitamin B2, zinc, phosphorus, and vitamin B12) [Rakıcıoğlu et al., 2015] and it is high in carbohydrates [Boirie et al., 2014]. Dietary imbalances in older adults may determine several systemic conditions, including, frailty [Boulos et al., 2016], sarcopenia [Relph, 2016], dependency [Yildiz et al., 2015], dementia [Naseer et al., 2016], sleep disorders [Yildiz et al., 2015], increased risk of falls [Masumoto et al., 2015], mortality [Tsai et al., 2016] and higher rates of hospitalization [Geurden et al., 2015]. As a way to counteract this problem, a range of dietary supplements including milk-based drinks fortified with different nutrients are commercially available. In Chile, a governmental program to improve the nutritional condition of elderly Chileans has been in place for several years [Dangour et al., 2005]. This program consists of a nutritionally enriched milk-based drink that is currently delivered monthly to more than 400,000 people, free of cost. The dairy product is composed by 10% lipids, 18% proteins, 28% lactose, 8% sucrose, vitamins and minerals. According to the users, the dairy product has high acceptability and is usually consumed twice per day [Masi and Atalah, 2008].

Regular milk consumption is associated with low caries experience [Petridou et al., 1996], probably due to the claimed anti-cariogenic properties of their components [Bowen and Pearson, 1993; Harper et al., 1987; Slomiany et al., 1986] and the antimicrobial activity of proteins, such as casein, albumin, lactoferrin and lysozyme [Johansson and Lif Holgerson, 2011]. Nonetheless, whole milk supplemented with sugars, like in the milk-based drink for elders in Chile, has the potential of inducing root caries at a level similar than a pure sucrose solution [Giacaman, 2014]. On the other hand, some unsaturated fatty acids have been reported to be anticariogenic [Giacaman et al., 2014]. Milk's fat seems to be important in reducing the cariogenic potential elicited by lactose. Indeed, milk reduced in fat content (skim milk with 0.05% fat) induces higher cariogenicity in vitro than milk containing all the fat (whole milk with 3.1% fat) [Giacaman, 2014]. Despite its sugars content, the milk-based drink for older adults distributed in Chile might present low cariogenic potential due to its high amount of fat (10%). Dental caries has been currently defined as a sugars-caused disease [Sheiham and James, 2015] and since the product contains a high amount of sucrose, it is plausible to speculate that the protective effect from fat may be overcome. In this context, the aim of this investigation was to determine the cariogenicity of the Chilean milk-based drink intended for older adults on root dentin caries development due to its high concentration of sugars.

Materials and Methods

Experimental Design

A previously validated experimental caries model with biofilms of *Streptococcus mutans* was used in this study [Ccahuana-Vasquez and Cury, 2010] with modifications [Giacaman et al., 2013]. This model allows the study of the effect of different substances on the biofilm and on dental demineralization. All the experiments were conducted in the Cariology Laboratory of the University of Talca. Biofilms of *Streptococcus mutans* UA159 (*S. mutans*) were grown on the surface of bovine root dentin slabs for 5 days. The initial Knoop surface microhardness (SH_i) of the slabs was assessed. Slabs were randomly assigned to one of the following treatments: Skim milk (Colún, La Unión, Chile; 0.05% fat, 4.5% total carbohydrates, 4.5% lactose), whole milk (Colún, La Unión, Chile; 3.1% fat, 4.6% total carbohydrates, 4.6% lactose), milk-based drink "Bebida Láctea Años Dorados" (Calo, Osorno, Chile; 1.25% fat, 7.5% total carbohydrates, 1% sucrose and 2.5% lactose) and milk-based drink supplemented with sucrose to simulate the actual consumption, according to studies of our group (10 g of sucrose added per serving; 6% of final sucrose concentration) [Castro, Unpublished data]. A group with 10% sucrose and another with 0.9% sodium chloride (NaCl) were used as positive and negative caries controls, respectively. Treatments were applied 3× per day for 5 min each. Biofilms were washed with 0.9% NaCl and relocated to a medium-containing well. Culture medium was changed twice per day, before the first treatment (8 am) and after the last treatment (5 pm). After the experimental phase, biofilms were separated from the slabs and analyzed for viable bacteria counts. Acidogenic potential from the biofilms was determined by pH measurements of the culture medium after 8, 24, 32, 56, and 80 h after the initial biofilm formation and the resulting area above the curve (AAC) formed was quantified [Hassan et al., 2015]. Final SH (SH_f) on the slabs was determined and compared with the SH_i to obtain the percentage of surface microhardness loss (%SHL). Slabs were prepared to measure cross-sectional microhardness and determine the percentage of mineral loss in each lesion [Featherstone et al., 1983]. The entire experiment was performed 3 times, with each condition in triplicate ($n = 9$).

Dentin Specimen Preparation

Bovine incisors were disinfected with a 5% NaOCl solution and stored in 0.9 % NaCl (w/v) until use for up to 30 days [Ccahuana-Vasquez and Cury, 2010]. Dentin slabs (6 × 4 × 1 mm) were prepared from bovine roots using diamond disks, a low-speed handpiece and polishing disks (Soflex, 3M, St. Paul, MN, USA). Pulpal surface of each root was roughly flattened, while the outer dentin was flattened and finely polished with disks (Soflex, 3M). SH_i was determined using a Knoop microhardness indenter (402 MVD, Wolpert Wilson Instruments, USA) by placing 3 indentations in the center of each slab, 100 μm apart from each other at 10 g for 5 s. Slabs were selected based on their SH_i, including only those of 45.5 ± 1.48 kg/mm² ($n = 54$). Slabs were randomly assigned to the treatments and suspended into wells of a 24-well culture plate, through a specially designed device made of stainless steel wire [Ccahuana-Vasquez and Cury, 2010] and sterilized with ethylene oxide [Thomas et al., 2007].

In vitro Biofilm Model – Salivary Pellicle Coating

Stimulated whole saliva was collected from 2 healthy volunteers (37 and 28 years old) by chewing paraffin strips for 5 min (Parafilm M; American Can Co., Neenah, WI, USA). Donors were not using antimicrobials, mouthwashes or any other medication known to affect salivary composition and flow at least 3 months before and during the experiments. To preserve protein integrity, whole saliva collected was mixed with absorption buffer (1:1) 0.1 M (50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$–$2H_2O$, 0–1 mM $MgCl_2$–$6H_2O$), containing phenylmethylsulfonyl fluoride 1:100 (v/v) as protease inhibitor. Previously sterilized specimens/wires were transferred to a well of a new plate, covered with 2 mL of ultrafiltered (0.22 μm) pooled human saliva and agitated (60 rpm) for 30 min at 37 °C. Saliva coating of the slabs allows the formation of an acquired pellicle-like structure on the dentin slabs, suitable for *S. mutans* initial adherence [Koo et al., 2003].

Inoculum Preparation and Biofilm Formation

Frozen stocks of *S. mutans* UA159 were reactivated on Brain Heart Infusion medium supplemented with 1% glucose (Merck, Darmstadt, Germany) at 37 °C and 10% CO_2 for 18 h until reaching an optical density (OD_{800}) of 0.8. One hundred μL of microbial culture was mixed with 50 mL of 1% sucrose-supplemented soy-trypticase medium without dextrose (Becton, Dickinson and Co, Sparks, MD, USA). An aliquot of 200 μL of the bacterial cultures with the medium was transferred to each well with the saliva-coated slab and grown for 8 h at 37 °C and 10% CO_2 to create an initial adhered biofilm. Slabs were transferred to another plate containing 2 mL of soy-trypticase medium without dextrose, supplemented with 0.1 mM glucose and 0.02 ppm F, for additional 16 h at 37 °C and 10% CO_2 until the completion of 24 h. After 24 h of biofilm growth, dentin slabs were exposed to the different treatments as previously described in the "experimental design" section.

Treatments

After 24 h of biofilm growth, dentin slabs were exposed to the different treatments 3 times per day (at 8:30 am, 12:30 pm, and 4:30 pm) for 5 min: 10% sucrose solution; 0.9% NaCl solution; milk-based drink; milk-based drink supplemented with sucrose; whole milk and skim milk. After each treatment, biofilms were washed 3× in 0.9% NaCl, and relocated to a medium-containing well. Culture medium was changed twice per day before the first (at 8 am) and after the last treatment (at 5 pm).

Biofilm Acidogenicity

To verify acid production by the biofilms in response to the tested treatments, culture medium pH was measured inside each well by an electrode (HI 1083B, Hanna instruments, Rumania) previously calibrated with pH 4.0 and pH 7.0 standards and coupled to a pH-meter (HI 9126–02, Hanna instruments, Rumania). Measurements of the spent medium were carried out once per day, after 8, 24, 32, 56, and 80 h of initial biofilm formation during 5 days. The AAC was calculated considering pH 6.87 as the cut-off point, based on the highest pH value obtained by the negative control with 0.9% NaCl. This cut-off point allows evaluating pH dynamics in the other groups.

Viable Microorganisms Analysis

After 5 days of biofilm growth, slabs were washed 3 times with 0.9% NaCl and placed in sterile tubes containing 1 mL of 0.9% NaCl. Slabs were vigorously vortexed for 30 s to detach biofilms from the slabs. This suspension was serially diluted in 0.9% NaCl (v/v) and plated on Brain Heart Infusion agar plates by the drop-technique (20 μL). After 24 h incubation at 37 °C and 10% CO_2, viable colonies were counted under a stereomicroscope (SMZ161, Motic, BC, Canada). Results were expressed as colony-forming units CFU/mL.

Dentin Demineralization

The SH_f of the dentin slabs was measured to estimate demineralization induced during the 5 days of the experiment. Measurements were obtained by a row of 3 sequential indentations placed in the center of each slab, 100 μm apart from each other, and 100 μm to the right from the initial indentations. Demineralization was estimated using the %SHL, which was calculated as: (mean SH_i – mean SH_f) × 100/SH_i.

Cross-Sectional Microhardness Analysis

Once %SHL was registered, slabs were longitudinally sectioned through their center producing 2 halves. One of them was embedded in acrylic resin, leaving the outer polished surface exposed. Slabs were indented in the left fourth, in the middle and in the right fourth of the exposed surface by a row of 12 indentations in each one of them, at 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, and 300 μm from the outer surface of the slabs at 10 g for 5 s [Featherstone et al., 1983]. The integrated mineral loss (ΔZ; vol % min × μm) was determined for each slab using a previously reported formula [Featherstone et al., 1983], which considered the average value of the 3 indentations at each depth and then using all 12 values.

