Khat and Oral Microbiota

A microbial study with relevance to periodontitis and dental caries

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I dedicate this work to

my mother Ruqaia Abdulghani and

my father Noor Al-hebshi

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List of publications

The thesis is based on the following papers, which are referred to in the text by Arabic numerals:

1- Al-hebshi N, Skaug N. Khat (*Catha edulis*) – an updated review. Addict Biol 2005; 10: 299-307. *

2- Al-hebshi N, Skaug N. Effect of khat chewing on selected periodontal bacteria in sub- and supragingival plaque of a young male population. Oral Microbiol Immunol 2005; 20: 141-146.

3- Al-hebshi N, Al-haroni M, Skaug N. Antimicrobial and resistance modifying activities of aqueous crude khat extracts against oral microorganisms. Arch of Oral Biol 2005; in press (Epub Oct 24).

4- Al-hebshi N, Nilsen Ø, Skaug N. In vitro effects of crude khat extracts on the growth, colonization and glucosyltransferases of *Streptococcus mutans*. Acta Odontol Scand 2005; 63: 136-142.**

Summary

Khat chewing is a highly prevalent habit in Yemen and East Africa. None of the few studies that investigated the effect of this habit on dental and oral health provides information about its influence on oral microbiota (Paper 1, a review). The **aim** of the study was to investigate the effects of khat chewing and aqueous khat extracts on a panel of periodontal and cariogenic bacteria, in a step towards better understanding of the relation of the khat chewing habit to periodontitis and dental caries. **Materials**: A total of 408 plaque samples obtained from 51 khat chewer and non-chewer young males, lyophilized crude aqueous khat extracts made from three cultivars, and a panel of 36 oral microorganisms. **Methods:** The plaque samples were analyzed by the DNA-DNA checkerboard hybridization method, comparing the prevalence and levels of 14 selected periodontal bacteria in sub- and supragingival plaque samples of khat chewers and non-chewers and of the khat chewing and non-chewing sides (Paper 2). For evaluation of antimicrobial properties of khat, the extracts were tested against 33 oral strains using the agar dilution method of the National Committee for Clinical Laboratory Standards (NCCLS) (Paper 3); in addition, the extracts were tested for their ability to potentiate activity of tetracycline and penicillin G against three resistant isolates (Paper 3). The extracts at various concentrations (0.125-2% w/v) were also evaluated for their effect on key virulence factors of *S. mutans*: planktonic growth, sucrose-dependent colonization, glucan synthesis, and glucosyltransferases (GTFs) production (Paper 4). **Results:** Khat chewing increased the prevalence or/and levels of a number of periodontal health-associated species, while it did not influence, and in some cases decreased, those of periodontal pathogens (Paper 2). Subsequent findings showed that the khat extracts demonstrated selective antimicrobial properties *in vitro*, with the majority of susceptible strains being periodontal pathogens; the extracts also potentiated the activity of tetracycline and penicillin G against the tested isolates (Paper 3). Concerning the virulence factors of *S. mutans*, the extracts inhibited formation of adherent biofilms while they enhanced planktonic growth, and inhibited synthesis of both types of glucans while

they unregulated GTFs production (Paper 4). **Conclusions:** Khat chewing does not seem to induce a microbial profile that would put the periodontium at risk of developing disease; it rather favors presence of species that are compatible with periodontal health. This may be attributed, at least in part, to the selective antibacterial properties of khat. Khat also has water-soluble ingredients, probably tannins, with cariostatic properties. In addition, there is preliminary evidence for the presence of resistance –modifying components.

Introduction

1. Khat

Khat, or *Catha edulis*, is an evergreen shrub of the plant family Celastraceae (Figure 1). It is widely cultivated in Yemen and East Africa, where its fresh leaves are habitually chewed for their amphetamine-like effects. This many-centuries-old habit is practiced by millions of people, and has been introduced to the western countries by immigrants. There is an extensive literature about khat, providing information about its history, botany, production, geographical distribution, chemistry and pharmacology, and exploring the social, economic, medical, psychological, and oral aspects of its use. Despite this extensive literature, studies that have investigated its effect on the different aspects of dental or/and oral health are much less than one may expect. Searching pubmed by using keyword combinations like "khat and oral" or "khat and dental" resulted in a total of only 10 hits (as for January 2005). These few studies focused on investigating the possible association between khat chewing and periodontitis, dental caries, and mucosal changes including malignancy. None of them touched upon the possibility of interaction between the habit and oral microbiota. Paper 1 provides an extensive up-to-date review on khat with emphasis on the pharmacological, medical and oral aspects of its use.

Figure 1. Three different khat cultivars from yemen (Nashat Al-hebshi)

2. Oral microbiota

2.1 The mouth as a microbial habitat

The oral cavity, like other parts of the gastrointestinal tract, possesses natural microflora. It has a number of features that makes it a unique microbial habitat. Teeth characteristically provide hard non-shedding surfaces that allow accumulation of large masses of microorganisms (dental plaque), especially in stagnant areas. Such accumulation is restricted on mucosal surfaces due to continuous epithelial desquamation; the only exception is the dorsum of the tongue that is highly papillated and thus supports higher densities of microbes (Marsh, 2000). Another important feature is that the oral cavity is continuously bathed with saliva, which has a profound effect on the ecology of the mouth. Saliva has a pH range (6.75-7.25) that favors growth of many microorganisms. Salivary components influence oral microbes by one of four mechanisms: aggregating microbes to facilitate their clearance from the mouth, adsorbing to teeth surface to form an acquired pellicle to which microorganisms can attach, serving as a primary source of nutrients, and mediating microbial inhibition or killing (Scannapieco, 1994). In addition to saliva, the gingival crevicular fluid (GCF), a plasma-derived fluid that flows through the junctional epithelium, provides microbes in the gingival crevice with nutrients and carries host immune components that play an important role in regulating the microflora therein (Marsh, 2000).

The oral cavity is not a homogenous environment. There are differences among sites in key ecological factors like adhesion ligands, pH, nutrients, redox potential, oxygen and temperature. Thus the lips, palate, cheek, tongue and the different teeth surfaces are distinct habitats, each supports a characteristic microbial community. Which species occupy a particular habitat depends on the habitat properties; however, metabolism of these species may modify the surrounding environment, making it suitable for other species to colonize. For example, early colonizers will deplete oxygen, lowering the redox potential and therefore providing a suitable environment for anaerobes. Thus, we find a bidirectional relation between the habitat and the microbial community within (Marsh, 2000).

Oral microbes are predominantly bacteria but fungi, viruses, mycoplasmas and even protozoa (Marsh, 2000) and archaea (Kulik et al., 2001) can also be found. Antony van Leeuwenhoek was the first to point out the diversity of oral microbiota when he examined a sample of his own dental plaque under his primitive microscope in 1683 (Theilade and Theilade, 1976). With cultivation-based techniques about 350 cultivable bacterial species have been detected in samples from the oral cavity (Moore and Moore, 1994). Amplification, cloning and sequencing of 16S ribosomal RNA genes have recently enabled the identification of a whole range of oral bacteria that have yet to be cultured. Paster et al. (2001) identified 182 novel clone phylotypes in subgingival plaque samples and estimated that total species diversity of the oral cavity to be between 500-600 species. In a very recent report, the number tops out at 700 (Pennisi, 2005). Much of this diversity exists in dental plaque. The genera that are found in the oral cavity are presented in Table 1.

Table 1. Bacterial genera found in the oral cavity (adapted from Marsh and Martin, 1999))

	Gram-positive	**Gram-negative**	
Cocci	*Abiotrophia* *Enterococcus* *Peptostreptococcus* *Streptococcus* *Staphylococcus* *Stomatococcus*	*Moraxella* *Neisseria* *Veillonella*	
Rods	*Actinomyces* *Bifidobacterium* *Corynebacterium* *Eubacterium* *Lactobacillus* *Propionibacterium* *Pseudoramibacter* *Rothia*	*Actinobacillus* *Bacteroids* *Campylobacter* *Cantonella* *Capnocytophaga* *Cantipedia* *Desulphovibro* *Desulphobacter* *Eikenella* *Fusobacterium*	*Haemophilus* *Johnsonii* *Leptotrichia* *Prophyromonas* *Prevotella* *Selenomonas* *Simonsiella* *Tannarella** *Treponema* *Wolinella*

* A new genus; not present in the original source.

