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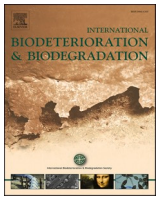

Anaerobic biodegradation of anthracene by oral Firmicutes isolates from smokers and its potential pathway

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ABSTRACT

Exposure to polycyclic aromatic hydrocarbons (PAHs) from tobacco smoke has been linked to many negative health effects. Studies on the biodegradation of PAHs by human microbiota and detailed pathways for their anaerobic biodegradation are scarce despite their importance in getting rid of these toxic compounds. In a previous study for our group, we determined the ability of oral bacterial isolates in the anaerobic biodegradation of anthracene as a model of PAHs. Three isolates with the highest anthracene degradation ability were selected for the present study which include *Limosilactobacillus fermentum*, *Veillonella parvula*, and *Streptococcus anginosus*. In this study, we aimed at exploring and elucidating the anthracene anaerobic biodegradation pathways in selected Firmicutes oral isolates. Metabolites throughout the pathway were detected by gas chromatography coupled with mass spectroscopy (GC-MS) using anthracene as sole source of carbon. After incubation for 3 days, anthracene was undetected in the supernatant of *L. fermentum* and *V. parvula*, while a residual of 3% of anthracene was detected in presence of *S. anginosus*. Results revealed that anaerobic biodegradation by *L. fermentum* and *V. parvula* started with hydroxylation and dehydrogenation producing 9,10- anthraquinone and ended up with simpler structures such as catechol, while *S. anginosus* hydroxylation for anthracene resulted in the production of 1,2-anthracenediol and ended up with catechol and phthalic acid. The biodegradation of anthracene by oral bacteria could convert it to other toxic metabolites such as anthraquinone and catechol which were reported to have potential carcinogenic effects. Moreover, fatty acids detected as biodegradation metabolites could be one of the causes of smokers' heart-related diseases. Thus, this study explored oral metabolites resulting from smoking under anaerobic conditions towards elucidating the role of oral microbiota in health and disease states.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds composed of two or more benzene rings (Lawal, 2017). Anthracene and phenanthrene, containing three fused aromatic rings, are the simplest two PAHs based on their structures as reported by the International Union of Pure and Applied Chemistry (IUPAC). They are mainly produced during the incomplete burning of various natural substances including tobacco (Karale et al., 2015). PAHs from tobacco smoke may accumulate in the lungs of smokers depending on the amount and duration of exposure as well as a variety of other host-related factors (Armstrong et al., 2004).

Algae, bacteria, and fungi have shown the ability to biodegrade PAH. This involves the breakdown of this complex compound through biotransformation into a less complex form which makes them eco-friendly (Gao and Gu, 2021; Sepehri et al., 2020). The biodegradation of PAH was extensively studied under aerobic conditions where the microorganism aerobically degrades PAHs through an oxidative attack (Aryal, 2021; Daou et al., 2021; Logeshwaran et al., 2022). However, the biodegradation of these PAH compounds under hypoxic conditions is usually adopted in the natural environment. The biodegradation of PAHs under hypoxic conditions is a slow process, and its biochemical mechanism has not yet been elucidated (Dhar et al., 2020). From all the studies carried out to date on biodegradation of aromatic compounds, such as phenols, cresols, anilines, benzoates, toluene, benzene, xylenes,

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nitroaromatic and chlorinated compounds, and many others, it can be concluded that anaerobic bacteria follow a strategy that is similar to that of aerobic bacteria (Haritash and Kaushik, 2009). Many facultative and/or obligate anaerobes can degrade PAHs in hypoxic regions using terminal electron acceptors alternative to oxygen such as nitrate, iron, manganese, and sulfate (Dhar et al., 2020; Zhang et al., 2020). Degradation of PAH usually occurs in two stages: from hydrocarbon molecule to central intermediates then from the destabilization of central intermediates to aromatic ring cleavage and biomass yield comprising the higher and lower degradation pathways, respectively (Ladino-Orjuela et al., 2016).

The majority of human microbiome is comprised of obligate or facultative anerobic bacteria and that exert essential metabolic functions for human health as biodegradation of xenobiotics. The oral microbial community residing in the human mouth forms the second most complex microbial community in the body after that of the colon (Dewhirst et al., 2010). It plays an essential role in oral and systemic health and the equilibrium between oral microbiota contributes to maintaining a healthy state. Members of the phyla *Firmicutes* and *Proteobacteria* represent almost 60% of oral microbiota; (33.2%) and (27.5%); respectively. Of the *Firmicutes* members predominant in the oral microbiota the genus *Streptococcus* (Class Bacilli), represents 19.2% of the total oral microbiota (Al-Mudallal et al., 2008), genus *Limosilactobacillus* (Class Erysipelotrichia) and genus *Veillonella* (Class Clostridia) represents together about 8.7% of the total oral microbiota (Zheng et al., 2020).

Due to the shortage of studying PAHs biodegradation by oral microbiota compared to studies carried out on PAHs environmental biodegradation and by gut microbiota, it was aimed in this study to explore the pathway by which anaerobic biodegradation of anthracene (a member of PAHs) by oral microbiota could run and to determine the metabolites of possible impact on human health. The relatively simple structure of anthracene makes it an ideal model for microbial biodegradation of PAHs studies (Karale et al., 2015). In this work, isolates recovered from oral rinse of a heavy smoker participant were tested for their ability to degrade anthracene and resulting metabolites were identified by GC-MS. Metabolite identification allowed the prediction of possible biodegradation pathways as well as the evaluation of the potential hazards of metabolites on smokers' health.