Statistical Analysis

Bacterial counts were expressed at $\log_{10}$. To determine whether the data had parametric distribution, the Kolmogorov-Smirnov test was performed. Mean values obtained from acidogenicity, dentin demineralization and counts of viable microorganisms were compared by analysis of variance followed by a Tukey post hoc test using SPSS 15.0 statistical software. Differences were considered significant if $p < 0.05$.

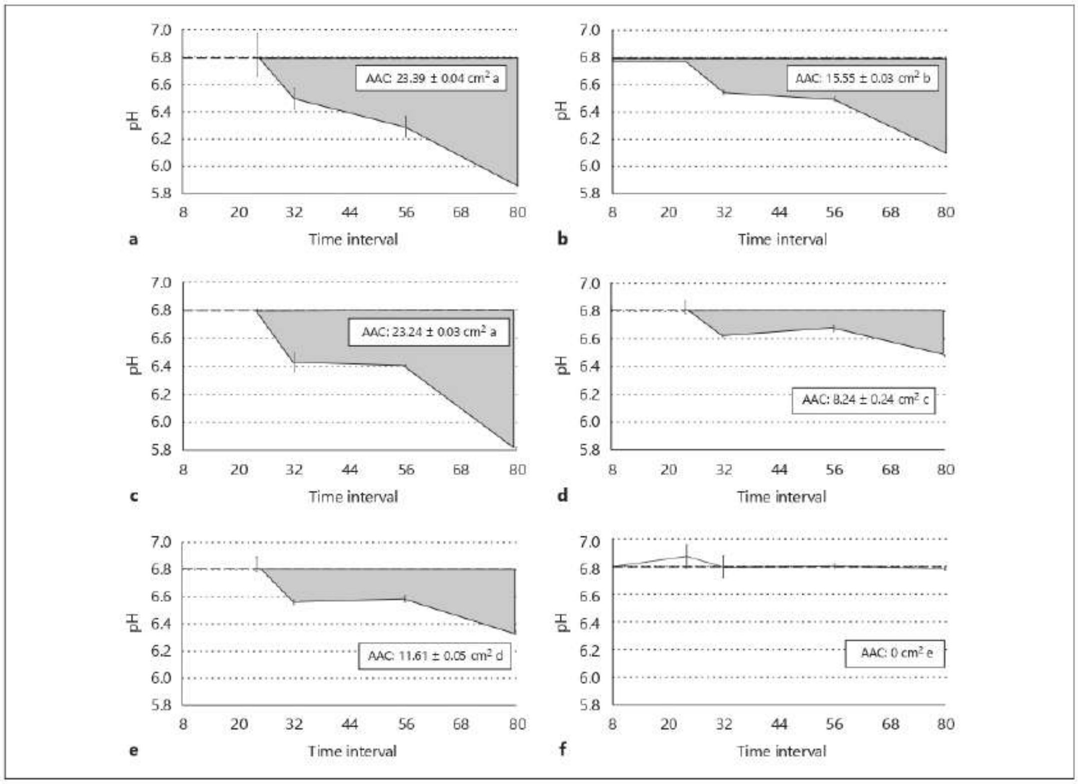

Fig. 1. Acidogenicity elicited from the *S. mutans* biofilms formed on dentin in response to different treatments: (**a**) sucrose 10%, (**b**) milk-based drink, (**c**) milk-based drink + sucrose 6%, (**d**) whole milk, (**e**) skim milk and (**f**) NaCl 0.9%. Plot shows mean pH registered in the spent culture medium and mean ± SD values for area above curve (AAC) in each experimental condition. Different letters represent significant differences among treatments by Tukey test ($p < 0.05$).

Results

The milk-based drink (Fig. 1b) showed lower acidogenicity than a 10% sucrose solution (Fig. 1a). When the dairy product was supplemented with sucrose (final concentration of 6%), a similar acidogenicity to 10% sucrose was observed. The milk-based drink showed higher AAC than whole (Fig. 1d) and skim milk (Fig. 1e). Among all experimental groups, whole milk showed the lowest acidogenic potential. Regarding viable microorganisms (CFU $\log_{10}$/mL), whole milk showed lower counts (p = 0.02) compared to the 10% sucrose solution (9.16 ± 1.08E−02), milk-based drink (9.1 ± 5.97E−02; p = 0.06) and milk-based drink supplemented with sucrose (9.12 ± 2.54E−02; p = 0.014), while no significant differences were found among these products ($p > 0.05$). Skim milk (9.07 ± 4.07E−02), however, presented values similar to all the dairy products. As expected, the lowest counts were found in the presence of 0.9% NaCl (Fig. 2).

The milk-based drink and the milk-based drink supplemented with sucrose induced lower demineralization than the 10% sucrose solution (Fig. 3), but the demineralization induced by the milk-based drink supplemented with sucrose was higher than that found in dentine slabs exposed to the dairy product, without sucrose (p = 0.0001). Skim milk induced higher demineralization in comparison with whole milk, but both milk types induced demineralization statistically lower than milk-based drink test groups ($p < 0.05$).

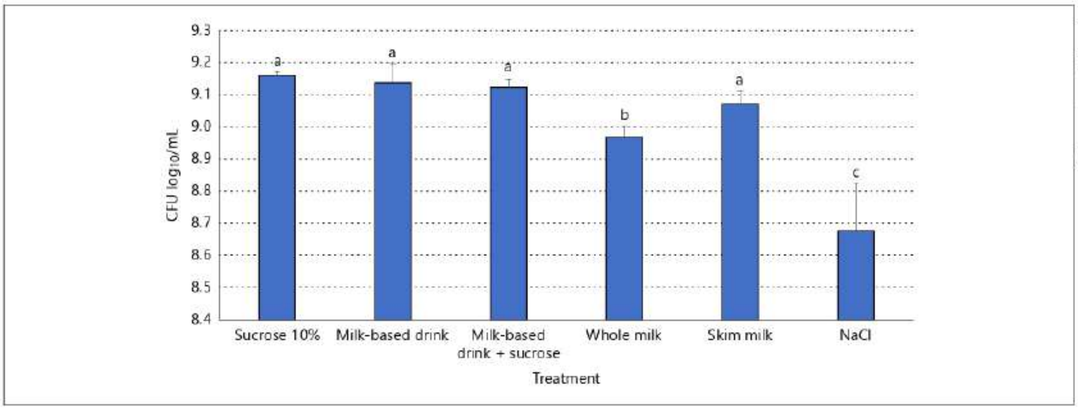

Fig. 2. Viable microorganisms from *S. mutans* biofilms. Bacterial cells retrieved from each treatment-exposed biofilm were counted and expressed as CFU $\log_{10}$/mL. Bars show mean counting obtained from 3 independent experiments, each in triplicate. Error bars indicate SD. Letters a–c represent significant differences among treatments by Tukey test ($p < 0.05$).

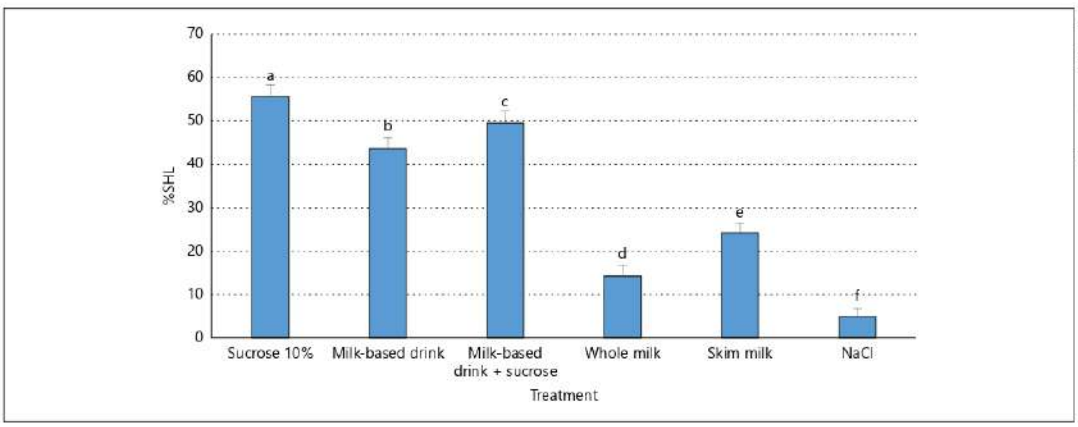

Fig. 3. Dentine demineralization induced by different treatments. Demineralization was assessed by surface hardness loss (%SHL) induced by treatments after 5 days. Bars represent mean %SHL of the slabs. Error bars indicate SD. Letters a–f represent significant differences among treatments by Tukey test ($p < 0.05$).

Regarding integrated mineral loss (ΔZ), demineralization induced by the milk-based drink was within the range of that induced by the 10% sucrose solution ($p = 1.00$) and skim milk ($p = 0.75$). The milk-based drink supplemented with sucrose was statistically higher in ΔZ than that induced by whole milk, skim milk and the negative control, but it was not different compared with the milk-based drink and the 10% sucrose solution. The lowest values of ΔZ were found for the negative control group (Table 1).

Discussion

The present study shows that the milk-based drink used for Chilean elderly and the same product with sucrose added to a concentration of 6% has slightly lower cariogenic potential than a 10% sucrose solution, but clearly a higher cariogenic potential than milk, either whole or skim. Data of %SHL followed a similar pattern than biofilm acidogenicity (Fig. 1, 3). There was more

Table 1. Integrated mineral loss (ΔZ; mean ± SD) of dentin in response to different treatments

Treatments	ΔZ (vol % min × μm)
10% sucrose	740.9±137.2[a, b]
Milk-based drink	728.1±26.9[a, b]
Milk-based drink + sucrose	897.7±49.2[a]
Whole milk	499.7±94.5[c]
Skim milk	549.8±32.2[b, c]
0.9% NaCl	287.8±70.7[d]

Superscript letters a–d represent significant differences among treatments by Tukey test ($p < 0.05$).

pronounced lesion formation in root dentin when exposed to skim milk (0.05% fat) as comparison to whole milk (3% fat). These results are consistent with previous findings from our group [Giacaman and Muñoz-Sandoval, 2014]. When whole milk was supplemented with sucrose (3% fat and 10% sucrose), however, a similar cariogenic potential than the control 10% sucrose solution was elicited. The latter suggests that milk's fat content is not capable of hampering the cariogenic potential of the whole milk when sugars are added. The milk-based drink for older adults evaluated in this study (1.25% fat, 7.5% total carbohydrates, 1% sucrose and 2.5% lactose) showed slightly lower cariogenic potential than the sucrose solution used as the control with a 10% concentration, even when the product was supplemented with sucrose (6% final concentration). As expected, demineralization of the sugars-added milk-based drink was higher than the same product without sugars supplementation. Altogether, these findings suggest that the fat concentration of milk and the milk-based drink might counteract, at least in part, the cariogenicity induced by sucrose confirming our previous findings. Thus, when fat is removed from milk, as it is the case in skim milk, protection is lost and thus, cariogenicity increases. There might be a threshold in which fat's protective effect is overcome by the intrinsic cariogenicity of sucrose. The exact point where this occurs is yet to be determined. The milk-based drink tested is manufactured with milk of a relatively low-fat content (semi-skim). In addition, the milk-based drink has a partial content of lactose and sucrose added (2.5% lactose and 1% sucrose), resulting in a product that has a considerably high cariogenic potential on root dentin.

