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Original Research

Investigating *Streptococcus pyogenes*-Produced Bioactive Secondary Metabolites by Gas Chromatography-Mass Spectrometry (GC-MS) and Evaluating Its Antimicrobial Effects

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Abstract;

Background:

With a prevalence of 15% to 30% in paediatric pharyngitis, *Streptococcus pyogenesis* is the most prevalent and consequential bacterial cause of this condition. Although the typical laboratory test for microbiologic confirmation of pharyngitis involves growing a culture from a throat swab specimen, this process takes a long time and means that adequate therapy is delayed. S. pyogenes pharyngitis can cause both immediate and long-term problems if not addressed. Here, we detailed the volatile metabolomes of Staphylococcus pyogenes and related oropharyngeal colonising bacteria. We present evidence to back future breath-based diagnostic testing for streptococcal pharyngitis and propose potential biomarkers that differentiate S. pyogenes from other species. Cellular activity, maintenance, and growth rely on metabolites, which are tiny molecules that take part in metabolic activities. Metabolites typically have concentrations spanning multiple orders of magnitude and a molecular weight ranging from 50 to 1500 Da. Metabolite sensitivity to many environmental factors contributes to the metabolome's high degree of dynamic and time-dependent nature.

Aims and Objectives:

The researchers in this lab set out to identify the antibacterial bioactivity of Streptococcus pyogenes's chemical components and learn more about their biological activity.

Method:

Isolation of *Streptococcus pyogenes* was accomplished by collecting swabs from children afflicted with streptococcal pharyngitis at Babylon Hospital for Women and Children. Following incubation of the samples in stationary culture at 37°C for 24 hours, headspace samples were taken. Every bacterial species was cultured in the same glassware using the same medium. Analysis and concentration of volatile substances: Gas chromatography-mass spectrometry was used to concentrate and separate volatile metabolites. The biological components, sometimes known as bioactive chemicals, were examined in this study using gas chromatography–mass spectrometry (GC–MS) methods. On top of that, an experimental laboratory was used to assess the efficacy of Streptococcus pyogenes's ethanolic extract against microorganisms.

Results:

We experimentally identified the presence of the following bioactive components using GC-MS analysis on *Streptococcus pyogenes*: 3-Methylbutyraldehyde, 1,1-Dimethoxynonane, 2-Ethyl-1-butanol, 2,2'-Diaminodiethylamine, diethyl 2-(aminomethylene)malonate, N-

ethenylmethanimine, Pyrazine, 2,5-diethyl, 5-Ethyl-2,3-dimethylpyrazine, 4H-imidazo[4,5-c]pyridin-4-one, 2,6-Dimethyl-1-heptene, 2-Nonanone, 5-ethyl. Streptococcus pyogenes was tested for its potential antibacterial effects against five harmful bacteria by analysing its secondary metabolites. This study looked at five distinct infections and how effectively the standard antibiotics AM-Amikacin and CTX-Cefotaxime worked, in comparison to an ethanolic extract of *Streptococcus pyogenes*. *Enterococcus faecalis* (16.00±0.32, 26.09±0.25, and 20.71±0.24), *Escherichia coli* (13.60±0.18, 19.54±0.22, and 17.00±0.20), *Bacillus subtilis* (17.81±0.20, 21.00±0.23, and 22.64±0.24), *Proteus mirabilis* (21.65±0.23, 28.07±0.25, and 25.00±0.24), and *Staphylococcus epidermidis* (19.00±0.22, 24.07±0.25, and 21.89±0.20). *Streptococcus pyogenes* metabolites were shown to show remarkable activity against *Proteus mirabilis*, with a mean value of (21.65±0.23).

Keywords: *Streptococcus pyogenes*, Secondary metabolites, Antibacterial, GC/MS.