2. Materials and methods

2.1. Bacterial isolation, identification, and phylogenetic analysis

Bacterial isolates used throughout this study were previously isolated from oral rinse sample of a heavy smoker on tryptone yeast extract cystine with sucrose and bacitracin (TYCSB), Lactobacillus selective agar (LBS) and Veillonella selective agar medium for the isolation of the species of *Streptococci*, *Lactobacilli*, and *Veillonella*, respectively (Moussa et al., 2021). Informed consent was obtained from the participant and approved by the Research Ethics committees of the Faculty of Pharmacy at October University for Modern Sciences and Arts (MSA) and Faculty of Pharmacy, Cairo University with approval IDs (M2/EC2/2016MS), and (MI 1722), respectively. Colonies showing typical morphology were selected for identification to the species level by 16S rRNA gene sequencing at the Macrogen® sequencing facility (Seoul, South Korea), using the ABI 3730xl System. The 16S rRNA gene sequences were subjected to similarity search using the nucleotide Basic Local Alignment Search Tool (BLASTn) from NCBI. The gene sequences with high similarities to those determined in the study were retrieved and added to the alignment and a phylogenetic tree based on neighbor-joining was built using Molecular Evolutionary Genetics Analysis (MEGA 11) and the tree was tested with bootstrap analysis (1000 replicates).

2.2. Assessment of anthracene biodegradation using 2,3-dicholophenolindophenol assay

The anthracene biodegradation ability of the three bacterial isolates was measured indirectly by observing the disappearance of the blue color of the 2,6- dichlorophenolindophenol (2,6-DCPIP) (Sigma-Aldrich, Steinheim, Germany) using the method detailed in Moussa et al. (2021). The assay was performed by culturing isolates at 37 °C for 8 days under anaerobic conditions in prepared iron-free W medium with additional anthracene (SDFCL, Mumbai, India) as the sole source of carbon, and following the biodegradation ability of the isolate by bleaching the blue color of the reagent DCPIP (Karale et al., 2015). Aliquots from the assay mixture on days 0, 1, 2, 7, and 8 were centrifuged at 8000 rpm for 15 min to avoid interference between bacterial growth turbidity and indicator color. The anthracene biodegradation was estimated by measuring the change in absorbance of the DCPIP indicator. Absorbance readings were converted to concentrations using a standard curve. The percentage of remaining DCPIP color intensity was calculated by comparing the change in color intensity after a given time with the initial value (final DCPIP indicator concentration/initial DCPIP concentration) x 100 (Chowdhury et al., 2017). The growth of bacteria was assessed by viable count on selective media as detailed in Moussa et al. (2021).

2.3. Analysis of anthracene biodegradation metabolites using gas chromatography coupled with mass spectroscopy (GC-MS)

Analysis of the anthracene metabolites was determined along the following steps.

2.3.1. Cultivation and sample preparation

The experiment was performed in a 50 ml tube containing 22.5 ml Fe-free W medium, 1.5 ml $FeCl3.6H_2O$ (150 μg/l), 1.5 ml 2,6-DCPIP (50 μg/l), 2.4 ml cell suspension (OD=1 at 600 nm), and 0.25 ml anthracene (1 mg/ml in dichloromethane) and incubated anaerobically at 37 °C for 3 days. Two controls were used: Control (1): 5 mg anthracene dissolved in 120 μl silylating reagent N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (Sigma-Aldrich, Steinheim, Germany) to assure that anthracene as a parent compound is detectable by GC-MS analysis, and Control (2): anthracene in the assay medium containing 2,6-DCPIP without bacteria was used to assure that anthracene in the assay mixture is also detectable.

After the incubation, the residual anthracene and metabolites resulting from biodegradation were extracted from the medium by ethyl acetate, the organic layer was collected, and the extraction step was repeated three times for better yield. The organic extract was concentrated by a Rota evaporator until dry weight (Tarafdar et al., 2017). Five milligrams of the dry weight were mixed with 120 μl silylating reagent, incubated at 60 °C for 30 min, and analyzed by gas chromatography-mass spectrometry (GC-MS) (Hadibarata et al., 2012).

2.3.2. Gas chromatograph (GC) injection conditions

Anthracene biodegradation metabolites were analyzed using Shimadzu GCMS-QP 2010 (Shimadzu Corporation, Kyoto, Japan) equipped with Rtx-5MS (30 m × 0.25 mm i. d. X 0.25 μm film thickness) capillary column (Restek, USA) and a split–splitless injector. The initial column temperature was kept at 70 °C for 3 min, then programmed to 315 °C at a rate of 10 °C/min, and finally kept constant at 315 °C for 6 min. Injector temperature was 280 °C. Helium was used as a carrier gas with a flow rate of 1.24 ml/min. Diluted samples (1% v/v) were injected in split mode (split ratio, 1:10) with a 1 μl injection volume.

2.3.3. Interpretation of GC chromatogram and mass spectrum analysis

The obtained chromatograms were transferred from GCMS-QP Solution Software post-run analysis (Shimadzu Corporation, Kyoto, Japan) to the Automated Mass-Spectral Deconvolution and Identification System (AMDIS) to be processed. The raw data were first cleaned up from

any background noise and interferences matrix using the AMDIS deconvolution procedure. The data were further processed by matching the mass spectra and retention indices of peaks with references to retention index and mass spectra from the National Institute of Standards and Technology (NIST) library, various literature reviews, and databases of PAH metabolic pathways (Matyushin et al., 2020).

The intensities of the quantification ions of detected metabolites were reported as peaks height (Area %) from the unique absolute ion intensity. The active hydrogen atom(s) of the PAH metabolite was replaced by a trimethylsilyl (TMS) group (Si(CH3)3, m/z 73) due to the derivatization by the silylating agent. The unique peaks that were detected in biodegradation samples chromatograms while absent in the two controls were considered as possible metabolites. The degree of biodegradation was determined by comparing the amount of anthracene remaining between the controls and samples (Hadibarata et al., 2012).