There is some evidence supporting that fatty acid-containing diets may have an anti-caries effect [Kabara et al., 1972]. Possible explanations for this effect derive from the fact that fatty acids are not metabolized by the dental biofilm bacteria to produce acids [Schuster et al., 1980]. In addition, these molecules have antimicrobial activity, inhibiting nutrient transportation through the bacterial cell membrane and preventing cell adhesion [Williams et al., 1982].

Dietary proteins, such as those found in dairy products, may also play an important role as anti-caries substances. Proteins may serve as a source of carbon for bacteria of the dental biofilm [Chappelle and Luck, 1957]. Protein catabolism releases urea as a by-product in saliva. Urea is converted into ammonia, which acts as a buffering system controlling pH of the dental biofilm [Burne and Marquis, 2000]. Although the tested milk-based drink has a slightly lower protein concentration (2.25%) than whole milk (3.15%), we hypothesized that the combined effect of proteins and the fatty acids may be synergic to counteract, in part, its cariogenic potential. When the bacterial cell counts were considered, only mild differences could be detected. Despite the content of putative antibacterial molecules in the milk-based drink from milk, such as casein, albumin, lactoferrin and lysozyme [Johansson and Lif Holgerson, 2011], biofilm cell viability seemed uncompromised. It is likely that the effect in caries of these molecules is not antibacterial, but rather bacteriostatic [Bowen and Pearson, 1993]. Yet, it is important to mention that whole milk exposure to the biofilm resulted in the lowest counts of cells, potentially exhibiting an antibacterial effect. This mild killing effect is consistent with our previous studies and others [Giacaman et al., 2016; Huang et al., 2011] showing that a higher fatty acid concentration may be antibacterial.

Demineralization of the dental substrate was evaluated by %SHL using microhardness as a proxy. Loss of surface hardness has been extensively used as a reliable methodology to evaluate demineralization [Zero, 1995]. Although the cross-sectional microhardness analysis is a validated technique used to analyze mechanical properties and mineral content of carious lesions, it has been reported that ΔZ does not respond linearly to increased sucrose concentrations [Aires et al., 2006]. Moreover, previous data showed a considerable degree of variability and lack of sensitivity on outcomes obtained by this method [Buchalla et al., 2008]. In this study, we did not find statistical differences in ΔZ scores. Whole milk, however, showed lower values, confirming the abovementioned protective effect of milk's fat. It might be possible that this technique is not sensitive enough to detect small differences in small lesions.

Despite that it is likely that milk-based drink users have other sources of fluoride exposure, such as water or some foods, we have decided not to incorporate fluorides in to the cariogenicity model. The reason is that the validated models have not included fluoride, but also based on our studies and clinical experience showing a low use of fluoridated products among older adults. Yet, further experimental studies should be designed incorporating this variable, key in the outcome in many cases. We acknowledge the limitations implied in this study, for example, the single-species approach, the lack of fluoride and saliva and other factors associated with root caries. Yet, this research was conceived as a "proof-of-principle," over which further studies, closer to the clinical reality, must follow.

Replacing whole milk for skim milk has become a popular choice among many people. This practice has been considered healthier than consuming whole milk. It has been demonstrated that regular consumption of whole milk in adults and older people is positively associated with health outcomes, nonetheless neverthe. For example, the use of whole milk has been associated with diabetes control [Yakoob et al., 2016], normal body weight [Vanderhout et al., 2016], reduced risk of ischemic stroke [Elwood et al., 2005], lower risk of coronary disease [Steinmetz et al., 1994] and protection against some types of cancer [Prentice, 2014]. In addition, it has been stated that regularly consuming whole milk induces a rapid and prolonged sensation of satiety, unlike the consumption of skim milk. Due to the lower fat content, skim milk would lead to increased intake of high carbohydrate foodstuff or sugars, to get the same feeling of satiety [Dougkas et al., 2011]. In an era of an aging population with people retaining more teeth until an older age, usually with exposed root surfaces [Mariño and Giacaman, 2014], these results are worrisome. A higher root caries risk in older adults has been reported [Saunders and Meyerowitz, 2005]. Moreover, older adults tend to add sucrose (2 teaspoons) to the milk-based drink (unpublished data). Hence, these results may be helpful to raise awareness within the dental community of the potential risk of consuming this type of product in people with exposed root surfaces. Furthermore, consumption of dietary supplements intended for older adults, similar to the milk-based drink used in Chile, is becoming popular. An aggressive advertising campaign has been promoting the consumption of fortified or specific nutrient-enriched dairy products, with similar or even higher sucrose concentration than the milk-based drink tested here. In this scenario, education to consumers is crucial for preventing root caries. Users and prescribing physicians must be aware of the dental risks implicit in consuming dietary supplements with sugars added. More than restricting consumption, users should be careful to avoid frequent consumption and pay attention to dental hygiene after its consumption. Simple preventive measures could include rinsing with water right after consumption and tooth brushing before bedtime.

In conclusion, the tested milk-based drink used as a dietary supplement for older adults in Chile shows a potential risk for root caries, higher than whole milk. These findings stress the importance of testing the cariogenic potential of the dietary supplements available to the elderly population.

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Disclosure Statement

The authors declare no conflicts of interest.

Author Contribution

R.J.C. and R.A.G. conceived the experiments and the research questions. R.J.C., R.A.G., R.A.A., and M.M. designed the experiments. R.J.C. performed all the experiments. R.J.C., R.A.G., R.A.A. and M.M. analyzed the data. R.J.C. and R.A.G. drafted the first manuscript. R.A.A. and M.M. critically revised the manuscript and all authors read and approved the submitted version.

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CHAPTER II - FLUORIDATION OF A MILK-BASED DIETARY SUPPLEMENT DRINK FOR THE ELDERLY TO REDUCE CARIOGENICITY

Study 3. Anti-caries effect of fluoridated milk-based drink consumed by older adults on an *in vitro* root caries experimental model

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Anti-caries effect of fluoridated milk-based drink consumed by older adults on an *in vitro* root caries experimental model

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ABSTRACT

Objective: This study aimed to evaluate the anti-caries effect of a fluoridated milk-based drink on a root caries model by assessing mineral loss and both biofilm microbial viability and acidogenicity under increasing concentrations of fluoride supplementation.

Design: *Streptococcus mutans* UA159 biofilms were grown on root dentin slabs for five days. The slabs were randomly assigned to following groups: milk-based drink (G1) and milk-based drink supplemented with 5-ppm NaF (G2), 10 ppm NaF (G3), and 20 ppm NaF (G4). A 10% sucrose and 0.9% NaCl solution were used as positive and negative-caries controls, respectively. Slabs/biofilms were exposed to the different treatments 3 times/day for 5 min. To estimate biofilm acidogenicity, the pH of the spent media was serially measured to calculate the area above the curve. Viable bacteria and dentin demineralization were assessed after the experimental phase. Results were compared using ANOVA followed by the Tukey test.

Results: G1 exhibited slightly lower acidogenicity than the positive caries control group ($p < 0.05$). G2, G3, and G4 induced lower acidogenicity than 10% sucrose and the non-supplemented milk-based drink. The lowest acidogenicity was found in G4 ($p < 0.05$). Fluoride-supplemented milk-based drinks (G2, G3, and G4) resulted in lower bacterial counts ($p < 0.05$) and induced lower demineralization ($p < 0.05$) than the positive caries control and non-supplemented milk-beverage (G1). There was a dose-dependent inhibition of demineralization with fluoride-supplemented milk-based drinks.

Conclusions: Fluoride supplementation of a milk-based drink for older adults may reduce its cariogenicity in root dentin.

1. Introduction

Increased life expectancy and improvement in oral health status in terms of longer retention of natural teeth in older adults is being witnessed globally (Griffin, Griffin, Swann, & Zlobin, 2004). This scenario poses an important challenge in relation to the prevention and management of carious lesions that could affect both the crown and root surfaces of these teeth. Many factors inherent to the elderly, such as decreased salivary flow, use of removable prosthetic devices, periodontal disease and gingival recession, may predispose exposed root surfaces to carious lesion development (Sugihara et al., 2010). Epidemiological studies have shown that up to 100% of older adults may present root carious lesions (Hayes, Burke, & Allen, 2017). Root caries is the primary cause of tooth loss among older adults, and the most significant determining factor for the onset of root caries is the high intake of dietary sugars (Sheiham & James, 2015; Sugihara et al., 2010).

Besides root caries, older adults worldwide are also affected by malnutrition (Kaiser et al., 2010), especially individuals who are institutionalized, of whom more than 60% are affected by this condition (Corish & Kennedy, 2000). In general, the typical diet of the elderly has low levels of micronutrients, fiber, and protein (Rakıcıoğlu et al., 2016) and is often accompanied with a high percentage of carbohydrates (Boirie, Morio, Caumon, & Cano, 2014). These individuals largely prefer soft and highly processed foods with low nutritional value (Castrejón-Pérez & Borges-Yáñez, 2014). To address this unfavorable nutritional status, public policies and private initiatives have been implemented based on supplementation of specific foods and beverages, such as milk, to enrich the daily diet of older adults (Visser, McLachlan, Maayan, & Garner, 2018). One example of this supplementation provided by the Chilean government to older adults is an easy-to-prepare

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milk-based drink ("*Bebida Láctea Años Dorados*"), which contains significant amounts of sugars that increased the risk for caries development on the exposed root surfaces (Castro, Giacaman, Arthur, & Maltz, 2019).