2.2 Dental plaque

2.2.1 Definition

Clinically, dental plaque is the soft, tenacious deposit that forms on tooth surfaces and which is not readily removed by rinsing with water (Bowen, 1976). Microbiologically, it can be defined as the diverse community of microorganisms found on the tooth surface as a biofilm, embedded in an extracelluar matrix of polymers of host and microbial origin (Marsh, 2004). Since it is now recognized that dental plaque behaves as a typical microbial biofilm (Marsh, 2004), the new definition of a biofilm by Donlan et al. (2002) can be adopted to redefine dental plaque as a microbially derived sessile community characterized by cells that are irreversibly attached to the tooth surface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.

Dental plaque has the general properties of a biofilm that make the involved microorganisms dramatically different from their planktonic counterparts. Such properties include open architecture, protection from host defenses, enhanced resistance to antimicrobial agents, neutralization of inhibitors, novel gene expression, coordinated gene responses, spatial and environmental heterogeneity, broader habitat range and more efficient metabolism (Marsh, 2004).

Of all oral microbial ecosystems, dental plaque has been the major focus of oral microbiological research probably because of its characteristic features as a complex polymicrobial biofilm and its association with dental caries and periodontal diseases. According to its location, dental biofilm can be classified into fissure, smooth surface, approximal, supragingival, and subgingival. Composition of dental biofilm varies among these sites due to differences in their biological properties.

2.2.2 Structure

Dental biofilm is primarily composed of microorganisms; one gram of wet plaque contains approximately 2×10^{11} bacteria. The intercellular matrix account for 20-30% of

the biofilm mass, and is principally made up of polysaccharides of microbial origin (glucans and fructans) (Carranza and Newman, 1996).

Structural studies of dental biofilm have traditionally been performed using conventional light and electron microscopy (Listgarten, 1976; Nyvad and Fejerskov, 1987; Takeuchi and Yamamoto, 2001). With these techniques, however, biofilm preparation (dehydration, fixation, and staining) may result in artifacts, e.g. shrinkage and distortion. The advent of confocal laser scanning microscopy (CLSM) provided researchers with a valuable tool for studying the structure of biofilms in their fully hydrated intact form, with the possibility of making thin optical sections that can be reassembled into 3-dimensional information (Lawrence et al., 1991). Wood et al. (2000), using CLSM, showed that plaque has an open heterogeneous architecture with fluid-filled pores and channels, some of which extend through the entire thickness of the biofilm, interspersing mushroom-like bacterial/matrix masses that have narrow attachments to the enamel. This is in contrast to the densely packed structure of mature dental biofilm when viewed by light or electron microscope (Listgarten, 1994). Combined with vitality staining, species-specific florescence-labeled antibody probes or florescence markers, CLSM has also been used to study dental biofilm vitality (Auschill et al., 2001; Arweiler et al., 2004), spatial arrangement of species in dental biofilms (Guggenheim et al., 2001), or mass transport in biofilms (Thurnheer et al., 2003), respectively. Results suggest that bacterial vitality vary across the biofilm, but studies were inconsistent with regard to the pattern of such variation (Auschill et al., 2001; Arweiler et al., 2004).

2.2.3 Formation

Formation of dental plaque is a dynamic process involving continuous attachment, growth, detachment, and reattachment of oral microorganisms, but can be divided into several stages. As delineated by Marsh (2000), these stages are:

a) Formation of the acquired enamel (or dental) pellicle - This conditioning film forms immediately by selective adsorption of mainly salivary, but also some microbial, molecules to the tooth surface. Molecules detected in the acquired

pellicle include albumin, amylase, carbonic anhydrase II, sIgA, IgG, IgM, lactoferrin, lysozyme, proline-rich proteins (PRP), statherin, histatin 1, and mucous glycoprotein 1 (Li et al., 2004). Some of these like PRP, amylase, mucins and statherin function as receptors for bacterial adhesins (Lamont and Jenkinson, 2000). Glucosyltransferases can also be found in the active form in the enamel pellicle where it synthesizes glucan that serves as a ligand for glucan binding proteins on streptococci.

b) Passive transport of microorganisms to the coated tooth surface by the flow of oral fluids.

c) Reversible bacterial adhesion - This results from long-range (10-20 nm) physico-chemical interactions between the bacterial surface and the pellicle-coated tooth. The interplay of repulsive electrostatic forces (both surfaces are negatively charged) and van der Waals attraction result in a weak net attraction. This can be augmented by cation bridging and hydrophobic interactions or further weakened by hydration forces (Lamont and Jenkinson, 2000).

d) Irreversible bacterial adhesion - This results from short-range (<1nm) stronger, specific stereochemical interactions involving bacterial surface components (adhesins) and cognate receptors on the pellicle. A common type of such interactions is what is called lectin-like adhesion, which involves binding of carbohydrate (glycosidic) receptors by bacterial polypeptide adhesins. Binding of glucan by bacterial glucan binding protien is one example (Lamont and Jenkinson, 2000).

e) Later colonization (coadhesion or coaggregation) - This involves adhesin-receptor interaction between approaching bacteria and already attached early colonizers, increasing the diversity of the biofilm. The cohesion process results in characteristic morphological structures such as corncobs and test-tube brushes (Listgarten, 1999), and may facilitate metabolic interactions.

f) Multiplication of the attached microorganisms - The bulk of the biofilm results from cell division of the attached cells (Listgarten, 1999). Metabolism of

microorganisms modifies the local environment and creates gradients in key parameters (oxygen, redox potential, pH, nutrients, metabolic end products) creating micro-environments that enable coexistence and growth of diverse bacteria with conflicting needs (Marsh and Bradshaw, 1999). Synthesis of extracellular polysaccharides also takes place, resulting in the formation of intercellular matrix. The spatial arrangement of the cells and intercellular matrix will determine the architecture of the biofilm (Marsh, 2004).

g) Active detachment - Bacteria within the biofilm can produce enzymes that break specific adhesins, enabling cells to detach into saliva and probably colonize elsewhere (Marsh, 2004).

2.2.4 Microbial homeostasis

Despite its diversity, once established, dental biofilm is characterized by a high degree of stability. Such stable community is referred to as climax community. It is maintained in spite of host defense and modest environmental stresses, like changes in saliva flow, diets, regular exposure to mouth rinses and tooth pastes, challenge by exogenous species and exposure to antimicrobials. This stability, referred to as microbial homeostasis, involves negative feedback mechanisms and results from a balance of dynamic synergistic and antagonistic microbial interactions. This state of homeostasis is of great importance to oral health as it insures that potentially harmful species stay at low numbers, and that dental biofilm retains its protective function in terms of colonization resistance (Marsh, 2000).

2.2.5 Microbial interactions

Microorganisms within dental biofilm are spatially arranged in close proximity to each other, which facilitates interactions among them. These interactions can be synergistic and thus beneficial to the involved population, or antagonistic. Ultimately, both contribute to the diversity and homeostasis of the biofilm (Marsh, 2000).

One group of beneficial interactions is necessary for nutrition acquisition (Marsh and Bradshaw, 1999). Bacteria within the biofilm have overlapping patterns of enzymes, the concerted action of which enables complete degradation of complex host molecules, for example mucin, liberating an array of nutrients (Bradshaw et al., 1994). Biofilm bacteria are also involved in formation of food chains and webs to completely catabolize dietary sugars and other nutrients. *Veillonella* spp., for example, utilize lactic acid which is an end product of dietary carbohydrate metabolism by streptococci and actinomyces, and produce acetic and propionic acid that are in turn consumed by other species. Similarly, *Fusobacterium* and *Prevotella* species provide *Campylobacter* spp. with hydrogen and formate, while black pigmented bacteria benefits from protohaeme released by the latter. Another form of nutritional interaction is the degradation of extracellular polysaccharides, e.g. fructans, by some bacteria in the biofilm (Marsh and Bradshaw, 1999).

