DAVID ANALYSIS OF PROTEOMICS DATASETS FROM EPIAIRWAY™ EXPOSED AT THE AIR LIQUID INTERFACE TO AEROSOLS OF JUUL™ AND SYNTHETIC COOLANT (WS-23)

By: Alisa Mileend Gadkari

A capstone project submitted for Graduation with University Honors

February 26, 2023

University Honors
University of California, Riverside

APPROVED

Dr. Prue Talbot
Department of Molecular, Cell, and Systems Biology

Dr. Richard Cardullo, Howard H Hays Jr. Chair
University Honors

Abstract

Flavor chemicals and coolants have been added to electronic cigarette fluids at very high concentrations. While many of these chemicals have been studied for ingestion, little is known about their inhalation toxicity. Two experiments were conducted to analyze the effects of aerosols produced by chemicals on EpiAirway™, a 3D tissue model that recapitulates human bronchial epithelium. The first experiment was conducted with JUUL™ "Tobacco" and JUUL™ "Menthol" ECs on EpiAirway microtissues using the Cultex exposure system. The second experiment focused on flavor chemicals, such as benzyl alcohol, triacetin, cinnamaldehyde, eugenol, and the coolant, WS-23, in the Vitrocell. After the proteomics data were generated, DAVID, an online bioinformatics database, was utilized to identify the effects of the chemicals on biological and cellular processes. The results indicate possible adverse effects following acute inhalation of these chemicals at high concentrations.

Acknowledgements

I would like to thank Dr. Prue Talbot and the Talbot lab for their mentorship and support, as well as the helpful feedback and discussion towards my Capstone project. I am extremely grateful for everything I have learned and the people I have met in this lab throughout my Capstone experience.

Table of Contents:

Abstract ….…….. 2

Acknowledgements ……. 3

Key ……..……. 5

Introduction…….. 6

Methods…… 10

Results……...14

Discussion……..26

References……..33

Key:

	Endomembrane System
	Immune Response
	Mitochondrion/ Cellular Respiration
	Metal/Ion binding
	Nucleotide Binding/Synthesis
	Programmed Cell Death
	Protein Degradation
	Redox Reaction
	Signal Transduction
	Transcription/Translation
	Cell Division
	Dermis
	Cell Adhesion/Cytoskeleton
	Cilium

Introduction

Vaping continues to be a worldwide epidemic among youth. As disposable devices evolve, new chemicals are being added with little to no information on inhalation toxicity. Health effects attributed to electronic cigarette (EC) usage can involve the respiratory system and can include bronchitis obliterans and acute eosinophilic pneumonia (Hui et al 2020; Omaiye et al. 2019). Vaping is associated with inhaling and exhaling the aerosols created when an e-liquid is heated in an EC. Typically, e-liquids contain solvents (propylene glycol and vegetable glycerin), nicotine, acids (benzoic acid), flavor chemicals, and more recently, synthetic coolants (WS-23, WS-3). These devices are presented in various shapes, such as a USB flash drive associated with JUUL™, but can take the shape of a pen, a cigarette, or box-mod devices. ECs are also referred to as "vapes," "vape pens," "mods," and "Juuls". The chemical concentrations in EC products ha 37ve increased significantly in recent years and are not well regulated (Omaiye et al. 2022). A study looked at 277 refill fluids and tested their cytotoxicity. 85% had concentrations of flavor chemicals over 1 mg/ mL, which is high for a consumer product, and 37% were above 10 mg/ml (Omaiye et.al 2019). Little is known about the long-term health effects of ECs. Users inhale complex aerosols that may contain dangerous chemicals such as acetaldehyde, cinnamaldehyde, diacetyl, and various other chemicals.

The JUUL™ vape used to be the most popular EC and dominated the market, however, new spinoffs are becoming attractive to young users (Smith, 2019). JUUL™ was known for its flavored cartridge-based ECs. Some of these flavors included Cucumber, Mango, Creme, and fruit, which appealed to young users because they mask unwanted tastes and smells (Strombotne 2021). The FDA banned flavored JUUL™ products to decrease sales in 2020 (Omaiye et al. 2022), and Menthol and Tobacco are the only flavors still sold today. JUUL™ as well as other

suppliers continue to alter and sell the products with increased concentrations of chemicals due to the ban failing to cover disposable, flavored EC products (Omaiye et al. 2022). JUUL™ as well as other manufacturers are combining nicotine with acid. This generates an aerosol that is not as harsh due to the reduction in free-base nicotine (Leventhal, 2021). This innovative method has allowed the concentrations of nicotine to increase to 61 mg/ mL (Omaiye et al. 2022). Due to these changes, we focused on the cytotoxic effects of JUUL™ menthol and JUUL™ tobacco on cells.

Two exposure systems were used, a Cultex *(Figure* i) and Vitrocell (*Figure ii*). To observe the effects of JUUL™ "Tobacco" and "Menthol", the Cultex was utilized with EpiAirway tissues. EpiAirway is a 3D human tissue model that contains differentiated airway epithelial cells (Zavala 2016). The Cultex generates an aerosol for an air-liquid interface exposure which is similar to an aerosol similar to those inhaled by EC users. In the Cultex, an EC is attached and vaped using a smoking machine. The heating of these chemicals produces the aerosols and is puffed directly onto the airway cells (Aufderheide 2013).

Figure i: Cultex RFS Exposure System

Since the FDA flavor ban does not include disposable products, disposable ECs have gained popularity, especially among the youth. Along with the rise of the disposable ECs, came the use of the synthetic coolants. WS-23 is a synthetic cooling agent that is often used in personal

hygiene products, edibles, and cosmetics. WS-23 does not add flavor and has little odor or taste, but it is used to create the cooling, "icy," sensation that is appealing to many EC users because it reduces the harshness of aerosols (Omaiye 2022). While JUUL™ also contains synthetic coolants, the concentrations are relatively low. However, WS-23 is now being found at much higher concentrations in newer disposable products such as Puff ECs (Yogeswaran 2022, Omaiye 2019). WS-23 coolant concentrations can be 450 times higher in Puff than JUUL™ (Omaiye, 2022). To understand the risk of synthetic coolants in EC products, the margin of exposure (MOE) can be calculated. If the MOE is below 100, it is considered high risk and may require regulation. The MOE for WS-23 in Puff products was below the threshold of 100 for all flavors except tobacco at 1 ml consumption per day (Omaiye 2022). This drastic increase in concentration of WS-23 is concerning, which is why it is important to observe the specific effects of WS-23 on cells.

Similarly, to analyze the cytotoxicity of coolants (WS-23) and flavor chemicals (eugenol, benzyl alcohol, triacetin, cinnamaldehyde), the Vitrocell exposure system (Figure ii) was used with EpiAirway tissues. The Vitrocell is an advanced in vitro exposure system, but differs from the Cultex. In the Vitrocell, a single chemical can be tested without heating and therefore, no reaction products are produced (Steiner 2017).

Figure ii: Vitrocell Exposure System

Experiments were performed in the lab to generate proteomics data In these experiments, Incubator and Clean Air controls were included. EpiAirway cells either remained in the incubator in the ”Incubator” control or were taken out of the incubator and exposed to air in the room Clean Air controls. When the proteomics data is generated, Incubator and Clean Air should produce little effect to confirm the air in the room is not having an effect on the cells or the results that follow. The extracted proteomics data from the JUUL Cultex and the Vitrocell experiments were analyzed using a Bioinformatics database called DAVID.

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) allows us to understand the biological meaning behind a large list of proteins through various functional annotation tools. It creates a database identifying proteins that share biological functions. DAVID uses an algorithm to condense a large list of proteins and group functionally related ones together (Huang 2007). Using statistical methods, the proteins sharing similar biological processes were grouped together. This allows us to understand what biological impact these chemicals are having on the EpiAirway. The functional annotation clustering tool in DAVID creates grouped clusters with the biological process and lets us see the frequency of certain processes that come up more than once (Huang 2007). These clusters were used to generate categories relating to biological processes and a frequency distribution of the data from JUUL Cultex and the Vitrocell experiments.