In this context, the anti-caries effect of fluoride at the level of both enamel and dentin is universally accepted (Buzalaf, Pessan, Honório, & ten Cate, 2011). Given its constitutive presence in the oral cavity, even at low concentrations, fluoride is capable of reducing net tooth mineral loss by balancing the oral demineralization/remineralization process (Cury & Tenuta, 2008). Clinical studies involving children and adolescents have demonstrated that the regular use of milk supplemented with fluoride may be an effective preventive measure against the onset of dental caries (Bánóczy, Rugg-Gunn, & Woodward, 2013). Along with this positive effect in the reduction of caries, there is evidence supporting the use of milk as a safe and low-cost public health measure (Bánóczy et al., 2013). A 4-year follow-up of complementary dietary programs based on fluoridated milk (0.75 mg fluoride/day) demonstrated a decrease in the DMFT index (i.e., decayed, missing due to caries, and filled teeth) between 24% and 27% in children who received the product (Weitz, Marinanco, & Villa, 2007). Similar results were obtained after 5 years of follow-up, in which fluoridated milk consumed daily by 8-year-old children resulted in substantially lower caries development compared with children in schools receiving milk without fluoride supplementation (Petersen, Kwan, & Ogawa, 2015). It is important to note that this decrease in caries experience ceased when the use of fluoridated milk was discontinued (Mariño, Villa, Weitz, & Guerrero, 2004). According to reports, when the use of fluoridated milk is discontinued, the experience of caries returned to its initial status and was comparable with community controls who never received fluoridated supplements (Mariño et al., 2004). However, different experimental conditions across studies, mainly related to different study designs, varying methods of assessing fluoride concentrations, and different exposure times, preclude definitive conclusions regarding the use of fluoridated milk for caries prevention (Yeung, Chong, & Glenny, 2015). Moreover, evidence regarding the anti-caries effect of fluoridated milk in older adults is lacking. Although some studies have shown that milk containing fluoride at a concentration of 20 ppm promotes re-hardening of both enamel and root dentin caries-like lesions in a dose-dependent manner (Arnold, Heidt, Ku, ntz, & Naumova, 2014; Lippert, Butler, Lynch, & Hara, 2012; Malinowski, Duggal, Strafford, & Toumba, 2012), other studies did not demonstrate the same benefit (Cassiano et al., 2017; R. A. Giacaman, Muñoz, Ccahuana-Vasquez, Muñoz-Sandoval, & Cury, 2012). These contradicting findings warrant further investigations.

Considering that milk-based drink may be cariogenic to root dentin (Castro et al., 2019), we hypothesized that the addition of low concentrations of fluoride (5, 10, and 20 ppmF) to these drinks may counteract their cariogenic potential. These concentrations were chosen based on the optimal effects reported in previous studies (Arnold et al., 2014; Giacaman et al., 2012; Lippert et al., 2012; Malinowski et al., 2012). Thus, the present study aimed to evaluate the anti-caries effect of a fluoridated milk-based drink on a root caries model by assessing mineral loss and both biofilm microbial viability and acidogenicity under increasing concentrations of fluoride supplementation.

2. Materials and methods

2.1. Experimental design

Streptococcus mutans UA159 biofilms were grown for 5 days on the surface of bovine root dentin slabs with known initial Knoop surface microhardness (Ccahuana-Vásquez & Cury, 2010) previously covered by salivary pellicle, and were exposed to the following treatments three times per day at defined times (08:30 a.m., 12:30 p.m. and 4:30 p.m.) for 5 min each: group G1: milk-based drink ("*Bebida Láctea Años Dorados*"; Calo, Osorno, Chile [1.25% fat, 7.5% total carbohydrates, 1% sucrose and 2.5% lactose, 0 ppm fluoride]); G2:, milk-based drink supplemented with 5 ppm NaF; G3: milk-based drink supplemented with 10 ppm NaF; and G4: milk-based drink supplemented with 20 ppm NaF. Solutions of 10% sucrose and 0.9% sodium chloride (NaCl) were used as positive and negative controls, respectively. All biofilms were dip-washed with 0.9% NaCl after each treatment and relocated to a medium-containing well. Counts of viable cells on biofilms and percentage of surface micro-hardness change were determined on dentin species after 5 days of biofilm growth. The acidogenic potential of biofilms was assessed daily through pH measurements of the culture medium after 8, 24, 32, 56, and 80 h of biofilm growth, and the area above the curve (AAC) of pH fall was determined. Three independent experiments were performed. Sample size calculation was based on data of our previous study (Castro et al., 2019). Considering a difference of about 40% in %SHL between milk-based-drink and whole milk, a power of 80% and a confidence interval of 95%, 2 slabs were necessary for each group per experiment resulting in total of 6 slabs per group. Sample size was increased 50% to account for any loses during mineral loss assessment resulting in 3 slabs per each group/experiment (n = 54 slabs).

2.2. Preparation of dentin specimens

Considering the similarities in dental caries lesion development between human and bovine teeth (Hara, Queiroz, Paes Leme, Serra & Cury, 2003), the latter were chosen for this study and disinfected with 5% sodium hypochlorite (NaOCl) solution before use. Briefly, dentin slabs ($6 \times 4 \times 1$ mm) were prepared from the roots of bovine teeth, using diamond disks mounted on an electric cutter. The pulp surface of each root was roughly flattened, and the buccal surface was flattened and polished with increasing grade sandpapers mounted on a polishing machine. Dentin slabs were finely polished using a low-speed handpiece and soft disks (Soflex, 3 M, St. Paul, MN, USA). An initial assessment of baseline surface micro-hardness (SHi) was performed in all slabs by placing three indentations in the center of each slab, 100 μm apart from one another at 10 g for 5 s through a Knoop micro-hardness indenter (402 MVD, Wolpert Wilson Instruments, USA). The Knoop micro-hardness number (KHN) of the three indentations was averaged for each slab, and those presenting a mean baseline micro-hardness of 42.32 ± 0.41 kg/mm^2 (n = 54) were selected for the study. After randomly assigning the slabs to the different treatments, slabs were mounted on stainless steel wires and sterilized with ethylene oxide (Thomas, Ruben, ten Bosch, & Huysmans, 2007)

2.3. *In vitro biofilm model*

S. mutans culture ($OD_{600} = 0.8$) on 1% glucose supplemented Brain Heart Infusion medium (BHI; Merck, Darmstadt, Germany) was diluted in fresh 1% sucrose-supplemented Soy Trypticase Medium (STM) without dextrose (Becton, Dickinson and Co, Sparks, MD, USA). An aliquot of this microbial suspension was transferred to each well of a 24-well plate containing sterilized dentin slabs. All slabs were previously covered by a salivary pellicle formed by stimulated whole saliva collected from an adult subject and processed as described previously (Castro et al., 2019). Saliva was collected from a healthy donor who signed a written consent prior to saliva collection in accordance with the Declaration of Helsinki (Comité de Bioética de la Universidad de Talca; #2014-009-RC). All 24-well plates were initially incubated for 8 h at 37 °C in a 10% CO_2 environment to create an initial adhered biofilm. The slabs were transferred to another plate containing 2 mL of fresh STM (without dextrose and supplemented with 0.1 mM glucose and 0.02 ppm), for an additional 16 h at 37 °C and 10% CO_2 until completion at 24 h (Castro et al., 2019). Treatments with the tested solutions were performed as described above. Culture medium was replaced by fresh STM twice per day, before the first (at 08:30 a.m.) and after the last treatment (at 4:30 p.m.). To assess the biofilm acidogenic potential, the pH of each well was determined at 8, 24, 32, 56, and 80 h of biofilm growth, and the area above the curve (AAC) of pH fall was calculated for each replicate as previously described, considering pH 6.8 as the cut-off point based on the average of the highest pH level obtained by the negative control (Castro et al., 2019).

2.4. Analysis

After 5 days of biofilm growth, the slabs were aseptically collected and vigorously vortexed for 30 sec. The resulting suspension was serially diluted in 0.9% NaCl (v/v) and plated on BHI agar using the micro-method described by Westergren & Krasse (commonly referred to as the "drop-technique") (Westergren & Krasse, 1978). Plates were incubated at 37 °C and 10% CO_2 for 24 h and viable colonies were counted using a stereomicroscope (SMZ161, Motic, British Columbia, Canada). Results were recorded as $\log_{10}$ of colony-forming units (CFU)/mL.

The final surface micro-hardness (SHf) was assessed for each slab by placing a row of three sequential indentations in the center of each slab, 100 μm apart from one another and 100 μm to the right from the initial indentations. Demineralization was estimated using the percentage of surface micro-hardness loss (%SHL), which was calculated using the equation:

$$(\text{mean } SH_i - \text{mean } SH_f) \times 100/SH_i.$$

2.5. Statistical analysis

The assumption of normal data distribution was verified using the Kolmogorov-Smirnov test, and the outcomes ($\log_{10}$CFU/mL, AAC and %SHL) were compared among the experimental groups by analysis of variance followed by Tukey post-hoc test. Correlation between AAC and mineral loss (i.e., %SHL) was estimated using the Pearson correlation test. All analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA). Differences with $p < 0.05$ were considered to be statistically significant.

3. Results

Biofilms treated with 10% sucrose solution exhibited the highest acidogenic potential compared with the other treatments. Milk fluoridation resulted in decreased biofilm acidogenicity in a dose-dependent manner, with the lowest area above the curve (AAC) value found in biofilms treated with the milk-based drink supplemented with 20 ppm NaF compared with other fluoride concentrations (Fig. 1).

The highest counts of viable microorganisms were found in biofilms treated with 10% sucrose solution and the non-fluoridated-milk-based drink, although the differences between them were not statistically significant. Supplementation of milk-based drink with fluoride reduced the viability of microorganisms compared with both sucrose solution and the non-fluoridated-milk-based drink. However, no differences in counts were found among the fluoridated milk-based drink (Table 1).

With regard to mineral loss, the highest percentage of surface micro-hardness loss (%SHL) was found in root dentin slabs exposed to 10% sucrose solution. Supplementation of milk-based drink with fluoride resulted in a marked decrease in %SHL compared with both sucrose solution and the non-fluoridated milk-based drink in a dose-dependent manner. The %SHL found on slabs exposed to milk-based drink supplemented with 10 ppm and 20 ppm NaF was statistically similar but statistically lower than those found in milk-based drink supplemented with 5 ppm NaF (Table 1). Pearson's test revealed a strong and positive correlation between area above the curve (AAC) and percentage of surface micro-hardness loss (%SHL) ($r = 0.85$; $p < 0.001$).