Introduction:

Roughly fifteen million people in the US seek medical attention each year for acute pharyngitis, making it one of the most frequent outpatient medical conditions. The majority of instances of acute pharyngitis, accounting for as much as 15% in adults and 30% in children, are caused by infections with Streptococcus pyogenes, a type of group A beta-hemolytic streptococcus. But differentiating between viral and bacterial causes of acute pharyngitis is difficult based on clinical presentation alone, which is a big problem for outpatient care of the condition [1, 2]. To lessen the likelihood of post-infectious acute rheumatic fever, it is advised that patients with tonsillopharyngitis caused by *Staphylococcus pyogenes* take an oral beta-lactam antibiotic, such as penicillin or amoxicillin. When diagnosing streptococcal pharyngitis, the usual course of treatment involves taking a direct sample from the back of the throat, which is then cultured and tested for streptococcal antigens at the point of care. Although S. pyogenes pharyngitis can be highly sensitively identified using antigen detection in conjunction with culture, results can be skewed due to insufficient oropharyngeal collection and cross-reactivity with other oral colonisers. The increasing prevalence of antimicrobial resistance can be attributed, in large part, to the inappropriate use of antibiotics, especially in cases of viral respiratory infections. Among children and adolescents aged 0–19, the majority of antibiotics given for pharyngitis and other respiratory symptoms may not be required, according to the CDC. One potential substitute for conventional microbiological testing makes use of the unique metabolic capacities of several bacterial species. Bacteria can be identified, classified, and discriminated against by-products of the substrates they use for growth, which can include additional chemical indicators such volatile organic compounds (VOCs) [3-5]. Analysing the gas in the headspace above bacterial colonies chemically allows one to detect the characteristic volatile compounds produced by many bacteria. Certain bacterial species are already known to produce distinct aromas, and these volatile organic compounds add to those scents. For instance, indole is responsible for the characteristic smell of cultured Escherichia coli, while 2-aminoacetophenone is responsible for the typical grape-like odour of Pseudomonas aeruginosa. While the exact biological roles of most bacterial volatile organic compounds (VOCs) remain unknown, new research implies that they could play a role in defence, growth promotion, and interbacterial communication. The objective identification of VOC biomarkers specific to organisms is made possible by very sensitive molecular detection methods based on gas chromatography-mass spectrometry (GC-MS) instruments. It is possible to culture many bacterial pathogens that infect humans in either liquid or solid media [6, 7]. This allows for simple volatile organic compound detection in axenic cultures before moving on to more involved in vivo systems. This wealth of data and technological advancements suggests that volatiles, such as those found in an infected

wound, urine, or breath, could one day be used for in-situ infection diagnosis.

Commercially available for the detection of *Helicobacter pylori* infection, volatile analysis of human breath has previously shown success in pilot studies for the detection of infections like pulmonary tuberculosis and malaria. This indicates that the diagnostic technique is valid and has widespread applicability. Oropharyngeal volatiles, when tested for streptococcal pharyngitis, may include host-associated volatiles activated by the infection as well as volatiles that represent the pathogen's metabolic activity. The oropharyngeal microbial ecology is complicated, and there are both internal and external sources of volatile organic compounds (VOCs) [8, 9]. This makes it difficult to evaluate oropharyngeal volatile composition in a clinical context. Purpose: This laboratory study aimed to investigate the antibacterial bioactivity of chemical compounds generated by *Streptococcus pyogenes* and to identify those with the highest biological activity.

Materials and Methods:

Environment for culture and processing of samples:

Isolated strains were used for this study from streptococcal pharyngitis in children treated at Babylon Hospital for Women and Children. After incubating samples in stationary culture at 37°C for 24 hours, headspace samples were taken. We used the same glassware and media to cultivate every bacterial species. Vapour compound concentration and analysis : Concentrated and separated volatile metabolites were analysed using gas chromatography-mass spectrometry. The metabolites were evaporated using a rotary evaporator set at 45 degrees Celsius. After that, the metabolites were isolated from the culture by removing them from the liquid