The other group of beneficial interactions is important for persistence of involved species under environmental stresses such as aeration, pH fluctuations, antimicrobials and host defenses (Marsh and Bradshaw, 1999). Experiments showed that anaerobes were able to persist and grow when aerated in presence of oxygen-tolerant species, but not in their absence (Bradshaw et al., 1996). The role of coaggregation was found to be important in this respect, especially in presence of a bridging species like *Fusobacterium nucleatum* (Bradshaw et al., 1998). Similarly with pH, species in mixed cultures were shown to withstand pH values that they could not tolerate in pure cultures (McDermid et al., 1986). Biofilms comprising complex mixtures of oral bacteria were found to have pH gradients and micro-zones: discrete areas of low pH surrounded by areas of neutrality (Vroom et al., 1999). Certain interactions can also contribute to the increased resistance of biofilm species to antimicrobials and host defenses. For example, an organism can be protected by being close to neighbors that produce β-lactamase or sIgA protease (Marsh and Bradshaw, 1999).

Antagonistic interactions also play an important role in determining the composition of dental biofilm, and maintaining its homeostasis. A classical example of

such interactions is the production of bacteriocins or bacteriocin-like substances. These are polypeptide antibacterial substances produced by some plaque species, e.g. mutacin by *S. mutans* and sanguicin by *S. sanguis,* to interfere with the growth of others. Antagonism is also manifested by production of other inhibitory factors such as organic acids, hydrogen peroxide, and diacetyl (Marsh and Bradshaw, 1999).

In addition to the above described biochemical and metabolic interactions, cells have also been found to communicate with each other within the biofilm using signal molecules (autoinducers). When such signaling is activated in response to cell density, it is called quorum sensing. Quorum sensing is mediated by competence stimulating peptides (CSPs) and a group of homoserine lactones in Gram-positive and Gram-negative bacteria, respectively. These molecules are often specific and thus serve intraspecies communication purposes. More recently, another communication system, called autoinducer system 2 (AI-2), has been described; it mediates interspecies communication. Signaling molecules are recognized by two-component signal transduction systems that are involved in control of gene expression, resulting in an altered phenotype such as increased competence for natural transformation and enhanced ability to form biofilms (Scheie and Petersen, 2004) .

2.2.6 Association with disease - plaque hypotheses

The fact that periodontitis and dental caries, the most prevalent diseases in humans, are dental plaque-mediated diseases is very well established (Theilade and Theilade, 1976; Sbordone and Bortolaia, 2003). However, despite 120 years of active research, there has been on-going controversy as to which bacteria within the biofilm are involved in causation of these diseases. Traditionally, there have been two hypotheses in this respect: the non-specific and specific plaque hypotheses (NSPH and SPH), first delineated by Loesche (1976). The NSPH assumes that the entire plaque flora elaborate noxious products that, if exceeding the host detoxification threshold, result in slow tissue destruction. Consequently, the hypothesis relies upon mechanical debribement of dental biofilm from the tooth surfaces for treatment and prevention; this non-specific plaque

mass reduction has been the paradigm of dental care for more than 100 years (Loesche, 1999). The SPH, on the other hand, states that only plaque with certain pathogens and/or a relative increase in levels of given indigenous plaque organisms causes infections. It therefore entails that treatment should be aimed at diagnosis and then elimination of causative organisms, involving an antimicrobial component. While there is evidence to support effectiveness of such an approach (Loesche et al., 1996; Loesche, 1997; Loesche et al., 2002), the current treatment paradigm dictated by the NSPH still predominates (Loesche and Grossman, 2001).

More recently, Marsh (1994) has proposed a third hypothesis: the ecological plaque hypothesis (EPH). According to this, species in the dental biofilm, including opportunistic pathogens in low numbers, represent a stable homeostatic microbial community maintained by a number of synergistic and antagonistic interactions and negative feedback mechanisms. Major environmental perturbations, e.g. pH or redox potential changes, are necessary to break the homeostasis; this favors overgrowth of cariogenic bacteria or periodontal pathogens and enhance expression of their virulence factors. The EPH implies that disease can be prevented not only by targeting pathogens, but also by an ecological approach that interferes with environmental stresses that can break microbial homeostasis of dental biofilms (Marsh and Bradshaw, 1997).

2.3 Microbiology of periodontitis

Studies during the golden era of microbiology (1880-1930) suggested four microorganisms as etiological agents of periodontitis: amebae, spirochetes, fusiforms and streptococci. Interest in searching for a specific etiological agent of periodontitis then faded for about three decades before it revived again in the 1960s and has survived up to the present (Socransky and Haffajee, 1994). Indeed, in the last 25 years, there have been more than 200 studies that have compared the bacteria associated with periodontal health to that associated with periodontitis, using culture techniques or molecular methods such as DNA probes and PCR (Loesche and Grossman, 2001). However, there have been inherent difficulties in identifying periodontal pathogens, principally because

periodontitis occurs at sites with pre-existing complex normal flora, making discrimination of opportunistic pathogens from host-compatible species a real challenge, especially that the pathogens may be carried in low numbers at health (carrier state) (Haffajee and Socransky, 1994; Marsh, 2000). In addition, many of the periodontal bacteria are difficult or impossible to cultivate. Furthermore, periodontal infections seem to be mixed in nature, involving more than one species, rendering evaluation of the etiology of periodontitis even more difficult. For this and others reasons, Koch's postulates have been replaced by a set of criteria to define periodontal pathogens. These criteria include 1) association (the species is found more frequently and at higher levels in disease compared to health), 2) elimination (elimination of the species is paralleled by remission of disease), 3) host response (presence of immune response against that species), 4) possession of virulence factors, and 5) induction of disease in animals (Haffajee and Socransky, 1994).

In light of these criteria, searching through the growing literature enabled researches pointing out some candidates as etiological agents of periodontitis (Haffajee and Socransky, 1994). There was a strong evidence to support a consensus implicating *Porphyromonas gingivalis* and *Bacteroids forsythus* (current name: *Tanerella forsythia*) as etiological agents of adult periodontitis (now classified as chronic periodontitis), and *Actinobacillus actinomycetemcomitans* as that of early onset periodontitis (now classified as aggressive periodontitis). There was also moderate evidence for the involvement of other species such as *Treponema denticula*, *Fusobacterium nucleatum, Prevotella intermedia/nigrescens, Peptostreptococcus micros, Eubacterium nodatum*, and *Campylobacter rectus* (Consensus report,1996).

It is now known that established and putative periodontal pathogens as well as other periodontal bacteria exist in bacterial complexes in subgingival plaque (Table 2). Species belonging to the so-called red complex, and to a lesser extent the orange complex, are strongly associated with the clinical signs of periodontitis. Bacteria of the other complexes do not show an association with periodontitis and seem to be compatible with periodontal health (Socransky et al., 1998). It is worth mentioning that periodontal

pathogens have also been detected in supragingival plaque in association with periodontitis (Ximenez-Fyvie et al., 2000a; Ximenez-Fyvie et al., 2000b).

Table 2. Microbial complexes in subgingival plaque (Socransky et al., 1998)

Red complex *Porphyromonas gingivalis* *Tanerella Forsythia* *Treponema denticula*	**Yellow complex** *Eikenella corrodens* *Capnocytophaga gingivalis* *Capnocytophaga ochracea* *Capnocytophaga sputigena* *Campylobacter concisus* *Actinobacillus actinomycetemcomitans*
Orange complex *Prevotella intermedia* *Fusobacterium nucleatum* *Fusobacterium periodonticum* *Prevotella nigrescens* *Petptostreptococcus micros* *Campylobacter rectus* *Campylobacter gracilis* *Campylobacter showae* *Eubacterium nodatum* *Streptococcus constellatus*	**Purple complex** *Veillonella parvula* *Actinomyces odontolyticus*
	Green complex *Streptococcus mitis* *Streptococcus oralis* *Streptococcus sanguis* *Streptococcus intermedius* *Streptococcus gordonii*

Recently, some attention has been given to the so far uncultivable bacteria; studies using 16S rRNA gene analysis suggest that certain as-yet-uncultivable species may also be involved in the etiology of periodontal disease (Dewhirst et al., 2000; Sakamoto et al., 2002). Outside the "bacteria and periodontitis" mainstream, studies also have lately been evaluating the role of other microorganisms in the etiology of periodontitis. Indeed, there is a growing body of data supporting the belief that viruses such as cytomegalovirus contributes to the severity of periodontal disease, probably by altering the host's immune control of plaque bacteria (Slots, 2004). Interestingly, there is a recent report implicating archeae as a possible etiological factor of periodontitis (Lepp et al., 2004).