4. Discussion

Data from the present study confirmed that milk-based drink increases caries risk on the exposed root surfaces (Castro et al., 2019). Without fluoride supplementation, although the percentage of surface micro-hardness loss (%SHL) of slabs treated with the non-fluoridated milk-based drink had been statistically lower than that of slabs treated with sucrose solution, the resulted demineralization induced by the

Table 1
Count of viable microorganisms ($\log_{10}$CFU/mL) on biofilms and percentage of surface hardness loss (%SHL) of root dentin slabs (mean ± SD) in response to different treatments [Sucrose 10%; G1 (milk-based drink); G2 (milk-based drink+5 ppm F); G3 (milk-based drink+10 ppm F); G4 (milk-based drink+20 ppm F) and 0.9% NaCl].

Treatments	$\log_{10}$CFU/mL	%SHL
10% sucrose	9.24 ± 8.01^{a}	49.71 ± 0.58^{a}
G1	9.14 ± 8.37^{a}	40.99 ± 0.41^{b}
G2	8.88 ± 8.38^{b}	7.40 ± 0.78^{c}
G3	8.79 ± 7.97^{b}	3.50 ± 0.26^{d}
G4	8.58 ± 7.49^{b}	$2.61 \pm 0.14^{d,e}$
0.9% NaCl	8.26 ± 6.89^{c}	1.62 ± 0.12^{e}

Different letters represent significant differences among treatments by Tukey test ($p < 0.05$).

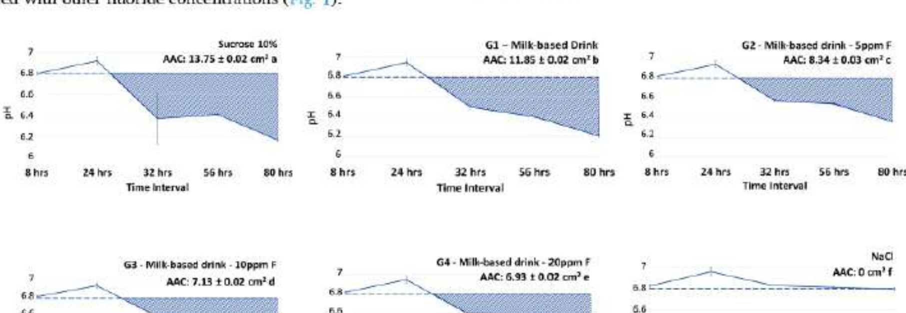

Fig. 1. Acidogenicity elicited from the *S. mutans* biofilms formed on dentin in response to different treatments: Sucrose 10%, G1 (milk-based drink), G2 (milk-based drink+5 ppm F), G3 (milk-based drink+10 ppm F), G4 (milk-based drink+20 ppm F) and 0.9% NaCl. Plot shows mean pH registered in the spent culture medium and mean ± SD values for area above curve (AAC) in each experimental condition. Different letters represent significant differences among treatments by Tukey test ($p < 0.05$).

non-fluoridated milk-based drink was as high as that of the 10% sucrose solution. As expected, data obtained from the negative caries control group are in agreement with a previous study that used the same biofilm model (Castro et al., 2019; Giacaman, 2014; R. A. Giacaman & Muñoz-Sandoval, 2014). This means that biofilms of the negative control group exhibited the lowest acidogenic potential, the lowest counts of viable cells, and the lowest percentage of surface micro-hardness loss (%SHL). In fact, the lack of a carbohydrate source frequently provided to biofilms in the negative control group accounted for the lowest acidogenic and cariogenic potentials (Fig. 1 and Table 1). As previously discussed by Castro et al. (Castro et al., 2019), despite the presence of anti-cariogenic molecules found in milk, such as casein, albumin, lactoferrin, lysozyme and fatty acids, the tested milk-based drink contained inherent sugars (e.g., sucrose and lactose), which counteracted any protective effect, *per se*, provided by the other milk components. This highlights the importance of modulating the cariogenic potential of milk-based drinks by supplementing them with fluoride. Supplementation of milk-based drink with low concentrations of fluoride was able to prevent root dentin demineralization, which confirmed our working hypothesis. Considering the high consumption of milk-based dairy products as nutritional supplements worldwide (Clerfeuille et al., 2013), especially by older adults and children (Singh et al., 2015) this accessible strategy to control/prevent carious lesion development appears beneficial, particularly considering the inherent sugars found on milk-based drinks.

As expected, exposure of teeth to low amounts of fluoride added to milk-based drinks resulted in decreased biofilm acidogenicity. *In vitro* models used to study remineralization of dental tissues have shown that milk supplemented with minimal fluoride concentrations (2.5 ppm) could be sufficient to promote remineralization of the root surface affected by superficial caries-like lesions (Arnold et al., 2014). At the community level, programs that use fluoridated milk target children and adolescents (Cagetti, Campus, Milia, & Lingström, 2013). In these programs, 0.5 to 0.75 mg fluoride/day (2.5 and 3.75 ppm of fluoride, respectively) was administered. In our work, we used higher doses of fluoride, starting from 5 ppm, to determine whether there is a dose-dependent effect, with the potential of increasing the protective effect that fluoride would give to milk-based products with added sucrose. The cariogenicity of milk-based drink is reduced by supplementing it with fluoride in a dose-dependent manner (Table 1), which is consistent with previous data demonstrating a fluoride dose-response effect on caries inhibition (ten Cate, 2013). Our study demonstrates that some anti-caries effect was evident with the lowest concentration of fluoride (5 ppm NaF) (Table 1).

Aside from exerting its main effect of modulating the physical-chemical interactions between tooth surfaces and the surrounding aqueous phase (Cury & Tenuta, 2008), fluoride may also modulate the acidogenic potential of *S. mutans*. Several mechanistic studies have suggested that fluoride is capable of inhibiting the enzymatic activity of enolase, which impairs the final conversion of glucose intermediates into lactic acid during glycolysis (Van Loveren, 1990). In an *in vitro* system used to analyze the effect of low doses of fluoride on the stability of complex oral microbial communities, it was determined that NaF at a concentration of 19 ppm resulted in a decrease in the amount of acids produced and, therefore, in the global decline in local pH. This environmental effect favors bacteria that are sensitive to low pH (i.e., less acidogenic and less acid-tolerant bacteria), which maintain their proportion within the biofilm and prevent highly- acidogenic and acid-tolerant bacteria, such as *S. mutans*, from proliferating (Marsh & Bradshaw, 1990). Other authors have confirmed the positive effects of low doses of fluoride on bacterial viability (Bowden, 1990; Bradshaw et al., 1990; Whitford et al., 1977) however, to obtain this positive effect, a high concentration of fluoride inside the biofilm (> 20 ppm) is required. This effect is well observed in the area above the curve (AAC) values with increased fluoride concentrations (Fig. 1). It is important to note that fluoride may be retained in both the solid and fluid phases of biofilms after consumption of fluoridated milk. When fluoride is incorporated in the mouth, its concentration drops rapidly due to the effect of clearance; however, in some subjects, it is possible to maintain a salivary concentration of this ion between 0.03 to 0.1 ppm for up to 6 h (Zero et al., 1992). It has been reported that to have a remineralizing effect in an active caries process, amounts as low as 0.03 ppm are sufficient (Featherstone & Zero, 1992). Therefore, by being trapped in biofilms, fluoride is able to exert a longer-term anti-caries effect (Vogel, 2011). Fluoride concentration in biofilm fluid is the major factor governing remineralization and it is here that fluoride exerts its major anti-caries effects (Vogel, 2011). *In vitro* studies have shown that fluoride concentrations of 0.1 to 2 ppm increase mineral deposition in demineralized dental tissues. This can occur directly on the demineralized enamel surface or within the biofilm itself, creating fluoride mineral deposits (Margolis & Moreno, 1990). Despite the fact that in many studies, the salivary fluoride concentration is considered as a reference after the application of some remineralizing agent, a higher concentration of this element can be obtained within the dental biofilm (solid and fluid phase) (Staun Larsen et al., 2017). However, although fluoride clearance from the oral cavity is expected to occur under clinical conditions due to the diluting and washing effect of saliva, it is likely that this clearance effect does not occur under the static biofilm growth induced by the root caries model used in this study. Furthermore, the lack of continuous flow over the biofilms contributes to a long-lasting effect of the acidic environment on the dentin surface. Under our test conditions, all acids produced by the biofilm remained trapped and concentrated in the surrounding culture medium. It may be possible that both the anti-caries effect and the decrease in the acidogenic potential of biofilms demonstrated in this study occurred to a greater extent than in clinical conditions. Moreover, the *in vitro* caries model used in this study was built on a mono-species biofilm formed by a highly acidogenic microorganism that permits the rapid development of caries-like lesions. Furthermore, caries-like lesions were developed under well-controlled conditions not typically found in the oral cavity. Nevertheless, even with a relatively high amount of inherent sugars, fluoridated-milk-based drinks may have been able to modulate *S. mutans* sugar metabolism, leading to less acid production over time. In turn, by decreasing the acidogenic potential of *S. mutans* biofilms, the fluoridated-milk-based drink was able to decrease mineral loss.

The potential anti-microbial effect exerted by fluoride has been debated for many years. It has been shown that when fluoride is present in the medium, *S. mutans* strains tend to be highly susceptible to growth inhibition (Beighton & Hayday, 1980). Early studies demonstrated that the reduction in acid production induced by fluoride was mainly due to the inhibition of the enzyme enolase, altering the glycolytic pathway (Hamilton, 1990). The results of more contemporary experiments suggest that fluoride exerts dual antimicrobial effects: fluoride interferes with the growth of *S. mutans* by inhibiting critical metabolic processes (direct effect) and reducing environmental acidification (indirect effect) of biofilms (Bradshaw, Marsh, Hodgson, & Visser, 2002). In fact, biofilms treated with fluoride-supplemented milk-based drink led to a decrease in the counts of viable *S. mutans* compared with both non-fluoridated milk-based drink and sucrose-treated biofilms (Table 1), which may indicate a slight antimicrobial effect induced by fluoride. Interestingly, it appears that the sensitivity of bacteria to fluoride is dependent on the surrounding pH (Bradshaw et al., 1990; Whitford et al., 1977). Thus, considering the acidogenic potential of biofilms treated with fluoridated-milk-based drinks decreased over time (Fig. 1), it is possible that the increase in the pH of the spent medium attenuated the antimicrobial effect induced by fluoride, which could explain the similar counts of viable cells among the biofilms treated with the fluoridated milk-based drinks. Under our testing conditions, it appeared that the inhibition of *S. mutans* acid production induced by low fluoride concentrations may be more important for an anti-caries effect than any decrease in bacterial viability.

