PRODUCTION OF CARCINOGENIC ACETALDEHYDE BY ORAL MICROBIOME

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ACADEMIC DISSERTATION

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To my mother

Contents

ABBREVIATIONS

ADH	Alcohol dehydrogenase enzyme
AIRE	Autoimmune regulator gene
ALDH	Aldehyde dehydrogenase enzyme
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
APECED	Autoimmune polyendicrinopathy-candidiasis-ectodermal dystrophy
CCUG	Culture Collection of University of Gothenburg
CFU	Colony forming unit
CHX	Chlorhexidine
CO	Control patient
CO_2	Carbon dioxide
CYP2E1	Cytochrome P450 2E1
DNA	Deoxyribonucleic acid
EBV	Ebstein-Barr virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
IARC	International Agency for Research on Cancer
IL	Interleukin
Km	Michaelis constant
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide phosphate
OD	Odds ratio
od	Optical density
OLD	Oral lichenoid disease
OLL	Oral lichenoid lesion
OLP	Oral lichen planus
OSCC	Oral cavity squamous cell carcinoma
PCA	Perchloric acid
PDC11	Pyruvate decarboxylase enzyme

PVL	Proliferative verrucous leucoplakia
ROS	Reactive oxygen species
r_s	Spearman's Rho
RR	Relative risk
SEM	Standard error of mean
UK NEQAS	United Kingdom National External Quality Assessment Service
WHO	World Health Organization
XDH	Xylitol dehydrogenase enzyme

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I. Acetaldehyde production from ethanol by oral streptococci. Kurkivuori J, Salaspuro V, Kaihovaara P, Kari K, Rautemaa R, Grönroos L, Meurman JH, Salaspuro M. *Oral oncology* 2007;43:181-186

II. Chronic candidosis and oral cancer in APECED-patients: Production of carcinogenic acetaldehyde from glucose and ethanol by *Candida albicans*. Uittamo J, Siikala E, Kaihovaara P, Salaspuro M, Rautemaa R. *International Journal of Cancer* 2009;124:754-756

III. Xylitol inhibits carcinogenic acetaldehyde production by *Candida* species. Uittamo J, Nieminen MT, Kaihovaara P, Bowyer P, Salaspuro M, Rautemaa R. *International Journal of Cancer* 2011;129:2038-2041

IV. Acetaldehyde production and microbial colonization in oral squamous cell carcinoma, lichen planus and lichenoid reaction. Marttila E, Uittamo J, Rusanen P, Lindqvist C, Salaspuro M, Rautemaa R. Manuscript.

Publication #II has been used as a part of dissertation of author Siikala E. Publication III. will be used as a part of the dissertation of author Nieminen MT.

ABSTRACT

Oral cancer is the seventh most common cancer worldwide and its incidence is increasing. The most important risk factors for oral cancer are chronic alcohol consumption and tobacco smoking, up to 80 % of oral carcinomas are estimated to be caused by alcohol and tobacco. They both trigger an increased level of salivary acetaldehyde, during and after consumption, which is believed to lead to carcinogenesis.

Acetaldehyde has multiple mutagenic features and it has recently been classified as a Group 1 carcinogen for humans by the International Agency for Research on Cancer. Acetaldehyde is metabolized from ethanol by microbes of oral microbiota. Some oral microbes possess alcohol dehydrogenase enzyme (ADH) activity, which is the main enzyme in acetaldehyde production. Many microbes are also capable of acetaldehyde production via alcohol fermentation from glucose. However, metabolism of ethanol into acetaldehyde leads to production of high levels of this carcinogen. Acetaldehyde is found in saliva during and after alcohol consumption. In fact, rather low ethanol concentrations (2-20mM) derived from blood to saliva are enough for microbial acetaldehyde production. The high acetaldehyde levels in saliva after alcohol challenge are explained by the lack of oral microbiota and mucosa to detoxify acetaldehyde by metabolizing it into acetate and acetyl coenzymeA.

The aim of this thesis project was to specify the role of oral microbes in the *in vitro* production of acetaldehyde in the presence of ethanol. In addition, it was sought to establish whether microbial metabolism could also produce acetaldehyde from glucose. Furthermore, the potential of xylitol to inhibit ethanol metabolism and acetaldehyde production was explored.

Isolates of oral microbes were used in the first three studies. Acetaldehyde production was analyzed after ethanol, glucose and fructose incubation with gas chromatography measurement. In studies I and III, the ADH enzyme activity of some microbes was measured by fluorescence. The effect of xylitol was analyzed by incubating microbes with ethanol and xylitol. The fourth study was made *ex vivo* and microbial samples obtained from different patient groups were analyzed.

This work has demonstrated that isolates of oral microbiota are able to produce acetaldehyde in the presence of clinically relevant ethanol and glucose concentrations. Significant differences were found between microbial species and isolates from different patient groups. In particular,

the ability of candidal isolates from APECED patients to produce significantly more acetaldehyde in glucose incubation compared to healthy and cancer patient isolates is an interesting observation. Moreover, xylitol was found to reduce their acetaldehyde production significantly. Significant ADH enzyme activity was found in the analyzed high acetaldehyde producing streptococci and candida isolates. In addition, xylitol was found to reduce the ADH enzyme activity *of C. albicans*. Some results from the *ex vivo* study were controversial, since acetaldehyde production did not correlate as expected with the amount of microbes in the samples. Nevertheless, the samples isolated from patients did produce significant amounts of acetaldehyde with a clinically relevant ethanol concentration.

INTRODUCTION

Oral cancer is the seventh most common cancer worldwide and has a poor prognosis, with a five year survival rate varying between 40-56%. The main risk factors are chronic alcohol consumption and tobacco smoking, in particular the conjoint use, which increases the relative risk for oral cancer multiplicatively. In developed countries, an estimated 75-80% of all oral cancers can be explained by the use of alcohol and tobacco.

Ethanol itself is not a carcinogen, but its first metabolite, acetaldehyde, is. The International Agency for Research on Cancer has recently classified acetaldehyde, associated with alcohol consumption, to be a group 1 carcinogen for humans (Secretan et al. 2009). Acetaldehyde is capable of causing sister chromatid exchanges, point mutations, and interfering with the DNA-repair system. It has also been reported that acetaldehyde forms DNA-adducts (Seitz and Stickel 2009).

Most acetaldehyde in the oral cavity is produced by some members of the oral microbiota. In studies where alcohol was consumed, salivary acetaldehyde levels were found to be markedly elevated (Homann et al. 1997). Acetaldehyde is produced mainly by the microbial alcohol dehydrogenase (ADH) enzyme. Alcohol is further rapidly metabolized in the liver into acetate by the low Km aldehyde dehydrogenase (ALDH2) enzyme. Aerobic bacteria that represent human colonic flora have a limited capacity to detoxify acetaldehyde (Nosova et al. 1996, Nosova et al. 1998). This appears to be the case also with regard to oral microbiota (Salaspuro 2011). As mucosal cells lack low Km ALDH enzymes (Dong 1996), these conditions favor the accumulation of acetaldehyde in saliva during and after an alcohol challenge.

APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) is a rare autosomal disease that constitutes part of the Finnish Heritage of Diseases. It is caused by mutations in the AIRE gene. APECED patients have a markedly increased risk for oral cancer. In fact, over 10 % of adult patients with APECED in Finland have been diagnosed with oral cancer. The cancer has been found in the place of chronic candidosis which is one of most typical symptoms of the disease. Candidosis has been shown to be related to carcinomas but the mechanism is yet to be discovered.

Most risk factors for oral cancer appear to be associated with an increased exposure of the oral cavity to acetaldehyde via saliva. The risk factors include alcohol, tobacco, poor oral hygiene,

and gene polymorphisms associating with enhanced acetaldehyde production from ethanol (Salaspuro 2003a, Salaspuro 2009a). Acetaldehyde exposure via saliva can, however, be markedly reduced. The number of oral microbes and acetaldehyde load from ethanol can be significantly decreased by using antimicrobial mouthwash containing chlorhexidine (Homann et al. 1997). Medical devices slowly releasing L-cysteine can be used for the binding and inactivation of acetaldehyde both in the mouth and achlorhydric stomach (Salaspuro et al. 2002, Salaspuro et al. 2006, Linderborg et al. 2010). Xylitol has been shown to have an "anticariogenic" impact and some inhibition in the metabolism of the *S. mutans* have been reported (Söderling 2009). However, the possible effects of xylitol on microbial acetaldehyde production are thus far unknown.

Review of the literature

1. Oral Cancer

Over 100 000 new oral cancers were diagnosed in Europe in 2006, making it the seventh most common cancer (3.2% of all cancers diagnosed) (Ferlay et al. 2007). The prevalence of oral cancer varies between different countries; in South-Central Asia, Melanesia and Central and Eastern Europe oral cancer is found more frequently (Jemal et al. 2011). The incidence for oral cancer in Europe is especially high in Hungary and France (La Vecchia et al. 1997). The worldwide incidence is increasing, only the incidence for lip cancer is decreasing (Scully 2011).

Oral cavity squamous cell carcinoma (OSCC), originating from the oral keratinocyte, accounts for over 90% of all oral cancers. Other malignant lesions of the oral cavity include, for example, sarcomas, salivary gland malignancies, and metastases from other cancers (Baykul et al. 2010). OSCC continues to portend a poor prognosis, with an estimated 5-year overall survival rate of 40-56% (Kademani et al. 2005, Scully and Bagan 2009). OSCC is typically found in the tongue, floor of the mouth, or gingivae (Silverman 2001), and is usually discovered as a red patch called erythroplakia, or white patch called leucoplakia, a nonhealing ulcerative lesion. In later stages, loosening of teeth, for example, may occur (Neville and Day 2002). Early OSCC manifestations are usually asymptomatic, but when the disease is in the advanced stage, the patients can suffer from pain, numbness, and additional difficulties in chewing, opening of the mouth, and swallowing. Sometimes an enlarged lymphatic node in the neck can be the first sign of the disease. Lymphovascular invasion typically takes place in the early stage of the disease and metastases can be found (Leon et al. 2000). A large primary tumor size is associated with increased cervical lymph node metastasis, increased risk of local recurrence, and poor survival (Woolgar 1999, Silverman 2001). The risk of developing a second OSCC primary tumor is relatively high (Silverman 2001).

1.1 Etiology of oral cancer

Carcinogenesis requires a series of events where a normal cell transforms into potentially malignant cell. Mutagenic changes in the cellular DNA can occur spontaneously, or due to different substances such as alcohol, tobacco, and UV-radiation. The main risk factors for oral cancer are alcohol consumption and tobacco smoking (La Vecchia et al. 1997, Poschl and Seitz 2004), which are estimated to cause 75-80% of all oral cancers (La Vecchia et al. 1997). Viral infections such as human papillomavirus (HPV), are sometimes able to cause mutagenic abnormalities as well. In addition, potentially malignant lesions, Betel, dietary factors, and poor dental hygiene, have been estimated to have a role in the etiology of oral cancer. A normal cell has many ways to protect itself from mutagenic changes, for example, the DNA repair system is able to correct changes in the DNA. In cases where correction fails, normal cells begin to apoptose, undergoing controlled cell death. Mutated cells may escape apoptosis, proliferate autonomously, and thus carcinoma can begin to grow (Scully 2011). Nevertheless, the host immune system should detect and destroy such abnormalities. Defects in the immune system, as seen in patients suffering from autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), can predispose to carcinomas.

1.1.1 Alcohol

Alcohol consumption is one of the main risk factors for oral cancer (Seitz et al. 1998, Salaspuro 2003b, Poschl and Seitz 2004, Boffetta and Hashibe 2006, Secretan et al. 2009), its effect is strongly dose-dependent (IARC 1986, IARC 1988, Pelucchi et al. 2008). Based on two meta-analyses, the relative risk (RR) for oral and pharyngeal cancer is increasing rapidly with the concordant overall increase in alcohol consumption. Based on the results of Bagnardi et al's meta-analysis, where 26 studies concerning oral and pharyngeal cancer were included, the RR for oral and pharyngeal cancer is increased to 1.75 when the daily use of ethanol is fairly low; 25 g, which corresponds to approximately two doses of alcohol. With a daily intake of 50 g of ethanol, the RR is 2.85, and when the intake is increased to 100 g, the RR is 6.01 (Bagnardi et al. 2001). In the meta-analysis by Zeka et al, the numbers are even higher; with daily ethanol dose of 0-48 g the RR is 1.5 and when the dose exceeds 48 g/day the RR is 7.2 (Zeka et al. 2003). These results are also confirmed by the latest meta-analysis (Tramacere et al. 2010).

Ethanol itself as a molecule is not a carcinogen, but its first metabolite, acetaldehyde, is

(Homann et al. 1997, Salaspuro 2003a, Langevin et al. 2011, Joenje 2011). Acetaldehyde associated with alcohol consumption is regarded as 'carcinogenic to humans' (IARC Group 1) (Secretan et al. 2009). Upon consumption of alcohol, acetaldehyde is produced immediately by the microbes in the oral cavity (Salaspuro 1996, Homann et al. 1997, Väkeväinen et al. 2000, Salaspuro 2003a, Salaspuro 2003b, Linderborg et al. 2011). The microbial production of acetaldehyde may continue in the oral cavity four hours even after the end of alcohol consumption. This is due to the even distribution of ethanol to the whole water phase of the human body, including saliva (Jones 1979, Jones 1983, Gubala and Zuba 2002).

Certain alcohol beverages are believed to be more carcinogenic, such as calvados (Salaspuro 2011). In the areas where calvados is being used such as southern France, oral cancer incidence is higher (La Vecchia et al. 1997, Scully and Bagan 2009, Launoy et al. 1997). Calvados contains much more acetaldehyde than many other commonly used alcoholic beverages (Linderborg et al. 2008). High acetaldehyde concentrations have also been found in fortified wines such as port wines and sherries (Lachenmeier and Sohnius 2008). Very high acetaldehyde concentrations were recently reported to be in strong fruit spirits, which are used frequently in East European countries. This may explain the particularly high incidence of oral cancer in these countries (Boffetta et al. 2011). On the contrary, the lowest acetaldehyde concentrations are found in well distilled vodkas and beer (Lachenmeier and Sohnius 2008).

A point mutation in ALDH2-gene provides strong evidence for the local carcinogenic action of acetaldehyde in the upper digestive tract via saliva. This gene mutation affects hundreds of millions of East Asians and results in decreased ALDH2 enzyme activity, in addition to decreased ability to detoxify acetaldehyde (Yokoyama et al. 2003). Consequently, ALDH2 deficiency results in markedly increased upper digestive tract cancer risk and elevated acetaldehyde levels in saliva after an alcohol challenge as will be discussed in subsequent chapters.

1.1.2 Tobacco

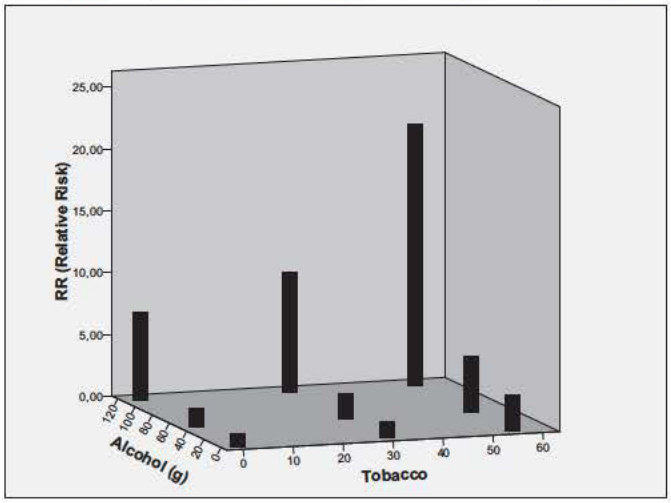
Tobacco smoking is an independent risk factor for oral cancer (IARC 1986). Warnakulasuriya et al. reviewed 11 different studies and calculated that the RR for upper digestive tract cancers in tobacco smokers varies between 1.6 and 20.7 (Warnakulasuriya et al. 2005). The amount of tobacco used has a strong impact on the RR. In a study by Moreno-Lopez et al., it is stated that when 1-20 cigarettes are smoked daily the RR for oral cancer is 3.15, but when more than 20

cigarettes are smoked daily the relative risk increases to 12.5 (Moreno-Lopez et al. 2000).

Carcinogenic acetaldehyde is one of the most toxic constituents of tobacco smoke and it can be found both in breath and saliva after smoking (Shaskan and Dolinsky 1985, McLaughlin et al. 1990, Smith et al. 2002, Salaspuro and Salaspuro 2004). Tobacco smoking also generates other carcinogens, such as tobacco specific nitrosamines and free radicals, which can impact on antioxidant enzymes such as catalase and glutathione reductase (Hoffmann et al. 1994, Brunnemann et al. 1996, Smith et al. 2002, Smith et al. 2003, Scully and Bagan 2009). However, acetaldehyde is the most abundant carcinogen in the tobacco smoke; given that its concentration is 1000 times higher than that of polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines (Hoffmann et al. 2001).

Alcohol and tobacco have a synergistic effect on cancer incidence, the cancer risk increases multiplicatively if both are used (Zeka et al. 2003, Hashibe et al. 2009) (Fig.1). The synergistic use of alcohol and tobacco results in synergistically increased acetaldehyde levels in saliva (Salaspuro and Salaspuro 2004).

Figure 1. The synergistic effect of alcohol and tobacco on cancer risk (modified from Zeka et al.). The cancer risk is strongly dose-dependent and the risk increases multiplicatively when alcohol and tobacco are both used.



1.1.3 Ultraviolet radiation

Ultraviolet radiation from the sun is a known risk factor for oral cancer, for lip cancer in particular. In a study by Kenborg et al., the OD for lip cancer was 1.67, if the patient had been working for more than 10 years outdoors (Gallagher and Lee 2006, Kenborg et al. 2010). Based on the review by Gallagher et al., there is sufficient evidence for a causal relationship between ultraviolet radiation from the sun and cancer of the lip. Radiation can cause mutations in DNA since it is able to enter the cell (Gallagher and Lee 2006).

1.1.4 HPV

Human papillomavirus (HPV) is reported to be the most important cause of cervical cancer and it has also been linked to oral cancer (zur Hausen 2009, Marur et al. 2010, Faridi et al. 2011). The role of HPV in cervical cancer is clear; more than 95% of biopsies from cervical cancer contain HPV (zur Hausen 2009). In oral cancer patients, the HPV prevalence has been 23.5% (Syrjänen 2010). Zur Hausen estimates that 25-30% of oropharyngeal cancers could be induced by HPV (zur Hausen 2009). A strong association of HPV with potentially malignant oral lesions, such as oral lichen planus and oral leukoplakia, was shown in a recent review (Syrjänen et al. 2011). Viral infection caused by HPV can occur when the virus enters the host cell. HPV infection type 16, in particular, can cause disruption in the host genome which might lead to mutagenic changes (Gillespie et al. 2009). The virus is able to inhibit the tumor suppressor p53 which enables the malignant growth of the tumor (zur Hausen 2002). HPV infection is most likely sexually transmitted and a wide number of sexual partners increases the odds for the infection (D'Souza et al. 2007). Nevertheless, controversial studies of the role of HPV in carcinogenesis are published. Based on a recent review, the role of HPV in oral cancer is not clear (Koshiol et al. 2010). The review concerns, however, only a Chinese population and it can be questioned, whether the results are consistent with other populations.

1.1.5 Potentially malignant lesions

Oral lichen planus (OLP) is a chronic systemic inflammatory disease caused by T-cell immunodysregulation (Al-Hashimi et al. 2007, Bidarra et al. 2008). Furthermore, Tao et al. showed that T regulatory cells (Treg cells) play a role in the pathology of OLP (Tao et al. 2010). Treg cells play a central role in maintaining immunologic tolerance to self and non-self, therefore changes in their action might expose patients to autoimmune diseases. The clinical

criteria for OLP issued by the World Health Organisation (WHO), indicates that mucosal lesions are usually bilateral, more or less symmetrical, and they contain different kinds of white lines (van der Meij and van der Waal 2003). Based on 26 different studies, the malignant transformation rate of oral lichen planus varies between 0.4% and 5.6% (van der Meij et al. 2006). In another study, where 229 patients with OLP were followed up for four years, four of the patients were diagnosed with oral cancer which makes the risk for carcinoma approximately 1.7% (Chainani-Wu et al. 2001). A review by Ramos-e-Silva et al., concludes that the possibility for OLP to develop into oral carcinoma is minor but exists clearly (Ramos-e-Silva et al. 2010). Oral lichenoid lesion (OLL) differs from OLP by its etiology though the clinical features resemble each other. OLP is typically bilateral unlike OLL. The etiology for OLL is believed to be type IV hypersensitivity reaction, for example allergy to dental filling material (Al-Hashimi et al. 2007, Muller 2011).

Leucoplakia is a clinical term concerning a white patch in the oral mucosa that cannot be characterized histopathologically, or via any other means, as anything else (McCullough et al. 2010, Kramer et al. 1978). Leucoplakia can be, for example, hyperkeratosis, dysplasia, or caused by tobacco, and it can develop a papillary surface and a severe form that is proliferative verrucous leucoplakia (PVL). PVL can progress into a verrucous carcinoma (Neville and Day 2002). Dysplastic changes can often be seen in leucoplakia and the risk for carcinoma is approximately 3.1% and equals that seen in lichen planus (Neville and Day 2002). Candida infection can be seen in leucoplakia, based on a study by Chiu et al., the recurrence rate of leucoplakia is increased in patients with candidosis (Chiu et al. 2011).

In a study by Wang et al., it was suggested that some oral, potentially malignant lesions, become malignant due to deficiency in the DNA repair system (Wang et al. 2007). Further studies are still warranted in order to establish whether this hypothesis is a fact, but it may at least in part explain the faith of some potentially malignant lesions.

1.1.6. Betel

Smokeless tobacco has also been found to increase the risk of oral cancer, including the habit of betel quit chewing for instance, which is fairly common in some Asian countries such as India, Pakistan, Bangladesh, China, and Thailand (Reichart and Nguyen 2008). The International Agency for Research on Cancer states that betel quid chewing, with and without tobacco, as ‘carcinogenic to humans (Group 1)’ (IARC 2004). The carcinogenic features caused by betel

quid chewing are possibly due to the areca nut which is the main component of betel quid. Areca nut contains arecoline which can induce mutagenic changes such as structural chromosomal aberrations and sister chromatid exchange (Chen et al. 2008). Nitrosamines derived from areca nut can also play a role in carcinogenesis (Zhang and Reichart 2007).

