Carcinogens Synthesized by Oral/Pharynx Microbes in Tobacco Smokers

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THESIS

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This thesis is dedicated to my parents. Without their support I would not have been able to accomplish half of the goals I've set for myself. Their support all these years have meant the world to me and I will forever be indebted to them. I truly love and appreciate them for everything they have done for me.

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EH

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

LIST OF ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionization
B[a]P	Benzo[a]pyrene
CE	Collision Energy
CytK	cyto-toxin K
CXP	Collision Cell Exit Potential
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DP	Ee-clustering Potentials
EP	Entrance Potentials
HPLC	high pressure liquid chromatographer
IARC	International Agency for Research on Cancer
IRB	Institutional Review Board
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid chromatography-mass spectrophotometer
MALT	Mucosal Associated Lymphatic Tissue
Nhe	non-hemolytic enterotoxin
OPC	Oropharyngeal Cancer
OSCC	Oral Squamous Cell Carcinoma
OTU	Operational Taxonomy Unit
PAH	Poly-Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction

PD	Periodontal Disease
rDNA	recombinant deoxyribonucleic acid
RT	Reactive Times
SD	Standard Deviation
ST	Smokeless Tobacco
TSNA	Tobacco-Specific N-Nitrosamines
WHO	World Health Organization

SUMMARY

Tobacco smoking has long been recognized as a risk factor for cancer and periodontal disease (PD). Both of these diseases are documented to be associated with changes in oral microbes. A second similarity between these diseases is an etiological association with inflammation. In the case of cancers such as oropharynx (OPC) or oral squamous cell carcinoma (OSCC) a host inflammatory activity occurs in response to hundreds of mutagens, toxins, and carcinogen mucosal DNA damaging agents. In the case of periodontal disease host inflammatory response occurs in response to microbe related proteins, lipids, and carbohydrates antigens. Our study suggests a tobacco product habit can alter healthy commensal microbe organization biofilm enhance survival of microbes that are not normally present in human oral biofilm and increasing cancer risk.. This altered microbe population can themselves release of mutagens, toxins and carcinogens. One group of carcinogens specified in this project is poly-cyclic aromatic hydrocarbons (PAH) as constituents of tobacco smoke these chemicals are recognized as type I carcinogens by the World Health Organization. Moreover, tobacco leaf products have been identified with a similar set of microbes that are capable of survival from tobacco smoke and additionally metabolize/degrade PAH, which occurs in nature during petroleum spills.

This study will briefly discuss PAH and their presence in the environment and tobacco products, as well as review the literature for microbes recognized to be linked to human diseases such as OSCC and PD and an association with tobacco product use. This thesis therefore provides novel information for risk for DNA damage to oral mucosa and oral carcinogenesis mediated by microbes.

1. INTRODUCTION

1.1. Background

Tobacco has been used by people for many generations in many forms. Today we often think of the household Camel or Marlboro brands of cigarettes, or smokeless dip popularized by baseball players when we think of tobacco, but tobacco is actually a native plant of North and South America. Historically tobacco has been used for medicinal, social, and religious reasons. It's uses ranged from being a hallucinogenic to treatment for tooth pain or a common cold cure and was consumed cured or uncured, smoked, eaten, and drank in a juice form. The importance of tobacco in the history of many cultures is financial, ritualistic, habitual, and medicinal.

The introduction of tobacco to Europe commercialized the product and fueled its trade. The popularity of the tobacco products has increased with marketing. In 1964 the Surgeon General wrote a report that assisted the government in regulating the advertisement and sales of cigarettes as the health hazard of smoking were studied and reported. The medical and dental community has long been interested in the relationship between tobacco and oral diseases as well as systemic diseases. There are many risks factors for oral and systemic diseases such as periodontal disease and cancer but studies on the subject have not been in agreement or conclusive and requires further discussion. [1]

1.2. Significance

A previous study by Dr. Belani found that the oral microbiome between smokers and non-smokers to be in fact quite different. Tobacco product use can result in creating a microenvironment for the oral cavity smoker. There are numerous published studies that show a correlation between tobacco product use and periodontal disease pathogenesis as

well as the related changes in the respective oral microbiome in each case. [2-12] This evidence appears to document a continuum for a relationship between oral disease, exemplified by periodontal diseases and tobacco use to result in a change in the microbiome at a variety of oral mucosa sites. Reports also demonstrate that tobacco product use is an independent risk variable for periodontal disease in itself. Furthermore, numerous published studies that show a correlation between tooth loss and increased risk for head and neck cancer. [13-18] Regardless of tobacco use, studies have found that oral health compared to a disease state, whether it is periodontal disease or oral cancer, can be characterized by an oral microbiome. [19-31] In this context we hope to find evidence that relationships have a positive correlation between tobacco product use and risk for periodontal disease as a product of selected microbe genera and species.

This study hopes to continue the understanding and identification of a unique set of microbes from tobacco smokers compared to non-smokers. We also add to the field another perspective of the microbes involved by selecting and identifying microbes that are able to metabolize and degrade poly-cyclic aromatic hydrocarbon (PAH) in smokers and non-smokers. Our finding from this study confirms a mechanistic relationship between selective microbe releases of PAH and DNA damaging chemicals after continual exposure to tobacco smoke in the oral/pharynx area of the oral cavity.

1.3. Specific Aims

The purpose of this study is to explore the differences in oral microbe biofilm of smokers compared to non-smokers, and then assess the degradation of poly aromatic hydrocarbons (PAH) by oral bacteria of smokers versus non-smokers. Having demonstrated the release and with known chemistry for PAH there is an increased

expectation that these microbes are active participants in oral carcinogenesis or at least enhance host tissue damage leading to carcinogenesis.

1.4. Hypotheses

We hypothesize that the microbiome of smokers and non-smokers is different. Furthermore we hypothesize that the continual exposure to tobacco smoke in the oral cavity of smokers will result in a novel set of microbes that are selective for metabolism and degradation of PAH, which are type I carcinogen.

2. REVIEW OF LITERATURE

2.1. PAH

2.1.1. Definition

Polycyclic Aromatic Hydrocarbons (PAHs) are chemical compounds that are formed from complete or incomplete combustion of organic compounds. The International Agency for Research on Cancer (IARC) has recognized 10 carcinogenic PAHs along with 53 other carcinogens present in cigarette smoke. [32] PAH's are ubiquitously present in the soil, water, and air and exist in many different forms and molecular weights. PAH exist in the soil from by-products of petroleum refineries, forest fires, and volcanic eruptions. Some of the PAHs that the IARC identified are benz-[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k[fluoranthene, dibenz[a,h]anthracene, dibenzo[a,e]pyrene, and 5-methylchrysene.[32]

PAH's are formed from combustion of organic compounds using heat as an accelerate. Combustion is also present in smokeless tobacco, but it is chemical in nature and does not require an external heat accelerate. Smokeless tobaccos are linked to risk for OSCC but it is less of a contributor to OSCC than tobacco smoke product use. However, the IARC lists 28 carcinogens present in smokeless tobacco which includes PAHs but the levels of PAH found in any product of smokeless tobacco will vary in parallel to type of tobacco leaf, harvest practices, processing and final market formulation.[33] This statement is exemplified by a study conducted by Stepanov. A 2-step extraction and purification procedure prior to gas chromatography-mass spectrometry was used to quantify different amounts of PAH in popular U.S. moist snuffs as well as spit-free tobacco pouches. These investigators lead by Dr. Stephen, concluded that spit-less tobacco had only trace amounts of PAH compared to the conventional moist snuff demonstrating that

manufacturing processes of smokeless moist snuff can be modified to reduce the amount of toxicants and carcinogens in the product.[34] Additionally this finding pointed to the possibility that variations in manufacturing might alter the presence of microbes present on the final tobacco product.

2.1.2. Degradation

PAHs are ubiquitous in the environment. They exist in the soil from by-products of petroleum refineries, forest fires, and volcanic eruptions. Often times biodegradation via microorganisms is used to degrade hazardous pollutants into carbon dioxide and water.[35] Much time and research has been spent in industry to study the degradation of hazardous pollutants. It has resulted in well documented microbial biodegradation pathways of PAH's and other hazardous pollutants as well as identification of specific bacteria that are able to metabolize these pollutants.

Bacteria need carbon sources for catabolic activity, and the dioxygenase enzyme; available in many different genera of microbes incorporates oxygen into the aromatic ring of the PAH to form a cis-dihydrodiol, after which through continued dehydrogenation, decarboxylation occurs forming epoxides. Cis-dihydrodiol and expoxides are the ultimate reactive oxygen species that are capable of DNA damage.[35]

Benzo[a]pyrene (B[a]P) is an example of a PAH that is found in coal tar, automobile exhaust fumes, cigarette smoke, and charbroiled food. The degradation of B[a]P and many other PAHs is documented on the Kyoto Encyclopedia of Genes and Genomes (KEGG). This database connects known information about molecular interaction networks, genes and proteins, as well as biochemical compounds and reactions.[36]

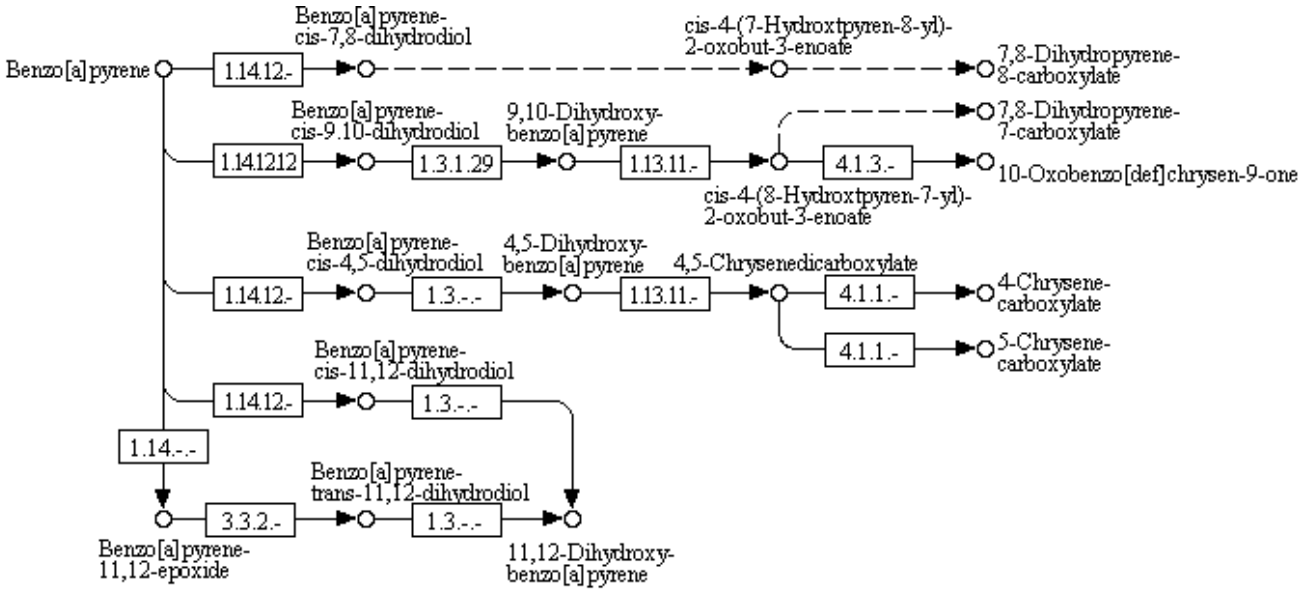


Figure 1. Degradation of Benz[a]pyrene as documented on the Kyoto Encyclopedia of Genes and Genomes.