2.4 Microbiology of dental caries

The first to establish the role of microorganisms in demineralization of enamel was Miller in 1890, who was able to demonstrate the ability of the mixed bacteria in saliva to

produce acid from fermentable carbohydrates in quantities sufficient to decalcify teeth (Kleinberg, 2002); however, he failed to identify a specific bacterium, providing the earliest basis for the NSPH of etiology of dental caries (Loesche, 1976). Despite Miller's findings, the 20th century witnessed extensive investigation of the causative agent(s) of dental caries. In fact, a systematic review of the literature from 1966 to 2000 revealed 2,730 publications in English on the role of bacteria in human primary dental caries (Tanzer et al., 2001). Generally, four groups of bacteria are frequently encountered in association with dental caries: mutans streptococci, lactobacilli, actinomyces and non-mutans streptococci.

In first half of the 20th century, lactobacilli were widely considered as the prime suspect in the etiology of dental caries (the lactobacilli era), largely because they were consistently isolated from deep carious lesions (Hamilton, 2000). This is no surprise, knowing that these bacteria are highly acidogenic and acidouric (van Houte, 1994). Lactobacilli have low avidity for teeth; they preferentially colonize dorsum of the tongue and are carried in saliva (Tanzer et al., 2001). Thus, they are not a requisite for development of lesions; however, given their frequent detection in large numbers in advanced caries, they are believed to be important for the progression of established lesions (Loesche, 1986; van Houte, 1994). Lactobacilli isolated from human dental caries include *L. acidophilus, L. casei, L. paracasei, L. rhamnosus,* and *L. fermentum (Martin et al., 2002).* In fact, a recent study, using molecular approaches, has detected in advanced carious lesions other lactobacillus species such as *L. gasseri, L. ultunensis, L. crispatus, L. gallinarum*, and *L. delbrueckii* as well as novel phylotypes, suggesting that lactobacilli in advanced caries are more diverse than previously thought (Byun et al., 2004).

Streptococcus mutans was first isolated from carious lesions by Clarke in 1924, but subsequently almost disappeared from the literature for more than three decades (Hardie and Whiley, 1999). Interest in *S. mutans* flared up in 1960s when it was used to demonstrate the infectious and transmissible nature of dental caries in experimental animal models (Keyes, 1960). Since then, *S. mutans* has become the primary focus of caries microbiology. Hundreds of cross-sectional, longitudinal, case control, and

interventional clinical studies have been conducted, providing strong evidence for the central role of S. mutans in the initiation of dental caries (Tanzer et al., 2001).

Recognizing the considerable serological and genetic heterogeneity of human and animal *S. mutans* strains, they were grouped into eight species collectively referred to as mutans streptococci (MS); the major species in humans are *S. mutans* (serotype *c,e,f*) and *S. sobrinus* (serotype *d,g*) with occasional isolation of *S. rattus* (seroype *b*) and *S. cricetus* (seroype *a*). *S. mutans* serotype *c* account for 70-100% of human isolates of MS (Loesche, 1986).

The biological and virulence properties of *S. mutans* have been the subject of an enormous number of studies, which have been comprehensively reviewed by several authors (Loesche, 1986; van Houte, 1994; Banas, 2004). Virulence factors of *S. mutans* are mainly adhesion, acidogenicity, and acid tolerance. *S. mutans* adheres to teeth surfaces by sucrose-dependent and sucrose-independent mechanisms. The former involves extracelluar glucosyltransferases (GTFs) for synthesis of glucans (glucose polymers) that mediate bacterial adhesion and contribute to biofilm formation (Loesche, 1986). S. mutans has three GTFs: GTF B, GTF C, and GTF D. The former two synthesize primarily water-insoluble glucans, while the latter synthesizes only water-soluble glucans; the activity of all three enzymes is required for optimal adherence of *S. mutans* (Ooshima et al., 2001). *S. sobrinus* also possesses 4 GTFs: GTF I, GTF T, GTF S, and GTF U; only GTF I synthesizes insoluble glucans (Nanbu et al., 2000). Sucrose-independent colonization of *S. mutans* involves interaction between cell surface polymers, such as the cell surface protein antigen PAc (also called antigen I/II, B, IF, P1 or MSL-1), and the acquired dental pellicle (Yu et al., 1997).

In spite of the strong association between MS and caries, there have been some reports of caries incidence in the absence of MS, or of very low caries prevalence in their presence in high numbers. These findings indicate that other species should not discounted as potential etiological agents on occasions (Hamilton, 2000). In fact, some recent studies have shown an association between non-mutans streptococci (non-MS) and carious lesions in humans, and have been able to demonstrate the ability of these non-MS

to generate acid at low pH with a capability of lowering the pH below 4.4 (Van Houte et al., 1991; Sansone et al., 1993; van Houte et al., 1996). Little information is known about the individual species of these "low pH" non-MS, but *S. mitis* may be an important member (Hamilton, 2000).

Actinomyces, especially *A. naeslundii* genospecies 1 and 2 (previously *A. viscosus*), have been consistently associated with root caries (Bowden et al., 1999); however, there is now evidence to suggest a polymicrobial etiology of root caries as recent studies have demonstrated increasing numbers of genera like *Prevotella*, *Capnocytophaga*, *Eubacterium*, *Corynebacterium* and *Clostridium* in association with root caries (Hamilton, 2000). Such diversity has also been observed recently, using molecular methods, in coronal caries (Chhour et al., 2005).

2.5 Oral habits and oral microbiota

Humans in different parts of the world developed certain oral habits. Smoking, coca leaves chewing in central and South America, betel quid chewing in Southeast Asia, and khat chewing in Yemen and East Africa are examples. Such habits have implications for oral health. Smoking, for example, is an established risk factor of periodontitis; approximately half of periodontitis cases have been attributed to either current or former smoking and up to 90 percent of refractory periodontitis patients are smokers (Johnson and Slach, 2001). Results from studies on betel or areca nut chewing suggest that it may exaggerate pre-existing periodontitis, while confer protection against dental caries (Trivedy et al., 2002). Studies on the oral effects of khat chewing are reviewed in Paper 1.

In line with this, and given the microbial origin of common oral diseases, some of the oral habits have been evaluated for their effect on oral microbiota. Most of such studies focused on smoking; while some of them showed little effect (Stoltenberg et al., 1993), others demonstrated that smoking resulted in an increase of certain periodontopathogens (Kamma et al., 1999). A more recent study, in which 7,271 subgingival plaque samples from 272 adults with different smoking histories were

analyzed with the DNA-DNA hybridization methods, showed that members of the orange and red complexes including *E. nodatum*, *F. nucleatum* ss *vincentii*, *P. intermedia*, *P. micros*, *P. nigrescens*, *T. forsythia*, *P. gingivalis* and *T. denticola* were significantly more prevalent in current smokers than in past or never smokers (Haffajee and Socransky, 2001). These differences were only found at sites with pocket depth <4 mm, suggesting the difficulty of demonstrating the effect of smoking (and probably of other oral habits) in deeper pockets. Betel quid chewing was found to increase the likelihood of subgingival infection with *A. actinomycetemcomitans* and *P. gingivalis* (Ling et al., 2001). On the other hand, areca nut was also shown to have antibacterial properties *in vitro* and this has been given as a possible explanation for its cariostatic role (Trivedy et al., 2002).

Up-to-date, the only published study on the microbiological aspects of khat is the one by Elhag et al. (1999) who showed that two compounds isolated from khat (22 ß-hydroxytingenone and tingenone) possessed potent antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus durans* and *Mycobacterium* species but not against *Escherichia coli* and *Candida albicans.* Notably, there is no single report about the possible effects of khat on oral microbiota.