Using GC-MS on an Agilent 789 to investigate the bioactive natural chemical components of Streptococcus pyogenes An instrument was utilised to conduct the analysis, which was executed using a GC-MS methodology. A DB-5MS column, bought from Folsom, California's J&W Scientific, was utilised for the gas chromatography. For this column, the following dimensions were taken: There is an internal diameter of 0.25 mm and a film thickness of 0.25 µm, with a diameter of 30 m. In contrast to the previous experiment [10], the furnace temperature remained constant throughout [10, 11]. We used helium as the carrier gas and maintained a constant flow rate of one millilitre per minute. The mass spectrometer (MS) source was fed directly with gas chromatography (GC) column effluent through a transfer line that was heated to 250 degrees Celsius. The ionisation process occurred at a voltage of 70 electron volts (eV) while the ion source was kept at a temperature of 230 degrees Celsius (°C). The measurement range encompassed 41 atomic mass units (amu) up to 450 amu.

Disclosure of compounds:

Mass spectral matching was used to assign possible identifications to chromatographic peaks. Peaks with a forward match score of ≥600/1,000 related to the 2011 mass spectral library of the National Institute of Standards and Technology (NIST) were specifically allocated as potential compound identifications .

The data were analysed using the freely accessible and interactive XCMS Online (20) at https://xcmsonline.scripps.edu. Ions with distinct mass-to-charge and retention-time ratios were considered to be metabolic characteristics. The following parameter settings were used for XCMS processing of GC-MS data: centWave for feature detection (Δ m/z = 100 ppm, minimum peak width = 5 s, maximum peak width = 10 s, and signal/noise threshold = 6); obiwarp for retention-time correction (profStep = 1); and parameters for chromatogram alignment, for example, mzwid = 0.25, minfrac = 0.5, and allowable retention time deviations bw = 10. Extracted ion chromatogram (EIC) areas were used for the relative measurement of metabolite characteristics. We were able to identify 1,420 distinct traits. By comparing the means of three separate groups, we were able to use multiple group analysis to find

metabolite characteristics with a statistically significant pattern of variation. Since identifying variations between bacterial species was the major aim of the study, medium analysis was omitted. We utilised the nonparametric Kruskal-Wallis test (P-value threshold 0.05) to assess the metabolite variation across the experimental groups [12, 13]. The molecules found via the Kruskal-Wallis test were utilised in Principal Component Analysis (PCA) to visualise variance among samples. The following features were not included in the principal component analysis (PCA): m/z > 150 a.m.u., retention time > 26 min, and P > 0.5. The main components and contaminants that exit the chromatographic column after 26 min are large masses, and chemicals associated to column bleed are also eliminated .

Assessment of the antimicrobial activity of compounds derived from secondary metabolites against five harmful pathogens.

The agar was prepared by boring five-millimeter-diameter holes with a sterile cork borer. Next, five distinct pathogens—*Enterococcus faecalis*, *Escherichia coli*, *Bacillus subtilis*, *Proteus mirabilis*, and *Staphylococcus epidermidis*—were tested using 25 microliters of sample solutions containing Streptococcus pyogenes metabolites and the conventional antibiotics AM-Amikacin and CTX-Cefotaxime.

Results and Discussion:

Streptococcus pyogenes, like S. pneumoniae, may colonise the respiratory mucosa, thus we wanted to see if there are enough metabolic changes to tell their volatile metabolome apart. We compared the profiles of volatile organic chemicals extracted from axenic cultures of clinical isolates of S. pyogenes in order to find potential biomarkers that are particular to this species. *S. pyogenes* is a successful pathogen that senses its environment and changes its metabolic status to survive, persist, and proliferate in a broad range of host environments. The present search covers the knowledge of those who used the established biochemical basis of metabolism to understand the underlying mechanisms of virulence. It should be mentioned that the initial research on S. pyogenes metabolism mostly concentrated on different parts of the pathogen's amino acid and carbohydrate metabolism. Despite their importance in secreting several cellular products, including numerous virulence factors, lipid metabolism and membrane transport have gotten comparatively little attention.