(http://www.genome.jp/kegg-bin/show_pathway?pag00624)

2.1.3. Cancer risk

Evidence for a link between PAH and accumulation in human tissues comes from measurements of PAH samples from the lung of 70 cancer free autopsy donors, the PAH concentrations were found to be higher in smokers with a dose-response relationship for greater smoking.[37]

PAH compounds are carcinogenic and mediate DNA damage. PAHs covalently bind to DNA bases to form DNA adducts. DNA adducts are promutagenic lesions that lead to mutations in oncogenes or tumor suppressor genes if not repaired. [38] PAH's transfer an aryl-alkyl group to DNA through activation of the dihydrodiol epoxide (dehydrogenase) mechanism resulting in modification of the exocyclic amino groups of deoxyadenosine and deoxyguanosine residues, and formation of an amino group attachment to deoxycytidine.[38]

For example, generation of a reactive oxygen species through metabolism of B[a]P produces a benzo[*a*]pyren-7,8-dihydrodiol-9,10-epoxide which is carcinogenic which can generate mutations. This occurs as Benzo[*a*]pyren-7,8-dihydrodiol-9,10-epoxide covalently bonds to guanine in DNA and disrupts the normal process of DNA replication thus inducing DNA damage. [39]

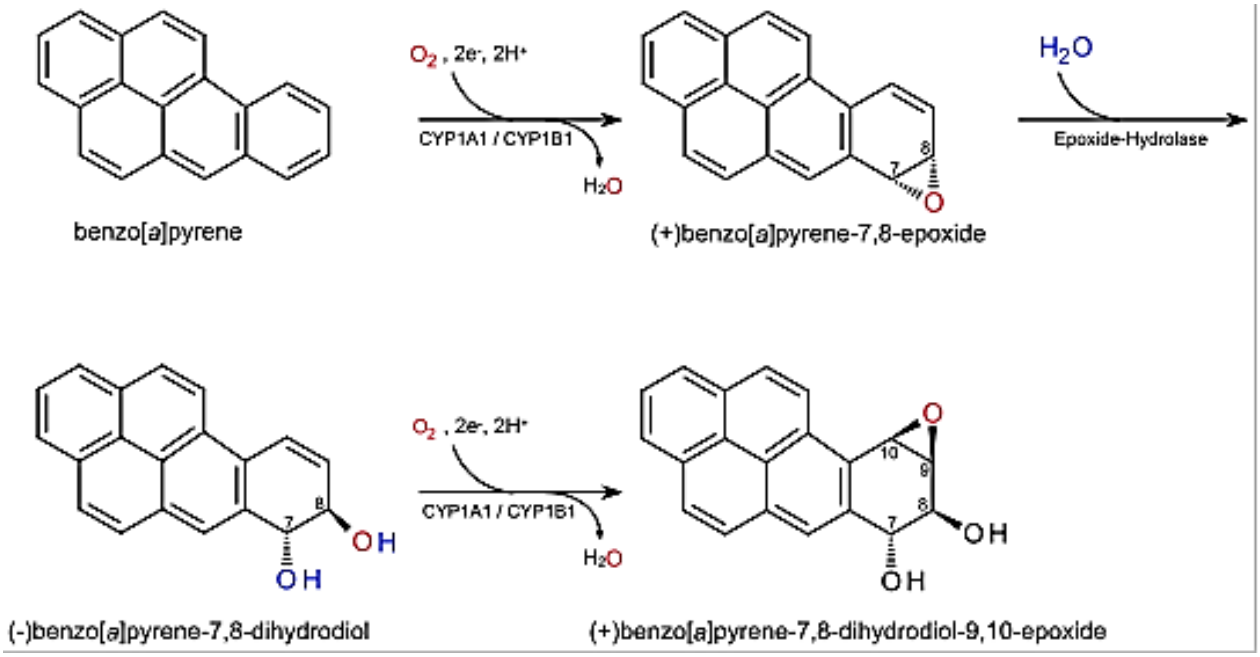


Figure 2. Metabolism of Benz[a]pyrene to produce an Epoxide. (http://commons.wikimedia.org/wiki/File:Benzo(a)pyrene_metabolism.svg)

2.2. Tobacco

2.2.1. Tobacco background

Smoked cigarettes and tobacco are used by people globally. The classic American cigarette can come metholated, filtered, unfiltered, flavored, or plain. Cigarettes manufactured in different parts of the world may be made up of different strains of

tobacco plants and/or be processed differently. Besides combustible tobacco products: cigarettes, cigars, hukka; there are multiple smokeless tobacco (ST) products which are another source of DNA damaging PAH and oxygen reactive generating species of nitrogenous compounds.

In some cultures smokeless tobacco (ST) has been commercialized for generations. A popular ST product in Sweden is snus and it is used by 29% of men and 6% of women aged 35-44 years of age [40]. Snus is made from dark air-cured ground tobacco mixed with salt and water and then processed with heat treatment and pasteurization. This process is said to make the end product of snus contain only limited number, genera and species microorganisms that have a survival capability to form spores under harsh conditions. Consequently low levels of tobacco-specific N-nitrosamines (TSNA) are found to be released or contain within snus.[40] ST products can be dry or moist but all are placed by the user in the vestibule area of the lower or upper lip.

In Sudan, the ST product which is popular is toombak. Toombak is produced from a native tobacco plant of the species nicotiana rustica. The preparation of Toombak includes harvesting the plant leaves, drying them, and then sprinkling them with water to ferment for a couple of weeks. This tobacco is then prepared into a course powder with sodium bicarbonate added to the mixture. It is generally used as a dip but can also be used in Hookahs. [41] Another form of ST exists in the Indian subcontinent and Southeast Asia and uses betal nut, acreca fermentation to produce the tobacco specific nitrosamine (TSNA), acrolein, which is further mixed with lime and tobacco leaf . An estimated 600 million people chew areca nut mixed with tobacco with variations on percentage of each

ingredient, metholation, and use.[42] All these tobacco products because of a plant source of orgin contain a variety of microbes that survive in association with the processing and ultimate chemistry of the leaf which is incorporated into the final market product.

2.2.2. Processing

An important factor that governs presence or absence of microbe in tobacco product is cultivation, soil conditions, geographic environment, harvest and processing methods.

Processing of tobaccos can be sun-, air-, flue-, or fire-cured. Sun-curing involves drying tobacco leaves in the sun, whereas, air-curing involves drying entire tobacco stalks on wooden staves in a well-ventilated barn[43] Flue-curing is accomplished by drying tobacco in an enclosed structure heated by circulating air produced by external heat sources and air-exchangers.[43] Fire-curing is accomplished by exposing tobacco to hardwood smoke from smoldering fires in an enclosed barn or building.[43] During fire-curing, smoke-related chemicals deposit on the tobacco leaf (e.g., PAHs, phenols, aldehydes) and other compounds increase in concentrations (e.g., TSNAs, N-nitrosated aromatic amines) [34, 44-47] Moist snuff, found in the United States, is made using both air-cured and fire-cured tobacco, and generally has higher PAH concentrations compared to snus, which does not contain fire-cured tobacco[48] Although, ten PAH compounds found in smokeless tobacco products have been designated as IARC group 1, 2A or 2B carcinogens [49]

In many cases, tobacco is aged following curing. Air-cured tobacco aging is completed rapidly (two months), in contrast to fire-cured, which generally takes three years. Fermentation and aging of tobacco is also common in the production of tobacco products such as cigars, hukka and some smokeless products (e.g., moist snuff and dry

snuff) [33, 50,51] During aging, chemical (Browning reactions) and bacterial-mediated reactions occurs that cause tobacco to have a more marketable flavor. [52]

2.2.3. Particles

The smoke of cigarette smokers also is found to contain particles which have a variety of energies, sizes, and charges. These particles may contain complexes of chemicals, fungal, or bacteria. The latter present in the tobacco leaf used for product market are survivors of combustion of the cigarette. Aside from chemicals released by bacteria and fungi, tobacco smoke also contains an estimated 5,300 chemicals that are formed during the burning of the tobacco [53], and one of these classes of chemicals are PAHs.

PAH's are hard to measure due to capture, modification in air, and quantity in the sample. Ding in 2007 was able to describe a technique in which mainstream cigarette smoke particulate was collected on glass fiber Cambridge filter pads through which the particulate was solvent extracted, and then purified and analyzed by liquid chromatography/atmospheric pressure photoionization along with mass spectrometry. [54] Evidence of additional immune responsive chemicals in tobacco smoke comes from a study by Hasday. In this study lipopolysaccharide (LPS) was measured and notable is the source of LPS which is derived from the bacterial cell wall. LPS was found in tobacco and the filter tip of cigarettes as well as the smoke after burning,[55] and demonstrated a specific group of bacterial toxins are present in tobacco smoke. [55]Larsson found the presence of LPS using gas chromatography-tandem mass spectrometry, as well as ergosterol, a fungal membrane lipid to be present in the tobacco and burned tobacco smoke[56, 57]. This technique indicated a further relationship between different types of microbes; in this case fungus and bacteria.

The effect of exposing oneself to high concentrations of respiratory, biologically active inflammatory stimulating LPS particles may contribute to the pathogenesis of local, oral inflammatory diseases of oral mucosa, such as benign nicotinic stomatitis, malignant OPC/OSCC or diseases of the periodontium (e.g., gingivitis, periodontitis) and more distant inflammatory pulmonary symptoms such as chronic bronchitis[55], which we will discuss in a later section.

Toombak, has been shown, using gas chromatography to have 100 times the level of TSNA compared to snuff or snus. These TSNA's include N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-butanone (NNK), N'(nitrosoanatabine (NAT), and N'nitrosoanabasine (NAB). Epidemiologic studies suggest that relative high frequencies of OSCC occur among toombak[58] dippers in Sudan because of these levels of TSNAs TSNAs.[41] The concentration of TSNA in saliva of toombak dippers was sufficient to produce rats tumors of the cheek and palate.[58]

In the New England Journal of Medicine in 1957 an article was published which showed the presence of Proteus vulgaris and Pseudomonas aeruginosa cultured from unopened cases of ST, snuff and these microbes were confirmed to be in the sputum of a patient with chronic bronchitis. [59] This early report begins to show that microbes associated with any tobacco market product can also be linked to human disease. Using this concept we reasoned that risk for tissue damage from tobacco product use of any kind may also be related to microbes that establish themselves in oral mucosa such as the tonsil-oropharynx.