Rationale

Periodontitits and dental caries are both infections due to ecologically driven overgrowth of opportunistic pathogens present in dental biofilm. Khat leaves are chewed and kept in the oral cavity for several hours daily, and it is possible that the various chemicals leaching from them may directly or indirectly affect oral bacteria including periodontal and cariogenic ones. This brings to mind many questions. Does khat chewing really result in any oral bacterial changes or shifts? If so, what are these changes or shifts? Are they relevant to periodontitis or dental caries?

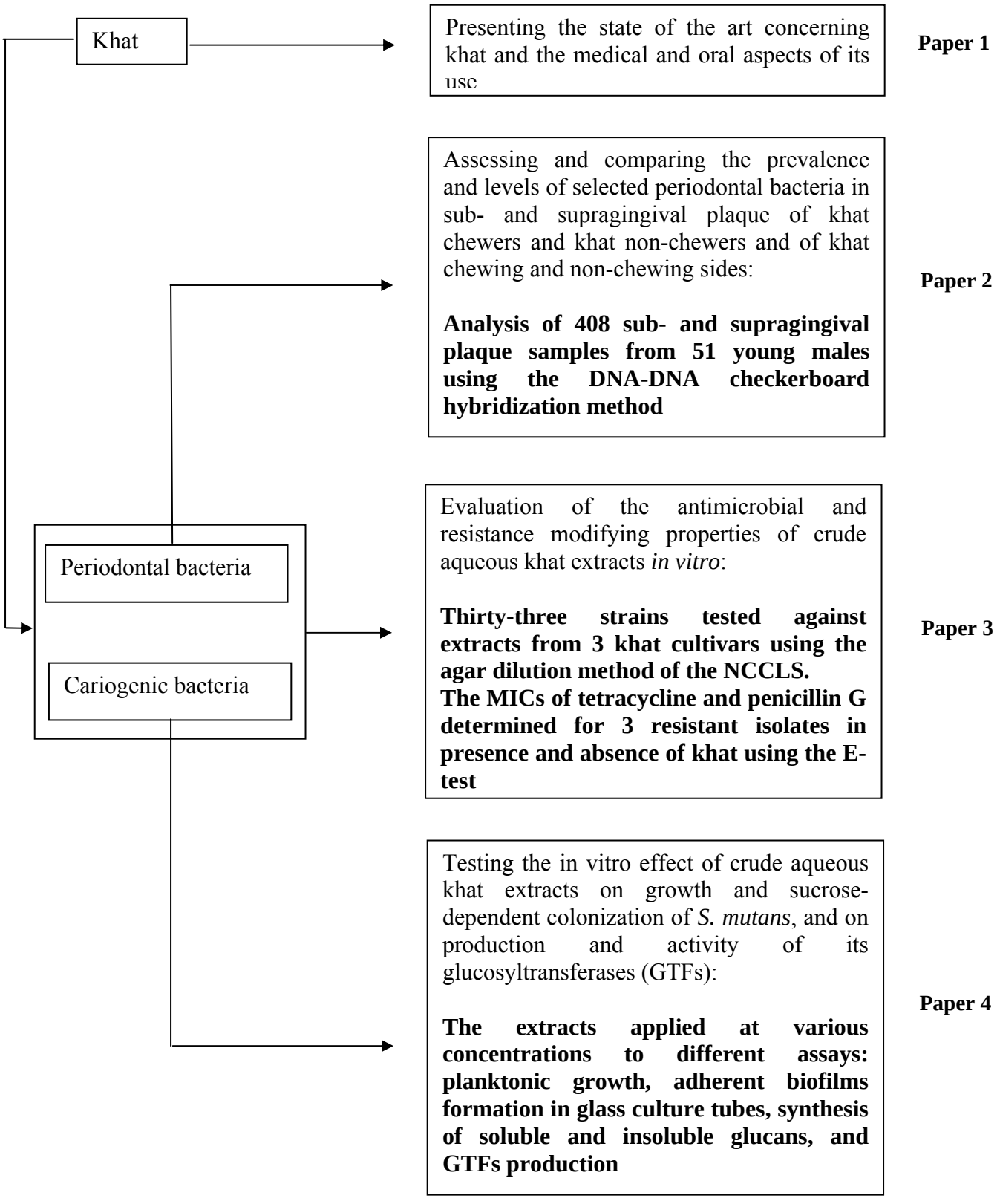
Answers to such questions shall improve our understanding of the yet unclear relationship between the khat chewing habit and these two diseases, and provide dental practitioners with some knowledge that may influence their assessment of khat chewer patients.

Study aim and objectives

The general aim of the current study project was to investigate oral microbiological effects of khat that may influence the pathogenesis of periodontitis and dental caries. The specific objectives were:

1- To study the effect of khat chewing on presence of selected health- and disease-associated periodontal bacteria in dental plaque by
   a) assessing and comparing the prevalence and levels of 14 periodontal bacteria in sub- and supra-gingival plaque of khat chewers and non-chewers, and
   b) assessing and comparing the prevalence and levels of 14 periodontal bacteria in sub- and supra-gingival plaque of khat chewing and non-chewing sides.
2- To evaluate the antimicrobial and resistance-modifying properties of crude aqueous khat extracts against a panel of cariogenic and periodontal bacteria *in vitro*.
3- To test *in vitro* effects of crude aqueous khat extracts on growth and sucrose-dependent colonization of *S. mutans*, and on activity and production of its glucosyltransferases.

Study Design



Recapitulation of the results

Paper 2

The khat chewers (n=29; mean age 23.7 years) and khat non-chewers (n=22; mean age 21.8 years) did not differ significantly in any of the clinical parameters measured; cigarette smoking was significantly more prevalent in the chewers. The khat-chewing sides showed a slightly but statistically significant lower mean pocket depth than did the khat non-chewing sides. Subgingivally, the khat chewers had significantly higher prevalence of *S. intermedius* and *V. parvula*, and significantly higher levels of the latter species and *E. corrodens*; these increases followed a khat chewing frequency-dependent manner. Consistently, the chewing sides demonstrated a significant increase in prevalence of the same three species; in addition, they also had significantly lower prevalence and levels of the pathogen *T. forsythia*. Supragingivally, there were no significant differences between the two study groups; however, the khat non-chewing sides showed significantly higher levels of *V. parvula* and *A. israelii*, and significantly lower prevalence of *C. gingivalis* and *F. nucleatum*.

Paper 3

Eighteen out of the 33 strains (55%) tested were sensitive to the extracts with MICs of 0.5-2% (w/v). Most of these were periodontal pathogens with *P. gingivalis* and *T. forsythia* being the most susceptible (MIC 0.5-1%); only *C. rectus* and one clinical strain of *F. nucleatum* were resistant; *A. actinomycetemcomitans* ATCC 43717 and the clinical strain were only sensitive to the *Hamdani* cultivar extract but showed marked growth reduction (MGR) at 2 % of the other two extracts. Periodontal health-associated bacteria were less susceptible with only five strains being sensitive at the highest concentration tested (2%). None of the cariogenic bacteria were sensitive. However, *L. acidophilus* showed MGR at 1%. The extracts were active against *Streptococcus pyogenes* (MIC 1-2%) but not against *Candida albicans* and *Staphylococcus aureus.* The presence of the khat extracts at a sub-MIC resulted in a 2-4-fold potentiation of the tested antibiotics against the resistant strains.

Paper 4

The khat extracts effectively inhibited formation of adherent biofilms by *S. mutans*. The minimum biofilm inhibitory concentration (MBIC) varied among the three khat cultivars (0.25-1% w/v). The extracts also inhibited synthesis of both glucan types, particularly insoluble glucans (average 85% inhibition at 1%), with similar differences among the cultivars. However, khat increased bacterial growth and, at sub-MBIC, also increased viability within biofilms in a dose-dependent manner; there were no inter-cultivar differences. In addition, the extracts resulted in 2.5-fold up-regulation of GTFs. Differences in the total tannins content of the three cultivars corresponded well to the differences in their biofilm formation and glucan synthesis inhibitory properties.