Methods

DAVID Analysis:

The functional annotation tool from the DAVID Bioinformatics Database was used to analyze lists of proteins that were affected by JUUL™ "Tobacco" or "Menthol" during exposure of EpiAirway in the Cultex. Significant proteins with iPvalues (adjusted p-value) < 0.05 were selected for analysis. The -ilogFC was adjusted to be positive by multiplying the column containing all the values by negative one. The data were sorted into two lists based on ilogFC where values that were positive were upregulated and negative values were downregulated proteins. It is important to note that output from DAVID does not take into account the directionality of the effect. This means that the clusters generated by DAVID cannot be interpreted to be upregulated or downregulated, but gives us an overall picture of the biological functions affected by the inputted protein IDs. The upregulated proteins were pasted into "Step 1: Enter Gene List". "Gene list" was selected as the list type under "Step 2: Select Identifier," UNIPROT_ACCESSION was selected followed by gene list under "Step 3: List Type". The submit button was clicked in "Step 4: Submit List" which was redirected to the gene list manager. The "homo sapien" filter was applied to limit the annotation to one species. Functional annotation clustering was selected for further analysis. Clusters with an enrichment score of $\geq$ 1.3 were then selected for significance with a medium classification stringency filter. The same process was then repeated for the downregulated list of proteins.

Unknown Protein IDs

After Step 4, there were occasions when protein IDs could not be identified by DAVID. This resulted as "unknown" under the "homo sapiens" filter. To identify the unknown proteins, "view

unmapped IDs" was selected. The individual protein ID was then reidentified by its protein name description which was provided by the master sheet. The protein name was inputted into uniprot.org where the updated protein ID could be found. The original Uniprot ID was then replaced with the updated ID located in the Master list separated based on whether it was upregulated or downregulated. This process was repeated until all unknown proteins were identified and the final list was reuploaded into DAVID to be analyzed.

JUUL™ "Tobacco/ Menthol":

After generating the list of significant proteins using the functional annotation tool, the clusters were grouped into categories based on commonalities between each individual cluster relating to biological function, processes, and organelles. Eight categories were created for the analyzed clusters which consisted of Cell Adhesion/Cytoskeleton, Cilia, Dermis, Endomembrane System, Mitochondrion /Cellular Respiration, Protein Degradation, Signal Transduction, and Transcription/Translation.

"Cell Adhesion/Cytoskeleton" included clusters involved in actin/cytoskeleton signaling or cell adhesion. "Cilia" included clusters involved in motility, cell projection, and ciliary-related diseases. Clusters assigned to the "Dermis" category pertained to the fibrous structure or function of the thick layer of living tissue. "Endomembrane System" included a group of membranes and organelles that work together to modify, package, and transport lipids and proteins. The organelles included ER, Golgi apparatus, plasma membrane, lysosomes, nuclear import and export. "Mitochondrion/Cellular Respiration" included mitochondria or clusters involved in energetics, cellular respiration, and transport into mitochondria. "Protein Degradation" included

clusters that encompass protein degradation pathways, such as ubiquitin, protease, and metalloprotease. Signal transduction clusters included signal transduction pathways that are not specified to a certain organelle or function. “Transcription/Translation” included any clusters involved in transcription or translation.

Vitrocell (WS-23, benzyl alcohol, cinnamaldehyde, eugenol, triacetin):

After generating the list of significant proteins using the functional annotation tool, the clusters were grouped into categories based on commonalities between each individual cluster relating to biological function, processes, and organelles. Nine categories were created for the analyzed clusters which consisted of Cell Adhesion/Cytoskeleton, Cell Division, Endomembrane System, Immune Response, Mitochondrion /Cellular Respiration, Metal/Ion binding, Protein Degradation, Signal Transduction, and Transcription/Translation.

“Cell Adhesion/Cytoskeleton” included clusters involved in actin/cytoskeleton signaling or cell adhesion. Clusters involved in “Cell Division" related to the cell cycle. “Endomembrane System” included a group of membranes and organelles that work together to modify, package, and transport lipids and proteins. The organelles included ER, Golgi apparatus, plasma membrane, lysosomes, nuclear import and export. “Immune Response” included clusters that are involved in the recruitment of the immune system or inflammation response. “Mitochondrion/Cellular Respiration” included the mitochondria or clusters involved in energetics, cellular respiration, and transport into mitochondria. “Metal/Ion Binding” involved clusters involved in metal or ion binding such as Ca+ and Mg+. Clusters involved in “Protein Degradation” included clusters that encompass protein degradation pathways such as ubiquitin, protease, and metalloprotease. “Signal Transduction” included clusters that utilized signal

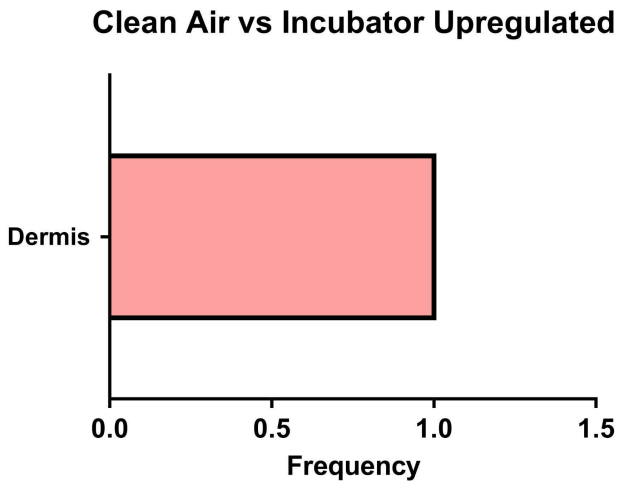
transduction pathways that are not specified to a certain organelle or function. The final category, “Transcription/Translation”, included any clusters involved in transcription or translation.

Generating PRISM graphs:

Each individual cluster was assigned to one category. Clusters that could fit into multiple categories were put into the category that best describes their specific biological function. Once all clusters were classified into their appropriate category, a frequency distribution was obtained for the Vitrocell data, and the JUUL data were graphed using the enrichment score from DAVID . This was carried out by identifying how many individual clusters were in each category. The frequency collected was used to generate graphs in PRISM software that illustrated the prevalence of each category in its respective comparison. A key was created with colors that corresponded to each category to help visualize trends. The JUUL graphs were labeled according to the original name of the cluster from DAVID, and were also color-coated and patterned to represent the upregulated and downregulated data on the same graph. The enrichment score was used because there was a small number of clusters or frequency. The enrichment score provided more information about the data than did the frequency in the JUUL experiment, whereas, the frequency distribution helped us analyze the data in the Vitrocell.