1.1.7 Dietary factors

Several epidemiological studies have been published on possible dietary correlates of oral cancer (La Vecchia et al. 1997, Riboli and Norat 2003, Pavia et al. 2006, Scully and Bagan 2009). Based on a recent review, the pooled RR for high vegetable consumption varies between 0.52 and 0.65 meaning that the intake of vegetables may be protective to oral cancer (Lucenteforte et al. 2009). In contrast, the consumption of red meat and eggs has been related to increased oral cancer risk, although a consensus view has not been reached in the literature (Levi et al. 1998, Franceschi et al. 1999, Tavani et al. 2000, Lucenteforte et al. 2009). The etiological basis behind dietary factors remains unclear and it has been suggested that current dietary factors are related to the socioeconomical status of the patients that can be linked to oral cancer (Garavello et al. 2008).

1.1.8 Poor dental hygiene

Poor dental hygiene has been associated with oral and esophageal cancer (Abnet et al. 2008). Based on two case-control studies made by Guha et al., the odds ratio (OD) for poor or average condition of the mouth and oral cancer was 4.51 (Guha et al. 2007). A larger amount of oral microbes (Abnet et al. 2005) and increased production of acetaldehyde from ethanol (Homann et al. 2001, Chocolatewala et al. 2010) have been suggested to contribute to the elevated cancer risk associated with poor dental hygiene. The role of microbes in the etiology of oral cancer has been pointed out as well in the review by Meurman (Meurman 2010). The author states that microbes may induce carcinomas due to ability to produce acetaldehyde but also for example due to act as tumor promoters.

1.1.9 APECED

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autosomal disease caused by mutations of the autoimmune regulator (AIRE) gene (Heino et al. 2001, Vogel et al. 2002, Mathis and Benoist 2007, Laakso et al. 2010). AIRE mutations mainly cause defects in T lymphocytes, which are central players in the immune system (Fierabracci

2011). Defects in the members of the Interleukin-family (IL) have also been found, such as the dysregulation of IL-7 (Laakso et al. 2011), that lead to autoimmune responses to various tissues, especially to endocrine glands. The most common features of the syndrome are chronic mucocutaneous candidosis, primary adrenocortical insuffiency and hypoparathyroidism. For a positive diagnosis, two of these are required (Husebye et al. 2009). The diagnosis can be hard to reach due to large variability of symptoms in patients. Other possible components are diabetes mellitus, gastrointestinal diseases, ectodermal dysplasia, keratoconjuctivitis, autoimmune hepatitis, vitiligo, alopecia, pernicious anemia, asplenia and dental, nail and tympanic membrane dystrophies (Perheentupa 2006, Husebye et al. 2009).

The disease is rare outside of the populations of Finland, Sardinia and Iranian Jews (Husebye et al. 2009), and it is part of the Finnish Heritage of Disease (Norio 2003). Over 10 % of all APECED patients in Finland over the age of 25 are diagnosed with OSCC. The age of the patients at the time of diagnoses has been reported to be 29-44 years which is significantly lower than OSCC patients in general (Rautemaa et al. 2007). The etiology behind carcinomas remains unclear, however, carcinomas often develop in the areas of chronic mucositis which might partly explain the carcinogenesis.

2. Oral microbiome

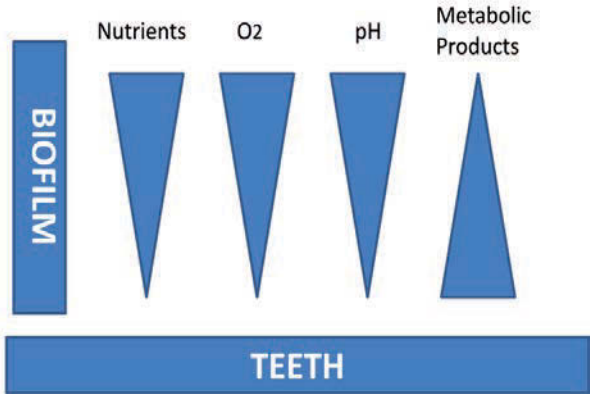
2.1 General

The oral microbiome is diverse and consists of a wide range of bacterial species, fungi, viruses, and sometimes even protozoa (Marsh and Martin 2005, Aas et al. 2005, Dewhirst et al. 2010). Over 750 different species have been identified by culture based methods and more are likely to be found. Up to 10^8 CFU/ml (colony forming unit/milliliter) bacteria can be found in saliva (Jenkinson 2005, Marsh and Martin 2009). The use of molecular methods has had an impact on our understanding of the oral microbiome (Dewhirst et al. 2010, Jenkinson and Lamont 2010). Pyrosequencing, in particular the use of PCR, has increased the resolution at which the microbiome can be analyzed (Keijser et al. 2008, Ghannoum et al. 2010, Jenkinson 2011).

Oral microbes readily form a biofilm on oral surfaces such as teeth or mucosa (Avila et al. 2009, Jenkinson 2011). By forming this three-dimensional structure, microbes gain multiple properties, including an improved protection against host defenses and new invading microbes. Salivary proteins that bind onto tooth surfaces, and form a pellicle, enable microbial binding, the first step

of biofilm formation. Mainly gram-positive bacteria, such as some species of streptococci and actinomyces, are first adsorbed to the pellicle and begin to multiply (Marsh and Martin 2009, Jenkinson 2011). These are often referred to as pioneer species, which thereafter create conditions suitable for other microbes. Respiration reduces the oxygen tension and increases the level of carbon dioxide resulting in hypoxic conditions (Marsh et al. 2011) (Fig.2). Most oral micro-organisms are facultative anaerobes and thrive under these conditions. For example oral streptococci can survive deep in a biofilm. In addition, bacteria, fungi, and mycoplasmas can also be found in oral biofilms and they comprise the main part of the oral microbiome. In addition, protozoa such as trichomonas species have been found in salivary samples, which have been linked to defects in the host immune system (Martinez-Giron and van Woerden 2011).

FIGURE 2. The metabolism of biofilm. In the deepest part of biofilms conditions are hypoxic. The metabolism of the pioneer species use nutrients and results in metabolic products. For example the concentration of potassium is higher and the concentration of sodium is lower within the biofilm compared to conditions at the surface. The pH level also decreases as a result of metabolic activity.



2.2 Bacteria

Oral streptococci, found all parts of the oral cavity, form an essential part of the oral microflora. They comprise up to 50% of the total cultivable flora and 15% of the total oral microbiome (Jenkinson and Lamont 2005, Keijser et al. 2008). Oral streptococci and especially *S. mutans* are the primary pathogens causing dental caries. Other gram-positive cocci such as enterococci, staphylococci, and micrococci, can be found in low numbers from several oral sites. The latter two are often found in the nasal flora in addition to the oral cavity (Marsh and Martin 2009).

Actinomyces and lactobacilli are the most commonly identified gram-positive rods in the oral cavity. Actinomyces species have been reported to be central in forming the dental biofilms and they comprise 8% of the dental plaque flora (Keijser et al. 2008). Lactobacilli are usually found in the oral cavity but they appear to comprise only 1% of the oral flora (Keijser et al. 2008).

Neisseria gram-negative cocci species are the most commonly detected in the oral cavity and in dental biofilm. The relative amount of Neisseria species in saliva has been reported to equal that of actinomyces species (Keijser et al. 2008). Oral Neisseria are rarely associated with diseases (Marsh and Martin 2009). Haemophilus and aggregatibacteria species are the most common gram-negative rods in the oral cavity. Haemophilus bacteria can be found in saliva, on epithelial surfaces, and in dental biofilm, contributing approximately 4% to the relative plaque amount (Keijser et al. 2008). Aggregatibacteria, especially *A. actinomycetemcomitans* has been associated with aggressive forms of periodontal diseases; their relative amount in a healthy oral microbiome is less than 1% (Keijser et al. 2008, Marsh and Martin 2009).

Although many oral micro-organisms are facultative anaerobic, obligate anaerobic gram-negative cocci and rods such as veillonella, eubacterium, prevotellae and fusobacterium spp. are commonly seen in the oral cavity as well. Veillonella, which comprises some 13% of plaque flora, can produce lactic acid and has been recognized to be one of the caries pathogens due to its ability to dissolve enamel (Marsh 1999, Keijser et al. 2008). Prevotella species are common especially in saliva; the relative amount is 20% (Keijser et al. 2008). Porphyromonas species and *Treponema denticola* have been linked to periodontal diseases and the relative amount of current bacteria increases in the absence of proper oral hygiene (Avila et al. 2009). *Fusobacterium nucleatum* has been shown to be able to invade epithelial cells. The bacterium also thrives in biofilms, the relative amount in plaque is 7% (Keijser et al. 2008, Avila et al. 2009)

Mycoplasmas are pleomorphic micro-organisms that can also be isolated from the oral cavity. The outer membrane of mycoplasmas is not rigid and therefore they differ from other oral microbes (Marsh and Martin 2009). They can be considered to be surface parasites, typically isolated as *Mycoplasma pneumonia*, which can cause infection in the upper respiratory tract (Razin 1996).

2.3 Fungi

Most of the fungi found in the oral cavity belong to the genus candida, although fungi of other genera such as aspergillus and saccharomyces can occasionally be seen (Marsh and Martin 2009, Ghannoum et al. 2010). Candida species are considered to be part of the oral microbiome and they can be found on any surface of the oral cavity. The most sensitive place for isolation of candida is the vestibular sulcus (Rautemaa et al. 2006).

Candida can reside in the oral biofilms and the proportion of candida in biofilms can be especially high in patients with oral candidosis (Thein et al. 2006, Rautemaa and Ramage 2011). In healthy people, the oral carriage rate of Candida is 75 % (Ghannoum et al. 2010). *Candida albicans* is by far the most commonly isolated yeast in the oral cavity (Marsh and Martin 2009). Other clinically relevant candida species include *C. tropicalis*, *C. krusei* and *C. glabrata* which are often referred to as "non-*Candida-albicans*-species" (Marsh and Martin 2009, Nieminen et al. 2009).

Oral candidosis is a superficial infection, which can be acute or chronic. Acute infection is usually caused by antimicrobial or local corticosteroid treatment, whilst chronic candidosis is more frequently seen in immunocompromised patients (Rautemaa and Ramage 2011). Chronic mucocutaneus candidosis is a rare form of the disease which has been diagnosed especially in APECED patients (Perheentupa 2002, Rautemaa and Ramage 2011). This form of candidosis has been linked to oral cancer (Rautemaa et al. 2007).

2.4 Viruses

Viruses differ in many ways from bacteria since they are not capable of reproducing themselves without the host cell. Viral infection initiates when virus enters the host cell and reacts with the cellular genome facilitating the commencement of Viral RNA and/or DNA production. Herpes simplex viruses, cytomegalovirus and human papillomavirus are the most commonly detected viruses in the oral cavity (Marsh and Martin 2009).

Herpes simplex viruses type 1 and 2 are the most common viruses in the oral cavity. Type 1 is the most common and it is the primary causative agent of cold sores. The herpes infection is usually acquired in childhood (Arduino and Porter 2006), after which the virus can remain latent in the trigeminal nerve ganglion and cause symptoms occasionally. Cytomegalovirus is part of the herpes virus family and is often detected in the saliva, whereas human papillomaviruses

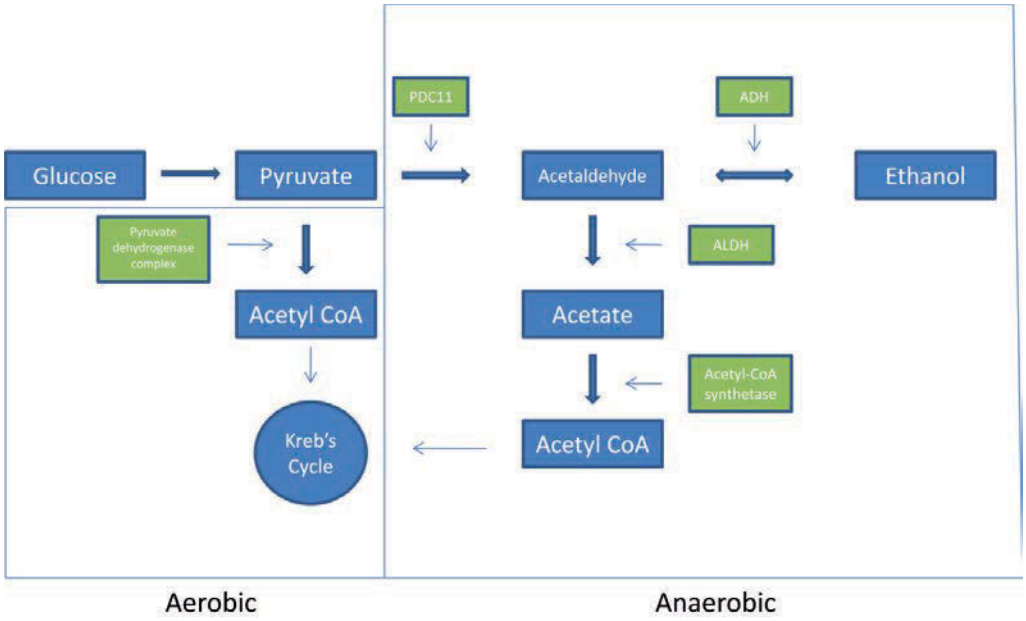
(HPV) can be found sometimes in the oral cavity. HPV has been connected to oral cancer, particularly HPV type 16 (Syrjänen 2010, Syrjänen et al. 2011, Termine et al. 2011).

Human immunodeficiency virus (HIV) can also be detected in the oral cavity. The most common oral manifestations of HI-virus are candidiasis, oral hairy leukoplakia and acute or chronic ulcerative gingivitis (Gennaro et al. 2008). The severe forms of HIV in oral manifestations are Kaposi's sarcoma (Leao et al. 2009) and lymphomas such as Non-Hodgkin's lymphoma. Ebstein-Barr virus (EBV) has also been associated with the lymphomas in the oral cavity (Sarode et al. 2009). EBV can cause severe disorders in immunosuppressive patients, similar to HIV patients (Dojcinov et al. 2010).

2.5 Energy metabolism of the oral microbes

Carbohydrates are the main energy source for oral microbes. They all are able to metabolize carbohydrates such as glucose in order to form ATP (Flores et al. 2000, Marsh and Martin 2009, Petranovic et al. 2010, Zhang et al. 2010) (Fig 3.). In the metabolic route, glucose is converted to pyruvate by glycolysis. The fate of pyruvate depends on the oxygen tension and the microbe. In *C. albicans*, pyruvate is directly metabolized into Acetyl CoA by pyruvate dehydrogenase complex in aerobic conditions, but under hypoxia the anaerobic route activates (Askew et al. 2009). In the latter reaction, acetaldehyde is formed. This formation of ethanol from glucose constitutes alcohol fermentation. In a recent study by Rozpedowska et al., it was shown that *C. albicans* is able to produce ethanol from glucose under anaerobic conditions, although the amount was found to be small (Rozpedowska et al. 2011). The authors stated that the metabolism of *C. albicans* prefers aerobic conditions. The atmosphere in the oral cavity is mostly microaerophilic and most likely both metabolic pathways are needed. Ethanol is toxic to microbes in high concentrations which could lead to metabolism avoiding ethanol accumulation.

Figure 3. The metabolism of glucose. Glucose is first metabolized into pyruvate in the reaction called glycolysis. The fate of pyruvate depends on the aerobic conditions. Both routes produce ATP but the end-products differ.



3. Ethanol metabolism

3.1 Distribution of ethanol

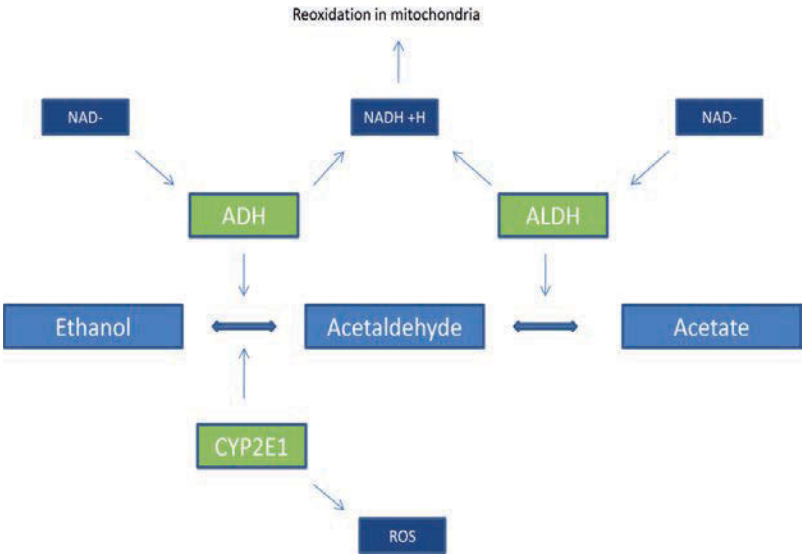
Ethanol is a water soluble substance with a small molecular size. Due to these characteristics it is absorbed by simple diffusion from the intestine without a transport mechanism (Crabb et al. 1987). The distribution of ethanol is equal for all of the body's liquid compartments, which results in equal concentrations of ethanol in saliva and blood (Jones 1979, Gubala and Zuba 2002).

3.2 Ethanol and acetaldehyde metabolism

The liver is the main site for ethanol metabolism in a healthy person (Seitz et al. 1994). Ethanol is metabolized via three pathways; by alcohol dehydrogenase enzyme (ADH), cytochrome P450 2E1 (CYP2E1) and by catalase. Metabolism by catalase plays a minor role in ethanol metabolism, however ADH and CYP2E1 metabolic pathways are of principal interest (Seitz and

Stickel 2007). These two pathways catalyze the initial oxidation of ethanol into acetaldehyde. Acetaldehyde is further oxidized into acetate by aldehyde dehydrogenase enzymes (ALDH) (Fig 4.).

Figure 4. Ethanol metabolism in humans. Ethanol is metabolized into acetaldehyde and further into acetate by reversible reactions.



The first reversible reaction where ethanol is oxidized into acetaldehyde via ADH is:
$CH_3CH_2OH + NAD \leftrightarrow CH_3CHO + NADH + H$. In this reaction acetaldehyde and a reduced form of nicotinamide adenine dinucleotide (NADH) are formed. When ethanol is metabolized into acetaldehyde via CYP2E1, the by-products of the reaction are reactive oxygen species (ROS).

ADH and ALDH can be found not only in the liver but also in digestive tract cells. In a study performed by Dong et al., biopsies of gingival, lingual mucosa, and tongue were analyzed by their ADH and ALDH activity. Results were compared to the activities of stomach, esophagus, and colon. All biopsies investigated were found to possess ADH and ALHD activity, though the low Km ALDH activities of oral samples were found to be under the detection limit (Dong et al. 1996). In a recent study by Koschier et al., a 3-D oral mucosal model, the 3-D EpiOral, was exposed to mouth washes containing alcohol. No acetaldehyde was found and the writers

therefore declare that oral mucosa does not possess ADH activity (Koschier et al. 2011). The conclusions are controversial when compared to earlier literature (Moreno et al. 1994, Dong et al. 1996, Jelski et al. 2002, Seitz and Stickel 2007, Jelski and Szmitkowski 2008), which may be due to the fact that there were deficiencies in the study protocol. For example the artificial model lacks a connective tissue layer which is needed for the proper metabolic activity of oral mucosa (Lachenmaier and Salaspuro 2011).

Microbes of the normal oral flora possess ADH activity and are responsible for the majority of acetaldehyde production in the oral cavity (Homann et al. 1997, Salaspuro 2003a, Salaspuro 2009a, Seitz and Stickel 2007).

3.3 Human ADH and ALDH enzymes

Human ADHs have been grouped into five classes I – V by Jörnvall et al. (Jörnvall and Hoog 1995). AHD I is named to be "the classical ADH" because it is responsible for the majority of enzymatic activity in the liver, but it can be found also in gastrointestinal tract, kidneys, and lungs (Jelski and Szmitkowski 2008). ADH I is composed of α-, β- and γ-subunits, which are encoded by ADH1A, ADH1B and ADH1C genes. Mutations in these alleles have been shown to be reflected in salivary acetaldehyde levels. Three different allelic forms of ADH1B have been found. ADH1B*2 allele results in approximately 40 times more active enzyme compared to enzyme encoded by ADH1B*1 allele. Two forms of ADH1C have been found. ADH1C*1 form leads to enzyme that is 2.5 times more active than ADH1C*2 (Bosron and Li 1986, Poschl and Seitz 2004, Jelski and Szmitkowski 2008). The ADH1B*2 allele is frequent in Asia and it has been announced to protect from alcoholism due to increased levels of acetaldehyde in blood after alcohol drinking. Increased blood levels of acetaldehyde can cause a flushing syndrome, which includes facial flushing, tachycardia, nausea, and even vomiting, after alcohol consumption (Enomoto et al. 1991, Higuchi et al. 1995, Seitz and Stickel 2007).

ADH1C*1 allele results in increased levels of acetaldehyde in saliva after an ethanol challenge, which has been suggested to lead to increased risk for upper digestive tract cancer (Visapää et al. 2004). The epidemiological data of the increased cancer risk is, however, controversial (Coutelle et al. 1997, Sturgis et al. 2001, Poschl and Seitz 2004). The discrepancy has been explained by differences in the geographic distribution of ADH1C genotypes in Europe and by the fact that the negative studies have generally included controls with minor or moderate alcohol

consumption and the positive studies with alcoholics or heavy drinkers (Homann et al. 2006). Class II ADH has been found in liver, class III exists in all tissues, and class IV mainly in the gastrointestinal tract. ADH V is poorly known (Jelski and Szmitkowski 2008).