2.2.4. Source of microbes

Tobacco product is merchandise of agricultural origin and for this reason naturally contains bacteria and fungi. Forgacs in 1966 was able to identify that all tobacco products purchased on the open market contained fungal mycelia, spores, and heat stable mycotoxins[60]. Microbes found in tobacco via cultures, biochemical test, microscopy, or genetic tests include these genera: *Acinetobacter, Bacillus, Burkholderia Closteridium, Klebsiella, Pseudomonas, Serratia, Campylobacter, Enterococcus, Proteus, Staphylococcus, Pantoea and Aspergillus.*[57] Zhao used 16S rDNA PCR-DGGE technology to further analyze bacterial communities on aging tobacco leaves and found that Bacillus megaterium and Bacteriovorax sp. EPC3 were present on leaf surfaces at 0-12 months. [61]

A review of the literature demonstrated 23 bacterial species linked to unburned tobacco products; 16, found after fermentation of fire-cured tobacco leaf; 42 identified in unaged flue-cured tobacco leaf; 15, aged flue-cured tobacco leaf; 9, air-cured tobacco leaf; 25 unburned tobacco leaf and 13, associated with chewing-snuff-snus-or unspecified ST products. Among fungal organisms were identified: 33, flue-cured tobacco leaf; 2 unspecified cure of tobacco leaf; 11 fermented tobacco leaf; 4, stored or loose tobacco leaf and 8 fungi found in cigarette filler.[51, 53, 56, 61-66]

Some bacteria are enhanced by ability to survive in association with tobacco use because they have a unique biology that enhances their survival. This could mean an opportunity for proliferation because of chemistry of tobacco leaf and product or biologic protection from harsh conditions such as capacity to form endospores. Endospore formation can involve a true spore as a dormant form of the bacterium.

For example under harsh conditions of curing and even pasteurization, endospores can survive for centuries. [67, 68] Endospore forming Gram positive bacteria such as *Bacillus*, or *Clostridium* genera are identified in tobacco products and may be transferred on to user mucosa surfaces.

Bacteria from the *Bacillaceae* genus exist in the soil and are mostly saprophytes but they can also function as animal parasites or pathogens to enhance their survival. *Bacilli spp*. bacteria are of particular interest because in addition to contributing to TSNA formation through nitrate and nitrogenous metabolism they are also engaged in PAH metabolism, and possible synthesis of Type I carcinogens. Moreover, both *B.pumilus* and *B. licheniformis* are identified in association with oral cancer; cutaneous anthracis-like reactions; oral mucosa lesions (e.g., gingivitis) and pneumonitis reactions. [4, 50, 51, 69, 70]

Members of the genus *Bacilli spp*. identified in tobacco products are capable of producing toxins. This capacity is exemplified by B.*thuringiensis* (Bt) which is a gram-positive and soil-dwelling bacterium that produces the Cry toxin. The Cry toxin is designated as a δ-endotoxin, which is a crystal protein used as a pesticide and is toxic to humans.[71, 72] Other *Bacilli spp*. that produces toxins that cause human disease are species *anthracis* and *cereus*. B.*anthracis* has not been identified among tobacco products but *B.cereus* is found in ST products and is often associated with food borne disorders such as diarrhea, intestinal cramps, vomiting and nausea following release of enterotoxins (hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe) and cytotoxin K (CytK). These enterotoxins are highly resistant to heat and to pH levels from acid to base. Although commonly associated with lower gastro-intestines disorders isolates are found in oral

tissues such as periodontal sulcus and this finding is of a particular concern because of the fore-stated survival of this microbe also occurs in ST tins.

Endospore forming bacteria in tobacco leaf also include: *Alkalibacillus* associated with bacteremia, infections of wounds, brain, kidney,arthritis [62, 73] ; *Anoxybacillus*: food contaminant (gelatin, milk); dairy processing[62, 74] and *Paenibacillus* with these identified activities or associations: oral non-tumor (abnormal neoplastic growth usually fibroma), polysaccharide-degrading enzymes ,and antimicrobials [62, 63, 69, 75, 76]

There are also several Candida species associated with tobacco products and many of these are also capable of PAH metabolism and/or nitrogenous metabolism of reactive compounds such as nitrosoamines.[77, 78] In addition to Candida species other fungi species associated with tobacco products include Aspergillus and Penicillium. [79]

2.2.5. Metabolisms/Degradation of PAH by bacteria derived from tobacco products

Different bacteria have been identified as existing in a variety of tobacco products capable of degradation of PAH; above we have commented . Gram negative bacteria identified with tobacco leaf or product include: *Pseudomonae, Ralstonia*, *Commamonas*, *Burkholderia, Sphingomonas*, *Alcaligenes spp* and these genera can also metabolize and degrade PAH. [62-64, 69, 73, 80-84] Besides gram negative bacteria, gram positive bacteria such as *Rhodococcus/ Nocardioides*, and *Mycobacterium, and Bacillus* strains are also identified to have a capacity to metabolize and degrade PAH and association with tobacco leaf or product. [35, 62, 63, 66, 69, 81, 85-89] These and many other examples of microbe genera found on tobacco leaf and/or tobacco product with a capability for synthesis of PAH are also capable of metabolism of nitrogenous compounds which include TSNA [90, 91]

Bacteria such as Porphyrmonas *gingivalis*, can use a variety of PAH substrates such as as fluorene (Fl), anthracene (Anth), phenanthrene (Phen), pyrene (Pyr), naphthalene (NAP) and benzo[a]pyrene (BaP)) to participate in this pathway. It is important to recognize that metabolism of some of these substrates include various reactive oxygen derivatives capable of DNA damage in plants and human cells; some are classified as Type I carcinogen (WHO) such as benzoate, which is the end product of this synthesis. Other derivatives include: 3,4 dihydroxy-fluorene; anthracene 9,10-dihydrodiol; 9,10-dihydroxy-anthracene; Phenanthrene-1,2-oxide; 3,4 dihydroxy-phenanthrene; pyrene-1,2 oxide; 1,6 and 1,8 dihydroxypyrene; Benzo[a]pyrene cis-9,10 dihydrodiol; 9,10 dihyroxy-benzo[a]pyrene; 4,5 dihydroxy benzo[a]pyrene; 11,12-dihyroxy-benz[a]pyrene). In addition, Naphthalene 1 or 2-Methylnaphthalene, or 2-Hydroxymethyl-naphthalene can be degraded to tyrosine, benzoate via salicylaldehyde to catechol, xylene or 4-hydroxycatechol. Recorded in the *Pseudomonas spp*. bacterium is expression of the nahE gene with activity by the trans-o-hydorxybenzylidenepyruvate hydratase or nmoAB gene with enhanced activity of 2-naphthoate monooxygnease reported. [92, 93]

Moreover, Cockrell et. al. and Rubinstein and Pedersen found *Bacillus spp*. in all ST products tested in their limited sample. B *licheniformis*, B.*firmis*, B.*circulans* as common isolates but others also identified: B.*brevis*, B.*sternothermophilus*, B.*megaterium* and B. *laterosporus*. *B. pumilus*, in fire-cured tobacco[50, 51] and still others observed a *B. anthracis*-like cutaneous lesion arising from exposure to these microbes which have a capacity to metabolize both nitrogen and PAH compounds to create reactive oxygen derivatives that damage DNA. One characteristic that enhances this

capacity is the expression of cytochrome P_{450} monooxygenase activity, which enabled the microbe to generate these reactive oxygen species[94, 95] Another gram positive, endospore-forming organism (*Brevibacillus*) is found in fermented fire-cured tobacco, and may be linked to cerebral infection[51, 96, 97]

Aspergillus species produce mycotoxins that damage integrity of mucosa. A variety of species from the Aspergillus genus are identified with tobacco and flue-cured tobacco leaves , loose-leaf chewing tobacco and stored leaves for ST, (e.g., *amstelodami; candidus, flavus, fumigatus, niger, ochraeus, repens, ruber, parasitus, tamari, and versicolor.)* Aspergillus species produce mycotoxins that damage integrity of mucosa. Recognizable is the risk for human pathology from both infections of C. *albicans* and Apergillus *fumigatus*. [51, 79, 98-100] It is interesting to note that there is reported to be a signal mediated interaction between Pseudomonas *aeruginosa* and Candida *albicans* which is thought to be accomplished by a phenazine/ PAH derivative. [101]

2.3. Periodontal Disease

2.3.1. Definition

Periodontal disease is an inflammatory disease of the supporting structures of the tooth that result in loss of normal architecture and function and damage to the supporting structures of the tooth such gingiva, periodontal ligament attachment, and bone. The diagnostic factor for periodontal disease is attachment loss but there are also many risk factors associated with periodontal disease ranging from bacterial plaque and calculus, oral hygiene, smoking, systemic diseases, host response, and genetics. Haffajee and Socransky show that the increase loss of epithelial attachment of the diseased tooth is associated with a change in microbes in the periodontal subgingival pocket compared to a

site without periodontal disease. Other studies also association of increases the risk for disease in distant non-oral tissues or organs to periodontal disease [102, 103].

2.3.2. Perio and smoking

One classic article about risk assessment for periodontal disease and presence of additional variables is an epidemiological survey designed by an Erie County study group that included cross sectional and longitudinal data. 1,426 subjects with attachment loss levels ranging from no attachment loss to severe attachment loss were studied. The systemic diseases correlated with attachment loss were diabetes and angina. Smokers with less than or equal to 15 pack-years had an odds ratio of 2.05 in having attachment loss compared to a 30 pack/year smokers with an odds ratio of 4.75.[104] Subjects that were older also had increased severity of attachment loss. Severity of risk factor such as diabetes, smoking, age, subgingival calculus and plaque were all positively correlated to severity of attachment loss and/or gingival inflammation. [104]

In Stockholm a retrospective study investigated patients with chronic periodontal disease in different age groups and their smoking habits. Smokers were found to have a higher prevalence of disease than non-smokers in all age groups at a risk ratio of 2.5.[105] Similarily, Machtei found that smokers had on average three times more clinical attachment level loss compared to non-smokers, with an adds ratio of 5.4 of increased risk of attachment loss compared to non-smokers. [106] Among Swedish dental hygienists with excellent oral hygiene, when comparing smokers, former smokers, and never smokers, the radiographic loss of periodontal bone height was investigated. It was found that in smokers, the loss of periodontal bone height increased with smoking exposure, and that smoking without any periodontal plaque influence is related to periodontal bone loss.[107]

A group in Iran completed a case-control study in the Golestan Province of Iran regarding the association between tooth loss/oral hygiene practices and esophageal squamous cell carcinoma. In this study, after matching the controls with esophageal squamous cell carcinoma cases for sex, age, and neighborhood, 78% of people with esophageal squamous cell carcinoma failed to practice regular oral hygiene compared to 58% of subjects without esophageal squamous cell carcinoma. A significant correlation was found between esophageal squamous cell carcinoma subjects and a high decayed, missing, or filled teeth (DMFT)[108] index. This is of course not a cause and effect relationship between poor oral hygiene and esophageal squamous cell carcinoma but that these two independent conditions may have similar underlying microbiology and genetic susceptibility.