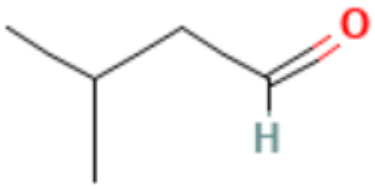
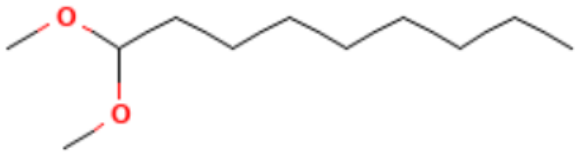
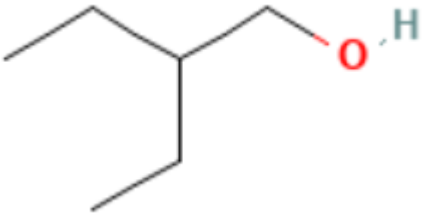
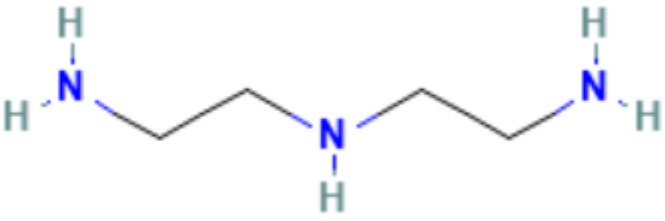
The GC-MS chromatogram of cultivated *Streptococcus pyogenes* also revealed the presence of several volatile chemicals, including a particular volatile organic molecule,

3-Methylbutyraldehyde, 1,1-Dimethoxynonane, 2-Ethyl-1-butanol, 2,2'-Diaminodiethylamine, diethyl 2-(aminomethylene)malonate, N-ethenylmethanimine, Pyrazine, 2,5-diethyl, 5-Ethyl-2,3-dimethylpyrazine, 4H-imidazo[4,5-c]pyridin-4-one, 2,6-Dimethyl-1-heptene, 2-Nonanone, 5-ethyl. We conclude that further research into volatile biomarkers in species of oropharyngeal streptococcal should be continued. While *Streptococcus pyogenes* cultures do contain some of the discriminant chemicals, such as pyrazine compounds, they are not typically seen in the exhaled breath of healthy humans. The present study examined the biological activity of five distinct pathogens using the standard antibiotics AM-Amikacin and CTX-Cefotaxime in conjunction with an ethanolic extract of Streptococcus pyogenes. *Enterococcus faecalis* (16.00±0.32, 26.09±0.25, and 20.71±0.24), *Escherichia coli* (13.60±0.18, 19.54±0.22, and 17.00±0.20), *Bacillus subtilis* (17.81±0.20, 21.00±0.23, and 22.64±0.24), *Proteus mirabilis* (21.65±0.23, 28.07±0.25, and 25.00±0.24), and *Staphylococcus epidermidis* (19.00±0.22, 24.07±0.25, and 21.89±0.20). *Streptococcus pyogenes* metabolites were shown to show remarkable activity against *Proteus mirabilis*, with a mean value of (21.65±0.23).

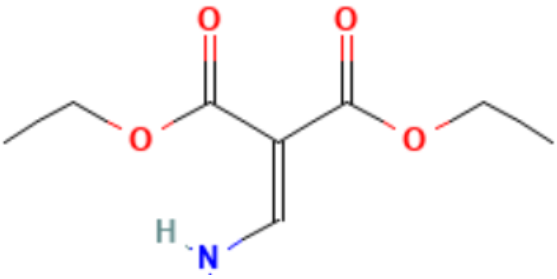
Table 1. Bioactive byproducts of *Streptococcus pyogenes* identified by gas chromatography-mass spectrometry