Interestingly, many studies have demonstrated the benefit of fluoridated milk for control of enamel caries-like lesions (Bánóczy et al., 2013; Itthagarun et al., 2011; Malinowski, Toumba, Strafford, & Duggal, 2018), little is known about the anti-caries effect of fluoride on root dentin. *In vitro* experiments have shown that milk supplemented with low doses of fluoride (2.5 to 5 ppm) are sufficient to obtain a positive effect in terms of remineralization or decrease of demineralization in enamel (R. A. Giacaman et al., 2012; Ivancakova, Hogan, Harless, & Wefel, 2003). However, when the amount of fluoride in milk increases to 10 ppm, a reduction in lesion depth and increased mineral content is observed, which indicates a potential protective effect of fluoridated milk on root caries (Arnold et al., 2014; Ivancakova, Harless, Hogan, & Wefel, 2005).

To our knowledge, the effect of fluoridated milk-based drinks on the prevention/control of root caries, especially in older adults, has not been investigated to date. Therefore, based on the data obtained in this study, we hypothesize that fluoridation of a milk-based drink may be clinically effective in terms of root caries control. This hypothesis needs to be confirmed either by *in situ* or in clinical studies. In relation to the age group that consumes dairy products, supplementing this food group with 10 ppm of fluoride is a measure that does not involve risks to the systemic health of the consumer. Furthermore, the doses used are in accordance with international recommendations for the maximum daily intake of fluoride (Bergman, Gray-Scott, Chen, & Meacham, 2009). Although the focus of this research was on root caries, it is important to note that, despite the safe and low doses of fluoride in milk supplementation, an exaggerated consumption of the supplemented food could carry a risk for dental fluorosis in children, in which the enamel is still developing (LeGeros, Glenn, Lee, & Glenn, 1985). For the target population of food supplementation analyzed in this study, and following the indications of consumption described on product packaging, adding the minimum amount of fluoride (5 ppm) would not carry risks to general health (Bergman et al., 2009).

5. Conclusions

Data from the present study permit us to conclude that the addition of at least 5 ppm NaF to a sugar-rich milk-based drink counteracts its cariogenic potential, leading to a reduction in both biofilm acidogenicity and root dentin mineral loss. Considering the recommendation for food supplements for older adults (World-Health-Organization, 2017), we envisage that the consumption of a fluoridated milk-based drink could reduce the burden of root caries in older adults. We acknowledge, however, that data from the present study cannot be directly translated into clinical practice. Clinical trials assessing the anti-cariogenic effect of fluoridated milk-based beverages in adults at risk of root caries development are required. Nevertheless, the consumption of fluoridated milk-based drink is a promising strategy for controlling root caries development in older adults consuming sugar-rich milk-based drinks.

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Study 4. Fluoridated milk-based drink and root caries development – an *in situ* study

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Short-title: Fluoridated milk and root caries

Keywords: Sucrose; Milk; Food supplement; Dentin; Demineralization; Fluoride; In situ; Dental biofilm

Declaration of interests

The authors declare no conflict of interest.

Introduction

The world is aging at an unprecedented rate and the older population over 60 years is expected to double by 2050 (ONU 2015). This phenomenon is considered one of the greatest epidemiological changes of the century, generating political, economic, socio-cultural and biomedical challenges (OMS 2015b). As part of these changes, natural teeth are retained longer in older adults, a condition that might be associated with increased risk for both dental caries and periodontal disease (Marino and Giacaman 2014; Nicolau et al. 2000). In this context, gingival recession that follows periodontal disease exposes the root, a reportedly more susceptible tissue than enamel to rapid demineralization, as dentin has a high organic content. Thus, root caries seems to affect preferably to older adults (Ekstrand et al. 2008). Furthermore, dental caries is the main cause of tooth loss among older persons, compromising their quality of life (León et al. 2014).

Among the consequences of tooth loss, functional masticatory impairment leads to a low-nutritional quality diet linked to a reduction in the consumption of fiber-rich foods (Castrejón-Pérez and Borges-Yáñez 2014) along with an increase in the intake of carbohydrate-rich foods with soft consistency (Boirie et al. 2014; Sugihara et al. 2010). Hence, malnutrition is commonly found in older adults (van Staveren and de Groot 2011). A number of dietary supplements designed for older adults, such as milk-based drinks (M-BD), have been developed to provide an adequate daily intake of nutrients (WHO, 2017). These non-specialized nutritional supplements usually contains varying amounts of proteins, fat, carbohydrates and significant amounts of sugar (20 to 36%) (Popkin 2006). It has been suggested that high-sucrose containing M-BD used as dietary supplement for older adults may be highly cariogenic, representing a potential risk for the onset of root caries (Castro et al. 2019).

A potential avenue to counteract the potentially deleterious effect of food supplements for elderly people is fluoride supplementation. We previously showed that supplementing M-BD with 5 ppm or 10 ppm fluoride decreased cariogenic potential reducing *in vitro* root dentin demineralization (Castro et al., 2020). In fact, regular consumption of fluoride-supplemented milk has been effective in reducing caries experience in children (Petersen et al. 2015; Weitz et al. 2007). The effect of fluoride supplementation of M-BD for older adults in root surfaces is unclear. We hypothesized that fluoride supplementation of M-BD at low concentrations decreases cariogenicity and prevent root caries.

Material and methods

Experimental design

A crossover split-mouth and double-blinded study was designed, divided into two experimental phases of 14 days each. Thirteen adult volunteers wore a removable palatal acrylic appliance containing bovine root dentin slabs. Slabs were exposed to a cariogenic challenge through *ex-vivo* sucrose exposure and volunteers were randomized according to the following treatments that had to be dripped onto the slabs: (1) deionized distilled water (DDW- control) and 10 ppm F-supplemented M-BD or (2) M-BD (control) and 5 ppm F-supplemented M-BD. A Seven-day wash-out phase was carried out between each phase. The inhibition of root dentin demineralization was assessed through changes on surface Knoop microhardness and by integrated mineral loss. Viable cells counts (total microorganisms, Mutans streptococci and Lactobacilli) and extracellular polysaccharide concentration were determined from the recovered biofilms. The experimental protocol of this *in situ* study was approved by the Bioethics Committee of the University of Talca, Talca, Chile (182017RCastro). Written informed consent was obtained from all subjects prior to the start of the study, according to the Declaration of Helsinki.

Volunteers

Thirteen volunteers (9 women and 4 men; mean age of 23.4 years $\pm$ 1.13) complied with the following inclusion criteria (Botelho et al. 2014): good general health, normal salivary flow (Ericsson 1959), absence of caries-lesions (ICDAS codes 3-6) and periodontal disease, full dentition, not having used antibiotics for at least the previous three months and not wearing any orthodontic appliance (fixed or removable). Subjects with inability to follow the study protocol as well as self-reported antibiotic- or fluoridated products use were excluded from the study at the recruiting phase. Sample size was calculated a priori, using G*Power (Faul et al. 2007) considering an medium average effect of 0.3, alpha risk of 0.05 and statistical power of 80% resulting in 128 samples, 32 slabs per study group. Considering 3 slabs per volunteer per study group, at least 10 volunteers should be enrolled. Considering 30% of drop-out, sample size calculation resulted in 13 subjects (n=39/study group).

Slabs preparation

One hundred-eighty dentin slabs (4x4x2mm) were prepared from disinfected bovine incisors, as previously described (Botelho et al. 2014). Baseline surface microhardness (SH_i) was determined using a Knoop microindenter (402 MVD, Wolpert Wilson Instruments, USA) by placing three indentations in the center of each slab, 100 µm apart from each other, at 10 g for 5 s. Slabs were selected based on their mean SH_i (41.49 n± 0.8 kg/mm^2). Slabs were sterilized with ethylene oxide (Thomas et al. 2007) and randomly distributed into the two experimental phases, making sure that mean SH_i was similar among the volunteers.

Palatal appliance preparation

Acrylic palatal appliances had 2 major slots (20 mm x 6 mm) on each hemi-arcade, located between the first premolar and second molar, which were further divided into 3 lateral slots (6 mm x 6 mm). Slabs were covered with a plastic mesh leaving a 1 mm space between the surface of the slab and the surface of the acrylic appliance to allow biofilm stagnation (Cury et al. 1997) (Figure 1) .

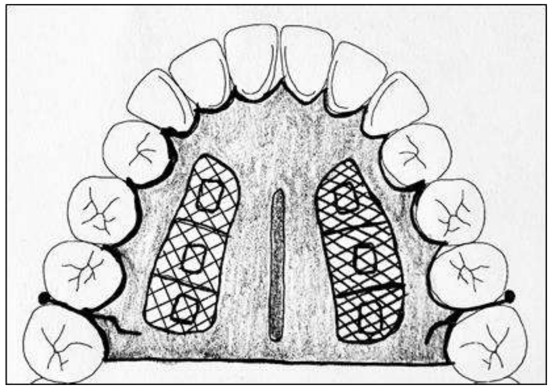

Figure 1. Diagram of the acrylic palatal device worn by the volunteers. Root dentine blocks were located in both sides of the appliance. A mid slot was designed to avoid spilling over of the experimental solutions.

Treatments

Fluoride-free toothpaste, a plastic box for *ex-vivo* intraoral acrylic appliance storage, sterile gauze and dripping bottles were provided to the volunteers. One of the bottles was labeled "Sucrose" (20% sucrose solution) and other two pre-coded bottles without labeling for the experimental solutions, to assure blindness. Volunteers were asked to drip extra-orally one drop of 20% sucrose solution onto each slab 8 times per day (8.30 am to 10.30 pm). At two specific times (8.30 a.m. and 8.30 p.m., simulating food intake times: breakfast and dinner), 5 minutes after dripping sucrose solution, a drop of the treatment solutions was applied onto each slab. After each dripping, volunteers were instructed to keep the palatal appliances inside a plastic box in a humid environment at room temperature for 5 minutes before placing it back inside the mouth. Any excess of the solution was dried out using sterile gauze to avoid carry over. Dripping bottles had to be manually shaken for 20 seconds before dripping. All solutions were prepared and replaced every 48 hours. Toothbrushing was done twice per day (morning and evening) with fluoride-free toothpaste, carefully avoiding brushing on the plastic mesh to avoid biofilm disruption. After the seven-day washout period, a new subset of slabs was provided at the beginning of each phase, so all the control/test solutions were applied by all the volunteers at the end of the study. Appliances had to be worn at all times (day and night) except for feeding, drinking and during toothbrushing. There was no dietary restriction. Compliance to the study protocol was assessed through mobile messaging (WhatsApp®) by the study coordinator. One of the researchers sent a remainder to each volunteer previous to each treatment time point. The volunteers had to reply to the remainder.