Another group in Japan studied the relationship between tooth loss and the risk of 14 types of common cancers as a large scale, case-control study of more than 15,000 subjects. Patients were evaluated for tobacco and alcohol intake and habit, vegetable and fruit intake, type of cancer, and number of remaining teeth. This study also showed associations between tooth loss and risk of epithelial cancers from the head and neck, esophagus, and lung [109]. Significantly this study demonstrates correlations between a number of variables which include tobacco use and oral hygiene which is characterized by tooth loss. These types of studies also points to a flaw. In general, there is a lack of mechanism to evaluate in a quantitative manner the association.

The effects of smokeless tobacco are often localized to the site of placement compared to the widespread periodontal destruction seen in cigarette smokers. Usually there is localized gingival recession with white mucosal lesions. [5] A Sri Lankan study

compared the periodontal status of male smokers, betal chewers and non-tobacco users. In a cross-sectional based study of 2277 males it was found that betal nut chewers had a higher plaque index and gingival index compared to smokers and non-tobacco users and the mean number of teeth loss in smokers and betal chewers were statistically similar and significantly more than non-tobacco users.[110]

An educational brochure from the American Cancer Society reads that chewing tobacco and snuff lead to " less sense of taste and ability to smell… dental problems such as receding gums, greater wear and tear on tooth enamel and more tooth decay."[111] In a review to determine if smokeless tobacco increases the risk of periodontal disease and or dental caries it was concluded that there is no association between smokeless tobacco use and dental caries however an association exists between smokeless tobacco use and gingival recession. [111]

The possibility that use of a tobacco product results in an increased risk of periodontal disease which may possibly extend to a selection of different types of microbes due to use of tobacco products is expected. [103, 112]

2.3.3. Periodontal disease and bacteria

A change in microbe biofilm from strict aerobes to facultative aerobes to facultative anerobes to more strict anerobic microbes occurs as periodontal disease severity increases. Socrasnky studied subgingival plaque samples from 185 subjects with and without periodontal disease. He found different bacterial communities to be present with disease and grouped them by complexes. The red complex bacteria are Porphyromonas *gingivalis,* Tannerella *forsythia*, and Treponema *denticola*.[113] Socransky associated this group of bacteria as most associated with severe periodontal disease. Orange complex bacteria such as Fusobacterium *nucleatum,* Prevotella *intermedia,*

Prevotella *nigrescens*, Peptostreptococcus *micros*, Streptococcus *constellatus*, Eubacterium *nodatum*, Campylobacter *showae*, Campylobacter *gracilis*, and Campylobacter *rectus*. All species in the orange complex also showed an association with increased periodontal pocket depths.[113] Liu et.al. state that periodontal disease samples also contain "virulence factors" that characterize an " adapted parasitic lifestyle" by the fore-mentioned microbes in a dynamic biofilm that overlies gingival mucosa.[28]

2.3.4. Periodontal and tobacco associated microbes

There are many genera and species of bacterial that exists in periodontal disease sites that have also been collected from tobacco smokers. A few of these have also been recognized to have the capacity to metabolize and degrade PAH.[114]

An extension of the Erie County Studies measured mean clinical attachment loss to determine the status of the periodontal condition and paper points were used to harvest periodontal pathogens to be identified using indirect immunofluorescence microscopy. In this Erie County Study it was found that *Bacteroides forsythu*s and *Porphyromonas gingivalis* were more prevalent in smokers than non-smokers. Both genera, *Bacteroides spp*.and *Porphyromonas spp*. are capable of metabolism and degradation of PAH and in this context they are specified to be periodontal pathogens. We contend metabolic activation of PAHs by these bacteria partly explain the increased risk of periodontitis is often found in smokers. [112] It has also been found that smoking leads to increased bacterial colonization of mucosal surfaces, however there is less known about the effect of smoking and changing the mucosal surface to increase its ability to attach bacteria. [7]

In another study using microbe identification; Mager examined 40 bacterial species on gingival surface or in saliva from smokers, non-smokers, healthy, and periodontally involved patients. The microbial profiles from the brush biopsies and saliva

samples showed that patients with periodontitis had more periodontal pathogens but smokers also had more P. *nigrescens*, F. *nucleatum* , and *Actinomycetes spp.* in their saliva. [7] The genus *Actinomycetes spp*. is also identified as capable of PAH metabolism and degradation.[115-117]

A review of the genera and species identified in subgingival samples from periodontal disease sites obtained from tobacco smokers confirm that there are many periodontal pathogens that are shared between periodontal subjects and smokers.[8]

For example, *Eubacterium nodatum* is a member of the orange complex of microbes identified by Haffajee and Socransky . This microbe is recognized to have the capacity to metabolize and degrade PAH.[114]

Another microbe member of the periodontal red complex, *Fusobacterium nucleatum* is also noted to participate in PAH metabolism and degradation (KEGG Pathway fnu00624). Furthermore F.*nucleatum* and Streptococcus *gordonii* are reported to cooperate during periodontal disease induction to facilitate adhesion and entry into the mucosa by F.*nucleatum* and as the host immune activity is depressed. Moreover, it is interesting to note both F.nucleatum and *S.gordonii* both have a capacity for PAH metabolism and degradation. (KEGG Microbial pathways).[118-120]

Furthermore, an important genus for microbe infection of the gingiva is *Porphymonas spp*.; represented in the subgingival site by the species member: *P. gingivalis*. This species is also noted to have a capacity for PAH metabolism and degradation [35, 121, 122] *P. gingivalis* has a methyltransferase activity to release 1-Methoxy-phenanthrene and synthesize, 1-Methoxy-pyrene. This pyrene derivative will be further oxidized to 1-Methoxypyrene-6,7 oxide , and 1-Hydorxy-6-methoxypyrene and again

through another methyltransferase produce 1,6 Dimethoxypyrene. It is recognized that pyrene and some derivatives are either Group 1 (e.g., benzo[a]pyrene) or 2A (e.g., pyrene) are possible carcinogens in humans (IARC, (2006) http://www.crios.be/ PAHs/ specific.classification/IARC.htm).

It is important to note that *Porphyromonas* members use a variety of PAH substrates such as Benzo[a]pyrene; pyrene; phenanthrene; anthracene, and fluorene. This activity is accomplished through expression of several genes such as, nahB and doxE. For example, some microbes are able to oxidize the methyl groups of the PAH, 7,12 dimethylbenzanthracene found in tobacco particulates to form hydroxymethyl derivatives [123] [124, 125]

Peptostreptococcus spp. (e.g. micros) is another periodontal pathogen that also participates in mixed anaerobic or microaerophilic infections with *Streptococcus sp*p. and *Staphylococcus spp*. P. micros are also found in respiratory; aero-digestive and urinary infections but this number constituents only about 4% of all Peptostreptococcus *spp.* infections. [126] This latter association between *P. micros* and other common oral infectious bacteria demonstrates the need to isolate selective species and to characterize the microbe ecology in which the species of interest occurs. In this specific case it is interesting to note that *Staphylococcus spp*. (e.g., Staph. *aureus* and *epidermidis*) and some *Streptococcus spp*. (e.g.,S.*sanguinis*) identified in the oral cavity and also associated with non-gingival sites, such as teeth (e.g., *S. mutans*) can also metabolize PAH. (KEGG Microbe PAH degradation pathways)

Although *Prevotella nigrescens or P. intermedia*, are members of the orange complex of bacteria detected in subgingival periodontal disease samples from cigarette

users, it is presently unknown if they can metabolize PAH. However, *P. ruminicola* a genetic close relative of these bacteria species was isolated from rumen samples and can metabolize and degrade PAH. (KEGG Microbe pathway). It is also recognized that the phylum/order of *Bacteroidetes* are genetically close relatives of the genus *Prevotella* and this latter genus of Bacteroides is well documented to metabolize and degrade PAH chemicals.[73]

A Bacteroides species identified in periodontal disease subgingival samples from tobacco smokers is B. forsythus. [73]

2.3.5. Periodontal disease and cancer risk

The link with oral cancer has been evaluated in many studies. However, in many of these studies oral cancers were grouped in with oropharyngeal cancers and therefore there has been no systematic approach to dissociate type of cancers, sites, stage, grade, age and variables of risk (e.g., tobacco smoking variables). The result is an associative link without details for mechanism for carcinogenesis. Although, it is recognized that incidence of periodontal diseases correlates to a high degree with poor-oral hygiene and loss of dentition [103]. These latter variables are also correlated with increased risk for oropharyngeal cancers. [127]

In one study it was reported that there is over a 5 fold risk increase of tongue cancer (e.g., OSCC) for each millimeter of bone loss due to periodontal disease.[127] However, a similar association with esophageal cancer, upper GI and gastric cancers, breast cancer, and pancreatic cancer was not clearly evident. In contrast, periodontal disease and lung cancer did seem to have some level of significant association although smoking is a high confounder of this relationship and there was no assessment of other factors such as radon exposure. [127] In another study Tezal et.al.; supplied an additional

level of correlation between severity of periodontal disease characterized by degree of bone loss and risk for tongue carcinoma. This association was further validated by several other studies that correlated in a similar manner a presence of cancer (e.g., OSCC) with periodontal diseases . [128-134] Taken together these studies represent thousands of at risk cancer patients with varying degrees of periodontal diseases. However, a high degree of correlation as defined by odds ratio of <1.0 between presence of periodontal diseases and risk for cancers of the oral cavity and head and neck has been obtained (e.g., (odds ratio = 2.63, 95% confidence interval = 1.1.68 - 4.14; $p < 0.001$) [132] The latter meta-analysis included 7 articles composed of 2 cohort and 6 case-control studies to reach these conclusions.

2.4. Tobacco products, oral health and disease

Correlative examples are reported that provides evidence for a difference between microbiome found with oral health and microbiome found with oral disease. These sets of data describe a microbiome for oral health as a result of good oral hygiene in the oral cavity and pharynx. [16] We will review in this section differences between health and oral conditions with periodontal disease and oral squamous cell carcinoma as well as the effect of tobacco use on these conditions.