2.4. Genomic analysis of anthracene degradation pathway in reference genomes using Bacterial and Viral Bioinformatics Resource Center (BV-BRC)

According to the results of phylogenetic analysis, the most similar strains with complete genomes were selected for analysis by the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (https://www.bv-brc.org/) (Olson et al., 2022). The three strains selected for study were *L. fermentum* JNU 532 (GenBank Accession: CP103400), *S. anginosus* FDAARGOS_1357 (GenBank Accessions: CP069892), *V. parvula* FDAARGOS 1046 (GenBank Accessions: CP065990). The BV-BRC generates a genome/pathway databases starting from the annotated reference genome representing the three strains, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

2.5. Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc., CA, USA) was used for statistical analysis. Multiple unpaired t-tests and multiple comparisons using the Holm-Šídák method were used to compare the change in bacterial growth. The readings were considered significant at $p < 0.05$.

3. Results

3.1. Bacterial identification and phylogenetic analysis

Sequencing of the 16s rRNA genes of isolates was used for their identification and bacteria were assigned to a species or genus level with similarity score between 90 and 99% as *Streptococcus anginosus* (SS-8), *Limsoilactobacillus fermentum* (SL-8), and *Veillonella parvula* (SV-8). Results in our previous study showed that the most prevalent bacteria in the host oral cavity were *S. anginosus* (1.7×10^7 CFU/ml) followed by *V. parvula* (7.2×10^6 CFU/ml) then *L. fermentum* (1.6×10^6 CFU/ml) (Moussa et al., 2021). The 16S rRNA gene sequences of the three isolates were added to the National Center for Biotechnology Information (NCBI) GenBank database under the accession numbers: OP896838,

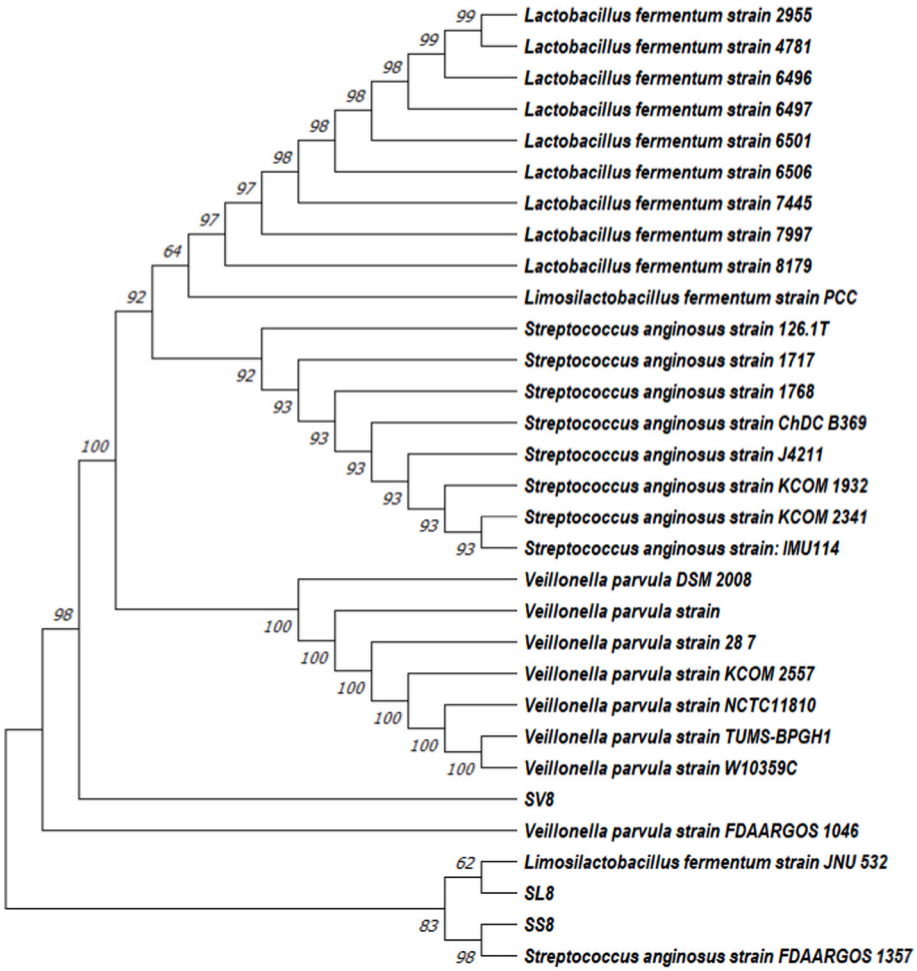

Fig. 1. Phylogenetic tree based on 16s rRNA sequence of the isolates: *Streptococcus anginosus* (SS-8), *Limosilactobacillus fermentum* (SL-8), and *Veillonella parvula* (SV-8). Isolates sequences were compared with sequences retrieved from NCBI GenBank for highly similar strains using Molecular Evolutionary Genetics Analysis version 11.0 (MEGA 11). The phylogenetic tree was constructed by neighbor-joining clustering method with a bootstrap value of 1000 replicates.