Results

a)



b)

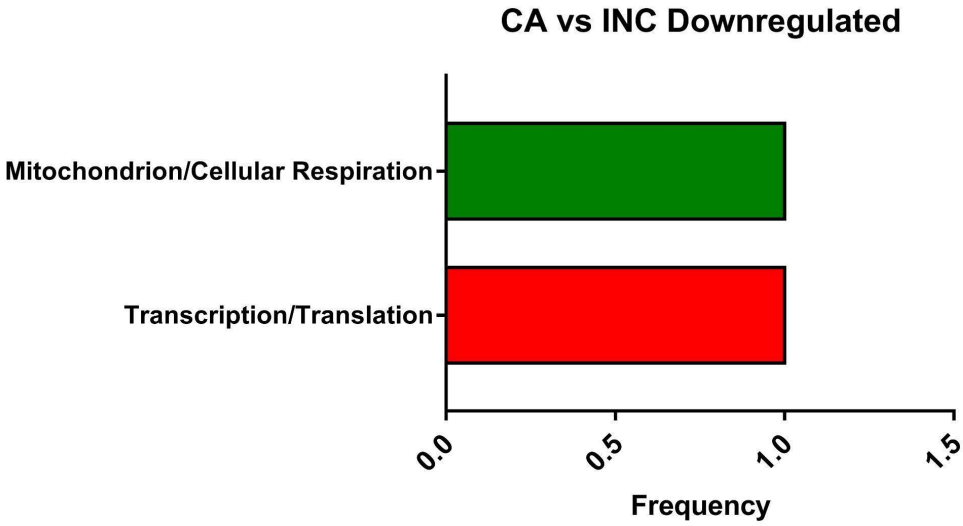


Figure 1: Frequency distribution for Clean Air (CA) versus the Incubator Control (INC). (a) upregulated and (b) downregulated

To determine the effect of putting transwells into the Cultex, CA vs INC *(Figure 1*) was analyzed first. The results showed an effect on the Dermis, Transcription/Translation, and

Mitochondrion/Cellular Respiration. The presence of little to no clusters indicates no potential confounding factors in the environment.

Cultex JUUL

Seven graphs were generated from PRISM for the four comparisons: Clean air versus Incubator Control, JUUL™ tobacco versus Clean Air, JUUL™ menthol versus Clean Air, and JUUL™ tobacco versus JUUL™ menthol. The specific categories affected by the chemicals were identified.

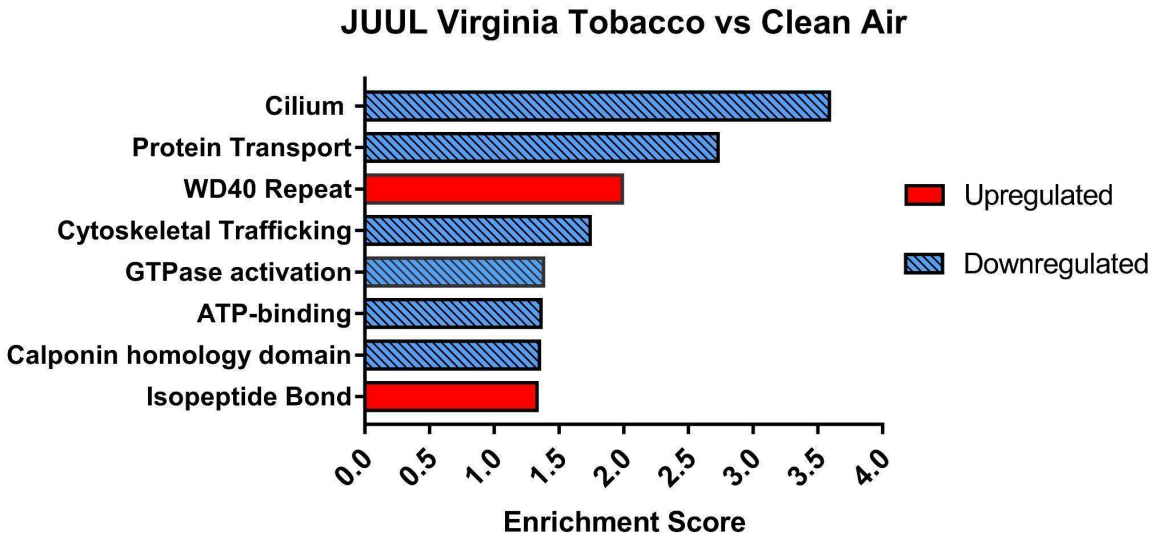


Figure 2: Enrichment scores for the biological processes affected by JUUL™ *Virginia Tobacco when compared to clean air. Solid bars (red) were upregulated proteins. Hatched bars (blue)were down regulated proteins.*

JUUL™ *Virginia tobacco vs Clean Air:*

JUUL™ Virginia tobacco (*Figure 2*) had an effect on the Endomembrane System, Protein Degradation, Cell Adhesion/ Cytoskeleton, Cilia, Signal Transduction, and Cilia. These

categories were only observed for JUUL™ Virginia tobacco versus Clean Air. Mitochondrion/Cellular Respiration was also observed in JUUL™ menthol (JMM) and the incubator control (Figure 1). Cilium presented with the greatest enrichment score of 3.59 and Isopeptide bond (Protein degradation) had the lowest enrichment score of 1.33. The enrichment score was used to rank biological significance and the higher the enrichment score, the lower the p-value (greater significance).

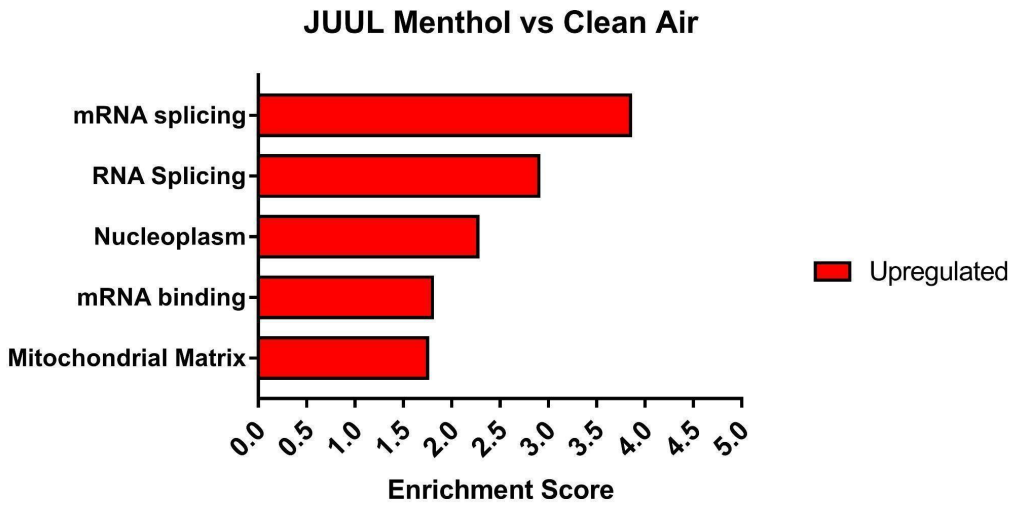


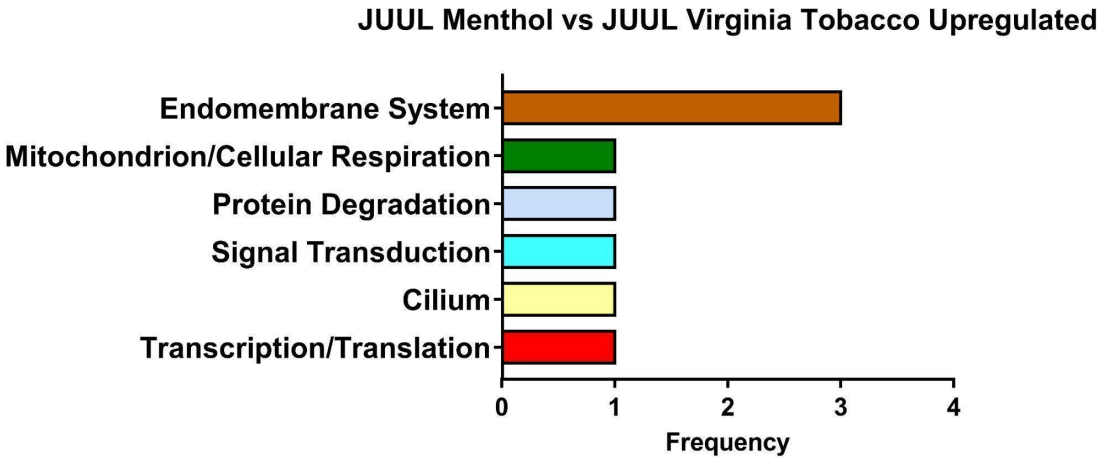
Figure 3: Enrichment scores for the biological processes affected by JUUL™ Menthol upregulated protein IDs when compared to Clean Air.