2.4.1. Periodontal disease and systemic disease

Stated above were a number of studies that show a relationship between use of tobacco products; increased incidence of periodontal diseases and specific microbe genera and species. This relationship is important because of systemic effects of tobacco smoke (e.g, concentrations of PAH chemicals) on the human body which affects metabolism of microbes located in biofilms at a variety of anatomic sites. Therefore it is

conceivable that tobacco smoke associated changes in microbe survival will have not only have local but distant effects on microorganism populations [1, 2, 6-12, 135-138]

It is important to recognize that taxonomically diverse microbes form a dynamic balance to maintain health. The loss of this balance between commensal bacteria and fungi or opportunistic microbe creates mixed bacterial-fungal infections that can increase mucosa deformity and loss of normal architecture. This process is associated with the release of virulence factors (e.g., aspartyl proteinases) other enzymes that require iron , and hydrolytic enzymes (e.g., phospholipases, proteases). When bringing in a factor such as tobacco smoke the species and dynamic balance of microbes needs to be considered to understand detrimental effects.

The phenomenon of colonization of potentially pathogenic species exists not only in the oral cavity, pharynx but the nasopharynx. A study compared the number of pathogenic species of bacteria as well as the aerobic and anaerobic bacteria that might possess interfering capabilities; as defined by ability to interfere with in vitro growth in the nasopharynx of smokers. They found that smoker had more potential pathogens isolated from nasopharyngeal cultures as well as fewer bacterial with interfering capabilities. [39] These finding may elucidate why many disorders have also been linked to uncontrolled proliferation of selective oral microbe genera and species, or linked to a depression or loss of regulation of immune responses such as periodontitis, diabetes, and angina. [122, 139-141]

In addition, tobacco product use is linked to a progression of vascular diseases in conjunction with enhanced risk for an aggressive loss of periodontal structures and suggestions for a selective microbial effect (e.g., aerobic to anerobic microorganisms) .

Among the microbes associated with periodontal conditions and vascular diseases is *Porphyromonas gingivalis*. This microbe is associated with enhancement of atherosclerosis in coronary arteries. Disclosed above, our review of KEGG microbial PAH degradative pathways showed a metabolic capability for release of PAH reactive derivatives after exposed to a variety of PAH substrates (e.g., fluorene, pyrene, benzo[a]pyrene, phenanthrene, anthracene). We further expected as a parallel finding, that periodontal associated microbe *Provotella intermedia* would be present, . Noted above was a capability to release PAH derivatives; because this characteristic is found in a close relative from this bacterial genus, *Bacteriodes spp*.

Anerobes appear to be important for the presence of severe periodontal diseases and development of vascular diseases and are also considered targets of tobacco product chemistry. *Treponema denticola*, is an anerobe associated with periodontal disease and possible enhanced risk for vascular diseases. Furthermore, anaerobic respiration among microbes is found in genera and species that are also capable of PAH metabolism and degradation. [142-146]

Indicated in the literature is a predominant role for Actinobacillus *spp*. (*Aggregatibacter*) actinomycetecomitans for periodontal infection. This microbe also has an adverse association with acute or stable coronary artery disease. [147] The family associated with this genus is Pasteurellacae and this family is also identified with PAH degradation and metabolism in soil and in aquatic environments. Presence of this activity in aqueous conditions is particularly interesting because of the aqueous conditions present in the oral cavity . [148-150]

In another study by Ohki et.al.; an investigation of microthrombi in vessels of patients with acute myocardial infarction by polymerase chain reactions (PCR); found a predominant presence of Aggregatibacter actinomcetecomitans, but present in smaller amounts was also *P.gingivalis* and *T. denticola* (respectively: 3.4% and 2.3% of the sites examined) [151]

Furthermore, patients who were tobacco smokers, and had severe periodontal disease, and diabetes, were at increased risk for atherosclerosis and congestive heart disease (CHD). Taken together these findings suggest a selection of microbe populations with a capacity for PAH metabolism and degradation and risk for vascular diseases[152]

2.4.2. Microbes in OSCC and periodontal disease

One study by Pushalkar S. et. al. compared oral microbiome in tumor and non-tumor tissues of patients with OSCC and detected several bacterial species (e.g., *Streptococcus spp* oral taxon 058, *Peptostreptococcus stomatis, Streptococcus salivarius, Streptococcus gordonii, Gernella haemolysans, Gernella morbillorum, Johnsonella ignava and Streptococcus parasanguis*). This finding is particularly interesting because Liu, et.al.disclosed similar sets of microbial genera linked to presence of periodontal diseases[28] Moreover, Liu et.al. used high-resolution functional information, KEGG Orthology, in order to uncover enriched metabolic functions to describe a "parasitic lifestyle" for the identified genera. Recognizable were metabolic pathways with linkage to PAH degradation and metabolism. These include benzoate degradation by hydroxylation; purine metabolism; tyrosine metabolism and fatty acid biosynthesis and metabolism.[28]

The citations given above indicate a growing realization that certain mciroorganisms are associated with human cancers, however their role is unclear. The

relationship of H. pylori with gastric cancer or MALT lymphoma seems causative, but H. pylori also appear to reduce the risk of esophageal cancer in other individuals[153]. Regardless of causation or association many bacterial species share the characteristic of highly site-specific colonization. This important characteristic may lead to ways of diagnosing and or treatment of many cancers and is the underlying important concept for the discovery we show in this study. Nagy in 1998 studied patients with OSCC and compared the biofilm samples from the center of the tumor lesion prior to antibiotic or tumor therapy to a sample taken on healthy mucosa. It was concluded that the sites of oral carcinoma harbored biofilm that had increased numbers of aerobic and anaerobic bacteria such as *Veillonella, Fusobacterium, Prevotella, Porphyromonas, Actinomyces, Clostridium, and Streptococcus spp.*, as well as Candida *albicans*.[154]

It is important to recognize that taxonomically diverse microbes form a dynamic balance to maintain health. The loss of this balance between commensal bacteria and fungi or opportunistic microbe creates mixed bacterial-fungal infections that can increase loss of viability and mucosal architecture as virulence factors (e.g., aspartyl proteinases) and enzymes (e.g., phospholipases, proteases) are released.

Fungal microbes such as, Candida *albicans* and other species also release an array of proteolytic enzymes and produce pseudohypae that invade into underlying oral epithelial mucosa. Furthermore, fungi can survive harsh conditions through spore formation, mannitol production, and melanogenesis. Candida species are also associated with oral malignancies and correlated to candida oral leukoplakia and oral epithelial dysplasia. [155-159] Additional evidence for association between C. *albicans* and OSCC was

provided by cross-reactivity by an antibody raised against C. *albicans* and found among tissues of OSCC.[160]

Pseudomonas *aeruginosa* in the oral cavity is an opportunistic infective microbe that is associated with acute inflammatory activity such as abscess formation and poor wound healing such as a ulceration but this bacterium is also an opportunistic microbe that may be present in premalignant and malignant lesion sites. Moreover *Pseudomonae* species are capable of PAH metabolism and degradation. Many species from this genus are identified in tobacco leaf and products. (P. *aeruginosa; fluorescens; fulva; oryzihabitans (previously Flavimonas oryzihabitans); putida and syringae* .[63, 161-163]

The most important finding which we referred to above; is the capability of identified microbes to metabolize and release reactive oxygen derivatives. This process can be broken down into several steps. Relatively large carbon sources can by used by both gram positive and gram negative bacteria associated with both periodontal disease and tobacco use to form intermediates of the PAH degradation metabolic pathway. These reactive chemicals take two forms: One is found as a product of nitrogenous metabolism and other is produced from PAH. The abundance of selective genera: *Bacteriodetes , Firmicutes, Actinobacteria, Fuseobacteria, and Proteobacteria spp*. that contain hundreds of species with these capabilities can not be overlooked as we seek to better understand their role in cancer induction.

Although the fore-mentioned identification of genera in association with OSCC was made there has beens no attempt to identify microbes linked in the presence of different levels of severity of periodontal disease. To close this gap we have provided comments on the close association of selected microbe genera and species with a PAH

metabolic capacity; and association with periodontal disease and tobacco smoking, which is expected to produce a release of reactive oxygen species to cause compromise of the oral mucosa, tissue damage, or DNA damage.

3. MATERIALS AND METHODS

3.1. Subjects

A total of 27 samples were collected from subjects that had various conditions of periodontal disease and presented to the University of Illinois at Chicago School of Dentistry for evaluation and treatment. We obtained samples from 17 tobacco smokers but systemically healthy subjects > 18 years of age. There were 7 females and 10 males in this group. They had varying ranges of periodontal status, periodontal treatment, medications taken, and systemic health. We also collected samples from 10 non-smokers also in good health. There were 7 females and 3 males in this group. Our control sample also had varying ranges of periodontal status, periodontal treatment, medications, and systemic health. All subjects in the smoker group had smoked for at least 5 years, and were currently smoking >10 cigarettes a day. All non-smokers were never smokers that hadn't taken systemic antibiotics in the last 3 months. Subjects were not excluded for factors such as age, sex, socio-economic level, systemic disease, medications taken, and degree of periodontal disease.

All subjects had a periodontal disease assessment in order to come up with a periodontal diagnosis. The clinical exam was completed and diagnosis determined following the procedure stated by Armitage.[164] The loss of attachment is the critical feature associated with the designation of severity. For example: Slight (1-2mm of loss of attachment), Moderate (3-4mm) loss of attachment), or severe (≥5mm) loss of attachment). Subjects in this study were chosen from a pool of patients that attend the periodontal and dental clinic at the University of Illinois, College of Dentistry. The consent was given after purpose and nature of the study was explained to each subject

and only after reading and signing of the consent form were these subjects entered into the study and samples taken. In all instances except for one subject which contributed repeat samples there was only one sample taken during the treatment period for periodontal condition. All subjects are monitored for periodontal condition change to continue on active periodontal therapy or to be placed on recall.

3.2. Molecular Techniques:

3.2.1. Microbe harvest

An oral cytology brush was used to obtain a surface sample from the pharynx of current tobacco smokers and current non-smokers. The brush was moved back and forth several times without any irritation or damage of mucosa. The brush is applied over the surface mucosa surface to capture microbes and biofilm by brushing back and forth at least ten times. After harvest of oral or pharyngeal microbes the sample is placed into phosphate buffered saline (1X, pH. 7.4). The sample is stored in a minus eighty freezer until a RNA extraction and analysis. Some samples from the oropharynx were also placed directly into bacterial growth broth and placed into culture to begin selection process after exposure to PAH composition as described below.

3.2.2. DNA and mRNA (16S) analysis and sequencing

The oral bacteria harvested from the oral pharynx area underwent DNA and mRNA (16s) analysis and sequencing for identification. The general procedure used to obtain DNA and identify microbes was according to a standard Epicentre method. (726 Post Road,Madison,WI 53713) This is based on a protocol for DNA extraction from bacteria (Cat. Nos. QEB0905T and QEB09050). For example: Bacteria (approximately 10^8) were centrifuged at 1,700 Xg (5,000 rpm) in a microcentrifuge tube for 3 minutes to pellet the cells. The bacteria pellet is washed once with 0.5 ml. of sterile water then

recentrifuged at 1,700 x g (5,000) for 3 minutes. A 1ml of lyse solution (lysozyme) was added to each tube and mixed gently by inversion. After incubation (1/2h to 1h) lysis was observed periodically. mRNA and DNA was used for PCR identification.