OQ383429, OQ389599 for *L. fermentum* (SL-8), *S. anginosus* (SS-8) and *V. parvula* (SV-8), respectively while pure cultures were deposited in the Culture Collection Ain Shams University (CCASU) with the numbers CCASU-2022-21, CCASU-2022-22, and CCASU-2022-23, respectively. The phylogenetic analysis of the three isolates relative to the most similar strains from the NCBI database showed that the most related strains for isolates SS-8, SL-8, and SV-8 were *S. anginosus* Strain FDAARGOS 1357, *L. fermentum* strain JNU 532, and *V. parvula* strain FDAARGOS 1046, respectively. The two Gram positive bacterial isolates SL-8 and SS-8 were closely related to each other representing a clade that evolve from a common node (Fig. 1).

3.2. Anthracene biodegradation potential by the oral bacterial isolates

Changes in isolate viable counts during anthracene biodegradation were measured during 8 days of incubation in presence of anthracene as the sole carbon and energy source. Biodegradation of anthracene was estimated indirectly by decolorization of the 2,6 DCPIP reagent. The decolorization of the redox indicator started from the first day of incubation in both *L. fermentum* and *V. parvula* while in *S. anginosus* the biodegradation reflected by indicator decolorization started later on the second day of incubation. After 8 days of incubation, *V. parvula* showed a higher decolorization of 59%, followed by *S. anginosus* (57%), and *L. fermentum* (51%). At the end of the second day of incubation which represented the time of the mid-log phase for the three isolates, the decolorization was detected at 42%, 30%, and 10% by *V. parvula*, *L. fermentum*, and *S. anginosus*, respectively (Fig. 2).

3.3. Analysis of anthracene biodegradation metabolites by GC-MS

The metabolic profile differences between *S. anginosus*, *L. fermentum*, and *V. parvula* in the presence of anthracene as the sole source of carbon, were analyzed using gas chromatography coupled with mass spectroscopy (GC-MS). Metabolites detected in both controls and samples were excluded. The metabolic profiles presented in chromatograms (Fig. S1) were analyzed after 3 days of incubation. A total number of 39 components were detected as presented in a heat map with different color codes presenting the concentration, from the absence to the most concentrated (Fig. 3). From these, three metabolites were common between the three microorganisms and at the same time absent in the controls media, these metabolites were arachidic acid, 1-monopalmitin, and catechol. The identified metabolites of anthracene biodegradation by the isolates are enlisted in supplementary data **(Table S1)** and corresponding spectral data for each metabolite are also provided in supplementary data (Fig. S2.). Anthracene, the parent compound, was eluted at 17.5 min and it was undetected in *L. fermentum*, and *V. parvula* supernatant while remaining in control (1), control (2), and *S. anginosus* with concentrations of 78.85%, 19.19%, and 3.17%, respectively.

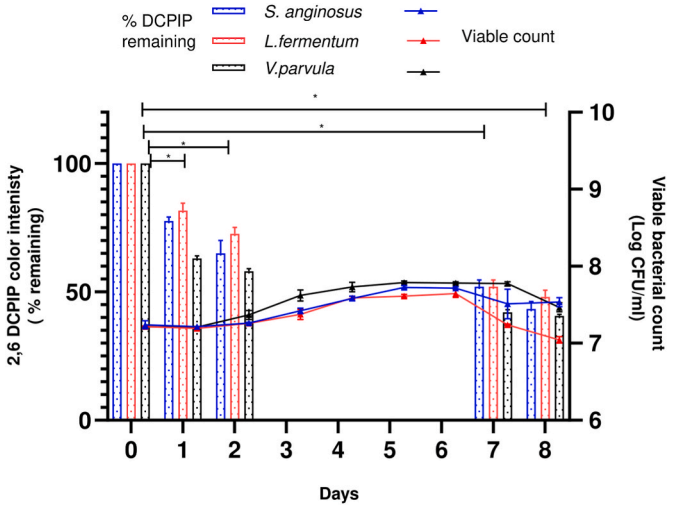

Fig. 2. Changes in viable count and anthracene biodegradation by *Streptococcus anginosus*, *Limosilactobacillus fermentum*, and *Veillonella parvula* isolated from a smoker participant over 10 days. Bacteria were incubated in Fe-Free W medium containing anthracene as the sole source of carbon. The viable count was determined by the drop plate method on a selective medium (shown as connected lines) and anthracene biodegradation was measured using a 2,6-dichlorophenol indophenol (2,6-DCPIP) colorimetric assay (shown as bars). Multiple unpaired t-tests and multiple comparisons using the Holm-Šídák method were used to compare the change in anthracene degradation. The readings were considered significant at *P < 0.05.

GC-MS analysis of the *L. fermentum* metabolized anthracene media extract provides eighteen metabolites where eight of these metabolites were detected in high amounts and these major eluting peaks were recorded at 10.43, 10.89,11.34, 19.76, 21.42, 21.64, 24.59, and 26.09 min and identified as glycerol, n-hexadecanoic acid, MSTFA, palmitic acid, oleic acid, stearic acid, 1-monopalmitin, and glycerol monostearate, respectively. Thirteen fatty acids representing 48.18% of total metabolites were detected, besides anthraquinone (2.7%) at R_t 17.8 with molecular weight (MWT) of 208 and fragments at *m/z* 180 (-CO), 152 (-CO), and 126, in addition to the presence of salicylic acid TMS.

Analysis of *V. parvula* metabolites resulted in the tentative identification of fatty acid metabolites (23.98%) ranging from short to long chains. These fatty acids were detected in concentrations higher than those detected in the control if present. The number of metabolites resulting from the biodegradation of anthracene by *S. anginosus* is nineteen identified metabolites, from which six fatty acids (51.25%), and six sugars (2.56%) through which sucrose represented the main identified sugar, besides the presence of traces of 1-methoxy-2 hydroxyanthracene, phthalic acid derivatives and unmetabolized anthracene (3.17%). As final and simple metabolites, we detected catechol and phthalic acid (Fig. 3).