JUUL™ *menthol vs Clean Air:*

Two categories, Mitochondrion/Cellular Respiration and Transcription/Translation, were present in JUUL™ menthol vs Clean Air (*Figure 3*). RNA splicing (Transcription/Translation) presented with the highest enrichment score of 3.85 and the Mitochondrial Matrix (Mitochondrion/ Cellular Respiration) with the lowest enrichment score of 1.75. The enrichment score was used to rank biological significance and the higher the enrichment score, the lower the p-value (greater significance). Both Mitochondrion/ Cellular Respiration and

Transcription/Translation were also observed in Clean Air versus Incubator Control and in JUUL™ Virginia tobacco versus Clean Air (*Figure 3*). This showed that JUUL™ menthol had an effect on Mitochondrion/Cellular Respiration and Transcription and Translation. JUUL™ menthol presented a unique category of Transcription/Translation as it was not observed in JUUL™ Virginia tobacco.

a)



b)

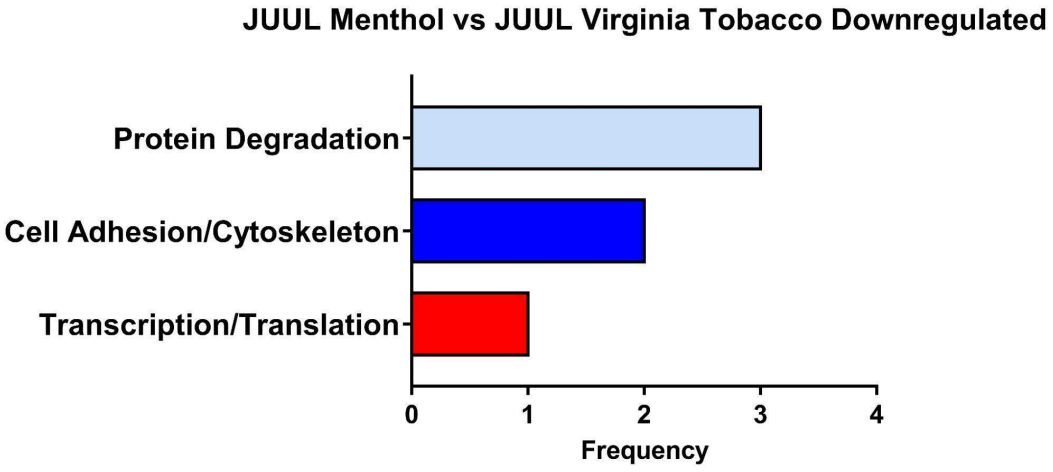


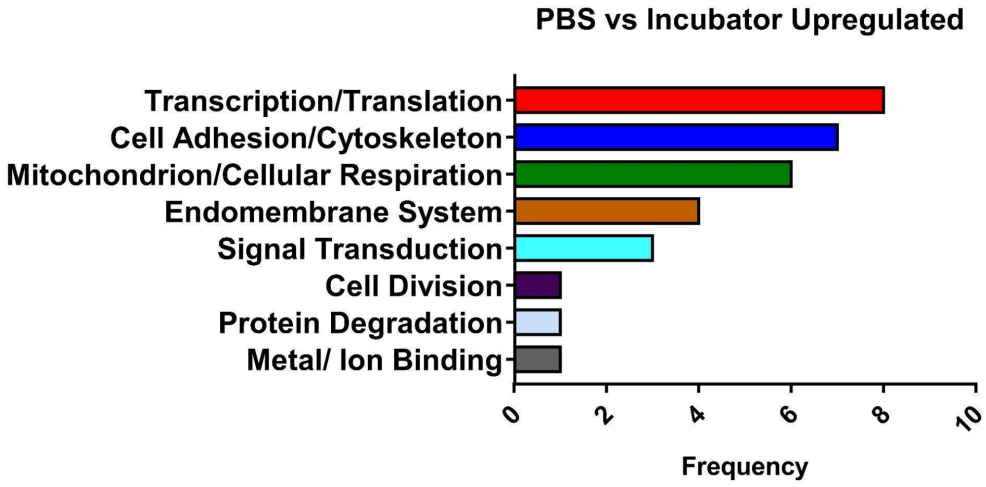
Figure 4: Frequency distribution of biological processes affected by JUUL™ *menthol and* JUUL™ *Virginia tobacco (a) upregulated and (b) downregulated in comparison to each other.*

JUUL™ *menthol vs* JUUL™ *Virginia tobacco*

When JUUL™ menthol and JUUL™ Virginia tobacco were compared to each other (*Figure 4*), Endomembrane System, Protein Degradation, Signal Transduction, and Cilia, Cell Adhesion/ Cytoskeleton, Mitochondrion/Cellular Respiration, and Transcription/Translation were observed. This comparison helped determine that the effects on Endomembrane System, Protein Degradation, Signal Transduction, and Cilia, and Cell Adhesion/ Cytoskeleton are due to JUUL™ tobacco, since the categories are not presented in JUUL™ menthol. Transcription/ Translation was present in JUUL™ menthol vs JUUL™ Virginia tobacco and Clean Air vs incubator (Figure 1), so it cannot be concluded that this category is due entirely to JUUL™ menthol or Clean Air.

Vitrocell:

a)



b)

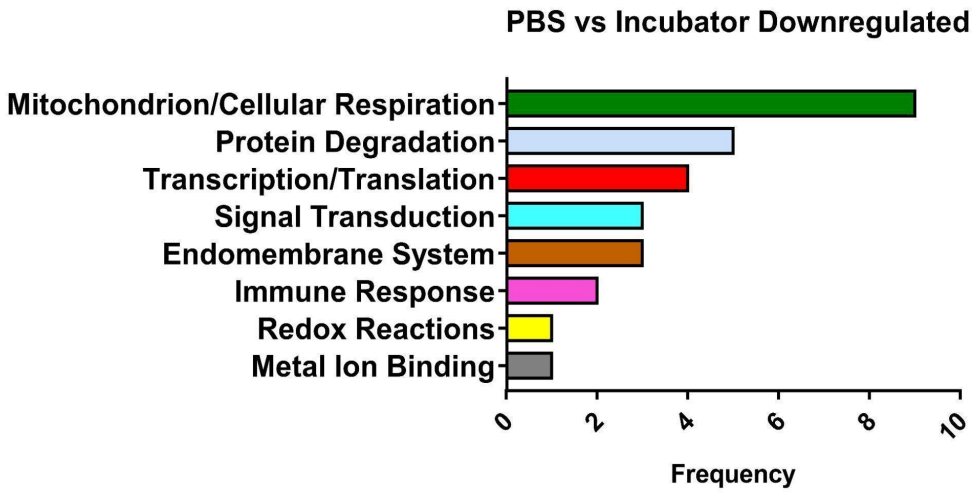
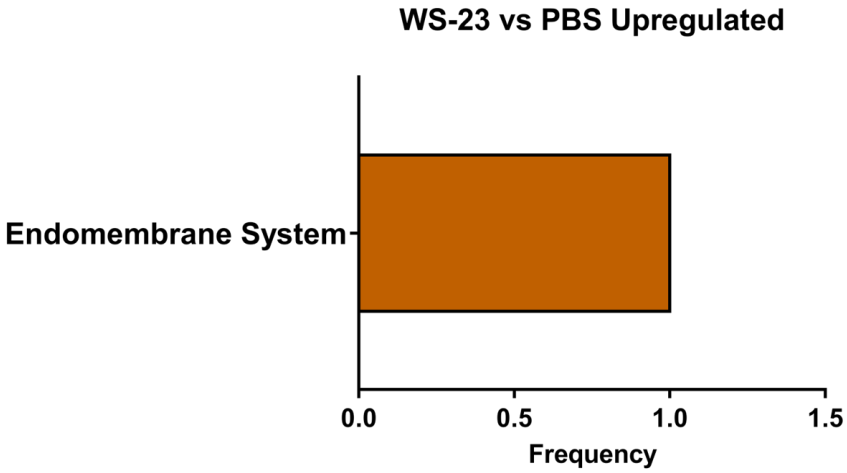