Discussion

1. Methodological considerations

1.1 Study subjects

Fifty-one males were recruited from among dental students/recently graduated dentists for the *in vivo* part of the study (Paper 2). Females were excluded on the basis of usually being light and occasional khat chewers, and considering the cultural restrictions on young unmarried women practicing the habit. Having such a fairly homogenous sample of dentists assured that the effect of potential confounders was minimized. Studies on the effect of an oral habit on oral microbiota usually adjust for the effect of disease severity by including it as an independent variable; however, this is confusing because disease severity is by principle a dependent variable (Figure 2); such an approach results in detection of a small or no effect. Conducting the study on young subjects enabled avoiding such mixing that would have been encountered if subjects with age related periodontitis were included. Furthermore, Haffajee et al. (2001) showed that smoking had an effect only on microbiota of pockets <4mm, suggesting that it is more logical to study the effect of an oral habit on microbiota in shallow pockets rather than established ones. The ultimate goal was thus to have a somewhat controlled semi-experimental model to test whether or not khat chewing *per se* affects the presence of periodontal bacteria in dental plaque.

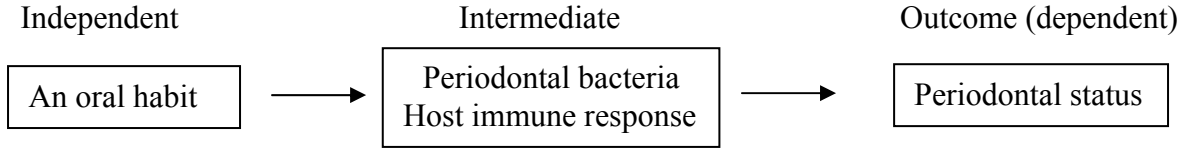


Figure 2. A simple schematic presentation of the causal pathway between an oral habit and periodontal status.

1.2 DNA-DNA checkerboard hybridization

Since its introduction, the DNA-DNA checkerboard hybridization method (Haffajee and Socransky, 1994) has been used by many researchers, and enabled analysis of huge

numbers of plaque samples. At Forsyth Dental institute alone, about 34,400 plaque samples were analyzed between 1993 and 1999 (Haffajee et al., 1999). Indeed, much of the current knowledge about the bacteriology of periodontitis and the microbial complexes in dental plaque came from studies that employed this method (Colombo et al., 1998; Haffajee et al., 1998; Socransky et al., 1998; Tanner et al., 1998; Papapanou et al., 2000; Ximenez-Fyvie et al., 2000; Ximenez-Fyvie et al., 2000; Socransky et al., 2002). The technique has the advantage of a fast identification of a wide range of bacteria (up to 40) in a large number of samples in one run, a low cost per sample, the possibility of striping and reprobing, and the ability to detect dead and uncultivable species. The sensitivity of the assay is in the range of 10^4 bacterial cells (Socransky et al., 2004). Like any other method, however, this technique has its disadvantages. Firstly, the detection is limited to species for which probes are available. Secondly, the technique employs whole genomic DNA probes, which may increase the possibility of cross-reactions between closely related species. Such cross-reactions may contribute to the higher detection rate obtained by DNA probes compared to culture methods (Papapanou et al., 1997). In a study, in which 40 probes were run against 80 species, 93.5% of all probe-heterologous species reactions exhibited cross-reactions amounting to <5% of the homologous probe signal (Socransky et al., 2004). It is possible then that weak signals may represent cross-reactions; therefore, signals corresponding to $<10^5$ bacterial cells were excluded in the current study to minimize false-positives.

During my attempts to maximize intensity of signals in the assay, I found that proper cross-linking of target DNA to the nylon membrane was one important factor. I could show that air-drying the membrane for 30 minutes after deposition of the samples, followed by exposure to 70mJ/cm^2 of 312-nm UV light gave the best results (Al-hebshi and Skaug, 2003). Nevertheless, and with many other efforts, I was not able to reach the optimal sensitivity, i.e. 10^4 bacteria cells, for all species. That is another reason why I excluded weak signals so that to have the same detection sensitivity for all species.

1.3 Antimicrobial susceptibility testing

The National Committee for Clinical Laboratory Standards (NCCLS) - now called Clinical and Laboratory standard institute (CLSI) – provides consensus standards and guidelines for various laboratory procedures including antimicrobial susceptibility testing. The agar dilution method used in my thesis to evaluate the antibacterial properties of khat (Paper3), is relatively time-consuming and labor-intensive, but allows testing of a large number of strains simultaneously, and in fact, was the most practical method to use with the khat extracts. Reproducible inoculation of plates was made possible by using an automatic multipoint inoculator. The use of broth microdilution or macrodilution method proved to be technically impossible because addition of the khat extracts resulted in formation of a precipitate that hampered reading the results. In addition, the NCCLS guidelines for the broth microdilution method for anaerobes are meant to be used with the *Bacteroids fragilis* group only.

The Epsilometer test (E test; AB Biodisk, Sweden) has been shown to be a reliable method for MICs determinations, with results comparable to those obtained by the NCCLS methods (Wust and Hardegger, 1992; Pierard et al., 1996). Though the proprietary antibiotic-impregnated E test strips are expensive, the procedure requires less labor and is easily carried out, and thus might be a preferable method for testing small numbers of strains (Best et al., 2003). Therefore, I preferred to use it to determine the MICs of tetracycline and penicillin G for the three resistant isolates.

1.4 The biofilm model

The biofilm model used in my study (Paper 4) is a simple but valuable tool for assessing sucrose-dependent colonization of *S. mutans*. Such colonization is a very important virulence factor of this bacterium (Loesche, 1986; Banas, 2004). However, I do appreciate that the situation *in vivo* is much more complex, in which other mechanisms of attachment are involved, a variety of species contribute to biofilm formation, and exposure to khat is not continuous. Therefore, I was convinced that khat should also be evaluated in an advanced biofilm model under more realistic conditions. B. Guggenheim

kindly accepted to test the extracts in the mutlispecies Zurich biofilm model (Guggenheim et al., 2001; Shapiro et al., 2002). In this model, six species representative of supragingival plaque (*S. sobrinus, S. oralis, F. nucleatum, Veillonella dispar, A. naeslundii,* and *Candida alblicans*) are used to generate biofilms over 64.5 hours on salivary pellicle-coated hydroxyapatite discs, in 24-well culture dishes incubated anaerobically at 37°C. The khat extract was tested in the feast-famine modification of this biofilm model (Van Der Ploeg and Guggenheim, 2004), which closely simulates the meal pattern of man. The biofilms were fed for 45 min 3 times daily, and in the remaining time, they were either subjected to the khat extract or incubated in saliva. The khat extract were applied at concentrations of 0.25% and 0.5% (w/v) for 3 hours, 4 times during the biofilm formation period (Figure 3).

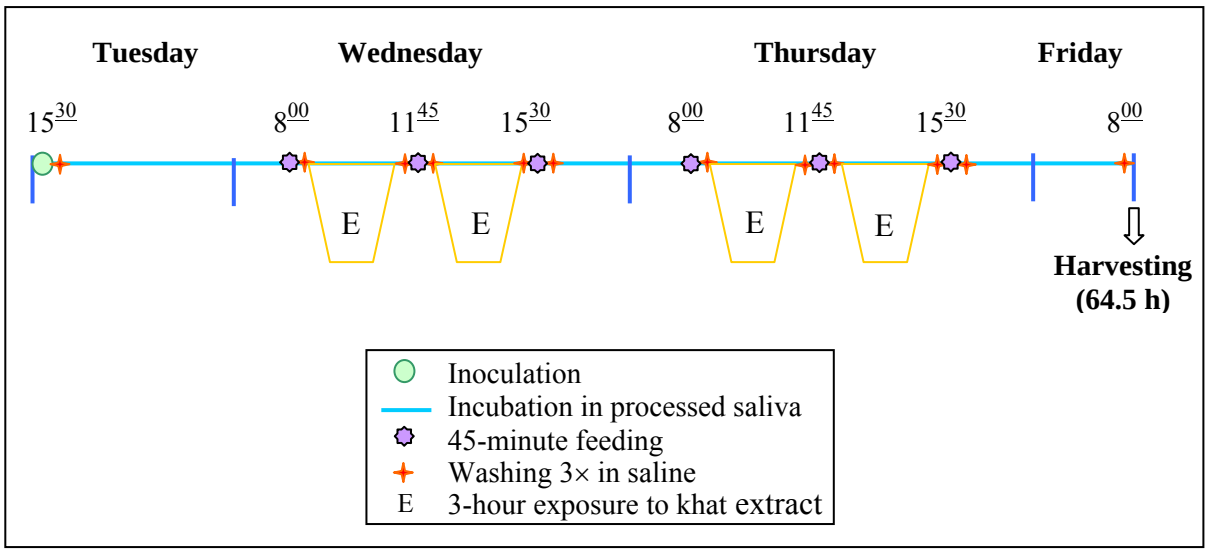


Figure 3. Schematic presentation of the experimental procedures used to test the effect of the khat extract on the formation of biofilms.