3.4. Predicted pathway of anthracene biodegradation by *Limosilactobacillus fermentum, Veillonella parvula and Streptococcus anginosus*

The tentative biodegradation pathway was predicted from the established structures of metabolites (Fig. 4). The anthracene molecule may be degraded in two different ways. The first pathway includes anthraquinone as an intermediate metabolite to catechol and that was detected in *L. fermentum* and *V. parvula* (Fig. 4a and b). The detection of anthraquinone suggested that the primary attack of anthracene was through the hydroxylase enzyme system, which leads to the formation of anthracene 9,10 diol, followed by a further and immediate attack by dehydrogenase enzyme system transforming intermediate to anthraquinone ending with catechol as a byproduct. In *L. fermentum,* salicylic acid was detected, suggesting the conversion of this intermediate to catechol by decarboxylation. The proposed pathway of anthracene degradation by *S. anginosus* involves the formation of anthracene-1,2-diol followed by methoxylation producing 1 methoxy 2- hydroxy anthracene, as well as late stages metabolites such as catechol and phthalic acid derivatives were detected, suggesting ring cleavage of anthracene as shown in Fig. 4 c. In the three strains under study, the detection of aliphatic fatty acids revealed further ring fission for catechol and phthalic acid followed by polymerizations. Among the aliphatic fatty acids detected are lauric, myristic, heptadecanoic, oleic, 13 octadecanoic, stearic, arachidic, and monopalmitic acids.

3.5. Genome analysis of reference strains revealing anthracene degradation pathway

Analysis of the reference genome of *S. anginosus* Strain FDAARGOS 1357, *L. fermentum* strain JNU 532, and *V. parvula* strain FDAARGOS 1046 were carried out using BV-BRC revealed the presence of genes

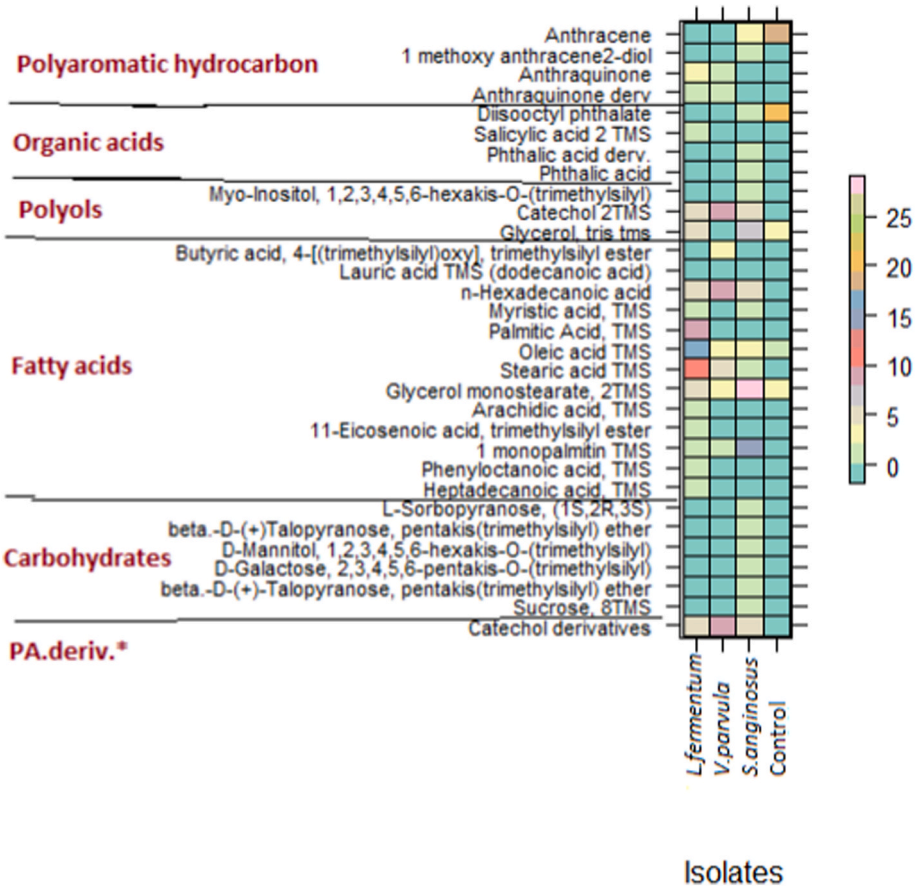

Fig. 3. Heat map representing all the breakdown metabolites during anthracene anaerobic biodegradation by *S. anginosus*, *L. fermentum* and *V. parvula* compared to assay control medium. Heat map was generated by RStudio using ggplot2 and reshape2 packages.*Pyruvic acid derivatives

coding for enzymes responsible for converting one or more of the intermediates in the anthracene degradation pathway. The analysis revealed the presence of genes coding for alcohol dehydrogenase in the three species and acetaldehyde dehydrogenase in all isolates except *V. parvula* which are involved in the steps of converting hydroxy anthracene to anthraquinone. Additionally, the gene coding for phenolic acid decarboxylase was detected in *L. fermentum* genome which confirms the presence of the anthracene degradation pathway that results in the production of salicylic acid in this bacterium as shown in Table 1.

4. Discussion

Oral microbiome dysbiosis resulting from cigarette smoking is not limited to changes in oral bacterial prevalence but it extends also to bacterial functional metagenomic pathways as detected by predictive computational metagenomic approaches as well as *in vitro* assays (Moussa et al., 2021; Wu et al., 2016). Anthracene is among the top 20 PAHs with high concentrations in cigarette smoking and represents 74.9 ng/cigarette (Hecht, 2011). Hydrophobicity of PAHs enables them to enter cells and some of the resulting biodegradation metabolites can bind to DNA and have mutagenic and possible carcinogenic effects, in addition to oxidative stress, and endocrine disruption (Bekki et al., 2013; Lawal, 2017; Lee et al., 2011). For all the previously mentioned toxic effects of this compound on human health, the degradation of this compound into a less toxic metabolite becomes essential.