Ten different human ALDH enzymes have been found. ALDH2 is responsible for most acetaldehyde oxidation (Seitz and Stickel 2007, Jelski and Szmitkowski 2008). The ALDH2 enzyme has two allelic forms, the normal allele is named ALDH2*1 and the mutated form is known as ALDH2*2. The mutated form has a limited capacity to oxidize acetaldehyde into acetate, which results in increased acetaldehyde levels. Homozygous mutation of ALDH2*2 has been found to protect from alcoholism in the same way as ADH1B*2 (Higuchi et al. 1995). A heterozygous form of ALDH2*2, instead, does not cause that severe flushing reaction, approximately 30-50% of the enzyme activity can still be reached (Poschl and Seitz 2004). Most importantly, ALDH2-deficiency results in elevated levels of acetaldehyde in saliva after alcohol challenge. After a moderate dose of alcohol, salivary acetaldehyde levels were found to be two to three times higher in patients with heterozygous mutant ALDH2*2 compared to patients with ALDH2*1 (Väkeväinen et al. 2000, Väkeväinen et al. 2001). These findings were confirmed by a Japanese group (Yokoyama et al. 2003).

The association of ALDH2 deficiency with increased upper digestive tract cancer risk was discovered by Yokoyama et al., who reported that the heterozygous form of the current enzyme is connected to a strong incidence of upper digestive tract cancers among Japanese alcoholics (Yokoyama et al. 1996, Yokoyama et al. 1998). These findings have been later confirmed in multiple studies and meta-analyses (Brennan et al. 2004, Wu et al. 2005, Hashibe et al. 2006, Chen et al. 2006, Asakage et al. 2007, Lee et al. 2008, Guo et al. 2008, Gianfagna et al. 2008).

3.4 Microbial metabolism of ethanol

Several gastrointestinal tract microbes have been found to posses ADH-activity (Salaspuro 1996, Jokelainen et al. 1996, Jokelainen et al. 1996b, Nosova et al. 1997, Salaspuro et al. 1999, Muto et al. 2000, Salaspuro 2003a), as evidenced by strongly increased salivary acetaldehyde levels during and after ethanol consumption (Jokelainen et al. 1996a, Homann et al. 1997, Väkeväinen et al. 2000, Homann et al. 2000, Homann et al. 2001, Salaspuro 2003b, Linderborg et al. 2011) In the study by Väkeväinen et al., it was demonstrated that isolates of *Neisseria* in particular have high ADH activity and therefore the ability to produce acetaldehyde in ethanol incubation

was significantly higher than other isolates analyzed (Väkeväinen et al. 2001). This is in line with the study by Muto et al., where the ability of Neisseria to produce a significant amount of acetaldehyde in ethanol incubation was shown (Muto et al., 2000). Väkeväinen et al. also discovered that *Streptococcus salivarius* differs from other streptococci analyzed by its high ADH activity since other streptococci analyzed were found to have low ADH activity (Väkeväinen et al. 2001).

Tillonen et al. showed that *C. albicans* strains isolated from the oral cavity are able to produce marked amounts of acetaldehyde from ethanol *in vitro* (Tillonen et al. 1999). Bertram et al reported that ADH1 is primary responsible for ethanol oxidation in *C. albicans* (Bertram et al. 1996), but also ADH2 has been found (Jones et al. 2004). ADH1 has been shown to be a bidirectional enzyme being able to catalyse the metabolism of ethanol into acetaldehyde as well as of acetaldehyde into ethanol (Bertram et al. 1996). The expression of ADH in *C. albicans* has been shown to differ in different growth phases and the activity of ADH is increased especially in the exponential growth phase (Bertram et al. 1996).

GI-tract microbes have also been found to possess some ALDH activity (Nosova et al. 1996, Nosova et al. 1998), but these were considerably lower than ADH activities. This might explain the accumulation of acetaldehyde both in the saliva and large intestine since ALDH is responsible for the reaction where acetaldehyde is further metabolized into acetate (Jokelainen et al. 1996, Jokelainen et al. 1996a, Salaspuro 2011).

4. Acetaldehyde as a carcinogenic substance

The International Agency for Research on Cancer (IARC) classified acetaldehyde recently as a group 1 carcinogen to humans (Secretan et al. 2009). Thus acetaldehyde included in beverages and formed endogenously during and after ethanol consumption is carcinogenic to humans (Salaspuro 2011). Acetaldehyde is a highly toxic, mutagenic, and carcinogenic substance that is able to facilitate the formation of DNA-adducts at a clinically relevant concentration (100 µM) (Theruvathu et al. 2005, Toh et al. 2010). These concentrations can be found in saliva after moderate ethanol consumption (Homann et al. 1997). Mutagenic 1,N^2-propanodeoxyguanosine adducts are formed in dividing cells when acetaldehyde is converted into crotonaldehyde in dividing cells by polyamines (Theruvathu et al. 2005). Acetaldehyde can cause point mutations in lymphocytes, induce sister chromatid exchanges, and interfere with the DNA-repair machine

(Obe et al. 1986, Woutersen et al. 1986, Dellarco 1988, Espina et al. 1988).

The most convincing evidence for the carcinogenicity of acetaldehyde can be derived from the uniform epidemiological and biochemical data concerning ADLH2-deficient alcohol consumers who have markedly increased risk for upper digestive tract cancers and an elevated level of acetaldehyde in their saliva after ethanol consumption (Yokoyama et al. 1996, Yokoyama et al. 1998, Väkeväinen et al. 2000, Väkeväinen et al. 2001, Yokoyama et al. 2003)

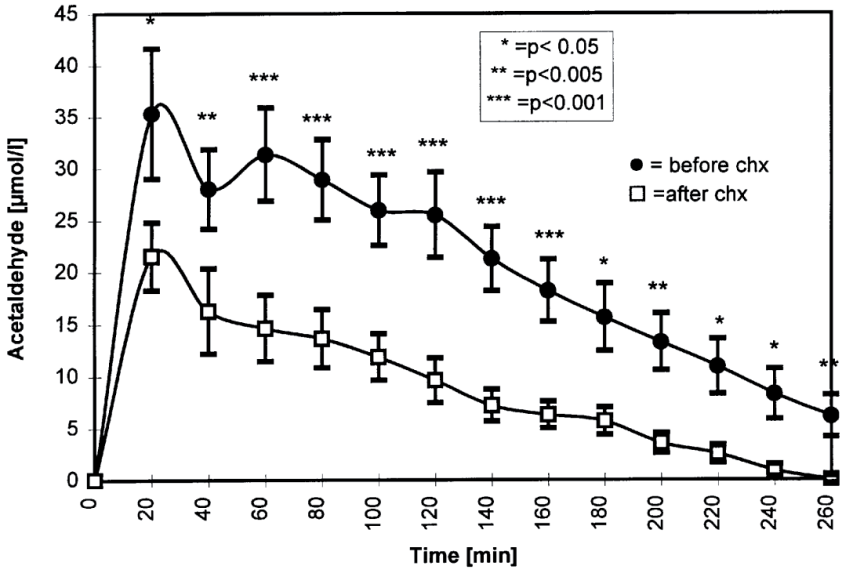
5. Regulation of local acetaldehyde concentration

The local acetaldehyde concentration in the mouth depends on many factors. Since the oral microbiome is the main causative agent producing acetaldehyde (Seitz and Stickel 2007, Salaspuro 2009a). One way to effect acetaldehyde production is to interfere with the oral microbiome, for example by reducing the amount of microbes which can be done by improving the oral hygiene (Homann et al. 2001). Xylitol is a sugar alcohol which has been shown to reduce the metabolism of certain oral microbes. Whether xylitol has an effect on acetaldehyde production will be discussed further. The oral mucosa may be responsible for acetaldehyde production to some extent due to its ADH enzymes (Seitz and Stickel 2007). Therefore mutations in the ADH enzyme have been shown to cause differences in salivary acetaldehyde levels (Visapää et al. 2004). A greater effect is associated with the mutation of ALDH2 enzyme because that is responsible for most of the metabolism of acetaldehyde to acetate (Väkeväinen et al. 2000, Seitz and Stickel 2007, Salaspuro 2009). Some oral microbes have been shown to have ADH activity (Tillonen et al. 1999, Muto et al. 2000) but the microbes seem to lack ALDH-activity or the current enzyme activity is minor (Nosova et al. 1996, Nosova et al. 1998, Salaspuro 2011). Therefore, acetaldehyde accumulation is seen in the saliva during ethanol challenge (Homann et al 1997). In addition, acetaldehyde binding agents, such as L-Cysteine, can be used for the reduction of acetaldehyde exposure in the mouth (Salaspuro and Salaspuro 2004). Naturally, the most effective way for the reduction of local acetaldehyde exposure via saliva can be achieved by avoiding alcohol and tobacco.

5.1 Chlorhexidine

Chlorhexidine is an antimicrobial agent typically used in mouthwashes. It has been shown to reduce the total count of microbes in the oral cavity (Homann et al. 1997). In the same study it was demonstrated that three days use of chlorhexidine mouthwash significantly decreases acetaldehyde production from ethanol *in vivo* (Fig. 5). The ability of chlorhexidine to decrease acetaldehyde production can be due to the effect of breaking the biofilm instead of reducing the total microbial count. Further studies are needed in order to establish why chlorhexidine reduces acetaldehyde production.

Figure 5. The effect of chlorhexidine. Chlorhexidine (chx) significantly reduces microbial acetaldehyde production during ethanol challenge *in vivo* (Homann et al. 1997). (License to use the figure, number 2731900176837)



5.2 L-Cysteine

L-Cysteine is a semi-essential amino acid and a normal component of human diet. It can be synthesized from methionine and serine via transsulphuration. L-Cysteine reacts covalently with acetaldehyde forming 2-methylthiazolidine-4-carboxylic acid (Sprince et al. 1974, Salaspuro et al. 2002). In a study by Salaspuro et al., L-Cysteine reduced the acetaldehyde produced during ethanol challenge by 59 ± 8% (Salaspuro et al. 2002). In that experiment, L-Cysteine-lozenge slowly releasing L-Cysteine was fastened under the lip of volunteers before a moderate dose of ethanol followed by acetaldehyde analysis from salivary samples. As mentioned earlier, tobacco smoke contains acetaldehyde, which dissolves into saliva during smoking (Salaspuro and Salaspuro 2004). L-Cysteine-lozenges containing only 5 mg of L-Cysteine are able to eliminate salivary acetaldehyde originated from tobacco smoking totally (Salaspuro et al. 2006).

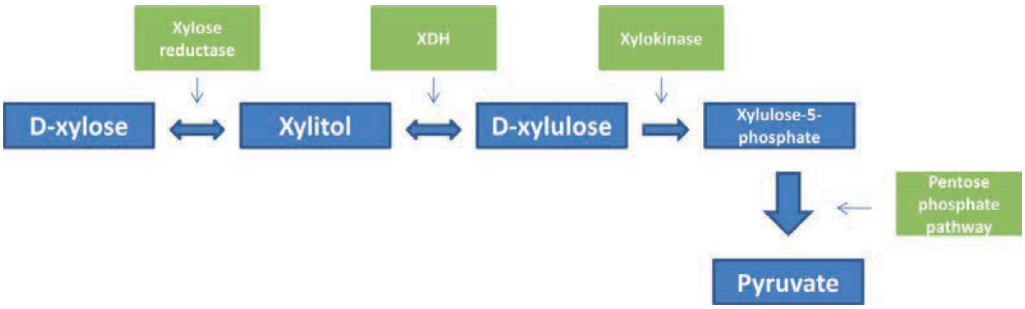
L-Cysteine can be used to eliminate acetaldehyde from achlorhydric stomach as well (Linderborg et al. 2010). Achlorhydria leads to achlorhydric atrophic gastritis that is the most important risk factor for gastric cancer (Sipponen et al. 1985, Aromaa et al. 1996, Salaspuro 2011). In an achlorhydric stomach, oral microbes are able to survive and to produce acetaldehyde during an ethanol challenge (Väkeväinen et al. 2000, Väkeväinen et al. 2001). The many ways for the reduction of the local acetaldehyde exposure in the upper digestive tract provides new possibilities for future intervention studies (Salaspuro 2011).

5.3 Xylitol

Xylitol is a sugar alcohol used for example in chewing gums. Xylitol has been shown to prevent caries partly due to its ability to inhibit the metabolism and decrease the counts of *S. mutans* (Ly et al. 2006, Söderling et al. 2011). *S. mutans* incorporates xylitol as xylitol-5-phosphate, which is dephosphorylated, this “futile xylitol cycle” consumes energy and inhibits growth. Furthermore, xylitol-5-phosphate can inhibit glycolytic enzymes leading to reduced acid production (Söderling 2009). Xylitol also has an inhibitory effect against otopathogenic bacteria (Uhari et al. 2000).

Xylitol can be formed from D-xylose which is a by-product of normal carbohydrate metabolism in yeasts. Xylitol is thereafter metabolized into D-xylulose (Fig.6).

Figure 6. Metabolism of xylitol. Xylitol is metabolized into D-xylulose by the enzyme XDH or into D-xylose by the xylose reductase. D-xylulose is further metabolized in the pentose phosphate pathway where the end-product is pyruvate.



D-xylulose is further metabolized into xylulose-5-phosphate which enters to the pentose phosphate pathway. The end-product of this pathway is pyruvate (Iablochkova et al. 2003, Jin et al. 2005) (Fig 3.). Xylitol is metabolized into D-xylulose by xylitol dehydrogenase (XDH) which closely resembles ADH (Persson et al. 1993). As a matter of fact, XDH is part of the ADH enzyme family and the NAD-binding parts of the enzymes are similar (Kotter et al. 1990, Persson et al. 1993). The possible effect of xylitol to disturb ethanol metabolism is, however, not yet known.

AIMS OF THE STUDY

The general aim of this study was to explore the ability of various members of the oral microbiome to produce acetaldehyde in the presence of clinically relevant ethanol and glucose concentrations. Furthermore the aim was also to find out whether by coincubating the isolates with xylitol the amount of acetaldehyde produced could be decreased.

Specific aims were as follows:

I. To measure the *in vitro* acetaldehyde production from ethanol by laboratory strains and clinical isolates of oral *Streptococci.*

II. To compare the amount of acetaldehyde produced by *Candida albicans* isolates obtained from patients with APECED, or oral cancer, and healthy controls from ethanol and glucose *in vitro.*

III. To examine the effect of xylitol, fructose and glucose on acetaldehyde production of *Candida* isolates from ethanol *in vitro.*

IV. To compare the amount of acetaldehyde produced *ex vivo* from ethanol by cultured microbial samples from patients with cancer or lichen planus and healthy controls.

MATERIAL AND METHODS

1. Material in the *in vitro* studies

1.1 Microbial strains and isolates

Altogether 94 laboratory strains and clinical isolates of microbes were used (Table 1). The laboratory strains were obtained from a culture collection (ATCC, American Type Culture Collection and CCUG, Culture Collection of University of Gothenburg) and external quality control specimens (UK NEQAS, United Kingdom National External Quality Assessment Service). Clinical isolates were isolated from patient samples by using conventional culture methods at the HUSLAB Laboratory of Clinical Microbiology of the Helsinki University Central Hospital (T-and HI-isolates), and Helsinki University Dental Institute (G- and L-isolates), and stored in the departmental depositories at -80°C. Identification was based on colony morphology, growth on chromogenic agar, microscopy, and biochemical reactions.

For the experimental analysis, streptococci strains and isolates were incubated on Brucella agar plates (Becton Dickinson, Maryland, USA) in CO_2-conditions for 48 h at 35°C (Alaluusua et al. 1984). Colonies from pure cultures were suspended in phosphate buffered saline (PBS, 6 M) and optical density (od) at 492 nm was adjusted spectrophotometrically (Multiscan RC, Labsystems, Helsinki, Finland) to 0.1. This od corresponds to $1x10^8$ colony forming units per millimetre (CFU/ml). Dilution plating was used to verify the microbial concentration.

Candida strains and isolates were incubated on Sabouraud dextrose agar plates (Lab M, Lancashire, UK) in aerobic conditions for 48 h at 37°C. Colonies from pure cultures were suspended in phosphate buffered saline (PBS, 6 M) and optical density (od) at 492 nm was adjusted spectrophotometrically (Multiscan RC, Labsystems, Helsinki, Finland) between 0.3 and 0.4. This od was found to correspond to $1x10^7$ CFU/ml. Dilution plating was used to verify the microbial concentration.

Table 1. Strains and isolates used in the studies.

Streptococcus **strains and isolates**	***Candida albicans*** **isolates from healthy patients**	***Candida albicans*** **isolates from APECED patients**	***Candida albicans*** **isolates from oral cancer patients**	**Non-** ***Candida albicans*****-strains and isolates**
S. anginosus ATCC 33397	*C. albicans HI-2501*	*C. albicans T-109*	*C. albicans T-1261*	*C. glabrata CCUG 32725*
S. anginosus T-40532	*C. albicans HI-2521*	*C. albicans T-343*	*C. albicans T-1275*	*C. glabrata G212*
S. constellatus ATCC 27823	*C. albicans HI-2530*	*C. albicans T-344*	*C. albicans T-1293*	*C. parapsilosis ATCC 22019*
S. constellatus T-42662	*C. albicans HI-2535*	*C. albicans T-355*	*C. albicans T-1298*	*C. parapsilosis G170*
S. intermedius ATCC 27335	*C. albicans HI-2543*	*C. albicans T-357*	*C. albicans T-1311*	*C. tropicalis ATCC 750*
S. intermedius T-41190	*C. albicans HI-2564*	*C. albicans T-359*	*C. albicans T-1323*	*C. tropicalis G9*
S. mitis ATCC 33399	*C. albicans HI-2579*	*C. albicans T-366*	*C. albicans T-1337*	*C. dubliniensis UK NEQAS 2/07*
S. mitis T-44744	*C. albicans HI-2580*	*C. albicans T-370*	*C. albicans T-1345*	*C. dubliniensis G130*
S. mutans ATCC 27175	*C. albicans HI-2656*	*C. albicans T-371*	*C. albicans T-1352*	*C. guillermondii UK NEQAS 9/ 06*
S. mutans L10	*C. albicans HI-2659*	*C. albicans T-372*	*C. albicans T-1396*	*C. krusei ATCC 6258*
S. mutans L13	*C. albicans HI-2677*	*C. albicans T-373*	*C. albicans T-1481*	*C. krusei T-880*
S. oralis ATCC 35037	*C. albicans ATCC 90029*	*C. albicans T-375*	*C. albicans T-1505*	
S. salivarius ATCC 13419		*C. albicans T-376*		
S. salivarius T-42104		*C. albicans T-384*		
S. sobrinus ATCC 33478		*C. albicans T-391*		
S. viridans T-47062		*C. albicans T-392*		
		C. albicans T-395		
		C. albicans T-436		
		C. albicans T-564		
		C. albicans T-695		
		C. albicans T-719		
		C. albicans T-816		
		C. albicans T-826		
		C. albicans T-916		
		C. albicans T-918		
		C. albicans T-923		
		C. albicans T-927		
		C. albicans T-931		
		C. albicans T-962		
		C. albicans T-967		
		C. albicans T-972		
		C. albicans T-975		
		C. albicans T-976		
		C. albicans T-926		
		C. albicans T-983		
		C. albicans T-985		
		C. albicans T-995		
		C. albicans T-1032		
		C. albicans T-1055		
		C. albicans T-1108		
		C. albicans T-1490		
		C. albicans T-1527		
		C. albicans T-1553		

2. Material in the *ex vivo* study

2.1 Subjects

The study was a prospective, where three different groups of patients being treated at the Department of Oral and Maxillofacial Surgery, Helsinki University Central Hospital, or at the Helsinki University Dental Hospital, during 2007-2011 were enrolled; 30 patients diagnosed with oral cancer (OSCC), 24 patients diagnosed with oral lichen planus or lichenoid lesion (OLD), and 30 healthy control patients (CO) (Table 2.). The subjects had not received any antibiotics within 7 days and those with HIV or hepatitis virus infection were excluded. All participants signed an informed consent and completed a questionnaire regarding their oral health and hygiene, cigarette and alcohol consumption (Appendix 1.).

2.2 Study design

Microbial samples were taken from oral mucosa by using the filter paper method (Rusanen et al. 2009). The lesion samples were taken from the site of active disease (OSCC/OLD) and control site samples from a contralateral site from healthy mucosa. After sampling the filter papers, diameter 13 mm, were immediately placed into sterile test tubes with 5 ml of sterile saline and processed within 60 min. For culture, the filter papers were agitated in saline for 30 s with five 3 mm glass beads. Then, 100 μl of the suspension was cultured on selective and non-selective media under anaerobic and aerobic conditions. Fastidious anaerobe agar (FAA; Fastidious Anaerobe agar (LAB-M LAB 90) supplemented with 5% horse blood) was used to enumerate the total cultivable bacteria. Lysed blood agar (BA; Trypticase soy agar (BBL 211047) and Mueller Hinton agar (BBL 212257) supplemented with 5% horse blood) was used for enumeration of total aerobic bacteria. Neomycin-vancomycin blood agar (NV; blood agar and neomycin sulphate (Sigma N-1876) supplemented with vancomycin (7.5 μ/ml), menadion (0.5 μg/ml) and sheep blood 5 %) was used to enumerate anaerobic gram-negative bacteria. Cysteine, lactose-and electrolyte deficient agar (CLED; C.L.E.D medium (BBL 212218) was used to select aerobic gram-negative fermentative rods. To detect yeasts Sabouraud Dextrose agar (SP; Sabouraud Dextrose Agar (Lab M), Bacto Agar (Difco) supplemented with penicillin (100,000 IU/ml) and streptomycin) was used. The BA, CLED and SP plates were incubated at 37°C for 48 h and the FAA and NV plates were incubated under anaerobic conditions at 37°C for 7 days. After culturing the suspension, the filter paper was removed from the saline and placed onto an

additional FAA plate for 30 s, removed and streaked and incubated under anaerobic conditions. This plate was used for acetaldehyde analyses.

After incubation, the number of bacteria and yeasts (CFU) and different bacterial colony morphology types were enumerated. The analyses were performed by two independent observers without knowledge of the sample type or interpretation by others. In cases of discrepancies, a consensus was reached by re-evaluation of the culture plates together. Gram stain was performed on all different colony morphology types from CLED and NV agars and the number of gram-negative colonies was recorded. The ratio of gram-negative to gram-positive bacteria and the ratio of aerobic to anaerobic bacteria were determined. For acetaldehyde analysis, each side of the filter paper was placed onto FAA media for 30 s and the media was incubated in a similar manner. The suspension for acetaldehyde analysis was prepared using the second FAA plate by injecting 3 ml saline into the media and gently scrubbing the microbes. The suspension was thereafter transferred into the sterile test tube and closed tightly.