Biofilm analysis

By the end of each 14-day phase and ten hours after the last exposure to the test solutions, slabs and biofilms were retrieved, as previously described (Botelho et al., 2014). Biofilms were suspended in 1 mL of sterile distilled water and used for both bacterial culturing and extracellular polysaccharides quantification, while dentin slabs were used for microhardness analysis.

For bacterial culturing, an aliquot from the biofilm suspension was serially diluted in 0.9% NaCl (v/v) and inoculated (20 µL) into Brain Heart Infusion agar plates (for total microorganism count), Mitis salivarius bacitracin agar (for Mutans Streptococci counts) and on Rogosa agar (for *Lactobacillus* spp counts) (BD, Sparks, USA) (Herigstad, Hamilton, & Heersink, 2001). Plates were incubated for 24 hours at 37°C at 10% CO_2. Colony-forming units (CFU) were counted under a stereomicroscope (SMZ161, Motic, British Columbia, Canada). Results were expressed as colony-forming units CFU/mL of biofilm suspension.

Polysaccharide concentration in the biofilms was assessed as previously described (Koo et al. 2003). An aliquot of the biofilm suspension (200 µL) was centrifuged (10,000 g during 5 min at 4°C) and the pellet was resuspended into 200 µL of 1M NaOH, homogenized (30 seconds vortexed) and centrifuged. Three volumes of cold 100% ethanol were added to each different supernatant fraction, incubated for 30 min at − 20 °C, and centrifuged, and the supernatant was discarded. The resulting pellet was washed with cold 70% ethanol and centrifuged again. Insoluble extracellular polysaccharide (IEPS) concentration was determined using the sulfuric phenol method (DUBOIS et al. 1951).

Results were normalized by biofilm dry weight (Koo et al. 2003) and expressed as percentage of IEPS by milligrams of biomass.

Hardness analysis

The surface hardness of the dentin slabs after the *in situ* phases (SH_f) was assessed. Measurements were obtained by a row of three sequential indentations placed in the center of each slab, 100 µm apart from each other, and 100 µm to the right from the initial indentations. Percentage of surface hardness loss (%SHL) was estimated as follows: %SHL= (mean SH_i - mean SH_f) x 100 / SH_i.

Slabs were then sectioned through their center producing two halves. One of them was embedded in acrylic resin, leaving the sectioned surface exposed. Slabs were indented (10 g for 5 s) in the left fourth, in the middle and in the right fourth of the exposed surface by a row of 12 indentations in each one of them distant 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275 and 300 µm from the outer dentin surface (Featherstone et al. 1983). The integrated mineral loss (ΔZ; vol % min x µm) was determined for each slab using a previously reported formula (Featherstone et al. 1983), which considered the average value of the indentations at each depth.

Statistical analysis

Normal distribution of data was checked by the Kolmogorov–Smirnov test. The outcomes of %SHL, IEPS, counts of viable microorganisms and integrated mineral loss were compared among the tested conditions by ANOVA followed by a Tukey post-hoc test, using SPSS v15.0 (IBM, NY, USA) statistical software for windows. Differences were considered significant if $p<0.05$.

Results

All volunteers completed the two experimental phases, without drop-out and full compliance. No statistically significant differences were observed among DDW, M-BD, 5 ppmF M-BD and 10 ppmF M-BD on viable total microrganisms (7,67E+08 ± 2,67E+08; 6,56E+08 ± 6,56E+08; 4,89E+08 ± 4,89E+08 and 4,83E+08 ± 4,83E+08), Mutans Streptococci (5,00E+08 ± 5,77E+07; 6,00E+08 ± 1,53E+08; 4,89E+08 ± 3,85E+07 and 3,85E+07 ± 2,12E+08) or Lactobacilli counts (3,15E+09 ± 1,82E+09; 2,85E+09 ± 2,85E+09; 2,65E+09 ± 1,53E+09 and 2,60E+09 ± 2,60E+09) ($p>0.05$) (Data not shown). The concentration of IEPS found on biofilms exposed to M-BD was statistically higher than on biofilms exposed to DDW, 5 ppmF M-BD and 10 ppmF M-BD which were similar among them (Figure 2).

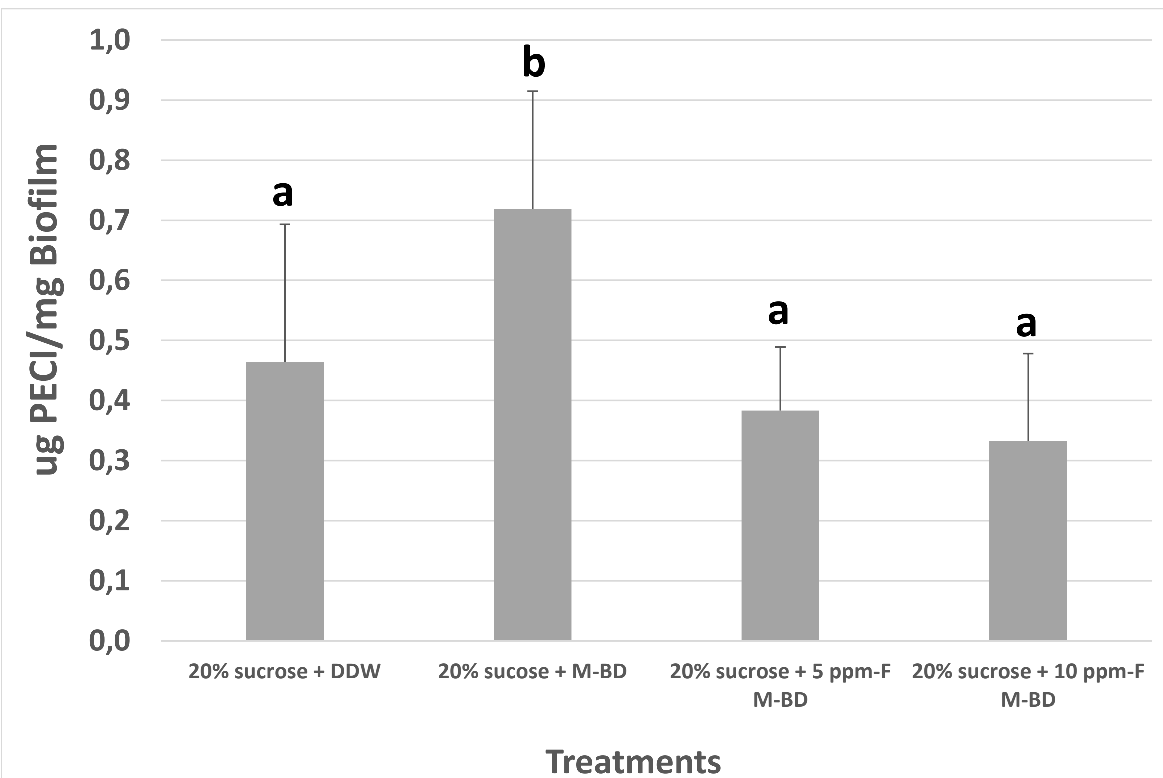

Figure 2. Concentration of insoluble extracellular polysaccharides (IEPS) on biofilms (n=13) by test conditions. Bars indicate mean values and error bars show SD. Different letters represent significant differences among treatments, $p<0.05$.

Regarding carious-like lesion development, there were statistical differences among all the tested conditions, with the highest mineral loss found on dentin slabs exposed to M-BD. A dose-dependent response was also found, with a %SHL on dentin slabs exposed to 10 ppmF M-BD lower than slabs exposed to 5 ppmF M-BD ($p<0.05$) (Figure 3). Likewise, slabs exposed to DDW and M-BD exhibited the highest integrated mineral loss (ΔZ). No differences were found on ΔZ between slabs exposed to 5 ppmF M-BD and 10 ppmF M-BD ($p>0.05$) (Table 1).

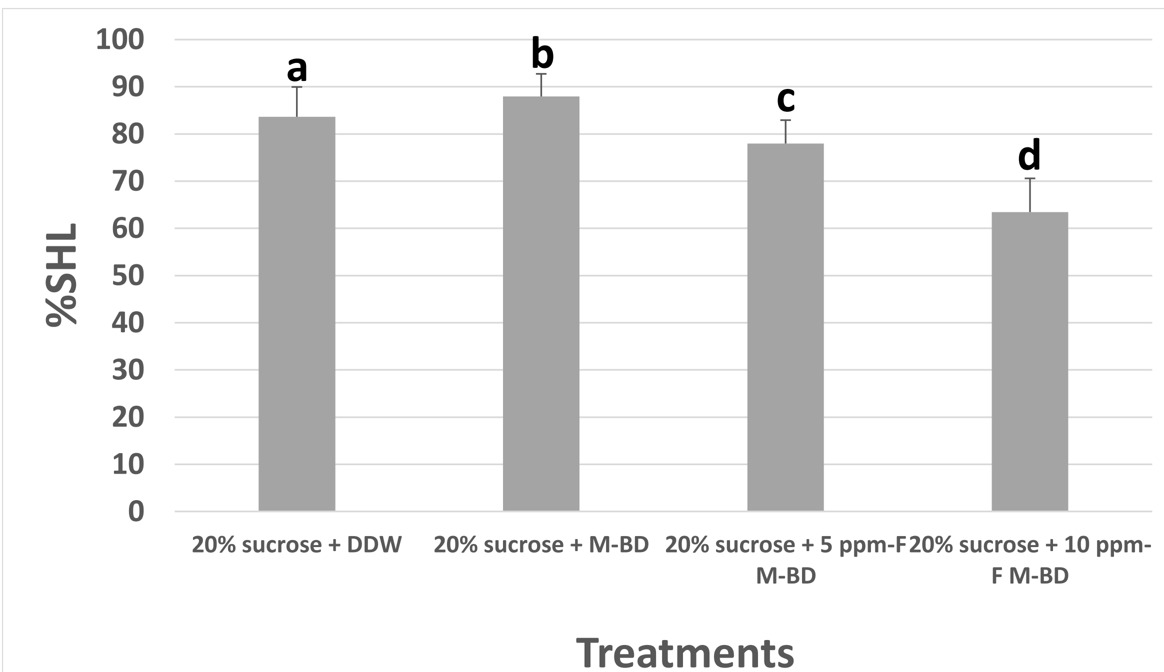

Figure 3. Percentage of surface hardness loss (%SHL) of dentin slabs by test conditions. Bars shown mean %SHL of the slabs. Error bars indicate SD. Different letters represent significant differences among treatments, p<0.05.