Microbial biodegradation represents the major mechanism for PAH degradation (Wartell et al., 2021) and since the oral microbiota is the first niche that comes in contact with cigarette smoke, our study aimed at studying the role of representative bacterial microbiota in the degradation of a model PAH (anthracene) (Hecht, 2011). The understanding of the biodegradation pathway of anthracene by oral microbiota will facilitate the understanding and explanation of certain physiological effects of cigarettes on smokers. Therefore, isolates from a heavy smoker which are exposed to high concentrations of anthracene were selected for studying anthracene biodegradation and possible resulting metabolites. The selected bacteria were *S. anginosus*, *V. parvula* and *L. fermentum* belonging to the most dominant oral phylum *Firmicutes* which represents 33% of the total oral microbiota. The relative volatility and solubility of low molecular weight PAHs as anthracene increase their solubility in water, and thus increase their susceptibility to biodegradation compared to high molecular weight PAHs (Pannu et al., 2003). Isolates' ability to utilize anthracene was confirmed by their continuous growth on anthracene-supplemented media as the sole source of carbon. Biodegradation ability was measured using the 2, 6-DCPIP assay which is considered suitable for hydrocarbon degradation studies as it can sensitively detect the primary oxidation of NADH to NAD+, which is related to hydrocarbon degradation by bacteria (Karale et al., 2015).

In our previous study carried out for comparing the anthracene biodegradation potential by oral bacterial isolates from smoker and non-smoker participants, we found that acclimatized *Streptococcus* sp. from smokers showed higher anthracene biodegradation ability while the biodegradation potential for *Limosilactobacillus* sp. and *Veillonella* sp. isolates was species dependent rather than dependence on isolation site conditions (Moussa et al., 2021). By the end of the eighth day of incubation for the three tested isolates, the decolorization of the DCPIP reagent was comparable. The binding of anthracene to live and dead lactic acid bacteria was previously reported by Yousefi and his colleagues, this removal process was variable according to PAH concentration and strain used (Yousefi et al., 2019). Another study carried out by Karale et al.

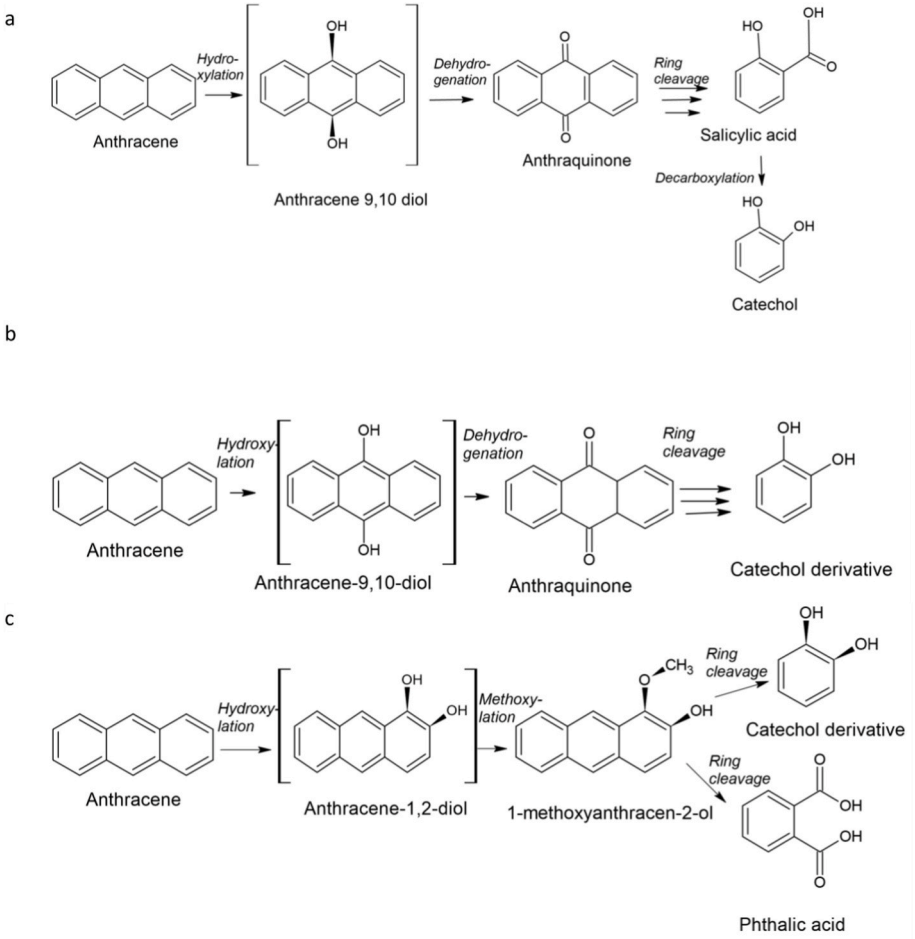

Fig. 4. Proposed anthracene biodegradation pathway by: A) *Limosilactobacillus fermentum, B) Veillonella parvula, C) Streptococcus anginosus*. Bacteria were grown in Fe-free W medium containing anthracene as the sole source of carbon under anaerobic incubation conditions at 37 °C for 3 days. The compound in brackets was not detected.