Figure 5: Frequency distribution of the Incubator control (a) upregulated and (b) downregulated

PBS vs Incubator

PBS vs Incubator had a significant number of clusters and categories. Both graphs (Figure 5a and 5b) presented with Transcription/ Translation, Mitochondrion/ Cellular Respiration, Protein Degradation, Endomembrane System, Signal Transduction, and Metal/Ion binding. Some categories that were unique to Figure 5a were Cell Adhesion/ Cytoskeleton and Cell Division. Similarly, categories unique to Figure 5b were Immune Response and Redox Reactions.

a)



b)

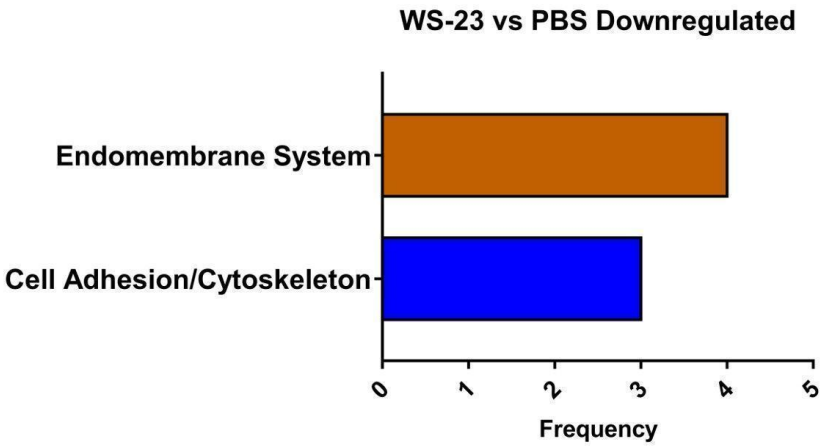


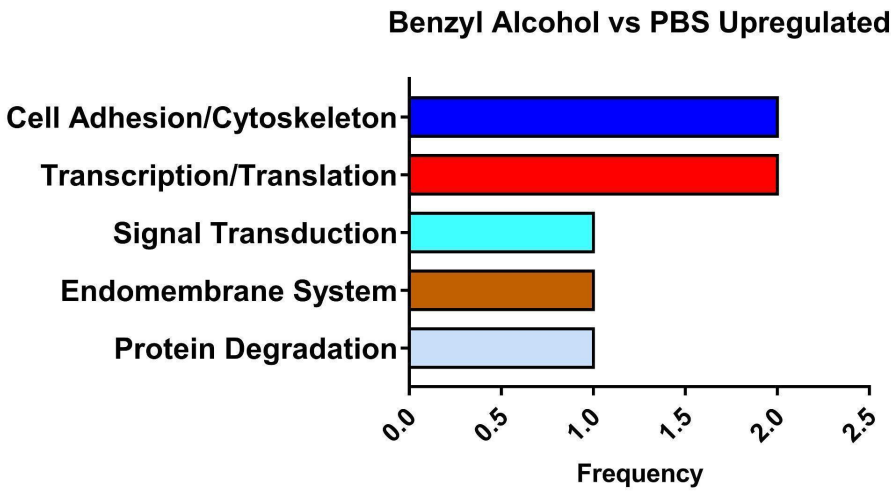
Figure 6: Frequency distribution of biological processes affected by the synthetic coolant WS-23 (a) upregulated and (b) downregulated

WS-23 vs PBS

Looking at *Figure 6,* WS-23 had an effect on the Endomembrane System and Cell Adhesion/Cytoskeleton. Both categories were present in BA vs PBS, PBS vs INC, EUG vs PBS,

TRI vs PBS, CAD vs PBS. Endomembrane System and Cell Adhesion/ Cytoskeleton were not unique to WS-23, suggesting these effects are very sensitive to different types of chemical treatment.

a)



b)

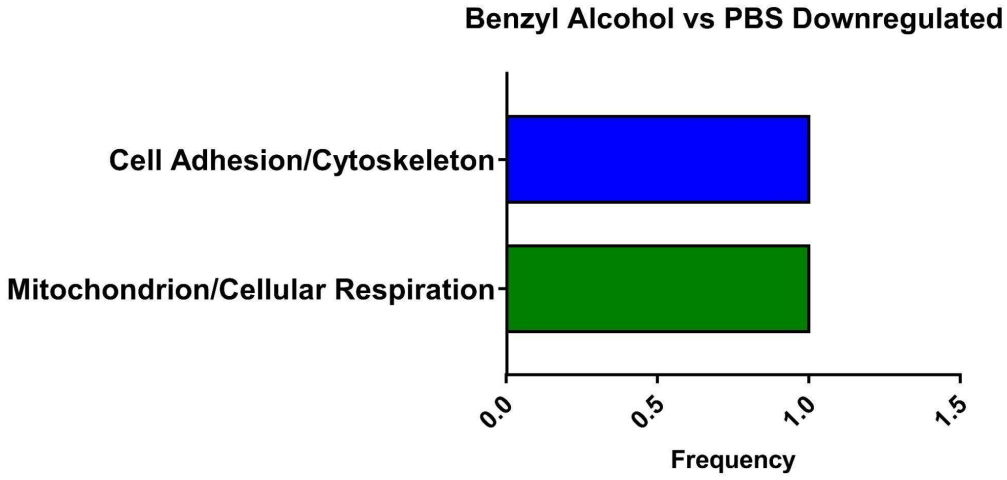
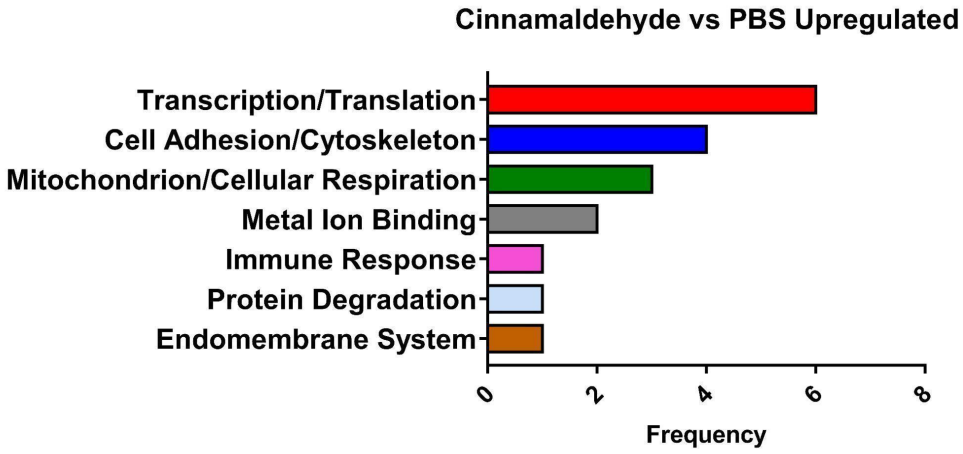


Figure 7: Frequency distribution of biological processes affected by Benzyl Alcohol. (a) upregulated and (b) downregulated

Benzyl Alcohol vs PBS

Benzyl alcohol affected Cell Adhesion/Cytoskeleton, Transcription/Translation, Signal Transduction, Endomembrane System, Mitochondrion/ Cellular Respiration, and Protein Degradation (*Figure 7*). These categories were also present in Benzyl Alcohol versus PBS, PBS vs INC, EUG vs PBS, TRI vs PBS, CAD vs PBS. Other flavor chemicals also had an effect on Cell Adhesion/ Cytoskeleton, Transcription/Translation, Signal Transduction, Endomembrane System, Mitochondrion/ Cellular Respiration, and Protein Degradation.