Isolates were identified by 16S rRNA gene amplification (amplicon sequencing, see below) with primers. For example, Eb246F (AGCTAGTTGGTGGGGT) and UA1406R (ACGGGCGGTGWGTRCAA) as the forward and reverse primers, respectively were used. PCR products were cloned, sequenced, and analyzed as described previously.[165]

A community structure was fabricated by single gene analysis and an operational taxonomic unit (OTU) listing 208 numbers of unique sequences with each OTU that indicates relative abundance of order, family, phylum and genus for tobacco smokers and 601 OTUs for non-smokers. This approach provides dominant taxa in each sample and relative abundance as indicated.

Genomic DNA was amplified using the Earth Microbiome Project barcoded primer set, adapted for the Illumina HiSeq2000 and MiSeq by adding nine extra bases in the adapter region of the forward amplification primer that support paired-end sequencing. The V4 region of the 16S rRNA gene (515F-806R) was amplified with region-specific primers that included the Illumina flowcell adapter sequences. The reverse amplification primer also contained a twelve base barcode sequence that supports pooling of up to 2,167 different samples in each lane.[166, 167] Each 25ul PCR reaction contains 12ul of MoBio PCR Water (Certified DNA-Free), 10ul of 5 Prime HotMasterMix (1x), 1ul of Forward Primer (5uM concentration, 200pM final), 1ul Golay Barcode Tagged Reverse Primer (5uM concentration, 200pM final), and 1ul of template

DNA. The conditions for PCR are also follows: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. The PCR amplifications are done in triplicate, and then pooled. Following pooling, amplicons were quantified using PicoGreen (Invitrogen) and a plate reader. Once quantified, different volumes of each of the products are pooled into a single tube so that each amplicon is represented equally. This pool is then cleaned up using UltraClean® PCR Clean-Up Kit (MoBIO), and then quantified using the Qubit (Invitrogen). After quantification, the molarity of the pool is determined and diluted down to 2nM, denatured, and then diluted to a final concentration of 4pM with a 30% PhiX spike for loading on the Illumina HiSeq2000 sequencer, and a final concentration of 4.0pM with a 10% PhiX spike for sequencing on the Illumina MiSeq. Amplicons were then sequenced in a 251bpx12bpx251bp MiSeq run using custom sequencing primers and procedures described in the supplementary methods of Caporaso et al 2012. [166, 167]

Sequence data were processed using the software package QIIME[166]. Raw sequence data from the Illumina MiSeq instrument were initially de-multiplexed using a separate index read using the split-libraries function, with default quality trimmed (split_libraries_fastq.py). Sequences from the entire dataset were then clustered into operational taxonomic units (OTU) - groups of sequences of at least 97% similarity - using the pick OTU function, implementing the UCLUST clustering algorithm (pick_otus.py). From this clustering, a biological observation matrix (BIOM) - a table of samples by taxa (OTU) consisting of number of sequences from each sample of each taxon - was generated. The BIOM was further processed to a series of BIOMs at various

taxonomic levels (phylum, class, order, family, genus) using the split_otu table script within QIIME (split_otu_table_by_taxonomy.py).

3.2.3. PAH exposure culture

Our next goal was to isolate and identify Oral and Pharynx Bacteria Based Upon Metabolism of PAH.

After harvest of oral or pharyngeal microbes and DNA./RNA identification and analysis. An alloquoted sample was placed in standard nutrient microbe growth broth medium with a PAH composition as a carbon source for the microbes to grow (37^0C) at room temperature. Our PAH test composition was : dibenz[a]pyrene DBP,benzo[a]pyrene B[a]P, Phenanthracene (Ph), Fluoranthrene (Fl), Napththalene (Nap), Pyrene (Pyr)and Chyrsene (Chyr)

Samples of the microbes grown in laboratory initially without fungicide to allow all microbe families to survive and to permit any interaction between fungus and bacteria that would be needed for growth.

Later samples were grown with fungicide to remove various species of *Candida*: Candida *albicans* was the predominant species. This sample was designated as follows: A 1-1 etc.for C.albicans and was grown only with PAH (50 microgram /milliter each PAH chemical recorded below).

Colonies of microbes were isolated by plating on to bacto-agar. Note was taken as to color, shape and contour with distribution of colonies after inoculum was observed. RNA was extracted from isolated colonies for identification (16S/23S RNA) using standard extraction procedure and methods described above. Species identification was subject to human oral microbe genome library comparison for identification and KEGG

pathway analysis to determine capacity for PAH and nitrogen metabolism, reduction and fixation.

3.2.4. Mass Spectrophotometric (Hybrid Linear Ion Trap LC-MS-MS) Approach to Assess PAH

A Liquid chromatography (LC)-Mass spectrophotometric (MS)-MS was developed for the detection of ten PAH's. 10 microliter (ul) of a PAH composition or single PAH control were injected on to a high pressure liquid chromatography (HPLC) system (Aligent 1100) coupled to a tandem quadruple 4000 Qtrap mass spectrometer (AB Sciex). Chromatography separations were performed on an Agilent Eclipse plus C18 column (3.5 micrometer (mm) 150 mm x 2.10 mm). The column temperature was held at 40^{0} C. Mobile-phase A was 0.1% formic acid in water, and mobile-phase B was 0.1% formic acid in acetonitrile. The gradient is shown in table 2:

The flow rate was 300 ul/min. The MS system was operated in positive atmospheric pressure chemical ionization (APCI) mode with a nebulizer current of 3 mA. Nitrogen was used as collision gas. The optimized source conditions were as follows (arbitrary units if not specified): Gas 1, 60; CUR, 30; CAD 12, and TEM, 400. The optimized compound conditions for the MRM transition for the ten PAH detected are shown in table 3. Data were processed with the Analyst software version 1.5.1 (AB Sciex).

In the results we summarize (table 4.) quadruple linear values (Q1, precursor ion selection and Q3, product trap) for each of the de-clustering potentials (DP); entrance potentials (EP); collision energy (CE); collision cell exit potential (CXP), and reaction times (RT) of 10 PAH derivatives.

Statistical Evaluation: In some instances we required a Student's T test and level of significance using ANOVA comparative analysis at a confidence limit of $p \geq .0.1$; which was performed.

4. RESULTS

This study tested the hypothesis that exposure to the mucosa of the oropharynx to tobacco smoke will result in a change in types of microbes that survive in the oropharynx and the metabolism by these microorganism will also change. We specifically exposed the microbes to poly-cyclic aromatic hydrocarbons to verify this change in metabolism by these microorganisms.

4.1. Subject analysis

Indicated above in our subject population of current smokers with periodontal disease were 7 females, with an average age of 51.0+/- 10.9 years, and 10 males, with an average age of 48.1+/-7.7 years. The average age for the total population of smokers was 49.7+/- 8.9 yrs. Female smokers had an average age of 30.5+/- 9.0 years and males smokers had an average age of 27.7 +/-10.0 years. Total number of smokers had a mean 28.8 +/-9.4 years of tobacco use. The mean percentage of periodontal depth > 5mm was: 16.4mm+/- 12.7 mm% for female and for males it was 13.8mm+/-12.3mm%. Total % PD >5mm was 15.0mm+/-12.6mm.

A companion comparison of % of PD > 5 mm was 9.3+/-11.6 mm % associated with localized (mild-moderate-severe) periodontitis, and 19.7+/- 12.1 mm % and associated with generalized (mild-moderate-severe) periodontitis. An additional comparison shows % of PD >5 mm totaled 15.7+/-6.4 mm% for non-smokers. In addition we recorded a slightly worse, 15.6+/-6.0 mm% for localized (mild-moderate-severe) periodontitis and 14.0+/-11.3 mm% for generalized severe periodontitis for non-smokers.

TABLE I

SMOKER AND NON-SMOKER DEMOGRAPHICS

(This table indicates the age, sex, pack/year smoking history, percent of probing depths greater than 5mm, and periodontal disease diagnosis.)

Age/Sex	Pack years	% PD>5mm	Periodontal Disease Diagnosis
59F	20	7	Localized severe periodontitis
63F	25	5	Localized severe periodontitis
39M	15	NA	No exam
47M	30	3	Localized severe periodontitis
51M	35	3	Localized moderate periodontitis
40M	40	9	Generalized moderate periodontitis
42M	23	NA	Localized severe periodontitis
55F	27	17	Generalized severe periodontitis
60M	35	25	Generalized severe periodontitis
35F	40	18	Generalized mild gingivitis
39M	11	NA	Localized moderate periodontitis
59F	22	2	Generalized severe periodontitis
38F	40	33	Localized severe periodontitis
52M	23	5	Localized severe periodontitis
56M	25	17	Generalized severe periodontitis
48F	40	33	Generalized severe periodontitis
55M	40	35	Generalized severe periodontitis
49.7 yrs	**28.8 pack yrs**	**15.0 % PD>5mm**	
Non-Smokers			
28F	NA	12	Localized severe periodontitis
28F	NA	10	Localized severe periodontitis
28F	NA	NA	Generalized mild gingivitis
59F	NA	20	Localized severe periodontitis
55F	NA	25	Localized severe periodontitis
47M	NA	17	Localized severe periodontitis
48M	NA	20	Localized severe periodontitis
32F	NA	6	Generalized severe periodontitis
66M	NA	10	Localized severe periodontitis
41F	NA	22	Generalized severe periodontitis
43.2 yrs.	**NA**	**15.7 % PD.5mm**	

4.2. Identification of Microbe Ecology

A focus on tobacco smokers using samples from the pharynx of subjects with periodontal disease permitted us to detect 68 operational taxonomy units that produced a <0.000 association value beginning with a cluster mean identification of 0.205 for the highest association value for an OTU. OTUs with an association of ≤0.10 shows a high degree of prevalence of bacteria from classes of *Firmicutes*, *Bacteriodetes* and *Actinobacteria* representating the top 4 cluster associations with respective genera associations of *Prevotella*, *Streptococcus*, *Veillonella*, and *Actinomyces*. (Table 3.) It is important to recognize that among this group all the bacteria that compose class; order; family and 20 genera an association of 85% is identified with PAH degradation. This was also confirmed through our comparison of smokers and non-smoker subjects with periodontal disease.