Compound	Formula	M.W
3-Methylbutyraldehyde	$C_5H_{10}O$	86.13 g/mol
1,1-Dimethoxynonane	$C_{11}H_{24}O_2$	188.31 g/mol
2-Ethyl-1-butanol	$C_6H_{14}O$	102.17 g/mol
2,2'-Diaminodiethylamine	$C_4H_{13}N_3$	103.17 g/mol
diethyl 2-(aminomethylene)malonate	$C_8H_{13}NO_4$	187.19 g/mol
N-ethenylmethanimine	C_3H_5N	55.08 g/mol
Pyrazine, 2,5-diethyl-	$C_8H_{12}N_2$	136.19 g/mol
5-Ethyl-2,3-dimethylpyrazine	$C_8H_{12}N_2$	136.19 g/mol
4H-imidazo[4,5-c]pyridin-4-one	$C_6H_3N_3O$	133.11 g/mol
1,3-Hexadien-5-yne	C_6H_6	78.11 g/mol
2,6-Dimethyl-1-heptene	C_9H_{18}	126.24 g/mol
2-Nonanone, 5-ethyl	$C_{11}H_{22}O$	170.29 g/mol

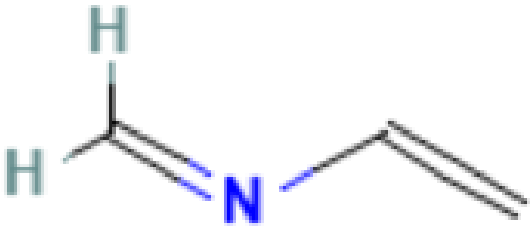
Table 2. *Streptococcus pyogenes* metabolite structures.

Compound	Structure
3-Methylbutyraldehyde	
1,1-Dimethoxynonane	
2-Ethyl-1-butanol	
2,2'-Diaminodiethylamine	