Table 2. Patients of the fourth study.

	Oral Cancer	Oral Lichen Disease	Control
Total number	30	24	30
Female:Male	12:18	16:8	19:11
Smokers (%)	9 (32%)	4 (19%)	9 (31%)
Alcohol consumers (%)*	24 (79%)	19 (91%)	26 (90%)
Heavy drinkers (%)**	5 (17%)	1 (5%)	2 (6%)

* excluding heavy drinkers

**) exceeds WHO levels for harmful alcohol consumption (288g alcohol per week in men and 192g per week in women)

3. Methods

3.1 Acetaldehyde measurement

Microbial suspensions were transferred into a gas chromatograph vials. Thereafter, PBS-buffer containing ethanol, glucose, fructose or xylitol was added and the vial was immediately sealed. In the first study, two ethanol concentrations were used; 11 mM and 1100 mM. In the second study, 11 mM ethanol and 100 mM glucose was used. In the third study, 12 mM ethanol and 110 mM fructose, glucose and xylitol were used. In the fourth study 22 mM ethanol was used. Samples were incubated for 30 min at 37°C in the studies 1-3 and 60 min in the fourth study. The reaction was thereafter stopped by injecting 50 µl of perchloric acid (PCA, 6 M) through the rubber septum of the test vial. Every sample was measured as a triplicate and the mean was used for the analysis. To measure the baseline and artefactual acetaldehyde, 50µl of PCA was immediately added to control vials and the suspension was equally incubated. The formed acetaldehyde was measured by headspace gas chromatography. The conditions for analysis were: Column 60/80 Carbopack B/5% Carbowax 20M, 2 m x 1/8" (Supelco Inc, Bellefonte, USA); oven temperature 85°C; transfer line and detector temperature 200°C; carrier gas flow rate (N_2), 20 ml/min (Jokelainen et al. 1994).

3.2 ADH-analysis

ADH-activity was measured by using fluorescence analysis with cofactor nicotinamide adenine dinucleotide (NAD) (Study I and III) and nicotinamide adenine dinucleotide phosphate (NADP) (Study I) (Holbrook et al. 1972). For the analyses, the microbes were first grown as described above. Streptococci cells were sonicated for 10 x 8s in an ice bath in the presence of a protease inhibitor cocktail (SIGMA, P 8340, Missouri, USA). Candida cells were lysed by glass bead vortexing in the presence of the same protease inhibitor cocktail. Five 1 min vortexing cycles and glass beads of 1.0 mm diameter were used. The samples were cooled on ice before each cycle.

Cell lysates were centrifuged for 5 min at 2900 g (Hettich EBA 20, Germany), the supernatants were collected and further centrifuged at 139700 g for 65 min at 4°C (Beckman Optima LE-80k Ultracentrifuge, USA). This supernatant was collected and used for the analyses. Cytosolic ADH activity was determined by measuring the fluorescence (ex 340 nm, em 440 nm) after addition of

ethanol or ethanol and xylitol (final concentration 100 mM) and NAD (final concentration 2.5 mM) at 25°C (study 1) and at 37°C (study 3 and 4) in 0.1 M glycine buffer (pH 9.6). Ethanol concentrations 0.68 to 2174 mM were used. ADH activity was determined by using Tecan SAFIRE monochromatorbased microplate detection system and Magellan Softwares V3.11 and V6.05 (Tecan Trading AG, Switzerland).To determine the enzyme activities the Lineweaver-Burk plot was used.

3.3 Statistical analysis

Results are expressed as means (±SEM) of at least three replicate measurements. Statistical significance of the differences between the acetaldehyde production of microbial isolates were analyzed by Wilcoxon Signed Rank Test by SPSS 12.0 (Study I), differences between the patient groups by two-tailed Mann Whitney U-test by Graph Pad Prism version 5.00 (study II and IV), differences within patient groups by two-tailed paired t-test by Graph Pad Prism version 5.00 (study II). The univariate analysis of variance was used for comparisons between species and experimental conditions. Spearman's rho (r_s) was used for the analysis of the correlations. The correlation was expressed with a 95% confidence interval and a P value. A P value less than 0.05 were considered as statistically significant. The generalized estimating equations model was used for comparisons between species and experimental conditions in the third study (III).

3.4 Ethical considerations

In the study four (IV) where patients were involved, the study protocol was approved by the ethical committee of the Helsinki University Central Hospital (§ 47/2007, 25.4.2007, Dnro 126/E6/07). Informed consent was obtained from all subjects.

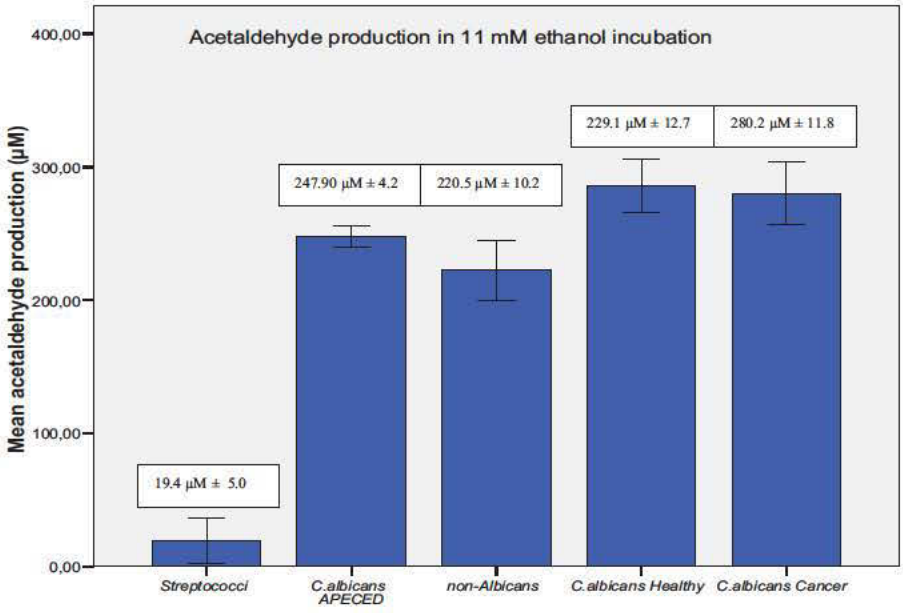
RESULTS

1. Results of the *In vitro* studies

1.1 Microbial acetaldehyde production from ethanol

The first three articles reported here were based on *in vitro* studies, where the ability of oral microbes to produce acetaldehyde from ethanol was measured. Almost all microbes were able to produce acetaldehyde during a 30-minute incubation and a large variation in the amount of produced acetaldehyde was found. Candida isolates were found to produce higher amounts of acetaldehyde from ethanol compared to streptococci isolates (Fig.7). The mean level acetaldehyde produced by candida isolates was more than 10 times higher than the mean level acetaldehyde produced by the streptococci (Fig 7).

Figure 7. Acetaldehyde production from 11 mM ethanol of all microbes analyzed *in vitro*, the incubation time used was 30 minutes. Streptococci (n=16), *C. albicans* (Healthy) (n=13), *C. albicans* (Cancer) (n=12), *C. albicans* (APECED) (n=44), *non-albicans* (n=12). The means (±SEM) and confidence interval of all groups presented. *Candida* isolates produced significantly higher amounts of acetaldehyde compared to streptococci isolates, ($p<0.0001$ in all groups).



1.1.1 Acetaldehyde production by oral streptococci from ethanol

The mean acetaldehyde produced from 11 mM ethanol was 19.4 µM (±5.0), and from 1100 mM ethanol 71.7 µM (± 15.04). Large variation and differences were found in the acetaldehyde production by analyzed streptococci isolates. Both isolates of *Streptococcus mutans* and the clinical isolate of *S. mitis* were not able to produce acetaldehyde in the incubation with 11 mM ethanol. Clinical isolate of *S. salivarius* was on the other hand able to produce mutagenic amounts of acetaldehyde in this ethanol concentration, 135.0 µM (±3.8 µM). Clinical isolate of *S. salivarius* was found to produce the significantly highest amount of acetaldehyde as well in the higher ethanol concentration; 426.3 µM (±12.6).

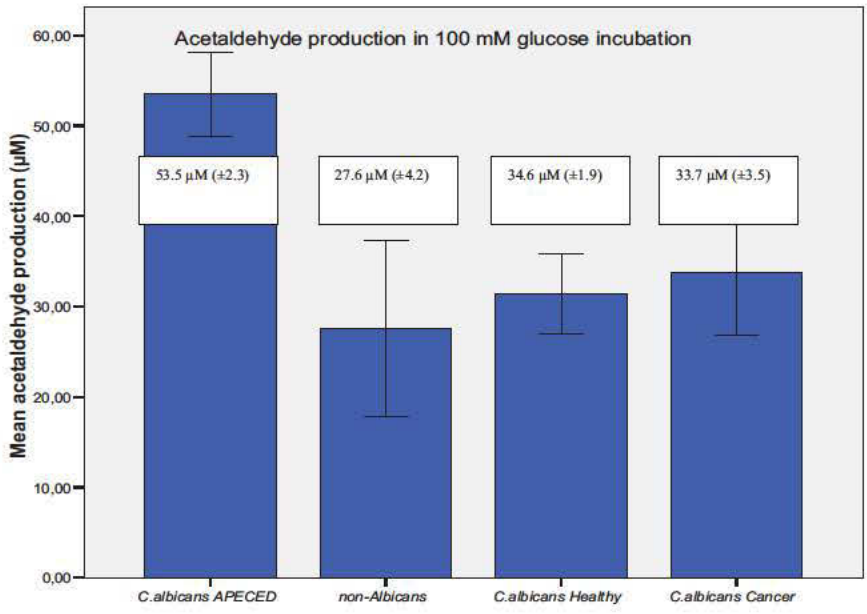
1.1.2 Acetaldehyde production by candida isolates from ethanol

The ability of Candida isolates to produce acetaldehyde from ethanol was analyzed in 11 mM incubation (Fig. 7). Large variation and differences were found in the acetaldehyde production by analyzed candida isolates. Both *C. krusei* isolates analyzed were found to be weak acetaldehyde producers; the average acetaldehyde produced being 53.7 µM (±9.1 µM). Of all isolates analyzed, *C. glabrata* was found to be able to produce the highest amount of acetaldehyde, 366.1 µM (±10.1 µM).

1.2 Acetaldehyde production by candida isolates from glucose and fructose

The ability of candida isolates to produce acetaldehyde from glucose was analyzed in studies II and III. All analyzed microbes were able to produce acetaldehyde from glucose. APECED isolates produced significantly higher amounts of acetaldehyde compared to isolates from oral cancer patients, healthy controls patients, or *C. non-albicans* ($p<0.0001$) (Fig.8). In study III, acetaldehyde production from fructose was also analyzed, in addition to the effect of glucose-ethanol coincubation and fructose-ethanol coincubation. The mean acetaldehyde produced from fructose was found to be small, 21.4 µM (±3.6 µM). The acetaldehyde produced in both coincubations was found to be smaller than in the incubation with ethanol alone (220.5 µM±10.2), but the difference was not significant. (Fig. 9) The mean acetaldehyde produced in ethanol-glucose incubation was 170.4 µM (±11.3) and in ethanol-fructose incubation 158.5 µM (±10.3).

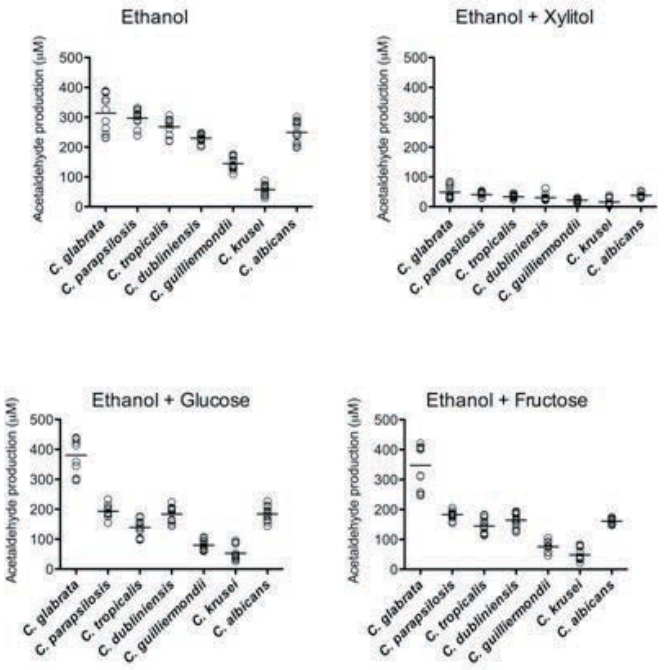
Figure 8. Acetaldehyde production from 100 mM glucose. Incubation time used was 30 min. The mean (±SEM) and confidence interval of each group presented.



1.3 The effect of xylitol

In the third study (III) the effect of xylitol was investigated by coincubating candida samples with xylitol and ethanol. Without xylitol, the mean acetaldehyde production within 30 minutes was 220 µM (±10.2), but when coincubated with xylitol, the mean acetaldehyde produced was only 32 µM (±1.7). The reduction of 84% was highly significant ($p<0.0001$) (Fig. 9). Acetaldehyde production was also analyzed in the absence of ethanol, but in this xylitol-incubation only 1.8 µM (±0.3 µM) acetaldehyde was produced.

Figure 9. The effect of glucose, fructose and xylitol on candidal acetaldehyde production in ethanol incubation.



1.4 Microbial ADH-activity

ADH-activity was measured in two studies (I and III). In the first study, ADH-activity was measured from four streptococci isolates; *S. salivarius* clinical isolate, *S. intermedius* clinical and laboratory isolates, and *S. mitis* laboratory isolate. The isolates were chosen due to their ability to produce a high amount of acetaldehyde compared to other analyzed streptococci. In the third

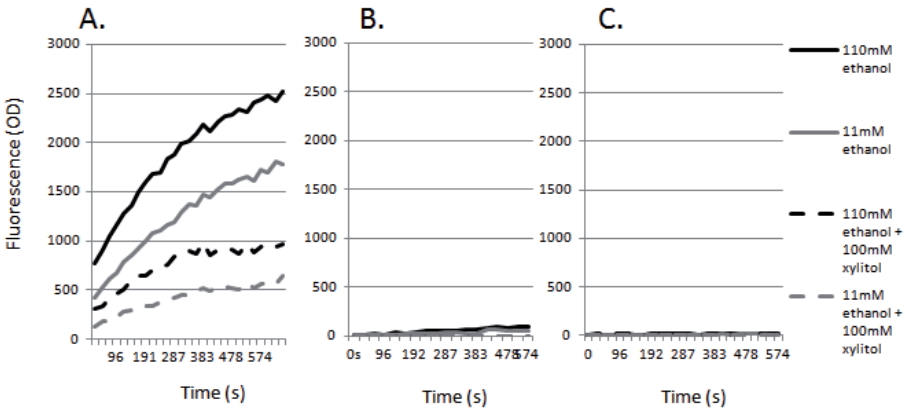
study, the ADH-activity of laboratory isolates of *C. albicans*, of *C. glabrata* and *C. krusei* was analyzed. The isolates were chosen because *C. glabrata* was found to be the highest acetaldehyde producer, *C. krusei* the lowest and *C. albicans* an average producer. Significant activity was measured in all analyzed streptococci, *C. albicans* and *C. glabrata*. ADH-activity of *C. krusei* was found to be low (Table 3.).

Table 3. Km-values of microbial ADH-enzymes.

Microbe	**Km-value (mM)**	**Acetaldehyde produced in 11 mM ethanol incubation (µM)**
S. salivarius T-42104	1.2	135.0
S. intermedius T-41190	not calculable	58.0
S. intermedius ATCC 27335	1490.0	46.3
S. mitis ATCC 33399	758.3	11.2
C. albicans ATCC 900029	0.16	219.7
C. glabrata CCUG 32725	2.83	366.1
C. krusei ATCC 6258	0.13	53.7

The effect of xylitol on ADH-activity was measured with candida isolates. Xylitol was found to reduce the ADH-activity of *C. glabrata* by 61% when coincubated with 110 mM ethanol for 10 minutes, and of *C. albicans* by 100%. No effect was seen in *C. krusei* (Fig.10).

Figure 10. ADH-activities of *C. glabrata* CCUG 32725 (A), *C. albicans* ATCC 90029 (B) and *C. krusei* ATCC 6258 (C). Activities were measured by using fluorescence analysis with cofactor nicotinamide adenine dinucleotide (NAD) after addition of ethanol, or ethanol and xylitol. Xylitol reduced the ADH activity of the *C. glabrata* and *C. albicans* markedly.



2. Results of the *ex vivo* study

Microbes cultured from all samples were able to produce carcinogenic levels of acetaldehyde from ethanol but no significant differences between the patient groups were found (Fig.11). The amount of acetaldehyde produced did not correlate with the amount of microbes cultured from the samples. The cultured microbial samples differed significantly between patient groups when the colony forming units were compared. Oral cancer patients had significantly more microbes in their samples compared to other groups (Fig. 12).

The number of aerobic bacteria was found to be significantly higher in OSC lesion sites compared to corresponding control sites ($p=0.0003$), both for sample sites of OLD patients (lesion $p=0.0002$, control $p<0.0001$) and control patients ($p<0.0001$). The number of anaerobic bacteria did not differ significantly between lesion and control sites in OSC patients. The difference was significant when the amount of anaerobes from the OSC lesion site was compared to both sample sites of OLD patients (lesion $p=0.0007$, control $p<0.0001$) and control patients ($p<0.0001$) The mean proportion of Gram-negative bacteria was found to be 8% of all bacteria in CO samples, 4% of the bacteria in OSCC lesions and 3% in OSCC control sites, 12% in OLD lesions, and 8% in OLD control sites. Thereafter it can be stated that the majority of bacteria were Gram-positive in all patient samples.

We analyzed whether the patients tobacco use, alcohol consumption, oral hygiene, or health would have an impact on acetaldehyde production but the only correlation found was the use of tobacco in oral cancer and lichen planus patients. The cultured microbial samples from smokers produced significantly higher amounts of acetaldehyde compared to samples from non-smokers ($p=0.0351$).

Figure 11. Acetaldehyde production by cultured microbial samples collected from patients with oral cancer, lichen planus and healthy controls. The incubation time used was 60 min. Mean and confidence interval presented.

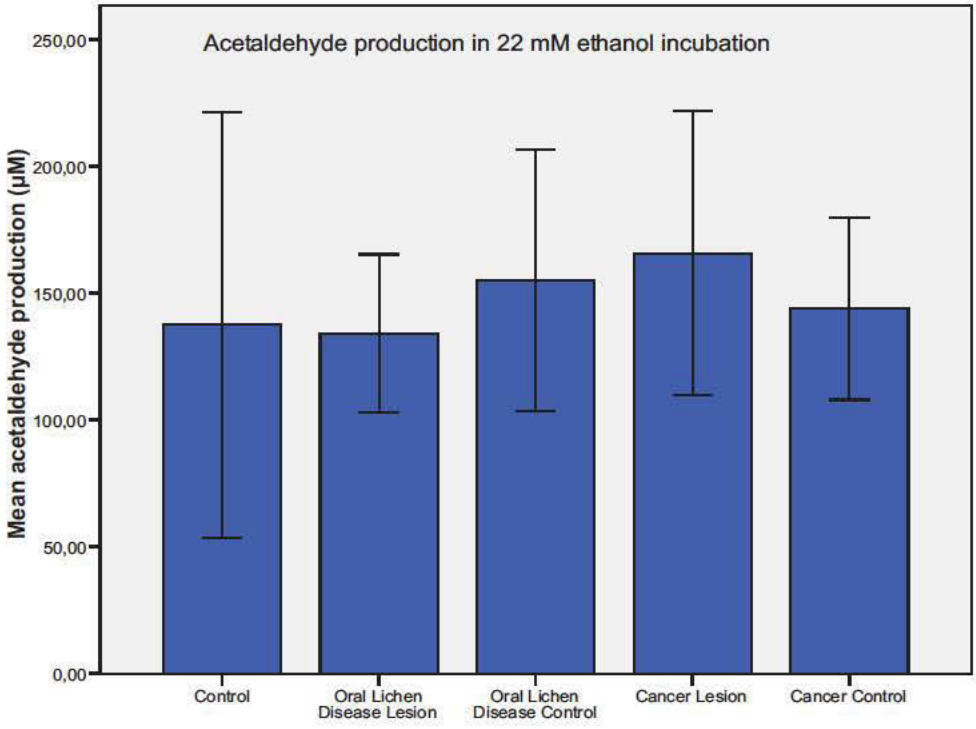
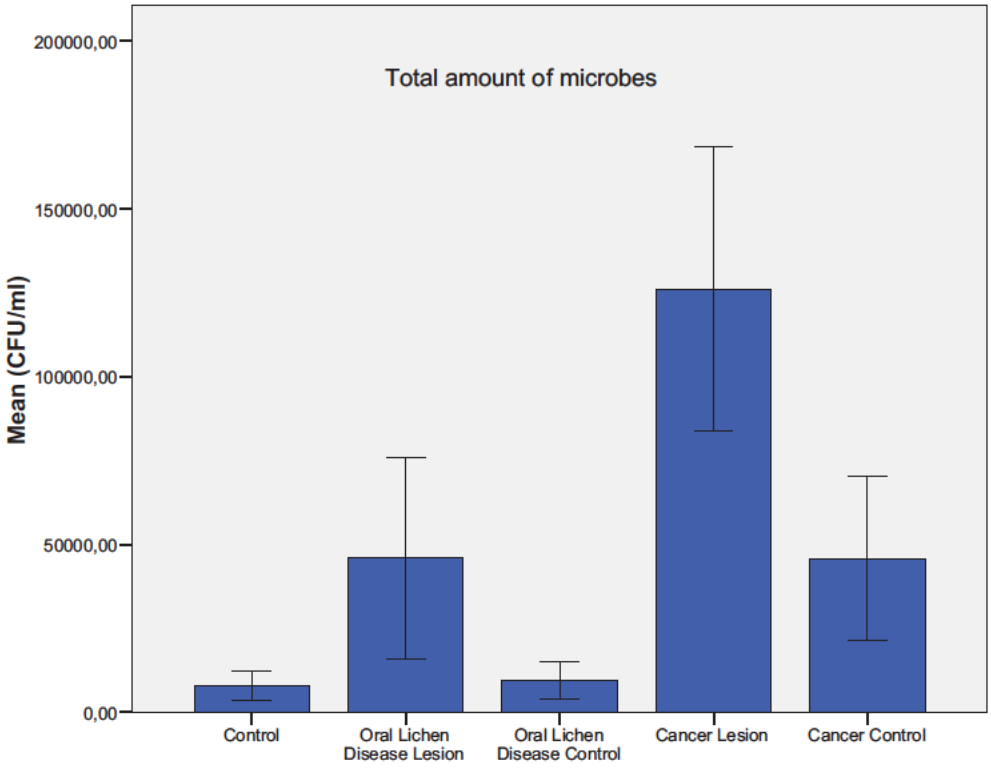


Figure 12. Total amount of cultured bacteria collected from patients with oral cancer, lichen planus and healthy controls. Microbes were calculated from FAA media incubated 7 days in anaerobic conditions. The mean and confidence interval presented.