a)



b)

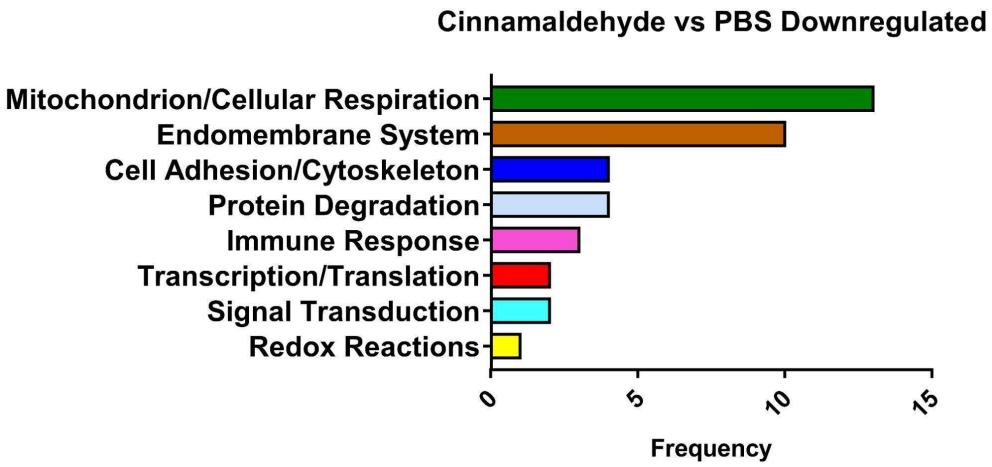
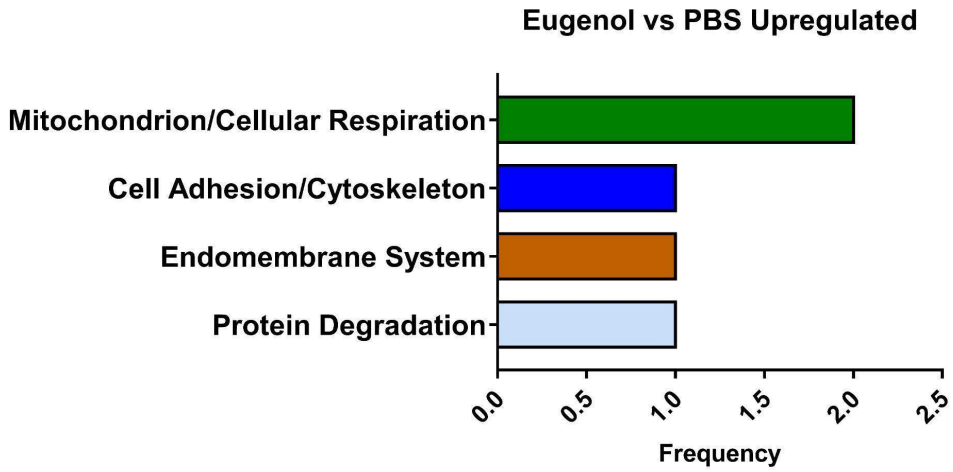


Figure 8: Frequency distribution of biological processes affected by cinnamaldehyde. (a) upregulated and (b) downregulated

Cinnamaldehyde vs PBS

Looking at *Figure 8*, cinnamaldehyde effected Transcription/Translation, Cell Adhesion/Cytoskeleton, Mitochondrion/Cellular Respiration, Metal/Ion binding, Immune Response, Protein Degradation, Endomembrane System, Signal Transduction, and Redox Reactions. Immune Response was also affected in PBS vs INC, CAD vs PBS, and TRI vs PBS. Metal/Ion binding was only present in the CAD vs PBS comparison.

a)



b)

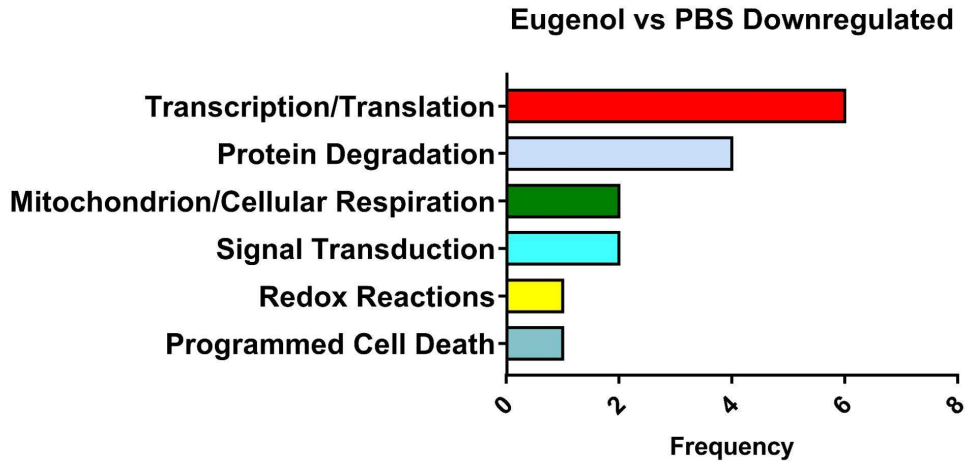
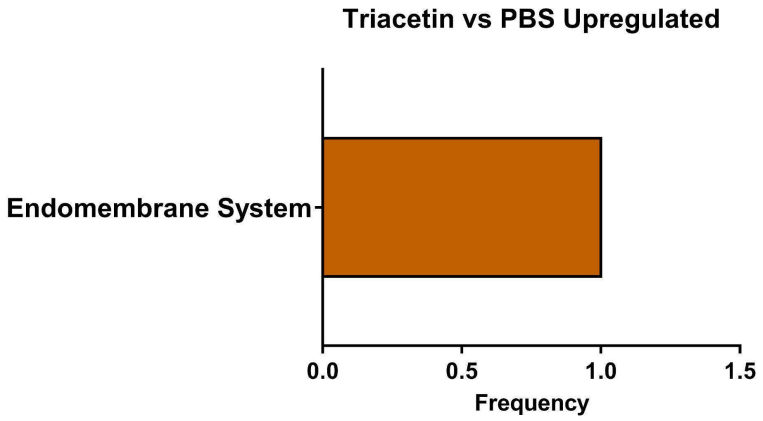


Figure 9: Frequency distribution of biological processes affected by Eugenol. (a) upregulated and (b) downregulated.

Eugenol vs PBS

Eugenol affected Transcription/Translation, Protein Degradation, Mitochondrion/Cellular Respiration, Signal Transduction, Endomembrane System, Redox Reactions, Programmed Cell Death, and Cell Adhesion/Cytoskeleton (*Figure 9*). Programmed Cell Death (*Figure 9b)* was only present in Eugenol. Redox Reactions was also present in CAD vs PBS and TRI vs PBS.

a)



b)

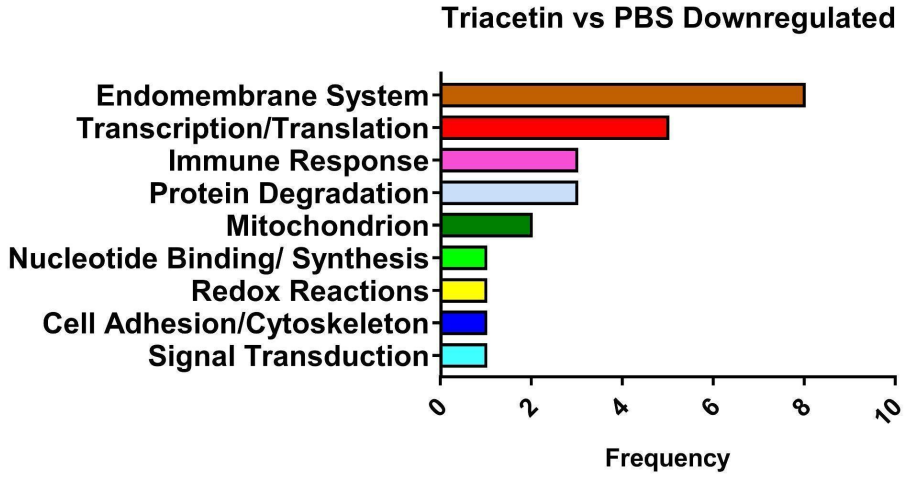


Figure 10: Frequency distribution of biological processes affected by triacetin. (a) upregulated proteins and (b) downregulated proteins.

