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Acid-producing capacity from sugars and sugar alcohols among *Lactobacillus* isolates collected in connection with radiation therapy

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ABSTRACT

Objective: To investigate the acid-producing capacity from sugars and sugar alcohols of oral *Lactobacillus* collected in connection with radiation therapy (RT) to the head and neck region.
Design: *Lactobacillus* were collected from the tongue, buccal mucosa and supragingival plaque in 24 patients before, during, and after RT. The acid-producing capacity of *Lactobacillus* isolates (n = 211) was analyzed using a colorimetric fermentation test in microtiter plates. Solutions containing 2% sugars (sucrose, glucose, fructose, lactose) or sugar-alcohols (sorbitol and xylitol) were used. After 24 h of incubation, bacterial acid-producing capacity was determined as strong (pH < 5), weak (pH ≥5–≤ 6) or low/absent (pH > 6). Data regarding intake frequency of sugar-rich products and products with sugar-alcohols was collected.
Results: The highest acid-producing capacity using the sugars was seen for isolates collected during RT. Sorbitol was fermented to a higher extent during and post RT, especially among isolates from plaque. *Lactobacillus* fermenting xylitol showed the highest acid-producing capacity during RT ($p < 0.05$). No statistically significant correlations between stimulated whole salivary secretion rate and acid-producing capacity, or between the intake frequency of sugar-rich products or sugar-alcohol containing products and Lactobacillus acid-producing capacity, were found.
Conclusion: The results suggest that *Lactobacillus* isolates, collected from the tongue, buccal mucosa and supragingival plaque, have a higher acid-producing capacity using sugars and sugar-alcohols during RT than one year post RT.

1. Introduction

Radiation therapy (RT) to the head and neck region results in a reduced salivary secretion rate when the major salivary glands are included in the radiation field (Baharudin, Khairuddin, Nizam, & Samsuddin, 2009). Pain in the mouth is common during radiotherapy (Trotti et al., 2003) and may lead to a diet rich in soft food items, which are rich in easily fermentable carbohydrates, favoring the growth of lactobacilli. Patients who have undergone RT to the head and neck region often harbor high numbers of lactobacilli, compared with healthy subjects (Al-Nawas & Grötz 2006; Almståhl, Wikström, & Fagerberg-Mohlin, 2008). This shift to a more acidogenic microflora is associated with a low oral pH (Almståhl, Wikström, Stenberg, Jacobsson, & Fagerberg-Mohlin, 2003) and an increased risk of caries (Spak, Johnson, & Ekstrand, 1994).

Approximately 100 different species of *Lactobacillus* are identified, out of which, e.g., *L. rhamnosus*, *L. salivarius*, *L. paracasei*, *L. casei*, *L. plantarum*, *L. vaginalis* and *L. fermentum* have been found in the oral cavity (Badet, Richard, & Dorignac, 2001; Munson, Banerjee, Watson, & Wade, 2004; Caufield, Li, Dasanayake, & Saxena, 2007; Simark-Mattsson, Jonsson, Emilson, & Roos, 2009; Almståhl, Carlén, Eliasson, & Lingström, 2010). The ability to produce acids during fermentation of carbohydrates varies greatly between the different species of *Lactobacillus* (Piwat, Teanpaisan, Dahlén, Thitasomakul, & Douglas, 2012). In a previous study by the authors, the acid-producing capacity of 50 *Lactobacillus* isolates, collected from supragingival plaque of irradiated patients, 3–5 years after RT, and from patients with primary Sjögren's syndrome, was tested (Almståhl, Lingström, Eliasson, & Carlén, 2013). The majority of the isolates lowered the pH to < 5.0 using sucrose, and all isolates using fructose. An ability to ferment the sugar-alcohol mannitol was found for 52% of the isolates, whereas 50% could ferment sorbitol and 36% xylitol. It is not known whether these isolates were already present in the oral cavity or if they were acquired during or after RT.

Knowledge regarding the acid-producing capacity for *Lactobacillus,* collected from other oral ecological niches such as mucosal sites, is

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lacking, as well as the bacterial adaptation to changed dietary habits over time of *Lactobacillus,* isolated from patients who have undergone RT to the head and neck region. If the *Lactobacillus* are favored by frequent intakes of both sugar-rich products and sugar-alcohols, this must be taken into account when giving dietary advice to patients undergoing treatment for cancer of the head and neck region.

The aim of the present study was to investigate the acid-producing capacity from sugars and sugar-alcohols of *Lactobacillus* isolates, collected from the dorsum of the tongue, buccal mucosa, and supragingival plaque in patients with cancer of the head and neck region, during and after RT.

2. Materials and methods

This study is part of a larger project entitled "Longitudinal changes in saliva, microflora, diet and oral status in relation to the quality of life in people receiving radiotherapy to the head and neck region". The project has been reviewed by the Regional Ethical Review Board at the University of Gothenburg (No. 682-07). The patients with cancer were recruited by Dr. Fagerberg-Mohlin, who was responsible for the odontological treatment of this patient category at the Department of Oral and Maxillofacial Surgery, Institute of Odontology, Sahlgrenska Academy, University of Gothenburg, Sweden. The patients were included consecutively. Inclusion criteria were: Age ≥18 years, ≥16 natural teeth, no removable prosthesis or implants including more than a single tooth. Thirty-three patients were included in the project. Age, gender, number of teeth and filled teeth were registered. All appointments were between 8 am and 11 am. Data was collected pretreatment, during treatment, six months and one-year post treatment.