DISCUSSION

Chronic alcohol consumption and tobacco smoking are generally accepted risk factors for oral cancer. They both result in increased salivary acetaldehyde level which has been concluded to be the main etiological reason behind upper digestive tract carcinogenesis (Salaspuro 2003a, Seitz and Homann 2007, Salaspuro 2009a, Seitz and Cho 2009, Seitz and Stickel 2009). Based on epidemiological, genetic, and biochemical evidence, acetaldehyde was recently declared to be a group 1 carcinogen for humans by the IARC (Secretan et al. 2009). All four studies included in this thesis showed that samples of oral microbiota are able to produce significant amounts of mutagenic acetaldehyde from clinically relevant ethanol concentrations. The phenomenon was detected both *in vitro* and *ex vivo.* The results support and expand the earlier literature on this topic (Jokelainen et al. 1996a, Muto et al. 2000, Väkeväinen et al. 2001, Väkeväinen et al. 2002, Salaspuro 2003a, Nieminen et al. 2009, Salaspuro 2009b).

Carcinogenesis may take place when a cell is exposed to mutagenic substances numerous times. Various genetic abnormalities have been found in upper digestive tract cancers, such as activation of oncogenes and inactivation of tumor-suppressor genes (Toh et al. 2010). A normal cell is able to protect itself from carcinogens, for example by a DNA repair system which corrects the mutagenic changes that have occurred. DNA repair systems are able to remove the DNA adducts which can be induced by acetaldehyde (Wang et al. 2007), however, if the mutagenic substance damages the DNA repair system and the cell remains viable, carcinoma may start to develop. This has been shown to happen with acetaldehyde (Espina 1988).

1. Acetaldehyde production by oral streptococci from ethanol

Oral streptococci can be found from the oral microbiome in everyone (Jenkinson and Lamont 2005, Keijser et al. 2008). Therefore the finding that some oral streptococci produce mutagenic amounts of acetaldehyde in clinically relevant ethanol concentrations may be important. Isolates of *S. salivarius*, in particular, appeared to be able to produce significant amounts of acetaldehyde. *S. salivarius* typically colonizes the mucosal surface of the oral cavity which is one of the sites where oral carcinomas can be found (Ruoff et al. 2003).

The ethanol concentration in saliva is particularly high during alcohol consumption but it

decreases after the consumption to the same level as seen in the blood (Jones 1979). Therefore the aim here was to measure acetaldehyde production under these two ethanol concentrations, 11 mM and 1100 mM. The smaller concentration reflects the one found in saliva in an average male after consuming 0.5 g ethanol per kg body weight. The latter concentration can be found in saliva immediately after a sip of an alcoholic beverage (Linderborg et al. 2011).

In accordance with *in vitro* acetaldehyde production from ethanol, the clinical isolate of *S. salivarius* possessed significant ADH-activity. The Km of the enzyme was found to be relatively low (1.2 mM), which indicates that the enzyme is already activated in very low ethanol concentrations. Such low ethanol concentrations can be found even in certain foods and non-alcoholic beverages which mean that acetaldehyde could be produced in the oral cavity even without drinking of official alcoholic beverages that contain over 2.8% of ethanol (Salaspuro 2011). This was an *in vitro* study and pure cultures of planktonic streptococcal isolates were used which is a limitation, since oral microbes readily form biofilms *in vivo*. Nevertheless, the results clearly show that certain isolates of this major group of oral microbes are able to produce acetaldehyde in clinically relevant conditions.

2. Acetaldehyde production by candida from glucose, fructose and ethanol

Chronic oral candidosis has been associated with carcinogenicity, especially in APECED patients (Rautemaa et al. 2007). Over 10% of the APECED patients over the age of 25 in Finland have been diagnosed with oral cancer at the site of candidal manifestation (Rautemaa et al. 2007). In this thesis project (study II) the acetaldehyde production of *C. albicans* isolates from APECED patients was analyzed and demonstrated that in glucose incubation, the isolates produced significantly higher amounts of acetaldehyde compared to isolates from cancer and healthy patients. The produced mean level of acetaldehyde in glucose incubation in APECED patient isolates was nevertheless fairly low (53.5 µM) compared to the level of same isolates produced in ethanol incubation (247.9 µM). APECED patients develop oral cancer at relatively young age, the mean age at diagnosis being 37 years. This suggests that alcohol and tobacco cannot be the only etiological factors. The fact that candida isolates are able to also produce acetaldehyde from glucose could partly explain the carcinogenesis in APECED patients.

On the other hand, many beverages and foodstuff contain minimal concentrations of ethanol and mutagenic concentrations of acetaldehyde. For example yogurts, kefir, some soya products, pickled vegetables, vinegars and even some fruits may contain marked amounts of acetaldehyde

(Salaspuro 2011, Uedelecker and Lachenmeier 2011, Lachenmeier 2011). Whether this has any relevance in carcinogenesis remains to be discovered, but it could partly explain the particularly high incidence of oral cancer especially in young APECED patients.

All analyzed candida isolates produced acetaldehyde from ethanol in studies II and III. This confirms the earlier findings of Tillonen et al., whom reported that many *Candida albicans* isolates obtained from saliva samples are able to produce significant amounts of acetaldehyde (Tillonen et al. 1999). A new finding from this work was the large difference in the acetaldehyde production capacity between candida isolates. *C. krusei* isolates were found to be low acetaldehyde producers and their ADH activity was also very low. On the other hand, *C. glabrata* isolates were found to produce six times higher levels of acetaldehyde as compared to *C. krusei*. When the acetaldehyde production was also analyzed from fructose, the mean acetaldehyde amount produced was found to be relatively low; 21.4 µM. The acetaldehyde production in ethanol-glucose and ethanol-fructose coincubations was found to be lower in the coincubations compared to incubation with ethanol. This might be due to a preference for sugars as an energy source over ethanol. The main energy sources for candida are carbohydrates such as glucose and fructose and especially in the aerobic atmosphere the metabolism prefers sugars.

3. The effect of xylitol on microbial acetaldehyde production

In the third study (III), it was discovered that xylitol strongly reduces the acetaldehyde production of different candida isolates *in vitro*. The mean reduction was 84%. Xylitol is used as a sweetener for example in chewing gums due to its "anticariogenic" features (Ly et al. 2006). Clinically relevant concentrations of both ethanol and xylitol were used in this study. The xylitol concentration used was equal to 17mg/ml, which can be found in the oral cavity during the chewing of xylitol-containing gums (Tapiainen et al. 2002, Lif Holgerson et al. 2006). Xylitol also significantly reduced the ADH activity of *C. albicans* and *C. glabrata* isolates. The reduction is most likely due to the resemblance in the structure of the enzymes responsible for xylitol end ethanol metabolism, XDH and ADH. The NAD-binding parts of the enzymes are similar (Persson et al. 1993). In addition, the possible competition from NAD might lead to the downregulation of ADH.

This was an *in vitro* study, thus similar to studies I and II, it is viable to question whether the laboratory circumstances correlate with the *in vivo* conditions in saliva. Oral microbes readily

form biofilms and the metabolism of microbes differs in the different parts of the biofilm, as do the oxygen tension and pH levels. The rinsing effect of saliva might have some impact as well. On the other hand, microbes in the oral cavity can be found in different metabolic phases. It has been discovered that candida which have been cultured for 16 hours are significantly more active in ethanol metabolism compared to the same isolates that have been cultured for 48 h (Marttila et al, unpublished data).

4. Acetaldehyde production of microbes cultured from patient samples

In the fourth study (IV), cultured microbial samples collected from patients with oral cancer, potentially malignant oral lesions, or healthy controls were used, and their capacity to produce acetaldehyde from ethanol *in vitro* was determined. The results were partly surprising; the total amount of microbes appeared not to correlate with the amount of acetaldehyde produced. No significant differences were found in the mean acetaldehyde production by the cultured microbial samples from three groups, though the number of colony forming units/microbial density varied significantly. On the other hand, all samples produced acetaldehyde under clinically relevant conditions. The ethanol concentration used in the incubation was 22 mM, which can be found in saliva of an average male after consumption of 0.7 g of ethanol per kg body weight. The method used is designed to sample microbes from oral mucosa quantitatively and qualitatively (Rusanen et al. 2009).

Only cultivable microbes were analyzed in this study, as acetaldehyde measurements can only be performed on viable microbes. This may have altered the proportions between microbes due to different growth rates. As described earlier, the use of molecular techniques has had an impact on the understanding of oral microbiome, since not all oral microbes can be cultured. Whether the uncultivable microbes would have an effect on acetaldehyde production is yet to be discovered. On the other hand, the method is unique because for the first time, acetaldehyde was analyzed in a lesion and sampling area in a specific way.

Mouthwashings of oral cancer patients have been found to produce more acetaldehyde than those of healthy controls (Jokelainen et al. 1996a). Ethanol concentration used was the same, 22 mM as well as the incubation time, 60 min. The difference might be due to different sampling methods used. Mouthwashing is not lesion specific and reflects planktonic microbes rather than those in biofilms. The clinical relevance of mucosa bound microbiome is that it could play a

more essential role in carcinogenesis than microbes in saliva. Furthermore, the patients in the study were found to have rather modest or poor dental hygiene. Poor dental hygiene correlates with increased acetaldehyde production *in vitro* in saliva samples (Homann et al. 2001). Nontheless, in our study no effect of poor dental status on the lesion-specific acetaldehyde production could be found.

The aim of this study was also to find out if acetaldehyde production by the colonizing microbiome could explain the malignant potential of oral lichen planus and oral lichenoid lesions (OLD). No differences were found in the acetaldehyde production between healthy controls and OLD patients. In the review by Gorsky et al., it was concluded that the malignant transformation of OLD does not associate with consumption of alcohol or tobacco (Gorsky and Epstein 2011). This hypothesis is also supported by the findings of Wang et al., which showed that potentially malignant lesions progress into carcinomas due to defects in the DNA repair systems (Wang et al. 2007). If the repair system is damaged, DNA adducts are not removed and carcinogenes may begin. Therefore one explanation for the carcinomas in the OLD patients could be acetaldehyde induced DNA adducts.

Another finding in this study was that the cultured microbial samples from smokers with oral cancer or lichen planus produced more acetaldehyde than samples from non-smokers. This is in accordance with earlier *in vitro* and *in vivo* findings showing that chronic smoking modifies oral flora to produce more acetaldehyde from ethanol (Salaspuro and Salaspuro 2004, Homann et al. 2000). Tobacco smoke contains acetaldehyde and repeated exposure to it might lead to selection of microbes that are capable of high rate acetaldehyde metabolism and are more tolerant to acetaldehyde.

Study IV findings thus support the hypothesis that lifestyle habits such as tobacco smoking and alcohol consumption have a greater role in the carcinogenesis than the individual characters of the oral microbiome. As presented in the literature review, alcohol consumption and tobacco smoking are reported to explain up to 80% of oral carcinomas (La Vecchia et al. 1997). Therefore, it can be stated that our results are congruent with the literature.

SUMMARY AND CONCLUSIONS

The main conclusions of this thesis are:

I. We were able to show that some isolates of oral streptococci and almost all candida spp. produce significant amounts of acetaldehyde in clinically relevant ethanol concentrations *in vitro*. Streptococci form an essential part of the oral microbiome and candida can be found in the oral cavity from a majority of people. Therefore these microbes may be responsible for the majority of local acetaldehyde exposure in the oral cavity. Furthermore, our results confirm the earlier findings suggesting that microbial ADH enzyme associates strongly with the acetaldehyde production capacity of oral microbes.

II. Candida isolates from APECED patients are able to produce significant amounts of acetaldehyde from glucose. APECED is a disease with exceptionally high risk for oral cancer. Candida isolates from APECED, oral cancer, and healthy patients were analyzed by their acetaldehyde production capability in ethanol and in glucose incubations. APECED isolates were able to produce higher amounts of acetaldehyde from glucose than the isolates from oral cancer and healthy patients.

III. Xylitol significantly reduces *in vitro* acetaldehyde production from ethanol in the analyzed candida isolates. The reduction was as high as 84% and is most likely due to the inhibition of their ADH enzyme. In the analyzed *C. albicans* and *C. glabrata* isolates xylitol also reduced the ADH activity significantly. Candida isolates were found to produce significantly lesser amounts of acetaldehyde when incubated with glucose and fructose compared to the amounts produced in ethanol incubation.

IV. Cultured microbial samples from oral cancer, OLD, and healthy control patients are able to produce significant amounts of acetaldehyde from ethanol *in vitro* but under the conditions used, no differences between the patient groups could be documented. The mean lesion specific acetaldehyde production was significantly higher than the minimum mutagenic amount of acetaldehyde. The findings support the concept that most oral cancers are more related to environmental factors, such as drinking and smoking habits, than to the individual characteristics of oral microbiota.

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Johanna Uittamo

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Appendix 1

KYSELYLOMAKE

Nimi:_____

Koehenkilön ikä:____

1. Miten usein olette käyttäneet alkoholia viimeksi kuluneen kuukauden aikana (ympyröi)?

1. joka päivä

2. Melkein joka päivä

3. viikonloppuisin yhtenä päivänä

4. viikonloppuisin kahtena päivänä

5. viikonloppuisin kolmena päivänä

6. 2-3 kertaa viikossa

7. kerran viikossa

8. 1-2 kertaa kuukaudessa

9. harvemmin

10. en lainkaan

2. **Paljonko joitte alkoholijuomia yleensä kerralla (ympyröi)?**

___1. 1-2 pullollista olutta/ long-drinkejä tai lasillisen viiniä

tai 1-2 ravintola-annosta väkeviä = 22.5 g

___ 2. 3-5 pullollista olutta/ long-drinkejä tai 5 lasillista

viiniä tai 3-5 ravintola-annosta väkeviä = 60 g

___ 3. 6-10 pullollista olutta/ long-drinkejä tai 1 ½ pullollista

viiniä tai ½ pullollista väkeviä = 120 g

___ 4. enemmän = 225 g

3. Milloin olette viimeksi nauttineet alkoholia?

4. Onko teillä jotain pysyvää lääkitystä?

__ Ei

__ Kyllä, mikä:_____

5. Nautitteko nyt tai oletteko viime kuukauden aikana nauttineet antibioottikuuria esim. hengitystieinfektioon, suolistosairauteen ym?

__ En

__ Kyllä, antibiootin nimi:_____

6. Kuinka monta savuketta poltatte päivässä?

____ 1. En tupakoi

2. ___ savuketta päivässä.

7. **Kuinka kauan olette polttaneet?**

8. Onko teillä koskaan todettu suun hiivasieni-infektiota?

1. Ei

2. Kyllä, kerran

3. Kyllä, useasti.

9. Kuinka monta hammasta teillä on?

10. Onko teillä hammasproteesia?

1. Kyllä

2. Ei

11. Kuinka usein harjaatte hampaanne?

1. harvemmin kuin kerran päivässä, kuinka usein?_____

2. kerran päivässä

3. kahdesti päivässä

12. **Milloin olette viimeksi syöneet/juoneet?**

13. Milloin olette viimeksi harjanneet hampaanne?

14. Mitä käytätte suun puhdistukseen?

1. Hammastahnaa

2. Hammastikkuja/ -lankaa

3. Suuvettä, mitä merkkiä?

15. Käytättekö Xylitol-tuotteita? Milloin viimeksi?

16. Onko jotain perussairauksia?

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Acetaldehyde production from ethanol by oral streptococci

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Summary Alcohol is a well documented risk factor for upper digestive tract cancers. It has been shown that acetaldehyde, the first metabolite of ethanol is carcinogenic. The role of microbes in the production of acetaldehyde to the oral cavity has previously been described in several studies. In the present study, the aim was to investigate the capability of viridans group streptococci of normal oral flora to produce acetaldehyde in vitro during ethanol incubation. Furthermore, the aim was to measure the alcohol dehydrogenase (ADH) activity of the bacteria. Eight clinical strains and eight American Type Culture Collection (ATCC) strains of viridans group streptococci were selected for the study. Bacterial suspensions were incubated in two different ethanol concentrations, 11 mM and 1100 mM and the acetaldehyde was measured by gas chromatography. ADH-activity was measured by using a sensitive spectroscopy. The results show significant differences between the bacterial strains regarding acetaldehyde production capability and the detected ADH-activity. In particular, clinical strain of *Streptococcus salivarius*, both clinical and culture collection strains of *Streptococcus intermedius* and culture collection strain of *Streptococcus mitis* produced high amounts of acetaldehyde in 11 mM and 1100 mM ethanol incubation. All these four bacterial strains also showed significant ADH-enzyme activity. Twelve other strains were found to be low acetaldehyde producers. Consequently, our study shows that viridans group streptococci may play a role in metabolizing

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ethanol to carcinogenic acetaldehyde in the mouth. The observation supports the concept of a novel mechanism in the pathogenesis of oral cancer.
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Introduction

The most important risk factors for upper digestive tract cancers are tobacco smoking, alcohol intake and poor oral hygiene.[1,2] They all result in increased acetaldehyde levels in saliva, which is believed to be behind carcinogenesis.[3–6] The mechanism leading to increased acetaldehyde levels in saliva after alcohol drinking is microbial acetaldehyde production, i.e. the oxidation of salivary ethanol to acetaldehyde by microbial alcohol dehydrogenase (ADH)-enzyme.[7–11]

According to the International Agency for the Research on Cancer (IARC) acetaldehyde is carcinogenic in vivo in animal studies and it is a possible carcinogen to humans.[12,13] The latter conclusion is supported by several epidemiological and biochemical studies. The mutagenic and carcinogenic changes caused by acetaldehyde can occur already in acetaldehyde concentration from 40 to 200 μmol/l, which can be found in saliva even after moderate ethanol consumption.[3,14]

Normal oral microflora contains a large scale of different bacteria. Over 750 cultivable species have been identified and even more species are probably to be found.[15] It has already been shown that some *Neisseria*-strains are able to produce significant amounts of acetaldehyde, probably via their high alcohol dehydrogenase (ADH) activity.[10] *Neisseria*-strains are considered to be part of the normal oral flora but they are found only in low numbers in the oral cavity.[16] However, the role of other oral bacteria is unknown, in this respect.

Viridans group streptococci, oral streptococci form the largest proportion of normal microflora in the oral cavity. Oral streptococci are gram-positive bacteria and they can be found in all parts of the mouth in all humans. In saliva they comprise even 50% of the total cultivable flora.[15] However, the ability of these bacteria to produce acetaldehyde from ethanol is not known. Therefore the aim of the present study was to investigate the capability of oral streptococci to produce acetaldehyde in vitro during ethanol incubation and, furthermore, to measure the ADH activity of the bacteria. The study hypothesis was that the voluminous group of oral streptococci might play a role in metabolizing ethanol to acetaldehyde.

Material and methods

Bacterial strains

Eight laboratory and eight clinical strains of viridans group streptococci were selected for the study (Table 1). The laboratory strains were obtained from the Institute of Dentistry's collection of the American Type Culture Collection (ATCC). The clinical strains were identified from patient samples by using conventional culture methods at the Research Laboratory of Institute of Dentistry of University of Helsinki and at the Laboratory of Clinical Microbiology of the Helsinki University Central Hospital. The identification was based on the colony morphology, staining and biochemical reactions.

Growth conditions

The bacteria were incubated on Brucella agar plates (Becton Dickinson, Maryland, USA) in CO_2-conditions for 48 h at 35 °C. A couple of colonies were suspended in phosphate buffered saline (PBS) and optical density (OD) of the solution at 492 nm was adjusted spectrophotometrically (Multiscan RC, Labsystems, Helsinki, Finland) to 0.1. This OD was found to correspond 1×10^8 colony forming units per millilitre (CFU/ml) by quantitative bacterial culture.