TABLE II

RELATIVE ORDER OF GENERA FOUND IN SAMPLES OF TOBACCO SMOKERS (PERIODONTAL DISEASE)

	Mean Cluster Identification Number
Bacteroidetes;(c) __Bacteroidia;(o)__Bacteroidales;(f)__Prevotellaceae;(g)__Prevotella	0.205
Firmicutes; (c)__Bacilli;(o)__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.174
Firmicutes;(c) __Clostridia;(o)__Clostridiales;(f)__Veillonellaceae;(g)__Veillonella	0.141
Actinobacteria;(c)_Actinobacteria;(o)_Actinomycetales;(f)Actinomycetaceae;(g)_ Actinomyces	0.103
Proteobacteria;(c) Gammaproteobacteria;(o) Pasteurellales;(f) Pasteurellaceae;(g)_ Haemophilus	0.069
Firmicutes;(c)__Clostridia;(o)__Clostridiales;(f)__Lachnospiraceae;(g)__Moryella	0.041
Fusobacteria;(c)Fusobacteria;(o)_Fusobacteriales;(f)_Fusobacteriaceae;(g)_Leptotrichia	0.047
Fusobacteria;(c)_Fusobacteria;(o)Fusobacteriales;(f)Fusobacteriaceae;(g)Fusobacterium	0.032
Actinobacteria;(c)_Actinobacteria;(o)_Coriobacteriales;(f)_Coriobacteriaceae;(g)__ Atopobium	0.027
Firmicutes;(c)_Bacilli;(o)_Lactobacillales;(f)_Carnobacteriaceae;(g)_Granulicatella	0.021
Firmicutes;(c)__Clostridia;(o)__Clostridiales;(f)_Veillonellaceae;(g)_Anaeroglobus	0.016
Firmicutes;(c)_Clostridia;(o)_Clostridiales;(f)_Lachnospiraceae;(g)_Oribacterium	0.014
Bacteroidetes;(c)Flavobacteria;(o)Flavobacteriales;(f)Flavobacteriaceae;(g) Capnocytophaga	0.009
Firmicutes;(c)Clostridia;(o)Clostridiales;(f)Veillonellaceae;(g)Selenomonas	0.008
Firmicutes;(c)_Bacilli;(o)_Gemellales;(f)_Gemellaceae;(g)_Gemella	0.008

Bacteroidetes;(c)Bacteroidia;(o)Bacteroidales;(f)Porphyromonadaceae;g) Tannerella	0.007
Bacteroidetes;(c)_Bacteroidia;(o)Bacteroidales(;f)Porphyromonadaceae;(g) Porphyromonas	0.005

We recorded for non-smokers over 500 OTUs however only 23 OTUs showed a mean cluster identification association $\leq$0.001. We note that the top 4 mean cluster associations in a range from 0.304 to 0.077 contained bacterial classes linked to PAH degradation and metabolism. For example Firmicutes, Proteobacteria, and Bacteriodetes are recognized to contain among order, family and genera bacteria with a capability for PAH degradation (Table 3b.)

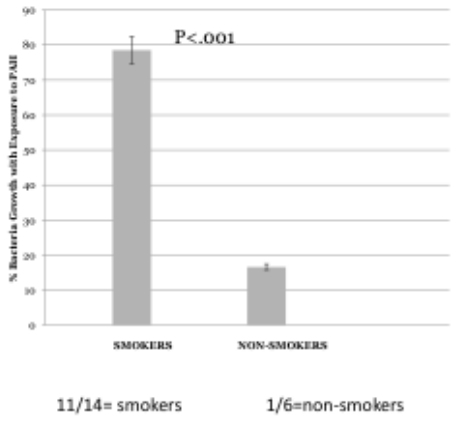
TABLE III

RELATIVE ORDER OF GENERA FOUND IN SAMPLES OF NON-TOBACCO SMOKERS (PERIODONTAL DISEASE)

	Mean Cluster Identification Number
Firmicutes;(c)__Bacilli;(o)__Lactobacillales;(f)__Streptococcaceae;(g)__ Streptococcus;(s)__infantis	0.304
Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__ Haemophilus;s__parainfluenzae	0.100
Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__ Melaninogenica	0.088
Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;Other;Other	0.077
Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Neisseria;Other	0.074
Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella;s__dispar	0.039
Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;Other	0.030
Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia;s__ mucilaginosa	0.027
Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;Other;Other	0.019
Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;Other;Other	0.017
Other;Other;Other;Other;Other;Other	0.012
Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia;s__ Dentocariosa	0.008
Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__	0.007
Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia;Other	0.005
Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__;s__	0.004
Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;Other;Other	0.004
Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinomycetaceae;g__ Parascardovia;s__	0.003
Firmicutes;c__Bacilli;Other;Other;Other;Other	0.003

4.3. Selective PAH metabolizing microbes

Tobacco smokers and presence of a specific bacteria ecology with a high level of PAH metabolic activity provides a context for us to suggest that microbes with a specific survival capability linked to exposure to PAH will exist in the oropharynx of tobacco smoker subjects in comparison to non-smokers. The percentage of microbes harvested from the subjects that were cultured in the presence of PAH and identified via RNA 16s identification. 11 of the 14 smokers had microbes that were able to survive in the presence of PAH. 1 of the 6 non-smoker cultures had bacteria that was able to grow in the presence of PAH.



Smokers (11): Candida *albicans*, Acinetobacter *junii*, Acinetobacter *baumannii*, Agrobacterium *tumefaciens*, Actinomyces spp, Bacillus *pumillus*, Bascillus *subtilis*, Kocuria *rhizophila*, Rhodococcus sp., Staphylococcus *epidermidis*, Staphylococcus sp.

Non-Smokers (1): Rhodococcus spp.

Figure 3. Bacterial growth in samples exposed to PAH.

4.4. Assessment of PAH Metabolism Using LS-MS-MS

We initially screened the supernatant containing the microbes for the presence of PAH after incubation as a sole carbon source composition of dibenz[a]pyrene DBP,benzo[a]pyrene B[a]P, Phenanthracene (Ph), Fluoranthrene (Fl), Napththalene (Nap), Pyrene (Pyr)and Chyrsene (Chyr). After removal of supernatant with PAH composition; microbes were incubated with an identical non-growth broth. This

supernatant containing only released PAH was analyzed using hybrid linear ion trap mass spectrophotometer (LS-MS-MS with quadruple linear trap).

Using the Ph 151 as a standard and sample 2 derived from C. *albicans* (Figure 3. A.) we detected the presence of 10 PAH derivatives after repeated exposure to our experimental composition stated above. To refine this result an additional sample from C.*albicans* and Acinetobacter *junii* was analyzed for Ph derivatives as identified in B. (Figure 3.) This process was repeated for Agrobacterium *tumefaciens* which was identified to release DB[a,l]P. Another microbe identified as Staphylocossus *hominis* is found to release B[a]P diol. In figure 3a. we present another microbe sample from Rhodococcus spp. associated with the release of DB[a,l]P . A sample from Staphylococcus *epidermidis* produced an oxygen reactive chrysene derivative 7-5 Methyl-chrysene-1,2 diol 239 and another reactive compound, benz[a] anthracene diol. Other identifications from Bacillus *pumilus*, C.*albicans* and Kocura *rhizophila*. Included release of B[a]P 8-OH 251.

These are examples of PAH that are reactive derivatives and capable of DNA damage and classified as Type I or II carcinogens by the international group: IARC. In contrast samples from non-smokers do not produce the PAH identification of microbes.

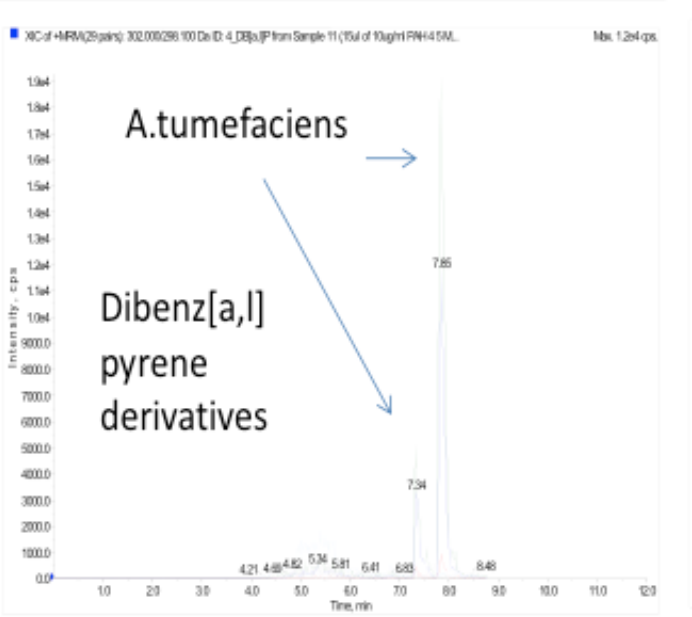


Figure 4. Mass spectrophotometric assessment (LC-MS-MS) of PAH metabolism. A. tumefaciens and PAH derivatives.

TABLE IV

MASS SPECTROPHOTOMETRIC ASSESSMENT (LC-MS-MS). MASS SPECTROSCOPIC CONDITION ANALYSIS AND IDENTIFIED METABOLIZED PAH DERIVATIVES

Q1	Q3	dwell time	ID	DP	EP	CE	CXP	RT
178	150	30	1_phenanthrene 151	75	8	72	6	6.6
178	176	30	1_Phenanthrene 176	75	8	52	6	6.6
195	165	30	3_Phenanthrene 1,2-diol 165	45	8	45	13	4.52/4.97
195	152	30	3_Phenanthrene 1,2-diol 152	45	8	41	13	4.52/4.97
302	298.1	30	4_DB[a,l]P	118	8	97	17	7.34/7.85
302	300.1	30	4_DB[a,l]P	118	8	63	17	7.34/7.85
317.9	287.1	30	5_DB[a.l]P-diol 287	90	8	84	16	5.87
317.9	261.1	30	5-DB[a.l]P-diol 261	90	8	108	16	5.87
335	289.1	30	6_DB[a,l] P-tetraol 335 to 289	82	5	40	15	4.63/4.91
335	307.1	30	6_DB[a.l]P-tetraol 335 to 307	82	5	20	15	4.63/4.91
370	276.1	30	6_DB[a,l]P-tetraol	55	6	75	15	4.63/4.91
258	239	30	7_5-Me chrysene-1,2-diol 239	79	9	51	11	5.29
258	226.1	30	7_5-Me-Chrysene-1,2-diol 226	79	9	86	11	5.29
263	245.1	30	9_B[a]A diol	56	8	26	8	4.48
263	202.1	30	9_B[[a]A diol	56	8	52	8	4.48
227.9	224.1	30	10_Chrysene	80	9	85	11	7.48
227.9	200.1	30	10-_Chrysene	80	9	80	11	7.48
241.9	237	30	11_5-Me Chrysene	67	10	92	17	8.08
241.9	239.1	30	11_5-Me Chrysene	67	10	64	17	8.08
243.9	189	30	12_1-OH-Chrysene	83	10	68	21	6.07
243.9	213	30	12_1-OH-Chrysene	83	10	78	21	6.07