Table 1
Genetic elements detected in reference genome of *L.fermentum* strain JNU532, *S.anginosus* strain FDAARGOS 1357 and *V.parvula* strain FDAARGOS 1046 coding for enzymes involved in anthracene degradation based on genome analysis by the BV-BRC.

Reference genome	RefSeq locus tag	Enzyme (EC number)	Genes	Function of the enzyme
Limosilactobacillus fermentum Strain JNU 532 (GenBank Accessions: CP103400)	NX839_05155	Alcohol dehydrogenase (EC 1.1.1.1)	*adhP*	Converting hydroxy anthracene to anthraquinone
	NX839_08620	Acetaldehyde dehydrogenase (EC 1.2.1.10)	*adhC*	
	NX839_01755	Phenolic acid decarboxylase (EC 4.1.1.-)	-	Convert 2 hydroxy phthalic acid to salicylic acid
Streptococcus anginosus strain FDAARGOS 1357 (GenBank Accessions: CP069892)	I6K87_3235	Alcohol dehydrogenase (EC 1.1.1.1)	-	Converting hydroxy anthracene to anthraquinone
	I6K87_07090	Acetaldehyde dehydrogenase (EC 1.2.1.10)	*adhC*	
	I6K87_04880	Acetyltransferase (EC 2.3.1.-)	-	Transferring group other than amino group
Veillonella parvula strain FDAARGOS 1046 (GenBank Accessions: CP065990)	I6I03_00720	Alcohol dehydrogenase (EC 1.1.1.1)	*yqhD*	Converting hydroxy anthracene to anthraquinone[a]

[a] (-) no annotated gene

(2015) reported degradation of anthracene by *Streptococcus* sp. causing a reduction up to 44% on the third day of incubation which is higher than the degradation by the same genus in our study reaching 10%. This difference in biodegradation between the two studies could be attributed to differences in either the strains or used medium or methods of measurement.

To elucidate the potential anthracene biodegradation pathways by the selected bacteria, the isolates were grown on a medium containing anthracene as the sole source of carbon and followed by metabolites analysis by GC-MS. This analysis tool was selected as being standardized for a long time, making their datasets much easier to process and more comparable than in LC-MS, in addition to the concept of retention indices introduced by Kovats (1965). The retention index replaced the variable absolute retention times by the assigned fixed retention indices using a series of aliphatic alkanes as retention markers across chromatograms.

In our work, we have detected different metabolites with a possible contribution to possible health effects on smokers. Interestingly, fatty

acids such as hexadecenoic acid (palmitic acid), tetradecanoic acid (myristic acid), linolenic acid, and octadecanoic acid (stearic acid) were detected in the tested samples and hexadecenoic acid represented 4.49%, 9.61%, and 4.24% of the total detected compounds of *L. fermentum, V. parvula*, and *S. anginosus,* respectively. Palmitic acid and hexadecane were previously reported as degradation products of anthracene and pyrene (Sekar and DiChristina, 201). The presence of common intermediates, such as ketoacyl-acyl-carrier-proteins between the de novo biosynthesis of fatty acid and PAHs could explain the presence of these metabolites in addition to a specific type of trans-acylase (PhlG) enzyme which catalyzes the metabolic divergence between the biosynthesis of fatty acid and PAHs, therefore, changes in fatty acid profiles may be interconnected with PAH metabolites. However, it is not clear which metabolic factors or genes determine the divergence between two metabolic pathways (Keum et al., 2008; Rehm et al., 2001). Fatty acid biosynthesis is essential for preserving membrane integrity in anaerobic bacteria and this might explain the direction of isolates for the production of long chain fatty acids utilizing anthracene (de Carvalho and Caramujo, 2018). These previously mentioned fatty acids may add a new reason for the increased incidence of myocardial infarction and heart diseases in smokers. It has been reported by Wang et al. (2017) that palmitic acid-induced myocardial inflammatory injury in animals and cell culture experimental models, through the Toll like receptor 4 (TLR4) accessory protein, furthermore, myristic acid has been reported as one of the most strongly related fatty acids to the high serum cholesterol and HDL levels (Tholstrup et al., 2003). Whereas palmitic and stearic acid was reported to exhibit antimicrobial effects (Chen et al., 2014).

As indicated by the GC-MS, certain metabolites were released in a high amount such as organic acids that could contribute to the harmful effect of toxins in cigarettes on smokers' health. A good interesting example is phenyl octanoic acid detected in *L. fermentum* which has a corrosive action so it could be one of the causes of smoker's teeth problems (**Hartnett et al., 2011**). Moreover, sugars were detected among *S. anginosus* metabolites despite the absence of sucrose in the culture medium as a result of depriving *S. anginosus* of sucrose which consequently affects the intracellular carbohydrate metabolism leading to degradation of intracellular polysaccharide (glycogen) (Costa Oliveira et al., 2021). Detection of catechol was alarming because it is classified by the International Agency for Research on Cancer (IARC) as a Group 2B, possible human carcinogen (https://monographs.iarc.who.in).