Experimental design

Acetaldehyde measurement

Bacterial suspension (250 μl) was transferred into a gas chromatograph vial. Thereafter, 250 μl of PBS-buffer containing ethanol was added and the vial was immediately tightly closed. Two different final ethanol concentrations were used, 11 mM and 1100 mM. Samples were incubated for 30 min at 37 °C and the reaction was stopped by injecting

Table 1 The laboratory and clinical bacterial strains used in the study

Laboratory strain	Identification/strain no.	Clinical strains	Identification/strains no.
S. anginosus	*ATCC 33397*	*S. anginosus*	*T-40532*
S. constellatus	*ATCC 27823*	*S. constellatus*	*T-42662*
S. intermedius	*ATCC 27335*	*S. intermedius*	*T-41190*
S. mitis	*ATCC 33399*	*S. mitis*	*T-44744*
S. mutans	*ATCC 27175*	*S. mutans*	*L10*
S. oralis	*ATCC 35037*	*S. mutans*	*L13*
S. salivarius	*ATCC 13419*	*S. salivarius*	*T-42104*
S. sobrinus	*ATCC 33478*	Viridans group streptococcus	*T-47062*

50 μl of perchloric acid (PCA, 6 M) through the rubber septum of the test vial. Every sample was measured as a triplicate and the mean was used for later calculations. To measure the baseline and artefactual acetaldehyde, 250 μl of the PBS-buffer was added to bacterium suspensions (250 μl) and the suspension was incubated as above for 30 min at 37 °C. To the suspension was then added 50 μl of PCA. The formed acetaldehyde was measured by gas chromatography as reported earlier.[17]

ADH-enzyme analyses

The ADH-activity of the bacteria was measured by using fluorescence analysis with both cofactors, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP).[18] To measure the ADH activity, a protease inhibitor cocktail (SIGMA, P 8340, Missouri, USA) was added to the bacterial suspension. The suspensions were sonicated for 10 × 8 s in an ice bath and then centrifuged

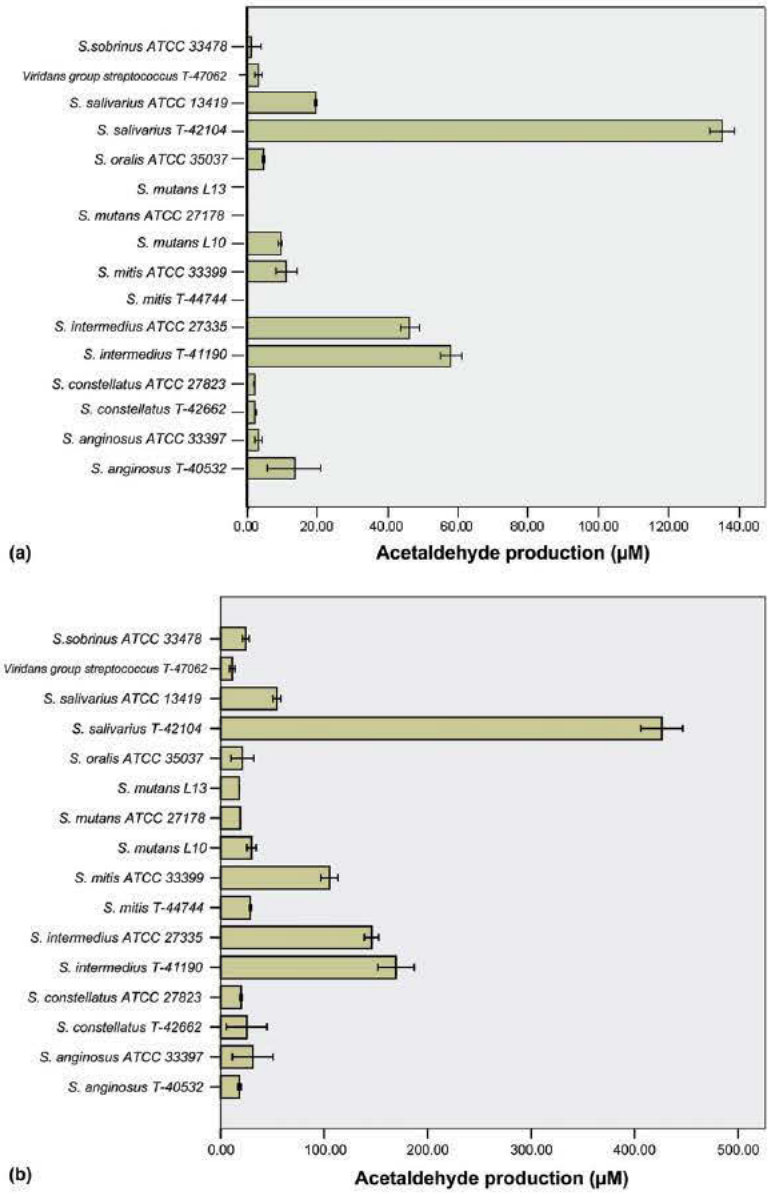

Figure 1 Acetaldehyde production in 11 mM (a) and in 1100 mM (b) ethanol concentration. The samples were incubated for 30 min and the bacterial concentration used was 1×10^8 CFU/ml.

with 39,000 rpm for 65 min at 4 °C. The supernatants were thereafter collected and used for the analyses. Cytosolic ADH-activities were determined by measuring the fluorescence, after addition of ethanol, of NAD (final concentration 2.5 mM) or NADP (final concentration 1 mM) at 340 nm at 25 °C in 0.1 M glycine buffer (pH 9.6). The ethanol concentrations used varied from 0.625 mM to 2200 mM.

ADH activity was determined by using Tecan SAFIRE monochromator-based microplate detection system and Magellan Software V3.11 (Tecan Trading AG, Switzerland). The Km-values of the enzyme activities were calculated.[19]

Statistics

Results are expressed as means (±SEM) of at least three different determinations. Statistical significance of the differences between the acetaldehyde productions in both ethanol concentrations used was analyzed by Wilcoxon Signed Rank Test using SPSS 12.0 (SPSS Inc. ©).

Results

Large differences and variations considering the amount of produced acetaldehyde could be detected among the tested 16 different viridans group streptococci.

Streptococcus salivarius T-42104 produced the highest amount of acetaldehyde in the both ethanol concentrations (11 mM: 135.0 μM, 1100 mM: 426.3 μM) (Fig. 1a and b). Also *Streptococcus intermedius T-41190*, *S. intermedius ATCC 27335*, *Streptococcus mitis ATCC 33399* and *S. salivarius ATCC 13419* produced significant concentration of acetaldehyde in both ethanol concentrations (Fig. 1a and b). The mean (and median) acetaldehyde production ±SEM of all the tested strains was 19.4 ± 5.0 μM (4.2 μM) in 11 mM and 71.7 ± 15.04 μM (27.4 μM) in 1100 mM ethanol concentration, respectively.

Significant ADH-activities were detected in the four bacterial strains, which were all high acetaldehyde producers. The respected Km-values for the ADHs were calculated for three strains and they showed significant variations, for *S. salivarius T-42104* the Km-value was 1.2 mM, for *S. intermedius ATCC 27335* it was 1490.0 mM and for *S. mitis ATCC 33399* 758.3 mM. For *S. intermedius T-41190* the calculation of the Km-value was impossible, because the ADH-activity increased linearly with all ethanol concentrations.

The effect of incubation time and concentrations of the bacterial suspensions to the produced acetaldehyde was tested with *S. salivarius T-42104*. Acetaldehyde production increased linearly with the ethanol incubation time with standard bacterial concentration as represented in Figure 2. Figure 3 shows the effect of bacterial concentration to the amount of produced acetaldehyde in standard incubation time.

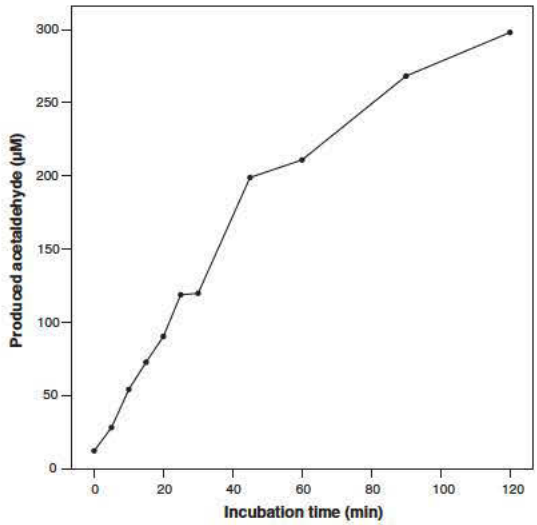

Figure 2 Time dependent acetaldehyde production of *S. salivarius T-42104* with 1×10^8 CFU/ml bacterial concentration in 11 mM ethanol concentration.

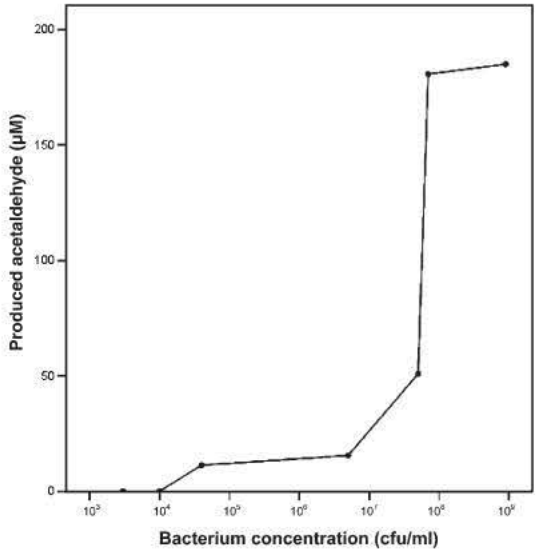

Figure 3 Acetaldehyde production in different bacterial concentrations with *S. salivarius T-42104* in 11 mM ethanol concentration after 30 min of incubation.

Discussion

Alcohol is one of the main risk factors for oral cancer. Alcohol itself is not carcinogenic, but it is oxidized to carcinogenic acetaldehyde in saliva by the ADH-enzyme of some oral microbes of the normal oral microflora.[5,10,20,21] Viridans group streptococci, oral streptococci are part of the normal microflora and represents facultative anaerobic bacteria. Under anaerobic conditions these bacteria can produce energy through fermentation of glucose to alcohol using their ADH-enzyme. However, when oxygen is available the ADH-reaction runs to the opposite direction and produces acetaldehyde from ethanol.[5]

Several studies have described the important role of microbes in the production of carcinogenic acetaldehyde to the oral cavity. It has been demonstrated in vitro that increased salivary acetaldehyde production is associated with poor oral hygiene and with an increased number of bacteria in saliva.[22] Moreover, some oral *Neisseria*-strains have been able to produce significant amounts of acetaldehyde in vitro.[10] In vivo, patients with oral cancer have increased number of bacteria of normal flora such as oral streptococci at their tumour sites. In addition, the tumour sites are frequently colonized by *Candida albicans*-strains, which have earlier been shown to be massive acetaldehyde producers.[11,23] This local acetaldehyde production could be decreased with an antimicrobial chlorhexidine treatment, which further highlights the important role of microbes in acetaldehyde production in the mouth.[3]

There is strong evidence of the carcinogenicity of acetaldehyde. It causes point mutations in human lymphocytes, induces sister chromatid exchanges and cross chromosomal aberrations. Acetaldehyde even interferes with the DNA-repair machine.[24–26] Most recently, it was demonstrated that polyamines are able to facilitate the formation of mutagenic DNA-adducts in biologically relevant acetaldehyde concentrations (50–100 μM).[4] The most convincing evidence for the in vivo carcinogenic influence of acetaldehyde is derived from epidemiological and biochemical studies on ALDH2-deficient Asians. Mitochondrial ALDH2-enzyme is responsible for most of the acetaldehyde oxidation to acetate. Among ALDH2-deficient subjects this enzyme is partly inactive, which results in the accumulation of acetaldehyde in saliva after alcohol drinking.[27] In Asian heavy drinkers ALDH2-deficiency associates with a 10-fold risk of oral cancer as compared to those with the normal ALDH2-enzyme.[28]

The concentration of ethanol in saliva reflects directly that of blood.[29] Therefore, it is possible to detect ethanol in saliva hours after drinking alcohol as salivary ethanol concentration decreases at the same speed as that in the blood. During and immediately after drinking, ethanol concentration in saliva is temporarily much higher because ethanol from alcoholic beverages dissolves directly to saliva. A concentration of 11 mM ethanol can be found in saliva of an average male after drinking 0.5 g alcohol per kg body weight and thus can be considered as clinically relevant. Therefore, concentrations 11 mM and 1100 mM were used in the study. As presented in the results, significant acetaldehyde production was detected at both ethanol concentrations. Accordingly, acetaldehyde production by the oral streptococci is evident already at the beginning of alcohol drinking. Similar results were obtained in a previous study where some *Neisseria*-strains were investigated for their acetaldehyde production capacity.[10]

To our knowledge, this is the first study where oral streptococci, which comprise a major part of the normal oral microflora,[15] were tested systematically for their acetaldehyde production capacity. Significant differences between the strains regarding acetaldehyde production capability and ADH-activity were detected. Based on our results it could be stated that certain oral streptococci are able to produce acetaldehyde in concentrations detectable also in saliva samples in vivo during ethanol challenge. The marked acetaldehyde producing capacity of the clinical strain of *S. salivarius* is a particularly important finding as the bacterium colonizes the mucosal surface of the oral cavity which rarely is colonized by other normal floras bacteria. The mucosal surface is the site where the oral cancer manifestations are frequently found.[30] This bacterium had also an extremely high ADH-activity and low Km which indicates that acetaldehyde may be produced in the oral cavity already in very low alcohol concentrations.

Four of the tested streptococcus strains showed ADH-activity. It remains to be examined, why some bacteria were able to produce acetaldehyde, but did not possess either NAD- or NADP-dependent ADH-activity. However, other described metabolic pathways than the ADH-mediated metabolism could account for the minor acetaldehyde production observed in these strains.[31] On the other hand, we were able to show that several oral streptococci are able to produce high amounts of acetaldehyde from ethanol in saliva and that this production is dependent on the activity and characteristics of ADH-enzyme of the bacteria.

In conclusion oral streptococci may contribute significantly to the normal individual variation of salivary acetaldehyde levels after alcohol drinking and thereby also to the risk of oral cancer. As the in vitro measured acetaldehyde production has been shown to correlate with that in vivo[3] *S. salivarius T-42104* could be considered as a harmful bacterium in heavy drinkers. Further studies are nevertheless warranted in order to assess the possible role of oral streptococci in the pathogenesis of oral cancer. The most obvious approach in the future will be the measurement of acetaldehyde production of microbial samples obtained from patients with premalignant changes such as oral leukoplakia and at the sites of diagnosed cancer.

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Letters to the Editor

Chronic candidosis and oral cancer in APECED-patients: Production of carcinogenic acetaldehyde from glucose and ethanol by *Candida albicans*

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Dear Sir,

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autosomal recessive disease caused by mutations of the AIRE (autoimmune regulator) gene.[1–3] It is associated with a limited T lymphocyte defect and an autoimmune response to various tissues, particularly endocrine glands. It mainly causes a set of three abnormal features; chronic mucocutaneous candidosis, hypoparathyroidism and adrenal insufficiency.[4] Most patients have chronic oral candidosis since early childhood. Of all the APECED patients in Finland that are beyond the age of 25, 10% have developed oral or oesophageal carcinoma at the site of chronic mucositis.[5] It is the only malignancy diagnosed in these patients. The age at cancer diagnosis was markedly low (29–44 years), significantly lower than for oral or oesophageal squamous cell carcinoma in general. The pathogenetic mechanism behind APECED associated oral cancer has so far remained unclear.[6–9]

The most important risk factors for upper digestive tract cancers are tobacco smoking, alcohol intake, and poor oral hygiene.[10–12] They all associate with increased acetaldehyde (ACH) levels in saliva, and there is strong evidence supporting the role of ACH as a common dominator.[13–18] The mechanism leading to increased ACH levels in saliva after alcohol consumption is local microbial ACH production, *i.e.* the oxidation of salivary ethanol (EtOH) to ACH by microbial alcohol dehydrogenase (ADH)-enzyme.[15–17] Previous studies have shown that *Candida albicans* can produce significant amounts of carcinogenic ACH in clinically relevant EtOH concentrations.[19]

It has been demonstrated that polyamines are able to facilitate the formation of mutagenic DNA-adducts in biologically relevant ACH concentrations (50–100 μM).[14] The local *in vivo* carcinogenic effect of ACH is derived from epidemiological and biochemical studies on aldehyde dehydrogenase-2 (ALDH2)-deficient Asians. The mitochondrial ALDH2-enzyme is responsible for most of the ACH oxidation to acetate. Among ALDH2-deficient subjects this enzyme is partly inactive, which results in the accumulation of ACH in saliva after alcohol drinking.[20] In Asian heavy drinkers ALDH2-deficiency is associated with a 10-fold risk of oral cancer when compared to those with the normal ALDH2-enzyme.[21] However, the relationship between glucose and ACH production by *Candida albicans* has not been investigated previously. *C. albicans* may play a role in metabolizing glucose into carcinogenic ACH in the mouth and could participate in the pathogenesis of oral cancer in non-alcohol drinkers. The aim of this study was to compare the production of ACH from EtOH and glucose by oral candida isolates from APECED patients with control isolates.

A total of 67 clinical oral isolates and one reference strain (ATCC 90029) of *Candida albicans* were selected for this study. Of these, 44 were isolated from 21 APECED patients during the years 1994–2007 (1–4 isolates/patients) mainly as reported earlier.[22] Twelve isolates from patients with oral carcinoma were designated as cancer-isolates and 11 isolates obtained from patients with oral candidosis but without any mucosal neoplasia were designated as control-isolates. The *C. albicans* isolates were identified from patient samples using conventional culture and identification methods at the Clinical Microbiology Laboratory of the Helsinki University Central Hospital. The identification of *C. albicans* was based on colony morphology on CHROMagar® Candida medium (CHROMagar, Paris, France), and the negative Bichro-Dubli® latex co-agglutination test result (Fumouze Diagnostics, Levallois Perret, France). *C. albicans* strains were subcultured on Sabouraud Dextrose agar (SP; Saboraud Dextrose Agar (Lab M), Bacto Agar (Difco) supplemented with penicillin (100 000 ui/ml) and streptomycin) for 48 hr at 35°C. Colonies were suspended in phosphate buffered saline (PBS) and adjusted to an optical density (OD) of 0.4 at 492 nm (Multiscan RC spectrophotometer, Labsystems, Helsinki, Finland) corresponding to 1×10^7 colony forming units per millilitre (CFU/ml) as controlled by dilution plating.

A total of 400 μl of the yeast suspension was transferred into a gas chromatograph vial. Thereafter, 50 μl of PBS-buffer containing EtOH or glucose was added and the vial was immediately closed tightly. The final EtOH concentration was adjusted to 11 mM and the final glucose concentration to 100 mM. Samples were incubated for 30 min at 37°C, and the reaction was stopped by injecting 50 μl of perchloric acid

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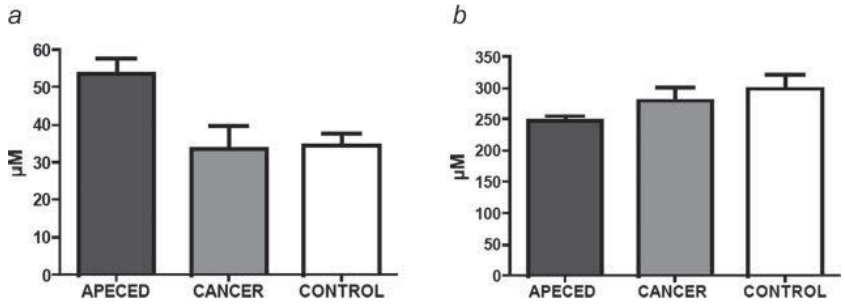

FIGURE 1 – The acetaldehyde production of *Candida albicans* isolated from APECED ($n = 44$), cancer ($n = 12$) and control patients ($n = 11$) in glucose (*a*) and ethanol (*b*) incubation. Mean (+SEM) µM of each group presented. When incubated in 100 mM glucose (*a*) the APECED isolates produced significantly higher amounts of acetaldehyde than the cancer isolates ($p < 0.0001$) and the control isolates ($p < 0.0001$). When incubated in 11 mM ethanol (*b*) the cancer and control isolates produced significantly higher amounts of ACH from ethanol than APECED isolates ($p = 0.0072$ and $p = 0.0003$, accordingly). The differences between the cancer and the control isolates were not significant.

(PCA, 6M) through the rubber septum of the test vial. Every isolate was assayed in triplicate and the mean was used for the analyses. To measure the baseline and artefactual ACH, 50 µl of PCA was immediately added to control vials and the suspension was equally incubated for 30 min at 37°C. The formed ACH was measured by gas chromatography as reported earlier.[23]

Results are presented as mean ± standard error of mean (SEM). Data was analyzed by using Graph Pad Prism version 5.00 (GraphPad Inc. San Diego, California, USA). The two-tailed Mann Whitney *U*-test was used for the comparisons between the patient groups. The two-tailed paired *t*-test was used for the comparisons within patient groups. *p* values of less than 0.05 were considered statistically significant.

When incubated in glucose the mean ACH production by the 44 *C. albicans* isolates from APECED patients was 53.5 µM (±2.3 µM). The means for the cancer and control isolates were 33.7 µM (±3.5 µM) and 34.6 µM (±1.9 µM), respectively (Fig. 1*a*). The APECED isolates produced significantly higher amounts of ACH than the cancer isolates ($p < 0.0001$) and the control isolates ($p < 0.0001$). The differences between the cancer and the control isolates were not significant. The mean ACH production by the reference strain of *C. albicans* ATCC 90029 was 38.02 µM ± 2.06 µM in glucose incubation.

When incubated in EtOH the mean ACH production of the APECED isolates was 247.9 µM (±4.2 µM). The means for the cancer and control isolates were 280.2 µM (±11.8 µM) and 299.1 µM (±12.7 µM) (Fig. 1*b*). The cancer and control isolates produced significantly higher amounts of ACH from EtOH than APECED isolates ($p = 0.0072$ and $p = 0.0003$, accordingly). The differences between the cancer and the control isolates were not significant. The mean ACH production by the reference strain of *C. albicans* ATCC 90029 was 157.43 µM (±1.57 µM) in EtOH incubation. All *C. albicans* isolates produced significantly more ACH in EtOH than glucose incubation ($p = <0.0001$).

From 14 APECED patients multiple isolates (2–4 per patient) from years apart were tested. There was no significant difference in the ACH production from EtOH or glucose between the earlier and more recent isolates from 6 patients. There were some differences between the earlier and more recent isolates from 8 patients, mostly when incubated in glucose, but there was no obvious temporal trend nor were the differences significant.