Triacetin vs PBS

Triacetin presented the most categories (*Figure 10*) and had effects on Endomembrane System, Protein Degradation, Transcription/Translation, Mitochondrion/Cellular Respiration, Cell Adhesion/Cytoskeleton, Redox Reactions, Signal Transduction, Immune System, and Nucleotide Binding/Synthesis. Nucleotide Binding/Synthesis (*Figure 10b*) was only present in the Triacetin group.

Discussion

In previous studies, various flavors and brands of ECs produced aerosols with cytotoxicity that correlated with high concentrations of chemicals and synthetic coolants (Omaiye et al. 2022). To further understand the cytotoxic effects chemicals have on lung epithelial cells, we used DAVID to identify and group the proteins that are involved in specific biological processes that were affected by JUUL™ menthol, JUUL™ tobacco, benzyl alcohol, cinnamaldehyde, eugenol, triacetin, and WS-23. The results of our analyses are summarized in Tables 1 (JUUL experiment with the Cultex) and 2 (individual chemical experiment in the VitroCell).

In the Cultex, the biological processes associated with the proteins involved in JUUL™ Virginia tobacco and JUUL™ menthol were identified. A prior study showed that aerosols produced by JUUL™ Virginia tobacco and JUUL™ menthol affected mitochondrial respiration and the electron transport chain by increasing proton leak, decreasing coupling efficiency, as well as a decreasing complex I, II, and IV (Lamb et al. 2020). Our analysis also showed that the upregulated (positive-value) proteins in JUUL™ Virginia tobacco affected processes related to the endomembrane system and protein degradation. The categories of Endomembrane System,

Protein Degradation, Cell Adhesion/ Cytoskeleton, Cilia, and Signal Transduction were not observed in JUUL™ menthol , however, were in JUUL™ Virginia tobacco down regulated proteins (negative-values). A study identified that both tobacco and menthol alter mitochondria/ cellular respiration and menthol flavored e-cigarettes affected mitochondrial respiration dysfunction in lung epithelial cells compared to tobacco (Rahman et al. 2020). We also observed that transcription/translation was affected by JUUL Menthol ™ , as it is not present in JUUL™ Virginia tobacco. The differences in the biological effects of JUUL™ menthol and JUUL™ Virginia tobacco on the lung epithelial cells could be due to varying compositions in each e-liquid. JUUL™ menthol had 1,391 ug/mL of menthol versus 118.44 ug/mL in JUUL Virginia Tobacco™ (Holt et al. 2021). The varying concentrations of chemicals in e-liquid could influence the different categories observed in JUUL™ menthol and JUUL™ Virginia tobacco, however, further analysis is necessary to validate this conclusion.

In the Vitrocell, WS-23, benzyl alcohol, cinnamaldehyde, eugenol, and triacetin were analyzed. WS-23 affected oxidative stress in lung epithelial cells or inhibited mitochondrial reductases as well as playing a role in modulating airway epithelial cell growth (Omaiye 2022, Yogeswaran et al. 2022). Our data showed that WS-23 did not affect Mitochondrion/Cellular Respiration but did have an effect on the Endomembrane System. Additionally, the downregulated graph suggests WS-23 had an effect on Cell Adhesion/ Cytoskeleton. This indicates that although not clearly indicated, Mitochondrion/Cellular respiration can be impacted by WS-23 and may not be shown due to a lack of significant clusters or data, as well as overlapping processes in the Endomembrane System and the Mitochondria being grouped together in DAVID. Benzyl alcohol is classified as a harmful alcohol that appears frequently in e-fluids (Omaiye et al. 2019). Benzyl alcohol had an effect on Transcription/Translation, Signal

Transduction, Endomembrane System, and Protein Degradation in the upregulated proteins and cell adhesion/cytoskeleton and Mitochondrion/Cellular Respiration. Cinnamaldehyde was one most potent flavor chemical and impaired the function of immune cells in the respiratory system (Omaiye et al. 2019). Cinnamaldehyde in e-fluid suppressed inflammatory cytokines, inhibits non-specific immune responses, and increases DNA strand breaks (Morris et al. 2021, Behar et al. 2015). Our data also supported the conclusion that cinnamaldehyde has an effect on the immune response as well as metal/ion binding in the upregulated proteins. The down regulated proteins showed an effect on signal transduction and redox reactions. Cinnamaldehyde seemed to impact Transcription/Translation as well as the Mitochondria/Cellular Respiration the most, with a frequency of 6 and 15 clusters, respectively. Eugenol as well as five other flavor chemicals resulted in cellular levels that were consistent with cell death (Sherwood et al. 2016). This is consistent with our downregulated data that showing programmed cell death was influenced by eugenol. Additionally, eugenol increased both oxidative stress at a high concentration as well as pro-inflammatory mediators in the immune response (Fetterman, 2018). Oxidative stress, which is associated with the mitochondria, was present in both the upregulated and downregulated graphs and had the highest frequency of clusters. Immune response, however, was not present in either graph, and our data did not suggest an effect on immune response. Our data showed eugenol also had a strong effect on transcription/translation as six clusters seemed to be associated with this category. It also influenced cell adhesion/cytoskeleton, protein degradation, and endomembrane system in the upregulated data in addition to signal transduction and redox reactions in the downregulated data. Triacetin is a frequently used chemical and is classified as an extreme irritant (Omaiye et al. 2019). It degraded under thermal conditions or heat to form acetic acid, formaldehyde, acrolein, and acetaldehyde. These have led to a decrease in respiratory

rates and inflammation as well as an increase in oxidative stress (Vreeke et al. 2018). The effects on the immune response and Mitochondria/Cellular Respiration were observed in the downregulated data. Our data also found that triacetin impacted other biological processes. Both the upregulated and downregulated data presented an endomembrane system with a frequency of 10 clusters relating to the endomembrane system. The downregulated data showed that triacetin also affected Transcription/Translation, Protein Degradation, Redox Reactions, Cell Adhesion/ Cytoskeleton, Signal Transduction, and Nucleotide Binding/Synthesis. Nucleotide Binding/Synthesis was a unique category that did not appear often, and this category could also be associated with transcription/translation as it only appeared one time.

Although the data are labeled as upregulated and downregulated for each comparison, DAVID cannot predict if a certain effect is being upregulated or downregulated. Upregulated and downregulated protein IDs entered into DAVID referred to the positive and negative values and the biological processes associated with each. To determine what biological processes are being upregulated or downregulated, the bioinformatics tool, Qiagen Ingenuity Pathway Analysis (IPA) must be used. Although DAVID allows for summarizing inter-relationships and identifying key functions associated with protein lists, it occasionally results in a cluster being placed in the most representative category, however, it could still have an impact on another biological process.