Screening of literature indicated a shortage of studies on the anaerobic biodegradation of anthracene as an important member of PAHs. Very little is known about their biodegradation pathways, catabolic enzymes and/or regulatory mechanisms under smoking conditions (Mallick et al., 2011). Degradation of PAH usually occurs in two stages: from hydrocarbon molecule to central intermediates then from destabilization of central intermediates to aromatic ring cleavage and biomass yield comprising the higher and lower degradation pathways, respectively (Ladino-Orjuela et al., 2016). The most studied pathways for anthracene biodegradation are those under aerobic conditions. In this pathway, oxygen not only acts as the final electron acceptor but also as a co-substrate for the hydroxylation and ring cleavage by mono and dioxygenase enzymes (Ghosal et al., 2016). However, the microbiota usually exists under anaerobic conditions, therefore the anaerobic biodegradation of aromatic compounds by microorganisms plays a major role in the removal of contaminants in these ecosystems. This pathway is more challenging than the aerobic one because the aromatic C–C, and C–H bonds are more stable in the absence of oxygen or anaerobic conditions (Yan and Wu, 2020). Anaerobic bacteria have developed alternative strategies for PAH degradation and ring opening instead of those relying on oxygen as electron acceptors. In anaerobic biodegradation of PAH, the terminal electron acceptors (TEAs) could be an oxidized inorganic compounds such as nitrate (NO_3^{-1}), manganese (Mn(IV)), iron (Fe(III)), sulfate (SO_4^{-2}) and carbon dioxide (CO2) instead of oxygen. Most studies reported that, under anaerobic conditions, the first steps of PAH biodegradation could be catalyzed by synthases, dehydrogenases, and carboxylases enzymes (Meckenstock and Mouttaki, 2011) or include hydroxylation reactions (Liang et al., 2014; Nzila, 2018). The detected metabolites in our study support the oxygen independent hydroxylation of PAH as the initial step for biodegradation as reported in previous studies (Caldwell and Suflita, 2000; Zhang et al., 2013). The detection of anthraquinone revealed that the upper pathway of anthracene degradation started by oxygen independent hydroxylation of anthracene producing anthracene-1, 2-diol by *Streptococcus anginosus.* The 1, 2 hydroxylation was previously detected in Gram positive bacteria such as *Rhodococcus* sp. and *Mycobacterium* sp. (Dean-Ross et al., 2001; Moody et al., 2001). In the next step, 1, 2-dihydroxyanthracene undergoes *meta* ring fission by a ring cleavage enzyme. The 9, 10-hydroxylation of anthracene was detected in *L. fermentum* and *V. parvula* supernatant. This pathway was previously detected in studies on bacteria such as *Mycobacterium* sp. strain PYR-1 (Moody et al., 2001) and *Pseudomonas* sp. JP1 strain (Liang et al., 2014) resulting in production of anthraquinone. Moreover, anthraquinone was also previously reported in the anthracene biodegradation pathway by fungi such as *Aspergillus fumigatus* (Ye et al., 2011), ligninolytic fungus *Polyporus* sp. (Hadibarata et al., 2012), and bacterium such as *Bacillus thuringiensis(Tarafdar* et al., *2017)*. Anthraquinone derivatives have been used for centuries in medical applications, for example, as laxatives, antimicrobial and anti-inflammatory agents, however the correlation between this compound and cancer is controversial (Malik and Mueller, 2016; Nida, 2019). Detection of salicylic acid derivatives among the identified metabolites of *L. fermentum* confirms a pathway of anthracene biodegradation towards TCA cycle (Swaathy et al., 2014). The two Gram positive bacteria under study showed diverse metabolic mechanisms for degradation of anthracene which play an important role in their adaptation and occupation to a wide range of environments (Mashima et al., 2021; Travkin and Solyanikova, 2021).

The scarce literature on anaerobic biodegradation of anthracene together with the genes involved in addition to lack of the genomic data for the isolates under study, makes it more difficult to predict the orthologous genes. The analysis of the whole genomic sequence data of reference strains using the BV-BRC tool showed the presence of the *adhc* gene in genome of *S. anginosus* and *L. fermentum* coding for the enzyme acetaldehyde dehydrogenase (E.C:1.2.1.10). Additionally genetic element coding for the enzyme alcohol dehydrogenase (E.C:1.1.1.1) was detected in the three isolates. *L. ferementum* genome revealed a gene sequence coding for phenolic acid decarboxylase enzyme (E.C:1.4.1.-). According to the BRENDA enzyme database, the acetaldehyde dehydrogenase enzyme converts acetaldehyde into acetyl-CoA. and alcohol dehydrogenase enzyme which converts alcohol containing compounds into aldehydes or ketones (Chang et al., 2021).

5. Conclusion

The anaerobic biodegradation of anthracene by oral isolates of *S. anginosus*, *L. fermentum*, and *V. parvula* occurs through different pathways which start with oxygen-independent hydroxylation. *S. anginosus* isolate possesses different pathways for anthracene biodegradation which enables them to adapt to the smokers' oral cavity. Biodegradation of anthracene by *S. anginosus*, *L. fermentum*, and *V. parvula* occurs at different rates but after a long time of incubation, their biodegradation results were comparable. The biodegradation of cigarette xenobiotics by oral bacteria results in the production of metabolites with possible carcinogenic or atherogenic effects. Detection of catechol and anthraquinone as products of biodegradation is alarming due to the possible carcinogenic effect of these two compounds. Besides, several fatty acids were detected which were previously reported to be linked with heart diseases. Thus, the biodegradation of anthracene by oral bacteria does not completely detoxify it but rather convert it to other toxic metabolites.

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Author contribution

Research design was done by Reham Wasfi, Nourtan Abdeltawab, and Salwa Megahed. Experiments were conducted by Hams Moussa and Reham Wasfi. Data were analyzed by Reham Wasfi, Hams Moussa, Nourtan Abdeltawab, and Reham Bakr. The manuscript was written by Reham Wasfi, Hams Moussa, Nourtan Abdeltawab, and Salwa Megahed. All authors read and approved the manuscript.

Ethics approval

Informed consent was obtained from participants and approved by the Research Ethics committees of the Faculty of Pharmacy at October University for Modern Sciences and Arts (MSA) and Faculty of Pharmacy, Cairo University with approval ID (M2/EC2/2016MS), and approval ID (MI 1722), respectively.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2023.105598.

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