In this study we were able to show that *C. albicans* isolated from APECED patients produced potentially mutagenic amounts of ACH when incubated in 100 mM glucose. The amount of ACH produced was significantly higher when compared to *C. albicans* strains isolated from groups of patients with oral cancer or from healthy controls. 100 mM glucose is equivalent to 18 g/L, which can commonly be found in food and drinks.[24] Consumption of sweeter products may lead to even higher ACH levels and prolonged exposure. 100 mM glucose has also been shown to increase biofilm formation and adhesion of *C. albicans* in the oral cavity that may enhance the local mucosal exposure to ACH.[24,25]

The mean age at diagnosis of oral cancer in Finland is over 60 years and oral cancer is very uncommon among healthy young adults.[26] In APECED patients, however, the mean age at diagnosis is only 37 years.[5] The early onset of oral cancer in APECED patients could be partially due to their immune defect and other intrinsic factors. However, both extrinsic and intrinsic factors may operate, and with many of the factors, only long exposure might be carcinogenic. Most APECED patients suffer from refractory oral candidosis since early childhood and the oral carcinoma typically develops at the site of chronic mucositis. This supports the role of *C. albicans* in the pathogenesis of the carcinoma.[5]

According to our results all *C. albicans* isolates analysed were capable of producing carcinogenic levels of ACH from EtOH. The isolates from cancer and control subjects produced significantly higher amounts of ACH than those from APECED patients but the clinical relevance of this difference is questionable since all isolates produced very high amounts. The 11 mM EtOH concentration used can be found in saliva for hours after social alcohol consumption or naturally in many products produced by fermentation.[17]

The main finding of this study is that *C. albicans* may play a role in metabolizing glucose to carcinogenic ACH in the mouth and could participate in the pathogenesis of oral cancer in non-alcohol drinkers. The observation supports the concept of a novel microbially mediated mechanism in the pathogene-

sis of oral cancer and could partly explain why chronic oral candidosis is carcinogenic in APECED patients and why they have a high risk for oral cancer at an early age.

Yours sincerely,

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III

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Xylitol inhibits carcinogenic acetaldehyde production by *Candida* species

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Acetaldehyde is a highly toxic and mutagenic product of alcohol fermentation and metabolism which has been classified as a Class I carcinogen for humans by the International Agency for Research on Cancer of the World Health Organisation (WHO). Many *Candida* species representing oral microbiota have been shown to be capable of marked acetaldehyde production. The aim of our study was to examine the effects of various sugar alcohols and sugars on microbial acetaldehyde production. The study hypothesis was that xylitol could reduce the amount of acetaldehyde produced by *Candida*. Laboratory and clinical isolates of seven *Candida* species were selected for the study. The isolates were incubated in 12 mM ethanol and 110 mM glucose, fructose or xylitol at 37°C for 30 min and the formed acetaldehyde was measured by gas chromatography. Xylitol significantly ($p < 0.0001$) reduced the amount of acetaldehyde produced from ethanol by 84%. In the absence of xylitol, the mean acetaldehyde production in ethanol incubation was 220.5 μM and in ethanol–xylitol incubation 32.8 μM. This was found to be mediated by inhibition of the alcohol dehydrogenase enzyme activity. Coincubation with glucose reduced the amount of produced acetaldehyde by 23% and coincubation with fructose by 29%. At concentrations that are representative of those found in the oral cavity during the intake of proprietary xylitol products, xylitol was found to reduce the production of carcinogenic acetaldehyde from ethanol by *Candida* below the mutagenic level of 40–100 μM.

Acetaldehyde is a highly toxic and mutagenic product of alcohol fermentation and metabolism. Recently, acetaldehyde was reclassified as a Class I carcinogen for humans by the International Agency for Research on Cancer of WHO.[1] Mutagenicity can take place at concentrations as low as 40–100 μM of acetaldehyde.[2] Microbes possessing alcohol dehydrogenase (ADH) activity can produce acetaldehyde by ethanol oxidation.[3] This reaction takes place in the oral cavity when consuming alcohol and it continues for as long as there is ethanol in saliva.[4] Due to the equal distribution of ethanol to the fluid compartments of the body its concentration in saliva is the same as in the blood and its clearance is nonlinear in both.[5] *Streptococcus viridans* and *Neisseria* group bacteria belonging to the core oral microbiota have been shown to be able to produce significant amounts of acetaldehyde from ethanol.[6,7] Furthermore, many *Candida* species representing the normal oral flora of the vast majority of the population have been shown to be capable of even more marked acetaldehyde production.[8–10] Microbial acetaldehyde production is one of the key mechanisms in the cumulative exposure to carcinogenic acetaldehyde.[11]

Candida species can produce significant amounts of acetaldehyde also by alcohol fermentation from glucose.[9,12] Glucose is first metabolized *via* this pathway into pyruvate and thereafter into acetaldehyde and ethanol. Fructose can similarly be metabolized into pyruvate or alternatively to glycerol. Glucose together with fructose constitutes the dietary disaccharide sucrose. Xylitol is a five-carbon nonfermentable sugar alcohol which is metabolized into D-xylulose by xylitol dehydrogenase (XDH) closely resembling ADH.[13] Sugars are the main carbon and energy source for yeast cells but they can also use alternative nutrient sources such as amino acids and proteins. Different nutrients run through multiple metabolic pathways whose enzyme activities are regulated mainly by availability of oxygen and cellular coenzyme balance.

It has been suggested that chlorhexidine oral rinses could be used in controlling microbial acetaldehyde production in the oral cavity.[4] Chlorhexidine is a broad spectrum

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Short Report

Table 1. *Candida* isolates used in the study

Species	Isolate/identification no.	Source
C. glabrata	CCUG 32725	Blood
	G212	Oral cavity
C. parapsilosis	ATCC 22019	Stool
	G170	Oral cavity
C. tropicalis	ATCC 750	Sputum
	G9	Oral cavity
C. dubliniensis	UK NEQAS 2/07	Pharynx
	G130	Oral cavity
C. guilliermondii	UK NEQAS 9/06	Nail
	G66	Oral cavity
C. krusei	ATCC 6258	Sputum
	T880	Pharynx
C. albicans	ATCC 90029	Blood
	HI2580	Oral cavity

Abbreviations: ATCC: American Type Culture Collection; CCUG: Culture Collection of University of Gothenburg; UK NEQAS: United Kingdom National External Quality Assessment Service.

disinfectant capable of significantly reducing the number of viable microbes and the amount of acetaldehyde produced during ethanol consumption.[4] However, it has multiple side effects and is not recommended for continuous use. The effect of various sugar alcohols and sugars on microbial acetaldehyde production has not previously been investigated. Therefore, the aim of our study was to examine the effects of glucose, fructose and xylitol in microbial acetaldehyde production during alcohol oxidation and fermentation. The study hypothesis was that xylitol could inhibit acetaldehyde production from ethanol by high acetaldehyde producing *Candida* species belonging to the core oral microbiota of humans.

Material and Methods

Microbial strains

Laboratory and clinical isolates of seven *Candida* species were selected for the study (Table 1). The laboratory strains were obtained from culture collections and external quality control specimens. Clinical isolates had been isolated from patient samples by using conventional culture methods at the HUSLAB Laboratory of Clinical Microbiology of the Helsinki University Central Hospital (T- and HI-isolates) and Helsinki University Dental Institute (G-isolates) and stored in the departmental depository in −80°C. Identification was based on colony morphology, growth on chromogenic agar, microscopy and biochemical reactions.

Acetaldehyde measurement

Yeasts were incubated on Sabouraud dextrose agar plates (Lab M, Lancashire, UK) in aerobic conditions at 48 h at 37°C as described earlier.[9] Colonies were suspended in phosphate buffered saline (PBS) and then the optical density of the solution at 492 nm was adjusted spectrophotometrically to 0.4 (Multiscan RC, Labsystems, Helsinki, Finland) corresponding 1×10^7 colony forming units per millilitre by quantitative microbial culture. Aliquots of 350 µl of this yeast suspension were transferred into gas chromatograph vials. Thereafter, 50 µl of PBS buffer containing ethanol (final concentration 12 mM) and 50 µl PBS buffer containing glucose, fructose or xylitol (final concentration 110 mM) was added and the vial was immediately sealed. The ability of microbes to produce acetaldehyde from ethanol, glucose, fructose or xylitol alone was analyzed by adding 50 µl of one of these and 50 µl of PBS buffer to the yeast aliquots. The samples were thereafter incubated for 30 min at 37°C. The reaction was stopped by injecting 50 µl of 6 M perchloric acid (PCA) through the rubber septum of the vial. Three parallel samples were processed and the experiment was repeated. The means were calculated and used for the statistical analysis. To measure the baseline and artefactual acetaldehyde, 50 ml of PCA was immediately added to control vials and the suspension was equally incubated for 30 min at 37°C. The formed acetaldehyde was measured by gas chromatography (Perkin Elmer Headspace sampler HS 40XL, Perkin Elmer Autosystem Gas Chromatograph equipped with Ionization Detector FID, USA) and subtracted from the initial values as reported earlier.[14]

ADH analyses

ADH activity was analyzed for the highest (*Candida glabrata CCUG 32725*), the lowest (*Candida krusei ATCC 6258*) and an average (*Candida albicans ATCC 90029*) acetaldehyde producer. The ADH activity was measured using fluorescence analysis with cofactor nicotinamide adenine dinucleotide (NAD) as described earlier.[6] For the analyses, the yeast cells were first grown as described above and then lysed by glass bead vortexing in the presence of a protease inhibitor cocktail (SIGMA, P 8340, Missouri, USA). Five 1 min vortexing cycles and glass beads of 1.0 mm diameter were used. The samples were cooled on ice before each cycle. Cell lysates were centrifuged for 5 min at 2,900*g* (Hettich EBA 20, Germany), the supernatants were collected and further centrifuged at 139,700*g* for 65 min at +4°C (Beckman Optima LE-80k Ultracentrifuge, USA). This supernatant was collected and used for the analyses. Cytosolic ADH activity was determined by measuring the fluorescence (ex 340 nm, em 440 nm) after addition of ethanol or ethanol and xylitol (final concentration 100 mM) and NAD (final concentration 2.5 mM) at 37°C in 0.1 M glycine buffer (pH 9.6). Ethanol concentrations 0.68 to 2174 mM were used. ADH activity was determined by using Tecan SAFIRE monochromator-based microplate detection system and Magellan Software V6.05 (Tecan Trading AG, Switzerland). The Lineweaver–Burk plot was used to determine the enzyme activities.

Statistical analysis

SPSS ver. 16.0 (SPSS, Chicago, IL, USA) was used for the statistical analyses. The results are expressed as means (±SEM)

Short Report

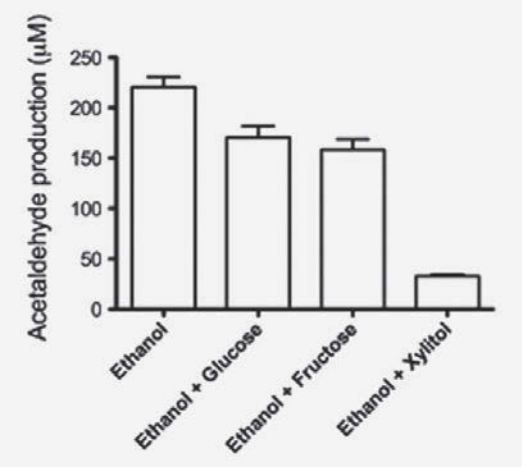

Figure 1. Mean acetaldehyde production (±SEM) by the 14 *Candida* isolates in different incubations. Final ethanol concentration used was 12 mM and final glucose, fructose and xylitol concentration was 110 mM. Samples were incubated for 30 min at 37°C and acetaldehyde production was measured by gas chromatography.

or as individual data points and means. The generalized estimating equations model was used for comparisons between species and experimental conditions. Replicate values for each isolate formed the subgroups in this model. Spearman's rho (rs) with a 95% confidence interval and the means of replicate values were used for the analyses of correlations. A p value less than 0.05 were considered as statistically significant.

Results

All *Candida* isolates were able to produce significant amounts of acetaldehyde. The mean acetaldehyde production in ethanol incubation at 30 min was 220.5 μM (±10.2) (Fig. 1). Xylitol coincubation reduced the amount of acetaldehyde produced by a mean of 84%. This was statistically highly significant ($p < 0.0001$). The mean acetaldehyde production in ethanol–xylitol incubation was 32.8 μM (±1.7). When the isolates were coincubated with glucose or fructose a less marked reduction was observed (Fig. 1). Coincubation with glucose reduced the amount of produced acetaldehyde by mean 23% and coincubation with fructose by mean 29% when compared to incubation with ethanol alone (p = NS). The mean acetaldehyde production in ethanol–glucose incubation was 170.4 μM (±11.3) and in ethanol–fructose incubation, 158.5 μM (±10.3). None of the isolates were able to produce significant amounts of acetaldehyde when incubated in the absence of ethanol. The mean acetaldehyde production in glucose incubation was 26.4 μM (±4.2) and 21.4 μM (±3.6) in fructose incubation at 30 min. In xylitol incubation, the mean acetaldehyde production was 1.8 μM (±0.3).

Large variation was seen in acetaldehyde production between the *Candida* species whereas within-species variation was less striking (Supporting Information Fig. 2). *C. glabrata* isolates were found to be the highest producers and *C. krusei* isolates the lowest producers of acetaldehyde under all conditions. In ethanol incubation, the *C. glabrata* isolates produced mean 366.1 ± 10.1 μM of acetaldehyde whereas the *C. krusei* isolates produced mean 53.7 ± 9.1 μM. The differences between the species within each experimental condition were statistically significant ($p < 0.0001$). *C. glabrata* isolates differed from the other *Candida* species by showing higher acetaldehyde production in ethanol–glucose and ethanol–fructose coincubation than in ethanol alone. In all *Candida* isolates acetaldehyde production in ethanol–glucose and in ethanol–fructose incubations showed a significant positive correlation [rS = 0.94 (0.81–0.98), $p < 0.0001$]. No correlation was found in acetaldehyde production between ethanol–xylitol incubation and ethanol–glucose or in ethanol–fructose incubation. *C. albicans* and *C. glabrata* isolates showed high ADH activities (V_{max} = 3.40 s^{-1}, K_m= 0.16 mM and V_{max} = 4.47 s^{-1}, K_m = 2.83 mM, respectively) whereas the ADH activity of the *C. krusei* isolate was low (V_{max} = 1.66 s^{-1}, K_m = 0.13 mM). Xylitol reduced the ADH activity of the *C. glabrata* isolate by 61% and of the *C. albicans* isolate by 100% when coincubated with 110 mM ethanol for 10 min (Supporting Information Fig. 3). When coincubated with 11 mM ethanol, the reduction was 66% in *C. glabrata* isolate and 100% in *C. albicans* isolate. In *C. krusei* no effect of xylitol on ADH activity could be detected.

Discussion

Xylitol was found to significantly inhibit candidal acetaldehyde production from ethanol. It reduced the production below the mutagenic acetaldehyde level of 40–100 μM for all *Candida* species tested.[2] In the absence of xylitol, the mean acetaldehyde production in ethanol incubation was high (220.5 μM). Xylitol has previously been shown to inhibit the metabolism of sugars by acidogenic oral bacteria and thus prevent tooth decay.[15] It is therefore incorporated in chewing gums and tablets as well as in health care products such as dentifrice and oral rinses. Five to six grams and three daily exposures are required for this clinical effect.[16] A xylitol concentration of 65 mM has been shown to have antimicrobial activity against otopathogenic bacteria.[17] In our study, the final concentration of 110 mM xylitol was used. This equals to 17 mg/mL readily available in the oral cavity during intake of xylitol products. Concentrations higher than 30 mg/mL can be detected in saliva during chewing xylitol-sweetened chewing gum and the levels have been found to remain significantly elevated up to 30 min.[18,19]

Xylitol strongly inhibited candidal ADH activity. It is possible that xylitol directly inhibits the ADH *via* blockage of the substrate binding site. XDH belongs to the ADH enzyme family and the NAD-binding part of XDH resembles that of the liver ADH and other enzymes within the family.[13,20]

Xylitol metabolism may also compete for the nicotinamide adenine dinucleotide (NADH) coenzyme leading to downregulation of ADH. Product inhibition and other regulatory circuits may also counter-regulate the ADH activity. The nature of the inhibition of ADH enzyme by xylitol remains to be established in future studies. Fructose and glucose reduced acetaldehyde production in ethanol incubation to lesser extent. This is likely to be due to catabolite repression and preference of sugars as carbon and energy source over ethanol. *C. glabrata* isolates were found to differ from the other *Candida* species by producing increased amounts of acetaldehyde when incubated with ethanol and fructose or glucose. This is not completely surprising as carbohydrate metabolism of *C. glabrata* differs in many ways from that of other candidal species and it is more adapted to fermentative anaerobic growth.[21,22]

In conclusion, our *in vitro* study shows that xylitol in concentrations that are comparable to those *in vivo* during intake of xylitol products reduce remarkably the production of carcinogenic acetaldehyde from ethanol by *Candida* representing normal oral flora. This appears to be caused by the xylitol-induced inhibition of the microbial ADH enzyme. Further studies are warranted to find out the potency of xylitol to control microbial acetaldehyde production *in vivo* after an alcohol challenge.

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Short Report

IV

Acetaldehyde production and microbial colonisation in oral squamous cell carcinoma, lichen planus and oral lichenoid lesion

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Short title: Acetaldehyde and microbes in oral carcinoma, oral lichen planus and oral lichenoid lesion

Key words: Acetaldehyde, oral cancer, lichen planus, lichenoid lesion, microbes

Abbreviations: OSCC oral squamous cell carcinoma, OLD oral lichenoid disease, CO control patient, CFU colony forming unit

Article category: Infectious Causes of Cancer

Our study provides novel findings regarding acetaldehyde production in association with microbial colonisation of oral squamous cell carcinoma, oral lichen planus and oral lichenoid lesion. Our study also provides unique information on the effect of smoking and ethanol consumption on the microbial colonisation and acetaldehyde production in oral malignant and potentially malignant lesions.

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ABSTRACT

The main risk factors for oral cancer are heavy drinking and tobacco smoking which both result in increased salivary acetaldehyde levels. In the oral cavity this carcinogen is mainly produced as result of microbial ethanol metabolism. Oral lichen planus and oral lichenoid lesion are potentially malignant mucosal disorders, and especially their atrophic and erosive forms have been linked to oral cancer. The aetiology of the malignant transformation remains unclear. The main aim of this prospective study was to explore the ability of the oral microbiome to produce acetaldehyde when exposed to clinically relevant amounts of ethanol. A total of 90 patients (30 oral squamous cell carcinoma, OSCC; 30 oral lichenoid disease, OLD; 30 healthy controls, CO) being treated at Helsinki University Central Hospital were enrolled in the study. Microbial samples for culture were taken from the mucosa using a filter paper method. The density of microbial colonisation was calculated and the spectrum analysed. Microbial acetaldehyde production from ethanol (22mM) was measured by gas chromatography. The majority (68%) of the cultures from the patient samples produced carcinogenic levels of acetaldehyde (>100 μM). The mean acetaldehyde production by microbes cultured from smoker samples was significantly higher (213 μM) than from non-smoker samples (141 μM) (P=0.0326). Acetaldehyde production did not correlate with the total microbial counts. In conclusion, the microbiota from OSCC and OLD patients as well as healthy individuals are able to produce carcinogenic levels of acetaldehyde and smoking increases the capacity for acetaldehyde production markedly.

INTRODUCTION

Oral cancer is amongst the most common cancers worldwide and its prevalence is increasing [1,2]. In Europe, over 100,000 new cases of oral cancer are diagnosed every year [3]. Over 95% of all oral cancers are squamous cell carcinomas (OSCC) [4]. Regardless of the advances in treatment modalities the mortality rates of OSCC patients have not improved and the 5-year survival rate is less than 50% [5]. The prominent risk factors for OSCC are tobacco (smoked and smokeless) and chronic alcohol consumption [2,6,7,8]. In addition, some oral mucosal diseases and conditions such as oral lichen planus and oral lichenoid lesion are potentially malignant [9,10,11]. Malignant transformation into OSCC is seen in approximately 2% of the cases in five-year follow-up [12].

There is a strong link between chronic inflammation and many types of cancers [13]. In line with this, atrophic and erosive types of oral lichenoid lesions in particular have been linked with malignant transformation [14,15]. Oral lichen planus is a chronic systemic inflammatory disorder and results from T-cell immune dysregulation whereas oral lichenoid reaction is typically compatible with a type IV hypersensitivity reaction [16,17]. In oral lichen planus, removal of DNA-damaged cells essentially depends on the activity of the tumour suppressor protein P53 and other proteins of the P53 family [18,19]. Mutations in the *TP53* gene are commonly detected in various human tumours, including OSCC [20].

Acetaldehyde has been linked in many studies to digestive tract cancers [21,22,23] and there is increasing evidence that acetaldehyde rather than alcohol itself is responsible for the carcinogenic effect of high alcohol consumption [24,25,26]. Acetaldehyde is the first metabolite of ethanol and has been shown to be carcinogenic both in animal models and *in vitro* in concentrations as low as 100 μM - a concentration that can be found in the saliva after moderate alcohol consumption [1,25,26,27,28]. It has been reclassified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen in association with alcohol consumption [29]. Acetaldehyde can also been found in cigarette smoke. It binds to DNA and forms DNA adducts, causes point mutations, DNA crosslinking and interferes with the synthesis and repair of DNA [24,25,27,30]. It has, for example, been shown to have a role in *TP53* mutations in oesophageal cancers [31]. Acetaldehyde may also induce inflammation and metaplasia of the tracheal epithelium and enhance cell injury [25].

Normal human saliva does not contain measurable levels of acetaldehyde. However, mutagenic concentrations of acetaldehyde are found in saliva during and after ingestion of alcohol as well as smoking [22,28,32]. Chronic smoking has been shown to modify the oral microbiome to produce more acetaldehyde from ethanol both in vitro and in vivo [32,33]. Furthemore, poor oral hygiene increases in vitro acetaldehyde production into saliva [34]. Microbes of the gastrointestinal tract and oral cavity produce acetaldehyde mainly by the oxidation of ethanol [35]. A spectrum of microbes, including oral streptococci, *Candida* and *Neisseria* spp., have been shown to be capable of producing significant levels of acetaldehyde [36,37,38,39]. Chronic oral candidosis has been associated with oral carcinoma in a number of studies [40,41,42,43]. Also, the neglect of oral hygiene and dental care has been associated with a higher incidence of OSCC [44].