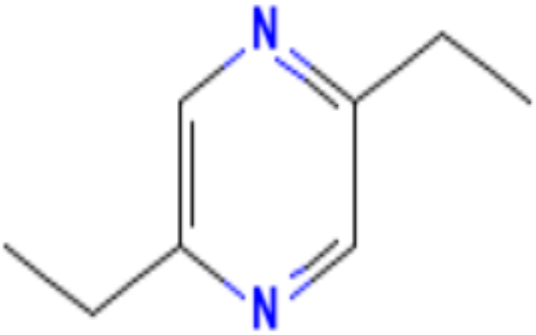
diethyl 2-(aminomethylene)malonate



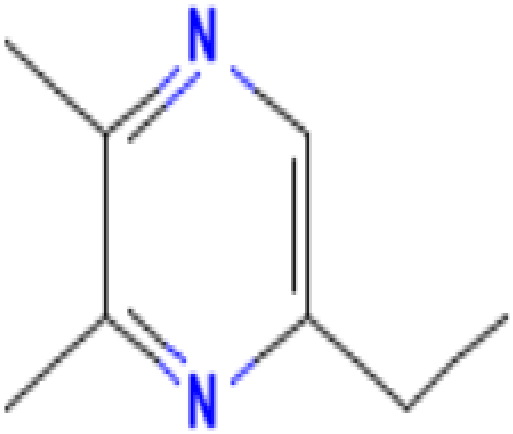
N-ethenylmethanimine



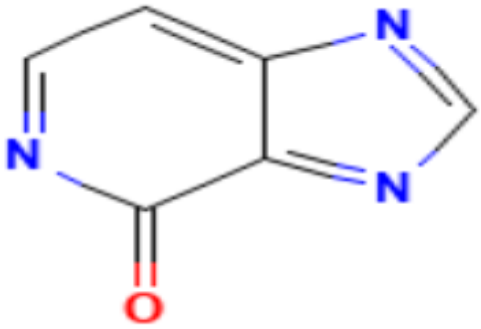
Pyrazine, 2,5-diethyl-



5-Ethyl-2,3-dimethylpyrazine



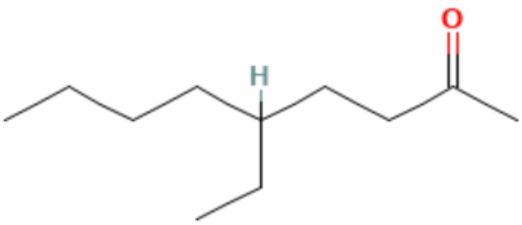
4H-imidazo[4,5-c]pyridin-4-one



1,3-Hexadien-5-yne

2,6-Dimethyl-1-heptene

2-Nonanone, 5-ethyl



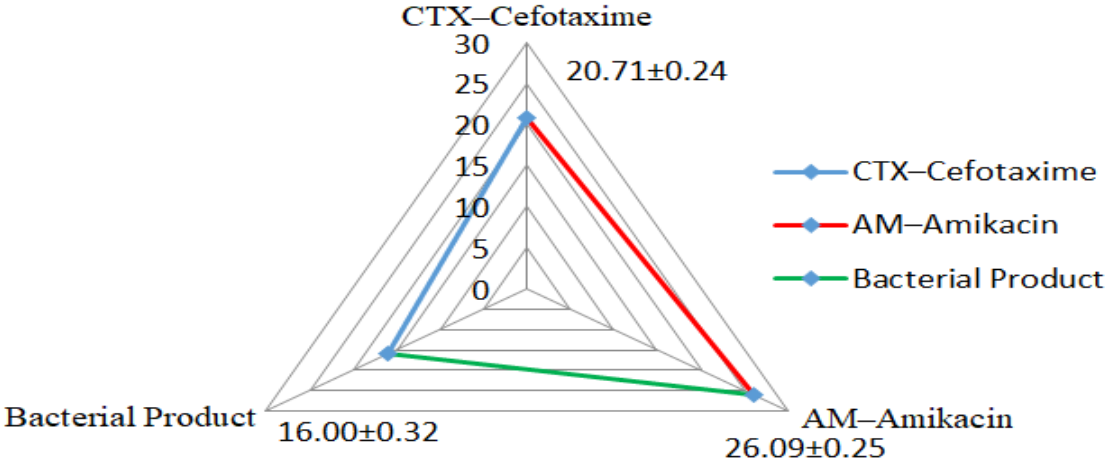


Figure 1. Bioactive secondary metabolites of *Streptococcus pyogenes* and standard antibiotics AM–Amikacin, and CTX–Cefotaxime against *Enterococcus faecalis*

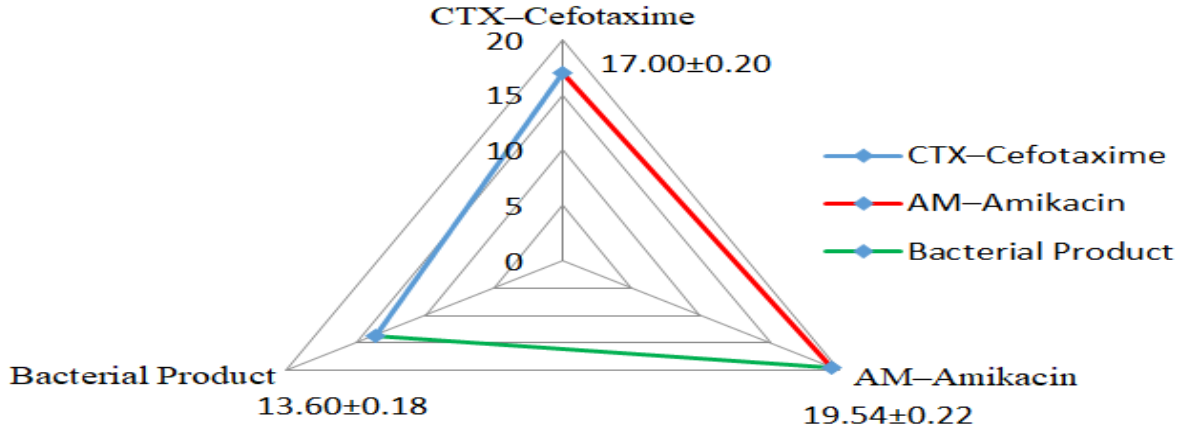


Figure 2. Bioactive secondary metabolites of *Streptococcus pyogenes* and standard antibiotics AM–Amikacin, and CTX–Cefotaxime against *Escherichia coli*