The harvested biofilms were then subjected to vitality staining and CFU counting on non-selective and selective media. There was no difference in percentage of living cells among control and khat-exposed biofilms (about 89% living cells in all). The effect of exposures on CFUs in the biofilms is presented in Figure 4. The total CFUs were slightly higher in biofilms exposed to the khat extract, and the numbers of *S. sobrinus* remained

almost unchanged. The khat extracts thus failed to exert biofilm inhibitory properties in this model. However, this should not be considered as an absolute contradiction to the results obtained with the *S. mutans* biofilm model for a number of reasons. First, formation of the mutli-species biofilm probably involves, in addition to sucrose-dependent colonization, other colonization mechanisms that are not affected by khat. Secondly, the Zurich biofilms were exposed to khat for relatively short periods while the *S. mutans* biofilms were formed under continuous presence of khat. Finally, adherence of formed biofilms was not challenged by vortexing as was done with the S. *mutans* biofilms.

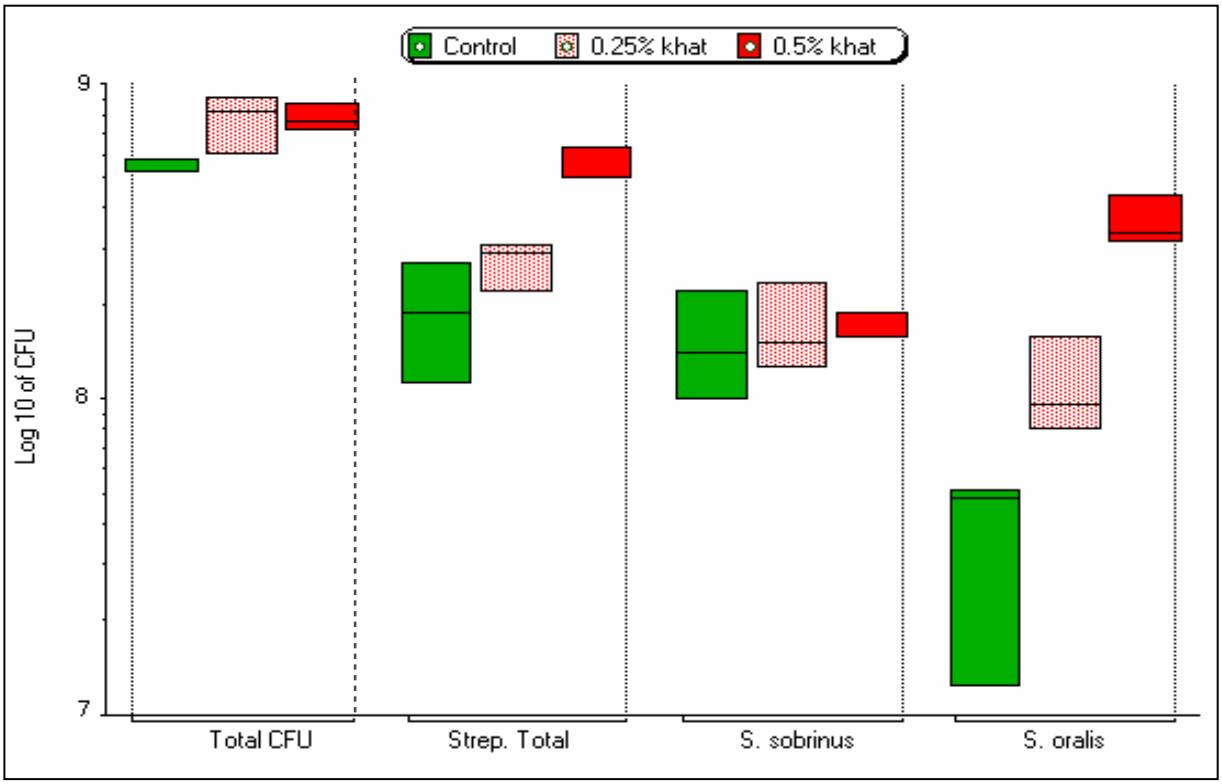


Figure 4. Box plots of CFUs in biofilms exposed to control and khat solutions for 3 hours, 4 times during the biofilm formation period. Box plots represent median and range of three determinations. The figure was provided by Dr. B. Guggenheim.

Apart from these primary results, some interesting and relevant secondary results popped up when proportions were calculated. *S. oralis* constituted only 9% of control biofilms while it made up 15 and 48% of biofilm exposed to 0.25 and 0.5% of the khat extract, respectively. Such striking increase in the proportion of *S. oralis* was paralleled with a drop in the proportion of non-streptococci from 66% in control biofilms to 29% in biofilms exposed to 0.5% khat; it is unfortunate that the results do not inform whether the four non-streptococcus species were equally or differentially affected. The proportion of *S. sobrinus* differed only slightly, being less (23%) in biofilms exposed to khat than in control biofilms (29%). These findings indicate that khat results in some shifts in the biofilm, probably by favoring growth of some species and/or retarding that of other species.

1.5 GTFs activity and production

The drawbacks of using a crude GTFs preparation in the glucan synthesis assay are discussed in Paper 4. Nevertheless, the preparation did allow reliable testing of the effect of the khat extracts on synthesis of soluble and insoluble glucans. All experimental setups and parallels were carried out using the same preparation so that variations due to use of different preparations were avoided. Quantification errors due to precipitation of khat carbohydrates along with glucans were adjusted for as described in the material and methods section of Paper 4.

Extracting the GTFs from khat containing-cultures in the GTFs production assay was the real technical challenge. Khat tannins, and probably other polyphenols, made complexes with proteins in the broth, interfering with their extraction, quantification, and electrophoresis. Therefore, I chose to test the effect of khat on GTFs production by including the extracts in agar rather than in broth. It was possible then to harvest bacteria from the agar surface and extract the GTFs with minimum interference from khat tannins.

2. General discussion

2.1 Motives and basis

The lack of information concerning oral microbiological effects of khat chewing was the major motive to conduct the current study. Dental practitioners in the regions where khat is chewed need to have a scientifically-based knowledge about possible oral effects of khat. In Yemen, dentists' views on this matter are largely anecdotal and, in many cases, are not different from those of the public. The studies that deal with this topic are few, and their results have not been disseminated among local dentists, probably because investigators were frequently foreigners. In this thesis, I reviewed results from previous studies (Paper 1) and added my own findings (Papers 2, 3, and 4), to elucidate the possible relationship between khat and oral diseases, specifically dental caries and periodontitis.

The very few published studies on the relation between khat chewing on the one hand and dental caries and periodontitis one the other provided a background for the current study. Information in the literature concerning the effect of khat chewing on the periodontium are contradictory; while one study showed that khat is detrimental (Mengel et al., 1996), others suggested the opposite (Hill and Gibson, 1987; Jorgensen and Kaimenyi, 1990). Therefore, it was not possible to formulate a one-sided hypothesis on the effect of khat on periodontal bacteria. The purpose thus was to provide microbiological information that would support one of the two views (Papers 2 and 3). Reports of low caries prevalence in khat chewers (Luqman and Danowski, 1976; Hill and Gibson, 1987) point to the possibility that khat has cariostatic properties at the microbiological level. This, together with studies on the inhibitory effects of some plant extracts on *S. mutans*, formed the hypothetical basis for Paper 4.