Table 1. Integrated Mineral loss (ΔZ; mean ± SD) of dentin slabs according to he treatments.

Treatments	Mineral Loss (ΔZ)
20% sucrose + DDW	1,024.3 ± 73.9 [a]
M-BD	1,183.6 ± 162.5 [a]
5 ppmF M-BD	592.4 ± 42.4 [b]
10 ppmF M-BD	746.7 ± 46.8 [b]

Means followed by different superscript letters differ statistically among treatments, $p<0.05$.

Discussion

Our results show that supplementation of a sugared M-BD with low doses of fluoride intended for older adults reduces both cariogenicity and the cariogenic potential induced by frequent exposure to sucrose. These findings are of importance due to increasing trend for root caries among older adults and the extensive use of milk-based supplements among this growing population worldwide.

Current evidence from *in vitro* studies suggests that milk supplementation with up to 10 ppmF may have some caries-protective effect on root dentin either reducing demineralization or enhancing remineralization, in a dose-dependent manner (Arnold et al. 2014; Ivancakova et al. 2005; Ivancakova et al. 2003). More recently, we reported that supplementation of M-BD with 5 or 10 ppmF decreased biofilm acidogenicity, *S. mutans* biofilms and root dentin demineralization (Castro et al. 2020). Although a slight antimicrobial effect of fluoride could be expected, our data showed no differences on the counts of viable cells among test conditions. Despite the fact that the tested fluoride concentration may have reduced the acidogenic potential from the biofilm, as we observed *in vitro* (Castro et al., 2020), acidification induced by the frequent exposure to sucrose (8x/day) may have overcame fluoride effect on bacterial acid production. Although the pH of the *in situ* formed biofilms was not assessed, it is plausible that frequent pH drops derived from frequent sucrose exposure led to an ecological pressure over the biofilm microbiota greater than any expected antibacterial effect induced by fluoride. Thus, it is reasonable that bacterial counts for total and cariogenic bacteria (Mutans streptococci and *Lactobacilli*) were similar among fluoridated- and non-fluoridated treatments. Moreover, although fluoride could have an antimicrobial effect on oral bacteria by inhibition of cellular enzymes or enhancing proton permeability of cell membranes in the form of hydrogen fluoride (HF) (Buzalaf et al. 2011), this effect occurs at higher concentrations than those found in topical fluorides (Maltz and Emilson 1982). As an example, concentrations of fluoride in saliva or oral biofilms, resulting from the application of a 1,500 ppm fluoridated toothpaste, are not sufficient to obtain the antimicrobial benefit (Lynch et al. 2004).

As previously described, the M-BD available and freely distributed in Chile for older adults is formulated with sucrose plus the inherent carbohydrate content in milk, reaching 8% final concentration (Castro et al. 2018). A previous *in situ* study showed that IEPS is synthesized in a sucrose concentration-dependent manner (Aires et al. 2006), which could explain the higher IEPS concentration found in biofilms exposed to sucrose followed by the M-BD compared to only sucrose (Figure 2). Moreover, the role of IEPS on the cariogenic potential of biofilms is well-know (Tenuta et al. 2006) which explains the higher %SHL found on those slabs exposed to the two non-fluoridated groups (DDW and M-BD) (Figure 3). Interestingly, the IEPS concentration of biofilms exposed to 5 ppmF- or 10 ppmF- supplemented M-BD was similar to the biofilms exposed to sucrose alone (20% Sucrose + DDW), but lower than the sucrose and M-BD group, suggesting that fluoride may induce a decrease in the cariogenic potential from food supplements based on milk intended for elderly people. Furthermore, the addition of fluoride to the M-BD was also effective in promoting remineralization of the newly formed carious lesions due to the sucrose challenge. Our results *in situ* are consistent with those previously reported *in vitro,* indicating that 5 ppm and 10 ppm fluoride-supplemented M-BD reduced the %SHL of dentin slabs in comparison to sucrose-only exposed ones in a dose-dependent manner (Castro et el. 2020). The mechanism underlying this effect seems not to be derived from antimicrobial or by altering bacterial metabolism, but physicochemical on demin/remineralization (Tenuta and Cury 2010). This protective effect of fluoridated M-BD was also detectable by ΔZ since slabs exposed to 5 ppm and 10 ppm fluoridated M-BD presented lower integrated mineral loss compared to non-supplemented M-BD and sucrose-only treated slabs (Table 1). In this assay, we could not observe the dose-response effect between 5 and 10 ppm of fluoride, which could be attributable to the fact technique sensitive of the ΔZ measurement. Indeed, some degrees of variability and lack of sensitivity have been previously reported in the literature (Buchalla et al. 2008).

Although the strategy of fluoridating milk has been reported as successful in children and adolescents (Bánóczy et al. 2013), its preventive effect may be lost when the program ceases (Mariño et al. 2004). This approach has been considered safe, as long as the fluoride supplementation is kept in low concentrations and under parental or school surveillance, being, moreover, a cost-effective (Bánóczy et al. 2013). In children, risk comes from the possibility of developing fluorosis, which does not apply in adults or older adults. According to current recommendations, adults should not have an intake of more than 4 mg of fluoride per day (Bergman et al. 2009). An average portion of the tested M-BD (200 mL) supplemented with 10 ppmF contains 2 mg of fluoride. Based on security and effectiveness, fluoridating milk-based food supplements for older adults may be a promising strategy to reduce the burden of dental caries, especially root caries.

While the results of this study offer a first look at a potential modulating effect of the cariogenicity of M-BD through fluoride supplementation, these findings need to be further explored under well-controlled clinical trials. Given the precarious oral health condition in older adults, there is an urgent need to dedicate more attention to possible ways of coping with oral health problems in this population. In elderly, tooth loss due to caries has a powerful negative effect. Aesthetic alterations (Hugo et al. 2007), functional impairment (Silva et al. 2010) and emotional stress (Okoje et al. 2012) related to tooth loss seriously affect people's quality of life (Gerritsen et al. 2010). If this cost-effective, safe and simple modification of the dairy product can control the potential harm elicited from milk-based supplements, these findings would be a real contribution to the quality of life of older persons.

In conclusion, supplementation of a milk-based product with low doses of fluoride (5 and 10 ppm) may reduce its cariogenicity, increasing at the same time the remineralization potential of root surfaces and contributing to control the increasing trend for root caries in elderly people.

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R. Castro: Contributed to conception, design, data acquisition and interpretation, performed all statistical analyses, drafted and critically revised the manuscript

R. Giacaman: Contributed to conception, design, data interpretation, drafted and critically revised the manuscript and gave final approval.

M. Maltz: Contributed to conception, design, data interpretation, drafted and critically revised the manuscript.

R. Arthur: Contributed to conception, design, data interpretation drafted and critically revised the manuscript.

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CONCLUDING CONSIDERATIONS

On the basis of the objectives proposed in this thesis, together with the relevant and current scientific information, revised to give structure to each of the scientific articles derived from it, valuable new knowledge has been generated.

There is broad acceptability and consumption of nutritional supplements within older adults. Although those supplements aim to enhance the nutritional status of their consumers, our data suggest that sugar-rich dairy products pose coronal and root surfaces at a risk for caries development even though almost 90% of the elderly consume this product ≤2cups a day. Therefore, Government, manufacturers and nutritionists should be aware of the potential risks of this product for caries development.

Our clinical and laboratory studies suggest that a slight modification in the formula of the milk-based drink, by incorporation of low-doses of fluoride significantly reduces its cariogenic potential against root dentin. The incorporation of fluoride to the product could represent a substantial benefit in terms of protection against caries improvement, generating a potential functional food.

Altogether, our data show that:

- Daily consumption of the milk-based drink distributed by the supplementary feeding program for the elderly is related to an increased risk for coronal and root caries lesions development.
- By using a root caries experimental model, the milk-based drink showed a potential risk for root caries due to its high acidogenic and cariogenic potentials.
- Small amounts of fluoride (5 and 10 ppm) added to the milk-based drink was able to decrease its acidogenic potential under an *in vitro* condition. Moreover, these same amounts of fluoride were able to decrease its cariogenic potential under both *in vitro* and *in situ* conditions, revealing an interesting approach to be used as preventive strategy for root caries management in older adults.

CONSIDERAÇÕES FINAIS

Com base nos objetivos propostos nesta tese, juntamente com a informação científica relevante e atual revisada para dar estrutura a cada um dos artigos científicos derivados da mesma, foram produzidos novos conhecimentos.

Há uma ampla aceitabilidade e consumo de suplementos nutricionais por adultos idosos. Embora esses suplementos visem melhorar o estado nutricional dos seus consumidores, os nossos dados mostram que os produtos lácteos ricos em açúcar colocam as superfícies coronárias e radiculares sob risco de desenvolvimento de cárie radicular mesmo que cerca de 90% dos idosos consumam ese produto numa quantidade menor que 2 copos por dia. Dessa forma, Governo, Indústria e nutricionistas precisam estar cientes do potencial risco de desenvolvimento de cárie devido ao consumo desse produto apresenta.

Nossos resultados demonstram que a adição de pequenas quantidades de flúor à uma bebida à base de leitereduz significativamente o seu potencial cariogênico sobre a dentina radicular. A incorporação do flúor à bebida pode representar um benefício substancial em termos de proteção contra cárie gerando um alimento potencialmente funcional.

De forma geral, nossos dados mostram que:

- O consumo diário da bebida à base de leite distribuída pelo programa de alimentação suplementar para os idosos está relacionado com um aumento no risco de desenvolver lesões de cárie coronária e radicular.

- Ao utilizar um modelo experimental *in vitro* de cárie radicular, observa-se que essa bebida á base de leite apresenta potencial de induzir lesão de cárie radicular devido ao seu elevado potencial acidogênico e cariogênico.

- Pequenas quantidades de flúor (5 e 10 ppm) adicionadas à bebida à base de leitefoi capaz de reduzir o seu potential acidogênico *n vitro*. Além disso, esas mesmas quantidades de flúor foram capazes de reduzir o potencial cariogênico da bebida em condição *in vitro* e *in situ*, sugerindo que essa abordagem pode ser utilizada como uma estratégia preventiva promissora para manejo de cárie radicular em idosos.

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