Table 1 Cultex JUUL summarizes the effects produced by the Clean Air versus incubator control, JUUL menthol, and JUUL tobacco. Clean Air seemed to have an effect on Mitochondrion/ Cellular Respiration, Transcription/Translation, and the Dermis that is observed in red. Mitochondrion/ Cellular Respiration and Transcription/Translation was also seen in JUUL menthol in blue, however, these effects could be due to Clean Air as it was also observed in the control and no other categories were present. JUUL Virginia tobacco did overlap with the Clean

Air control in producing the effect on Mitochondrion/ Cellular Respiration seen in red, however, it also had significant effects on other biological processes. These include Endomembrane system, Cell Adhesion/ Cytoskeleton, Protein Degradation, Signal Transduction, and Cilium.

Table 2 represented the effects produced by PBS, WS-23, benzyl alcohol, cinnamaldehyde, eugenol, and triacetin. In red, it was observed that PBS, the control, had an effect on many biological processes. Additionally, the various chemicals added were modulated by PBS and the effects of each chemical are observed in blue. For example, WS-23 produced an effect on the Endomembrane System and Cell Adhesion/ Cytoskeleton, however did not impact any other categories. Table 2 allows us to visualize the effects produced by each chemical in comparison to the PBS.

Through proteomics analysis, or a large-scale study of proteins, we were able to determine what simultaneously is occurring in the cell or the general biological processes, however, it does not tell us what is specifically going on. Other studies will need to be conducted to identify the more specific effects created by certain chemicals.

Table 1-Cultex JUUL:

Categories	Clean Air vs INC	JUUL Menthol vs Clean Air	JUUL Virginia Tobacco vs Clean Air
Endomembrane System			
Cell Adhesion/Cytoskeleton			
Protein Degradation			
Mitochondrion/Cellular Respiration			
Transcription/ Translation			
Signal Transduction			
Cilium			
Dermis			

*Table 1: Summary of biological processes affected by JUUL Virginia Tobacco™ and JUUL Menthol™ in the Cultex (*red represents effects produced by the control; blue represents effects produced by the chemicals)*

Vitrocell:

Categories	PBS vs INC	WS-23 vs PBS	Benzyl Alcohol vs PBS	CAD vs PBS	EUG vs PBS	TRI vs PBS
Endomembrane System						
Cell Adhesion/Cytoskeleton						
Immune Response						
Protein Degradation						
Mitochondrion/Cellular Respiration						
Transcription/ Translation						
Redox Reactions						
Signal Transduction						
Nucleotide Binding/Synthesis						
Metal/ Ion binding						
Cell Division						
Programmed Cell Death						

*Table 2: Summary of biological processes affected by PBS, WS-23, benzyl alcohol, cinnamaldehyde, eugenol, and triacetin in the Vitrocell (*red represents effects produced by the control; blue represents effects produced by the chemicals)*

References

Aufderheide, Michaela, et al. “Improvement of the CULTEX® Exposure Technology by Radial Distribution of the Test Aerosol.” Experimental and Toxicologic Pathology, Urban & Fischer, 3 Mar. 2017, https://www.sciencedirect.com/science/article/abs/pii/S0940299317300544.

Behar, Rachel Z et al. “Distribution, quantification and toxicity of cinnamaldehyde in electronic cigarette refill fluids and aerosols.” Tobacco control vol. 25,Suppl 2 (2016): ii94-ii102. doi:10.1136/tobaccocontrol-2016-053224

Esther E. Omaiye, Wentai Luo, Kevin J. McWhirter, James F. Pankow, and Prue Talbot Chemical Research in Toxicology 2022 35 (8), 1344-1358 DOI: 10.1021/acs.chemrestox.1c00423

Fetterman, Jessica L., et al. “Flavorings in Tobacco Products Induce Endothelial Cell Dysfunction ...” Flavorings in Tobacco Products Induce Endothelial Cell Dysfunction, 27 July 2018, https://www.ahajournals.org/doi/10.1161/ATVBAHA.118.311156.

Holt, Alaina K., et al. “A Retrospective Analysis of Chemical Constituents in Regulated and Unregulated e-Cigarette Liquids.” Frontiers, Frontiers, 27 Sep. 2021, https://doi.org/10.3389/fchem.2021.752342.

Hua My, Shouq Sadah, Vagelis Hristidis, and Prue Talbot (2020) Health Effects Associated with Electronic Cigarette Use: Automated Mining of Online Forums. Journal of Medical Internet Research, 22(1):e15684) doi: 10.2196/15684

Huang, Da Wei, et al. “The David Gene Functional Classification Tool: A Novel Biological Module-Centric Algorithm to Functionally Analyze Large Gene Lists - Genome Biology.” BioMed Central, BioMed Central, 4 Sept. 2007, https://genomebiology.biomedcentral.com/articles/10.1186/gb-2007-8-9-r183.

Lamb, Thomas et al. “Pod-based menthol and tobacco flavored e-cigarettes cause mitochondrial dysfunction in lung epithelial cells.” Toxicology letters vol. 333 (2020): 303-311. doi:10.1016/j.toxlet.2020.08.003

Leventhal, Adam M et al. “Effect of Exposure to e-Cigarettes With Salt vs Free-Base Nicotine on the Appeal and Sensory Experience of Vaping: A Randomized Clinical Trial.” JAMA network open vol. 4,1 e2032757. 4 Jan. 2021, doi:10.1001/jamanetworkopen.2020.32757

Morris, Anna M et al. “Effects of E-Cigarette Flavoring Chemicals on Human Macrophages and Bronchial Epithelial Cells.” International journal of environmental research and public health vol. 18,21 11107. 22 Oct. 2021, doi:10.3390/ijerph182111107

Omaiye, E.E., McWhirter, K.J., Luo, W. et al. High concentrations of flavor chemicals are present in electronic cigarette refill fluids. Sci Rep 9, 2468 (2019). https://doi.org/10.1038/s41598-019-39550-2

Omaiye, Esther & Luo, Wentai & McWhirter, Kevin & Pankow, James & Talbot, Prue. (2021). Flavor chemicals, synthetic coolants and pulegone in popular mint-flavored and menthol-flavored e-cigarettes. Tobacco Control. 31. tobaccocontrol-2021. 10.1136/tobaccocontrol-2021-056582.

Shawna Vreeke et al. “Triacetin Enhances Levels of Acrolein, Formaldehyde Hemiacetals, and Acetaldehyde in Electronic Cigarette Aerosols.”ACS Omega 2018 3 (7), 7165-7170 DOI: 10.1021/acsomega.8b00842

Sherwood, C.L., Boitano, S. Airway epithelial cell exposure to distinct e-cigarette liquid flavorings reveals toxicity thresholds and activation of CFTR by the chocolate flavoring 2,5-dimethylpyrazine. Respir Res 17, 57 (2016). https://doi.org/10.1186/s12931-016-0369-9

Stallings-Smith, Sericea, and Taylor Ballantyne. “Ever Use of E-Cigarettes Among Adults in the United States: A Cross-Sectional Study of Sociodemographic Factors.” Inquiry : a journal of medical care organization, provision and financing vol. 56 (2019): 46958019864479. doi:10.1177/0046958019864479

Strombotne, Kiersten et al. “Do JUUL and e-cigarette flavors change risk perceptions of adolescents? Evidence from a national survey.” Tobacco control vol. 30,2 (2021): 199-205. doi:10.1136/tobaccocontrol-2019-055394

Yogeswaran, Shaiesh, et al. “Synthetic Coolant WS-23 Increases e-Cigarette Generated Aerosolized Acellular Reactive Oxygen Species (ROS) Levels.” BioRxiv, Cold Spring Harbor Laboratory, 1 Jan. 2022, https://doi.org/10.1101/2022.06.20.496866.

Zavala, Jose, et al. “Assessment of Biological Responses of EpiAirway 3-D Cell Constructs versus A549 Cells for Determining Toxicity of Ambient Air Pollution.” Inhalation Toxicology, U.S. National Library of Medicine, 2016,

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4913276/. [Original source: https://studycrumb.com/alphabetizer]