2.2 Khat and periodontal bacteria *in vivo*

Results from the *in vivo* study (Paper 2) indicate that khat chewing generally favors presence of periodontal health-associated bacteria, while discouraged that of some periodontal pathogens. Most prominent was the strong association between khat chewing

and presence of *V. parvula, S. intermedius,* and *E. corrodens* in subgingival plaque; striking also was the significantly lower prevalence and levels of the pathogen *T. forsythia* in the khat chewing sides compared to the khat non-chewing sides. Consistent with this "healthy" microbial profile was also the significantly higher levels of *V. parvula* and *A. israelii*, and significantly lower prevalence of *F. nucleatum* in supragingival plaque of the khat chewing sides. In addition, there were also some tendencies ($0.05 > p$ value ≤ 0.1) that fitted well with the main results. Subgingival plaque of the khat chewing sides tended to have lower prevalence of the pathogens *P. gingivalis*, but higher prevalence of *A. israelii* and increased levels of *V. parvula*. Supragingivally, there was somewhat higher prevalence of *S. intermedius* in the khat chewers compared to the khat non-chewers, and in the khat chewing sides than in the khat non-chewing sides; the khat chewers and the khat chewing sides also tended to have lower prevalence of *A. actinomycetemcomitans.*

Since the probe species were selected to represent the color-coded microbial complexes (Table 2), the results obtained may allow speculation as to the association between khat chewing and other periodontal species. Such speculation is based on the fact that members of the same complex tend to coexist (Socransky et al., 1998). Consequently, it can be concluded that khat-chewing sides may harbor lower levels of *T. denticula,* based on findings concerning the other two members of the red complex. Similarly, it is likely that khat chewing is associated with members of the green complex because it showed a strong, dose-dependent association with *S. intermedius*. In fact this latter suggestion is supported by findings from the Zurich biofilm experiments, in which exposure of biofilms to 0.5% of the khat extracts resulted in a striking increase in the proportion of the biofilm made up by *S. oralis* (a green complex member) from 9% to 48%.

2.3 Khat and periodontal bacteria *in vitro*

In an attempt to provide some sort of explanation to these findings, we conducted an antimicrobial study (Paper 3), in which the khat extracts were tested against 33 oral

strains using the agar dilution method of the CLSI (formerly NCCLS). To a great extent, the results were consistent with those of the *in vivo* study. For example, *T. forsythia*, which showed an inverse relation to khat chewing, was among the most susceptible strains to the khat extracts. *V. parvula, S. intermedius,* and *E. corrodens*, with which khat chewing was strongly associated, were not sensitive to the extracts and did not even show any growth retardation. Although some streptococci were sensitive at the highest concentration tested (2% w/v), all of them showed better growth at low concentrations of the extracts compared to controls. Thus, the extracts were mainly active against periodontal disease-associated bacteria. In one experimental setup and accidentally, I observed that the blackish spots of *P. gingivalis* on the khat-containing plates were speckled with whitish colonies that increased in number as concentration of khat increased until that whole spot became whitish; the control plates showed the usual homogenous black pigmented spots. Gram-stained smears revealed the presence of very low numbers of contaminating unidentified gram positive cocci in control spots; however, as khat concentration increased, the contaminant cells increased in numbers and at 1% (w/v) there was no evidence for presence of *P. gingivalis* and the spot consisted only of the contaminant cocci. This observation indicates that the microbial shifts induced by khat (Paper 2) can be explained, at least in part, by its selective antimicrobial properties (Paper 3). However, other mechanisms may also be involved. For example, it is possible that khat contains substrates that enhance growth of certain bacteria such as streptococci.

2.4 Further elaboration

The *in vivo* and *in vitro* findings discussed so far bring to the surface the issue of "prebiotics". A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve the host health (Gibson and Roberfroid, 1995). The term "prebiotic", as the definition indicates, is used in the context of gut microbiology. I found no study that uses the term in connection with oral microbiota (Pubmed search).

However, the essence of this prebiotic approach, i.e. the maintenance of a homeostatic situation characterized by dominance of health compatible-species, is central to the ecological plaque approach recently proposed to prevent dental plaque-related diseases (Marsh and Bradshaw, 1997). It is therefore possible that khat possesses components with "prebiotic" properties, which if isolated may serve therapeutic purposes.

Though not within the context of the current discussion, it is worth mentioning that medically important bacteria such as *S. pyogenes* and *S. pneumonia* were also sensitive to the khat extracts. In addition to the selective antimicrobial properties, Paper 3 also provided preliminary evidence for the presence in khat of components that modify bacterial resistance to antibiotics such as penicillin and tetracycline. It is premature, however, to make speculations as to how this may influence oral microbiota based on this preliminary information. More studies are therefore required.

2.5 Khat and cariogenic bacteria *in vitro*

Unlike periodontal bacteria, none of the cariogenic ones was sensitive to the khat extracts at the concentrations tested although *L. acidophilus* showed marked growth reduction at 1% w/v (Paper 3). In studying the effects of the extracts on virulence factors of *S. mutans* (Paper4), it was found that the extracts resulted in a dose-dependent increase in planktonic bacterial growth and, at low concentrations, enhanced viability in the biofilms. However, two key virulence factors were inhibited in a dose-dependent manner: sucrose-dependent colonization and glucans synthesis. It was previously shown that presence of glucan matrix is essential to the pH-lowering ability of *S. mutans* (Van Houte et al., 1989). Indeed, an *in vitro* study demonstrated that maximum demineralization of enamel occurred when the artificial plaque consisted of 95% extracelluar polysaccharides and only 5% bacteria (Zero et al., 1986). Therefore, while khat favors growth of *S. mutans* (Paper 4), as it does with some other streptococci (Papers 2 and 3; the Zurich experiment), it also interferes with glucans synthesis and thus with *S. mutans* ability to colonize tooth surfaces and probably with its pH-lowering ability. As discussed in Paper 4, these anticariogenic properties are probably due the tannins present in the extracts. We

identified some of these tannins as proanthocyanidins (condensed tannins), namely prodelphinidine and procyanidine (Al-Hebshi et al., 2005)

Western blot analysis showed that the khat extracts resulted in up-regulation of GTFs production by *S. mutans*. However, coomassie blue-stained one-dimensional polyacrlyamide gel electrophoresis revealed that a number of other extracellular and cell-bound proteins were, on the other hand, down-regulated. This can be clearly seen in the 50- to 75-kDa region. Glucan-binding proteins (Gbps) are cell surface-associated proteins with molecular weights that fall in that range; GbpA is 74 kDa while Gbps B and C are 59 kDa (Russell et al., 1985; Smith et al., 1994; Sato et al., 1997). They are believed to contribute to adherence and carcinogenicity of *S. mutans* (Matsumura et al., 2003). It seems that these proteins were down-regulated by the extracts, but this has to be confirmed by Western blot analysis.

2.6 Final remarks

I would like to end this discussion by emphasizing that this work is not an attempt to give a good image of the khat chewing habit; I do appreciate that khat is a drug of abuse that has potential adverse economical, social and medical effects. However, this should not prevent us from objectively looking at the other side of the coin, i.e. investigating potential beneficial aspects. Of particular importance in this regard would be the possibility of isolating biologically active components that can be used therapeutically without harming the patient. This may be ironic, but in light of the previously reported cytotoxic activities of methanolic khat extracts and the antimicrobial, prebiotic and anticariogenic properties shown in this study, such a proposal is not implausible.

Conclusions

1- Khat chewing-induced bacterial shifts in gingival plaque are compatible with periodontal health.

2- Khat leaves and twigs contain water-soluble constituents possessing selective antimicrobial properties against oral microbiota. This explains, at least in part, the microbial shifts observed *in vivo*.

3- Khat leachables, probably tannins, have anticariogenic properties manifested as inhibition of *S. mutans* glucan synthesis and sucrose-dependent colonization.

4- There is preliminary evidence for the presence in khat of components that can modify microbial resistance to antibiotics.

5- Therefore, at the microbiological level, khat seems to play a preventive role in the pathogenesis of periodontitis and dental caries.

Future perspectives

The primary focus of a future work would be to perform an activity-guided fractionation of khat extracts, aimed at isolation and identification of the components that are responsible for the activities reported in this study. The active components would then be evaluated in mixed cultures and *in vitro* biofilm models. They may also be tested for their toxicity against human epithelial cells and fibroblasts!

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