2.1. Intake of sugar-rich products and sugar-alcohols

A 39-item questionnaire was used to collect data on the patients' intake of sugar-rich products and products containing sugar-alcohols, such as fluoride tablets, sugar-free tablets/chewing gums, saliva-stimulating sprays, and gels for dry mouth relief. The patients were asked to fill in how often they ate/drank/used each item on a 6-grade scale, with the following answer alternatives: Several times per day, once a day, several times a week, once a week, once a month, seldom/never.

2.2. Microbial sampling

Microbial samples were collected according to Almståhl and Wikström (2005) from the tongue, buccal mucosa, and from supragingival plaque. Before sampling the mucosal sites, saliva was dried off using sterile swabs. For sampling of the tongue, a plastic spatula with a round hole (1.5 cm in diameter) was used to standardize the sampled area. A cotton pellet, which had been immersed in sampling fluid VMGA I (Möller, 1966), was swept back and forth over the area inside the hole and was then transferred to a bottle with transport media VMGA III (Möller, 1966). For sampling of the buccal mucosa, a cotton pellet was first immersed in VMGA I (Möller, 1966), and then swept back and forth three times over an area of approximately 2 cm^2 near the orifice of the parotid gland, and then transferred to VMGA III. Supragingival plaque was collected using sterile toothpicks (TePe Björk, TePe Munhygienprodukter AB, Malmö) from four interproximal sites between the first and second molar in each quadrant, and transferred to VMGA III. When a site was missing, the sample was taken from the nearest site available.

2.3. Stimulated whole salivary secretion rate

The patients were informed to avoid eating, drinking (except water), tooth brushing and smoking one hour prior to their appointment. They were instructed to chew on a piece of paraffin until it was soft and to swallow once. Thereafter, the patient continued to chew on the paraffin wax and all saliva produced was collected in a test tube for three minutes.

2.4. Microbial analysis

The samples in the VMGA III were incubated in 36 °C for 30 min and then shaken on a Whirlimixer for 10 s. One hundred microliters of the sample were inoculated on Rogosa-SL agar plates (Difco Laboratories, Detroit, Michigan, USA), using the three stroke method (Dahlén, Linde, Möller, & Öhman, 1982) and incubated in 90% CO_2 and 10% N_2, in 36 °C for three days.

2.5. Quantification of lactobacilli

Growth of the lactobacilli was assessed as no growth, very sparse growth (< 10 colonies), sparse growth (> 10–100 colonies), moderate growth (growth in the second stroke), and heavy growth (growth in the third stroke).

2.6. Isolation of isolates

On plates showing at least sparse growth, one colony of the dominant *Lactobacillus* isolate/s (one or two different isolates), identified by colony morphology and Gram-staining, was transferred to a new Rogosa agar plate and incubated as previously described. After incubation, all cells were harvested and transferred to a Cryobank tube and stored at −70 °C.

2.7. Cultivation

Before the fermentation test, the *Lactobacillus* isolates were transferred from the Cryobank tube and cultivated on Rogosa agar plates, as described above. After 48 h of incubation, one colony was transferred to broth (Brain Heart Infusion (BHI – Bacto™) containing 1% glucose (pH 7.4). The tubes were incubated over night in 90% CO_2 and 10% N_2, at 36 °C and harvested at the mid-exponential phase.

On the day of the experiment, the cultured bacterial cells were centrifuged (Hettich EBA 35, Hettich Lab Technology, Tuttlingen, Germany) for 5 min at 1300*g*. To eliminate traces of glucose from the culture medium, the pellet was washed twice with Phosphate buffered saline (PBS) pH 7.1 and diluted to an optical density (λ = 650 nm) of 1.0 (Novaspec II Spectrophotometer, Pharmacia Biotech, Ace Lab Systems, St. Louis, MO, USA).

2.8. Fermentation of sugars and sugar alcohols

A slightly modified version of the fermentation assay, described by Hedberg, Hasslöf, Sjöström, Tvetman, & Stecksén-Blicks, 2008, was used to test the acid production from sugars and sugar alcohols. Fifty microliters of carbohydrate-free culture medium and 50 µl of 2% solution of various sugars (sucrose (Difco, USA), lactose (Merck, Germany), fructose (Merck), glucose (Difco), sorbitol (Merck) and xylitol (AB R. Lundberg, Sweden)), were mixed in duplicate wells in microtiter plates (Nunc, Denmark), together with 10 µl of a mixture (5 µl bacterial solution and 5 µl indicator solution (0.3% Bromocresol purple, Svenska Finkemikalier)). The pH indicator turns yellow at pH 5.2 and purple at pH > 6.8. PBS was used as a negative control. The microtiter plates were incubated in 90% CO_2 and 10% N^2, at 36° C for 24 h. After inoculation, the color of the wells was registered by photography using a digital camera (Canon EOD 450D) connected to a computer. The pH of the wells was measured for different colors and a visual scale was created as a reference. The acid-producing ability was determined by the authors (HR and AB), by use of the visual scale as yellow (strong acid-producing capacity, pH < 5), red/brown (weak acid-producing capacity, pH ≥5–≤6), or purple (low/absent acid-producing capacity, pH > 6). Duplicated wells were used for each isolate and the test was