The oral microbiome is diverse and it is continuously being modified by the host immune responses, such as chronic inflammation in the underlying mucosa. Acetaldehyde production by oral microbes colonising healthy, chronically inflamed or malignant oral mucosal surfaces has not been studied. All previous studies on acetaldehyde production by microbes from the oral cavity have been performed using saliva samples or mouthwashes [21,28,32,33]. This prospective study was designed to analyse the oral microbial colonisation of patients with OCSC and oral lichenoid disease and compare these to that of healthy individuals. The overall aim was to explore the ability of the oral microbiome in the different patient groups to produce acetaldehyde when exposed to clinically relevant levels of ethanol using a site-specific sampling method. Secondly, we wanted to evaluate the effect of alcohol consumption and smoking on the microbial colonisation and acetaldehyde production.

MATERIALS AND METHODS

Study design

A total of 90 patients (30 with oral squamous cell carcinoma, 30 with oral lichen planus or lichenoid lesion, 30 healthy controls) being treated at the Department of Oral and Maxillofacial Surgery, Helsinki University Central Hospital or at the Helsinki University Dental Hospital during 2007-2011 were enrolled (Table 1). After clinical assessment microbial samples for acetaldehyde measurement were taken using the filter paper sampling method [45]. Patients who had received antimicrobial therapy within the past 7 days, those with HIV or hepatitis virus infection were excluded. All participating subjects signed an informed consent before inclusion. Subjects filled in a questionnaire regarding their oral health and hygiene as well as cigarette and alcohol consumption. There were no statistical differences in the patient demographics or smoking and drinking habits between the study groups. The study has been approved by the Ethics Committees of the Helsinki University Central Hospital and the Helsinki Municipal Health Centre.

Subjects

Patients with oral squamous cell carcinoma. Of the 30 patients with clinically and histopathologically diagnosed oral squamous cell carcinoma (OSCC group) 12 were female and 18 were male. The anatomical locations of the lesions were the tongue (n=5), the floor of the mouth (n=5), the palatinum (n=3), the sulcus (n=2), the gingiva (n=10) and the tonsil (n=1). The mean age of the OSCC patients was 65.6 years (range 39-85). The mean age for the females was 68.8 years (range 53-85) and for males 63.4 (range 39-80 years). Nine (32%) of the OSCC patients were smokers. They smoked mean 9 cigarettes per day (range 4 to 20) and had smoked for mean 10 years (range 2 to 25 years). Twenty-four (79%) OSCC patients consumed alcohol and five (17%) were heavy drinkers, all male (Table 1). One OSCC patient declined to fill in the questionnaire.

Patients with oral lichen planus or lichenoid lesion. Of the 30 patients enrolled into the study with the clinical diagnosis of oral lichenoid disease 24 were histopathologically confirmed as oral lichen planus (n=10) or lichenoid lesion (n=14) and these were included in the study (OLD group). Of these, 16 were female and 8 were male. The anatomical locations

of the lesions were the tongue (n=7) and on the cheek (n=16). The mean age of the OLD patients was 54.0 years (range 24-74). The mean age for the females was 57.0 years (range 29-72) and males 47.7 years (range 24-74). Four (19%) OLD patients were smokers (1 oral lichen planus, 3 oral lichenoid lesion). The smokers smoked mean 12 cigarettes per day (range 5 to 20) and had smoked for mean 8 years (range 0.5 to 13). Nineteen (91%) consumed alcohol and one male (5%) was a heavy drinker (Table 1). Three OLD patients declined to fill in the questionnaire.

Control patients. Of the 30 generally healthy patients with no mucosal lesions, included as the control group (CO) 19 were female and 11 were male. The mean age of the CO patients was 30.4 years (range 19-56). The mean age for the females was 32.3 years (range 20-56) and males 27.2 years (range 19-37). Nine (31%) of the control patients were smokers. They smoked mean 9 cigarettes per day (range 4 to 20) and had smoked for mean 10 years (range 2 to 25). Twenty-six (90%) control patients consumed alcohol and two (6%) were heavy drinkers, both female (Table 1). One control declined to fill in the questionnaire.

Collection of microbiological samples

The microbiological samples were taken from the oral mucosa using a hydrophilic mixed cellulose ester MFMillipore Membrane filter (GSWP01300; Millipore inc., MA, USA, pore size 0.22 μm, ∅ 13 mm) [45]. The filter was placed gently on the oral mucosa for 30 seconds, with the glossy side of the filter paper placed against the mucosa, after which it was placed into a sterile test tube containing 5 ml of sterile saline [45]. Two samples were collected from each OSCC and OLD patient: one from a representative mucosal lesion and another from a clinically healthy contralateral site. Samples from the CO patients were obtained from the buccal mucosa. The microbiological samples were collected during 2007-2011 by authors E.S., J.U., or P.R.

Microbiological analyses

The microbiological samples were immediately taken to the laboratory, Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki. All samples were cultured within one hour of collection by E.S. or J.U. Before culture, the samples were agitated for 30 seconds with four sterile ∅ 3mm glass beads. Samples were diluted 10-fold and 100 μl of the dilution and the neat suspension were cultured on selective and non-

selective media under aerobic and anaerobic conditions. Fastidious anaerobe agar (FAA; Fastidious Anaerobe agar (LAB-M LAB 90) supplemented with 5% horse blood) was used to enumerate the total cultivable bacteria. Lysed blood agar (BA; Trypticase soy agar (BBL 211047) and Mueller Hinton agar (BBL 212257) supplemented with 5% horse blood) was used for enumeration of total aerobic bacteria. Neomycin-vancomycin blood agar (NV; blood agar and neomycin sulphate (Sigma N-1876) supplemented with vancomycin (7.5 μ/ml), menadion (0.5 μg/ml) and sheep blood 5%) was used to enumerate anaerobic gram-negative bacteria. Cysteine-, lactose-and electrolyte deficient agar (CLED; C.L.E.D medium (BBL 212218) was used to select aerobic gram-negative fermentative rods. To detect yeasts Sabouraud Dextrose agar (SP; Saboraud Dextrose Agar (Lab M), Bacto Agar (Difco) supplemented with penicillin (100,000 iu/ml) and streptomycin) was used. The BA, CLED and SP plates were incubated at 37°C for 48 h and the FAA and NV plates were incubated under anaerobic conditions at 37°C for seven days. For the acetaldehyde analyses, each side of the filter paper was placed onto a FAA plate for 30 seconds and plates were evenly streaked and incubated as described above.

After incubation the numbers of bacteria and yeasts were enumerated (colony forming units, CFU) as described before [45]. Gram stain was performed on all different colony morphology types from CLED and NV agars and the number of gram-negative colonies was recorded. The ratio of gram-negative to gram-positive bacteria and the ratio of aerobic to anaerobic bacteria were determined.

Measurement of acetaldehyde production

Microbial colonies on the FAA plate were carefully scraped and washed off with 3ml of sterile saline. Aliquots of 400 μl of the suspension were transferred into parallel gas chromatograph vials. Then 50 μl of PBS-buffer containing ethanol (final concentration 22mM) was added, and the vials were immediately sealed. Samples were incubated for 60 min at 37°C and the reactions were stopped by injecting 50 μl of 6 M perchloric acid (PCA) through the rubber septum of the vial. Control vials where PCA was added prior to ethanol were used to measure background acetaldehyde and ethanol levels. Three parallel samples were processed and the mean values were used for statistical analysis. The formed acetaldehyde levels were measured by gas chromatography (Perkin Elmer Headspace sampler

HS 40XL, Perkin Elmer Autosystem Gas Chromatograph equipped with Ionization Detector FID, USA) [28].

Statistical analysis

Data was analysed by using GraphPad Prism version 5.00 (GraphPad Inc. San Diego, California, USA). The two-tailed Mann Whitney test and the One-way ANOVA, Bonferroni post test and Fisher's exact test were used for the comparisons between groups and Spearman's rho (r_S) was used for the analyses of correlations. P-values of less than 0.05 were considered statistically significant.

RESULTS

Acetaldehyde production

The majority (68%) of the cultures from the patient samples produced mutagenic levels of acetaldehyde (>100 μM). This included 76% of all OSCC lesion samples, 72% of OSCC control samples, 61% of OLD lesion samples, 67 % of OLD control samples and 60% of samples from control patients (*P* = ns). The mean level acetaldehyde produced by all samples was 158 μM (range 13-1000 μM; Table 2). There were no significant differences in the acetaldehyde production between samples from OLD patients diagnosed histologically with lichen planus and lichenoid reaction.

The mean acetaldehyde production by microbes cultured from smoker samples was significantly higher (213 μM, range 40-1000) than from non-smoker samples (141 μM, range 13-589) (*P*=0.0326). The cultures from the lesion sites of smoker OSCC and OLD patients produced significantly higher amounts of acetaldehyde than those of non-smoker OSCC and OLD patients (*P*=0.0351) (Figure 1). The difference was not significant between the acetaldehyde production in the control sites of smoker and non-smoker patients. The cultures from the OSCC lesion sites of smoker patients produced 228 μM (range 102-532 μM) acetaldehyde while cultures from the lesion sites of non-smoker OSCC patients produced mean 158 μM (range 13-503 μM). Cultures from OSCC control sites of smokers produced mean 135μM (range 50-258 μM) and of non-smokers mean 193 μM (range 48-589 μM)

acetaldehyde. The microbes cultured from the smoker OLD patient lesion and control sites produced mean 125 μM (range 44-206 μM) and 308 μM (range 99-616 μM) acetaldehyde, accordingly, where as the non-smoker OLD lesion and control sites produced mean 122 μM (range 35-271 μM) and 129 μM (range 41-271 μM) accordingly. The microbes cultured from smoker control patient samples produced mean 233 μM (range 40-1000) and non-smoker control samples mean 102 μM (range 23-194 μM) acetaldehyde. No significant differences were found between the levels of acetaldehyde produced by cultures from samples collected form alcohol consumers and non-drinkers in any of the patient groups or in samples from lesion or control sites.

Microbial findings

As determined by colony forming units (CFU) per sample, significantly higher number of microbes were present in OSCC lesions compared to the healthy mucosa of OSCC patients (P=0.0005), OLD lesions (P<0.0001), the healthy mucosa of OLD patients (P<0.0001), and the mucosa of CO patients (P<0.0001) (Table 2). OSCC patients had generally a denser colonisation of microbes on their oral mucosa, as significantly higher number of microbes were also detected from samples taken from their healthy sites compared to those from control patients (P=0.0004) or healthy mucosa of OLD patients (P=0.0010). There was no significant difference in the microbial colonisation density in OLD lesion or control sites as compared to control patients, nor between OLD patients histologically diagnosed with lichen planus and lichenoid reaction. There was no correlation between acetaldehyde levels and the total amount of cultivable microbes in any patient group or sample site.

Significantly higher number of aerobic bacteria (CFU/sample) were cultured from OSCC lesion sites compared to the corresponding control sites of OSCC patients (P=0.0003), lesion sites of OLD patients (P=0.0002), control sites of OLD patients (P<0.0001) and control patients (P<0.0001) (Table 2). There was no significant difference in the number of anaerobic bacteria (CFU/sample) between lesion and control site of OSCC patients. However, the lesion sites of OSCC patients had significantly higher amounts of anaerobes as compared to the lesion sites of OLD patients (P=0.0007), control sites of OLD patients (P<0.0001) and control patients (P<0.0001) (Table 2). The control sites of OSCC patients had significantly higher amounts of aerobes and anaerobes than the control sites of OLD patients (P=0.0008 and P=0.0199, respectively) and the control patients (P<0.0001 and P=0.0087, respectively)

(Table 2). The differences between the amount of aerobic and anaerobic bacteria in lesion and control sites of OLD patients were not significant. The majority of bacteria cultured from samples from all patient subgroups were Gram-positives: mean 4% of the bacteria in OSCC lesions and 3% in OSCC control sites, 12% in OLD lesions, 8% in OLD control sites and 8% in controls were Gram-negatives.

Samples from lesion sites had a higher frequency and density of candidal colonisation than the control sites or samples from control patients. *Candida* was cultured from 27% of the lesion and 10% of the control sites of OSCC patients, from 8% of the lesion and 4% of the control sites of OLD patients and from 3% of the control patients (Table 2). The samples cultured from the lesion sites of patients with *Candida* colonisation produced mutagenic amounts of acetaldehyde significantly more frequently than cultures of patients with no candidal colonisation (P=0.0008).

Non-smoker lesions had higher microbial density than smoker lesions (CFU/sample). This was the case with the samples from lesion sites of OSCC (smokers mean 568,000 vs. non-smokers mean 802,000), the control sites of OSCC patients (smokers mean 193,000 vs. non-smokers mean 313,000), OLD lesions (smokers mean 165,000 vs. non-smokers mean 207,000) and the samples from the control sites of OLD patients (smokers mean 41,000 vs. non-smokers mean 49,000). However, the smoking control patients had higher microbial numbers than the non-smoking control patients (smokers mean 56,000 vs. non-smokers mean 32,000). A significantly higher number of microbes were isolated from OSCC lesions but not from control sites of patients who did not consume any alcohol (mean 1,400,000) compared to patients who consumed alcohol (560,000, P=0.00063). No significant difference was found between drinker and non-drinker control patients. It was not possible to perform these statistical comparisons for the OLD samples due to the small number of non-drinkers in this patient group (Table 1). There were no significant differences in the total number of microbes or the proportions of aerobes and anaerobes or Gram-positive/negative ratio between samples from smokers and non-smokers and drinkers and non-drinkers.

DISCUSSION

Microbes cultured from all patient subgroups in this study were able to produce significant amounts of acetaldehyde in the presence of 22mM ethanol. The mean amount of acetaldehyde produced (158µM) clearly exceeded the mutagenic concentration of 100µM [25,27]. The ethanol concentration used in this study is clinically relevant as it corresponds to the salivary ethanol concentration for an average male after consumption of 0.7g ethanol per kg body weight. In the present study, the measurement protocol for microbial acetaldehyde production was the same as in our previous studies except that the sampling area rather than the amount of microbes was standardised [38,39]. There were significant differences between the subgroups in the number of microbes cultured from the samples but the acetaldehyde production did not correlate with the total microbial counts. We have previously shown that many *Candida* spp. are high acetaldehyde producers [39]. Interestingly, in the present study *Candida* colonisation was significantly more common in the cultures producing mutagenic concentrations of acetaldehyde (>100µM) than those which did not. In addition, *Candida* colonisation frequency and density was higher at OSCC and OLD lesion sites compared to control sites and control patients. However, *Candida* was not detected in all samples producing high amounts of acetaldehyde. Mixed bacterial flora was also found to be capable of high acetaldehyde production. This is in line with the association of poor oral hygiene and increased acetaldehyde production into saliva and higher incidence of OSCC [33,44].

Oral lichen planus and oral lichenoid lesions are potentially malignant chronic inflammatory mucosal conditions with malignant transformation rates of 0.4-4.9 % [11]. Microbes cultured from 61% of the lesion sites and 67% of the control sites of OLD patients produced carcinogenic levels of acetaldehyde. This is of interest as the malignant transformation of the OLD does not appear to be restricted to the site of the lesion [10]. Oral lichen planus is a systemic disease and even the healthy appearing mucosa is diseased. A higher proportion of Gram-negative anaerobic bacteria were detected in OLD lesion samples than in other sample types. OLD lesions also had the highest *Candida* colonisation density of all sample types. However, the acetaldehyde levels produced by the microbes cultured from OLD lesions did not differ significantly from the other subgroups indicating that the Gram-negative anaerobes or *Candida* do not determine the acetaldehyde production capacity of the local microflora alone. This is in line with our previous results on the high acetaldehyde production capacity of oral streptococci and other Gram-positive bacteria [38]. All in all, a spectrum of oral

microbes is capable of high acetaldehyde production and contributing to the malignant transformation in the potentially malignant mucosal conditions.

Smoking is one of the best-described risk factors for OSCC and smoking increases salivary acetaldehyde levels markedly. Chronic smoking has been shown to modify oral flora to produce more acetaldehyde from ethanol both *in vitro* [34] and *in vivo* [32]. Furthermore, active smoking results in an additional 200-400µM peak acetaldehyde concentration in saliva that lasts for as long as smoking continues.

All previous studies on oral microbial acetaldehyde production have been performed using uncultured saliva samples or mouthwashes [21,28,32,34]. This does, however, only give information on the metabolic state of the microbes at the time of sampling. In the present study the microbes were cultured prior to ethanol exposure and acetaldehyde measurement. Therefore any differences detected in their metabolism are likely to be due to genetic changes rather than a response to a temporary exogenous stimulus. Interestingly, microbes cultured from samples from smokers produced significantly higher amounts of acetaldehyde than those of non-smokers. The difference was most significant when lesion sites were compared. In addition, microbes cultured from the control sites of smoker OLD patients produced more than twice the amount of acetaldehyde of the microbes from the corresponding control sites of non-smoking OLD patients. Repeated exposure to acetaldehyde in cigarette smoke may lead to the selection of microbes that are capable of high rate acetaldehyde metabolism and are more tolerant to acetaldehyde.

Smoking and alcohol consumption have a synergistic effect on the cancer risk of the upper digestive tract. Combined use increases the relative risk exponentially [46] and associates with a synergistic exposure of the upper gastrointestinal tract to carcinogenic acetaldehyde [32]. In the present study no significant differences were found between the levels of acetaldehyde produced by microbes isolated from drinkers or non-drinkers. However, the non-drinker flora from the lesion sites of OLD patients produced higher amounts of acetaldehyde than that of the drinkers. It has been suggested that the mucosa in OLD may be more susceptible to mutagens in tobacco and alcohol further increasing the carcinogenic effect of acetaldehyde in OLD tissue [47].

The samples collected from OSCC lesions contained significantly higher microbial counts compared to the contralateral healthy mucosa. This is in accordance with the results of Nagy *et al.* [48]. However, in their study microbial samples were collected using sterile swabs. Although swabbing is a representative sampling method the sampling area is difficult to standardise and it is semi-quantitative at its best. The filter paper method used in the present study was developed for quantitative site-spesific sampling and gives detailed data on colonisation per surface area [45]. Surprisingly, the microbial counts of both the lesion site and the healthy mucosa of OSCC patients were significantly higher than those of OLD or control patients. The high density of microbial colonisation of OSCC lesions especially with anaerobes is most likely due to the uneven and partly necrotic tissue in cancerous lesions favouring the growth of anaerobic bacteria.

For the measurement of acetaldehyde production the microbes were cultured under anaerobic conditions. In the oral cavity microbes are mostly found in locations with limited levels of oxygen such as subgingival pockets or moist mucobuccal folds, and oral microbes are adapted to these conditions. The low oxygen pressure favors the fermentative metabolic pathways of the microbes enabling them to readily produce acetaldehyde. The risk for malignant transformation of precancerous lesions has been shown to correlate with the anatomical location of the lesion and the lateral tongue and floor of the mouth have been reported as high-risk areas [49]. The floor of the mouth, in particular, is a location with reduced oxygen pressure: covered with a thick layer of saliva and the tongue resting against it.

Oral carcinoma is a multifactorial disease with no single clearly recognisable cause. For cancer to develop, the altered cells need to escape the surveillance of the host DNA repair mechanisms and the immune system. Deficiencies in the DNA repair system have been linked to oral premalignant conditions [50]. Mutations in *TP53* are commonly detected in several human tumours, including OSCC. Overexpression of *TP53* is found in oral lichen planus [19] and acetaldehyde has been shown to be able to impair the function of p53 [31]. Local microbial acetaldehyde production is one mechanism which may contribute to the malignant transformation in OLD.

In conclusion, our results show that microbes cultured from samples from OSCC and OLD patients as well as healthy individuals can produce carcinogenic levels of acetaldehyde from ethanol. Smoking increases the capacity for acetaldehyde production markedly. This is most

prominent in OLD patients and their apparently healthy mucosa. This is in accordance with the fact that in OLD not only the lesion site, but also the healthy appearing mucosa, is potentially malignant.

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TABLES

Table 1. Patient demographics and smoking and drinking habits. There were no statistical differences in the patient demographics or smoking and drinking habits between the study groups.

	OSCC	OLD	CO
Total number	30	24	30
Female:male	12:18	16:8	19:11
Smokers	9 (32%)	4 (19%)	9 (31%)
Non-drinkers	6 (21%)	2 (10%)	3 (10%)
Alcohol consumers	23 (79%)	19 (91%)	26 (90%)
Heavy drinkers*	5 (17%)	1 (5%)	2 (7%)
Non-responders	1 (3%)	3 (13%)	1 (3%)

OSCC, oral squamous cell carcinoma patients; OLD, oral lichenoid disease patients, CO, control patients
Percentages of smokers and drinkers given of all responders in each group.
* Exceeds WHO levels for harmful alcohol consumption (288g alcohol per week in men and 192g per week in women)

Table 2. Microbial colonisation (CFU/sample) and acetaldehyde production (µM) in the different patient groups and sites presented as means (range). Significantly higher amounts of microbes (CFU/ml) were detected in lesions of OSCC patients compared to other sites. The mean CFU/sample in *Candida* (**) has been calculated from positive samples. The proportion of *Candida* positive samples of each sample type is given in parenthesis.

Patient group	ACH production	Total amount of microbes	Aerobic bacteria	Anaerobic bacteria	*Candida***
OSCC lesion	182 (13-532)	721,000* (32,000-2,368,000)	484,000 (18,000- 164800)	344,000 (3,000-815,000)	4,600 (27%) (0-36,000)
OSCC control	173 (48-589)	272,000 (1,800-1,280,000)	175,000 (750-704,000)	190,000 (0-614,000)	380 (10%) (0-800)
OLD lesion	134 (35-336)	193,000 (1,300-1,000,000)	184,000 (600-1,250,000)	56,000 (250-534,000)	34,000 (8%) (0-68,000)
OLD control	155 (41-616)	47,000 (400-218,000)	34,000 (650-163,000)	18,500 (1,000-78,000)	50 (4%) (0-10)
CO	158 (23-1000)	39,400 (1,200-240,000)	26,000 (800-154,000)	21,000 (850-102,000)	50 (3%) (0-50)

OSCC oral squamous cell carcinoma patient, OLD oral lichenoid disease patient, CO control patient

FIGURE

Figure 1. Samples isolated from the lesion sites of smoker OSCC and OLD patients combined produced significantly higher amounts of acetaldehyde than samples from the lesions sites of non-smoker OSCC and OLD patients (*P*=0.0351). Each dot represents the mean of triplicate measurements and horizontal line represents the mean value of each subgroup.