4.5. Summary of Results:

We have shown:

1) Sequencing based 16S RNA identification confirmed the presence of selective species of microbes.
2) An innovative sole carbon source of PAH composition approach (e.g., dibenz[a]pyrene DBP,benzo[a]pyrene B[a]P, Phenanthracene (Ph), Fluoranthrene (Fl), Napththalene (Nap), Pyrene (Pyr)and Chyrsene (Chyr) was used to isolate microbes from tobacco smoke samples obtained from the pharynx.
3) Capability to release PAH derivatives into medium/supernatant from microbes that formed growing colonies was accomplished using a LS-MS-MS spectrophotometer.
4) 10 PAH derivatives were identified and linked to specific microbe

5. DISCUSSION

Previous reports have linked genera and species that are associated with tobacco products at various stages of development for the market and this relationship is summarized in table 6. Our findings show a heretofore specific link between specific sets of microbes, human oropharynx and tobacco product use. We acknowledge previous reviews that identified a link between tobacco product use and enhanced risk for periodontal disease in our background but we also demonstrate unrecognized and a neglected predominant role for microbes to cause DNA damage as a consequence of metabolism and degradation of PAH compounds derived from exposures to the oral pharynx, and cavity environment. This finding supports a broader view for microbe metabolic and physiologic activities in concert with a host immune response that promoting tissue breakdown.[168-170]

To accomplish our aim and obtain results we made modifications of standard techniques to isolate microbes with a strong capacity for growth in a tobacco smoke rich PAH environment. This adjustment offered the opportunity to observe colony growth, identify microbes (16S RNA) and assess PAHs released after incubation with a PAH composition; as a sole carbon source. Besides visual observation of colony growth and 16S RNA identification of the microbes, our collaboration with Linear Hybrid Mass Spectrophotometer (LS-MS-MS) experts from Dr. Amin's laboratory at Pennsylvania State confirmed presence of PAH metabolism by oral microbes and identified 21 PAH derivatives.

There has been recognized to be present an "oral healthy microbiome" that is distinguished from persons with an "oral non-healthy microbiome".[28-31]

A unique biofilm has also been identified for specific stages of periodontal disease and in various anatomic sites of the oral cavity such as the oropharynx [28] and possible association with oral cancers. [19-22, 31, 69, 70, 154, 171-174]

In addition there appears to be a consensus that tobacco use increases risk for periodontal infections and oral cancer; although some hold this risk is not as large as others report. However, this is a rationale for this type of study because we provided new information as to microbe ecology and speciation capable of altering micro-environmental relationships between microbial biofilm derived from tobacco product use and oral mucosa. Furthermore, our results provide some support for the thought that oropharynx is a site of increased risk for cancers because the oral microbiome at this site differs from other anatomic sites. Specifically this refers to risk to contain more genera capable of releasing PAH derivatives but with a likely enhancement of DNA damage by specific microbe species that survive because of a capacity to metabolize PAH. This metabolic activity has additional consequences which is to enhance human tissue breakdown and participate in inflammatory diseases found among tobacco product users such as periodontal diseases and immunosuppressed individuals[1, 2, 4-12, 19-22, 31, 69, 70, 154, 171-183]

Moreover, there is an ever growing literature that offers an opportunity to link a variety of microbes which we noted to be present in the oropharynx to tobacco cultivation, harvest, processing, or marketed tobacco product. Speciation also showed these selective microbe to have a capability to metabolize chemicals inherent in a process of tobacco product production or use.[62 63, 64, 69, 73, 80-84]

In the background and result section we have cited reports of microbes connected to tobacco product use and an ability to metabolize and degrade PAH but perhaps more importantly is an association of these microbes with those found in periodontal disease sites and oral cancer sites. Notable in our results was the high incidence of these microbes identified in microbial order, class , family and genera which had the characteristic for PAH metabolism.

This identification was also interesting because we recognized that a high mean cluster identification included the presence of *Bacilli spp*. and *Clostridium spp*. that have the ability to survive harsh environments such as tobacco smoke. Bacilli spp. are also identified with tobacco products and have an capacity for PAH metabolism and degradation evident in environmental pollution sites[35, 121, 184-187]

Our examination of specific microbes further identified a selective growth with PAH for Agrobacterium *tumefaciens*. This microorganism had only received limited association with human tissues and disease risk. Agrobactrium *tumefaciens,* is used during plant cultivation to transfer genetic information using various plasmids. In addition, one of these plasmids, Ti can transfer genetic information from one plant to another to cause tumor formation (e.g., callus). In addition this microorganisms has been shown to transfer plasmid information into human cells (e.g., HeLa, HPV 18 infected cervical carcinoma). This suggests Agrobacterium tumefaciens can act as a source for cell disruption which would be amplified by a capacity to release reactive PAH derivatives such as, DB[a,l]pyrene.[188, 189 63]

It is also reported that *Agrobacterium spp*. and Agrobacterium *radiobacter* are found in association with tobacco aged flue cured tobacco leaf and air-cured tobacco leaf.

Another microbe identified with an extensive literature associated with cancer development is C.*albicans*. This fungus was observed to be a predominant growing microbe that required us to use fungicide to observe the growth of less robust microbes. However, C.*albicans* has repeatedly been linked to cancer development and tobacco product use. However, the role for this fungus during carcinogenesis remains unclear. For example, one set of studies has identified the production of nitrosamines in a laboratory setting while other studies indicated the ability of C.*albicans* to colonize oral keratinocyte and mucosa and produce a loss of normal architecture. This process suggests an abnormal host inflammatory response identified by immune factors (e.g., antibody cytokines, lymphokines,etc) as part of the possible contribution by C.*albicans* for cancer carcinogenesis. In accordance with our data we suggest that C.*albicans* plays a role in DNA damage and increased risk for mutation (e.g., bulky adduct formation) in mucosa as reactive PAHs are released. In addition, our identification of PAH release can provide a biomarker approach to verify this relationship and assess metabolic activity tied to growth in clinical settings. This approach would also provide a better understanding of a role for fungus during carcinogenesis with invasion (e.g., pseudohypae identification) into mucosa structures.[51, 79, 98-100]

Moreover, interaction between fungi and other microbes forming a biofilm is also thought to promote disease virulence activities.[101]

Other microbes identified such as the *Acinetobacter spp*. are known to be associated with tobacco product use, association with periodontal infection and oral cancers.[62-64, 69, 70, 190]

However, this genus capability to synthesize PAH was not previously known and because these microbes were isolated from the oropharynx it is suggested more attention is needed to understand the role of this genus related to human diseases. Acinetobacter spp. also is shown to produce a tissue emulsifier and respond directly to human behavior such as exposure to alcohol products which is consistent with an environmental responsive microbe.[191, 192]

It is unknown to our knowledge if PAH degradation capability in humans has been previously reported for *Micrococcus spp.*, which is capable of PAH metabolism . *Micrococcus spp*. is similar to microbe genera identified in soil which are associated with contaminated petroleum sites and metabolize/degradation of PAH. [193-196]

Rhodococcus spp. is a another genus of aerobic non-sporulating nonmotile gram positive bacteria related to *Mycobacteria* and *Corynebacteria spp.*[197, 198] Rhodococcus spp. is reported in aged and un-aged flue-cured tobacco leaf and associated with oral cancer, brain abscess and encephalitis[63, 69, 199-202]

Rhodococcus spp. can metabolize a wide range of compounds such as steroids, acrylamide, toluene, herbicides, PCBs, acrylic acid, quinolone, pyridine, catechols, benzoate and PAH. [203] Chemically PAH are similar to poly-cyclic structures associated with steroids (e.g., naphthalene). The biochemical mechanism is as follows: oxygenation of aromatic rings to form diols (two alcohol groups) with predicable chirality, and stero-specificity of cyclic rings to result in a cleavage with intra/extradiol mechanisms, to open the ring and exposing the substrate to further metabolism. Presence of " Box and Paa" pathways illustrate the prevalence of non-oxygenolytic ring-cleavage strategies in aerobic aromatic degradation processes. Functional genomic studies also establish *Rhodococcus*

spp with high numbers of homologous enzymes with few redundancies needed for promotion of PAH oxidative degradation. For example, the multiplicity of ring-cleaving dioxygenases in certain *Rhodococcal spp*. isolates is attributed to cryptic (e.g., infrequent rare) aromatic catabolism of different terpenoids and steroids that are similar to PAH structures.[204, 205] The biology cycle of *Rhodococcus spp* also involves the use of large linear plasmids and these plasmids are linked to a fast growth rate.[197]

Staphylococcus spp. such as *Staphylococcus hominis and epidermidis* has been reported in various human diseases such as oral cancer, toxic shock, food poisoning and septicemia in immune-compromised individuals at various sites (e.g., urinary tract infection, endocarditis, pneumonia, wounds). *Staphylococcus spp*. are coagulase negative gram positive spherical bacteria. Species of *Staphylococcus* are often commensal and found in oral cavity biofilm and on skin where they can be abundant. These bacteria produce acid from glucose, fructose, trehalose and glycerol and therefore contribute to a lower pH microenvironment in contact with oral mucosa. A lowering of pH we suggest may increase stem cell like cell proliferation and in context with PAH release contribute to malignant transformation risk.[206] This cellular physiologic response may become important as *Staphylococus spp*. (e.g. *hominis*) release PAH derivatives throughout the oral cavity. Reported are 7 species of *Staphylococcus spp*. associated with various forms of tobacco product. These include Staphylococcus *aureus* found in unaged flue-cured; unburned cigarette leaf, loose-leaf chewing tobacco, dry and moist snuff. Staphylococcus *cohnii*; fermented fire-cured tobacco, and unburned cigarette; Staphylococcus *epidermidis*; unburned cigarette tobacco , moist and dry snuff; Staphylococcus *sciuri*; unburned cigarette tobacco, air-curing tobacco; Staphylococcus *maltophilia*; unburned

cigarette tobacco; Staphylococcus *saprophyticus*, unburned cigarette tobacco, flue-cured tobacco, air-curing tobacco and Staphylococcus *hominis*, associated with air-curing tobacco. [62-64, 69, 70, 89, 154, 190, 207-209]

6. CONCLUSION

We identified that tobacco smoke induced an environment that promoted the growth of selective bacterial genera that had a capacity to degrade PAH. This finding is significant because some PAH are well documented Type I carcinogens and linked to increased risk for OSCC. To achieve this finding an innovative approach was used that offered the opportunity to isolate and identify microbes from tobacco smokers. These microbes form a unique set of microbes with a capacity to release PAH derivatives; increase possible risk for DNA damage at various mucosa sites after exposure to an appropriate PAH containing environment such as tobacco smoke.

Several of the microbes identified where not previously associated with oropharynx mucosa or human head and neck tissues.

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