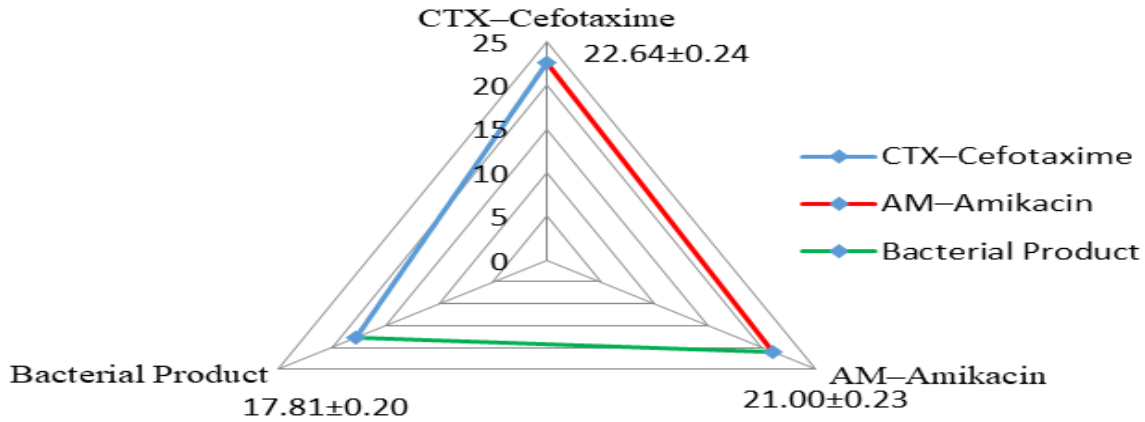


Figure 3. Bioactive secondary metabolites of *Streptococcus pyogenes* and standard antibiotics AM–Amikacin, and CTX–Cefotaxime against *Bacillus subtilis*

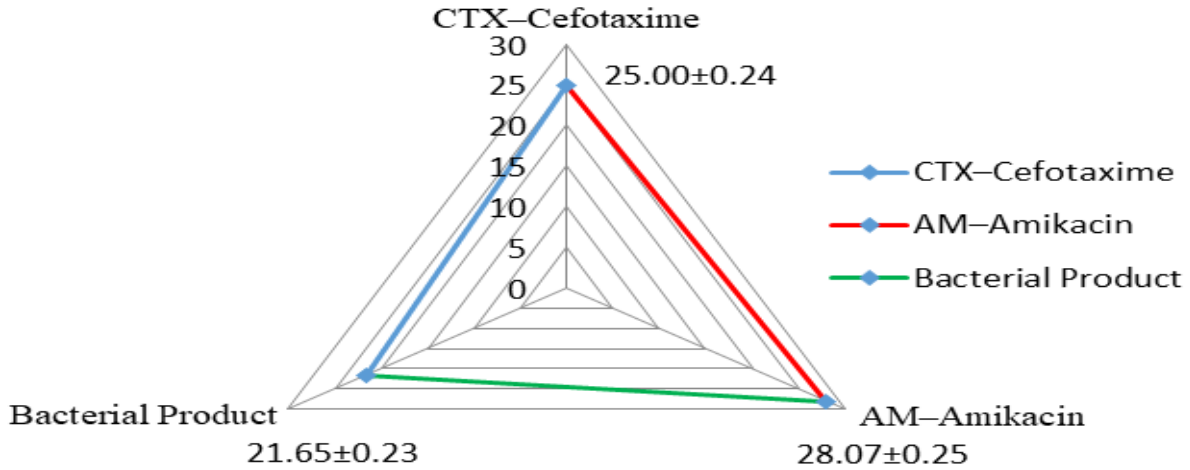


Figure 4. Bioactive secondary metabolites of *Streptococcus pyogenes* and standard antibiotics AM–Amikacin, and CTX–Cefotaxime against *Proteus mirabilis*

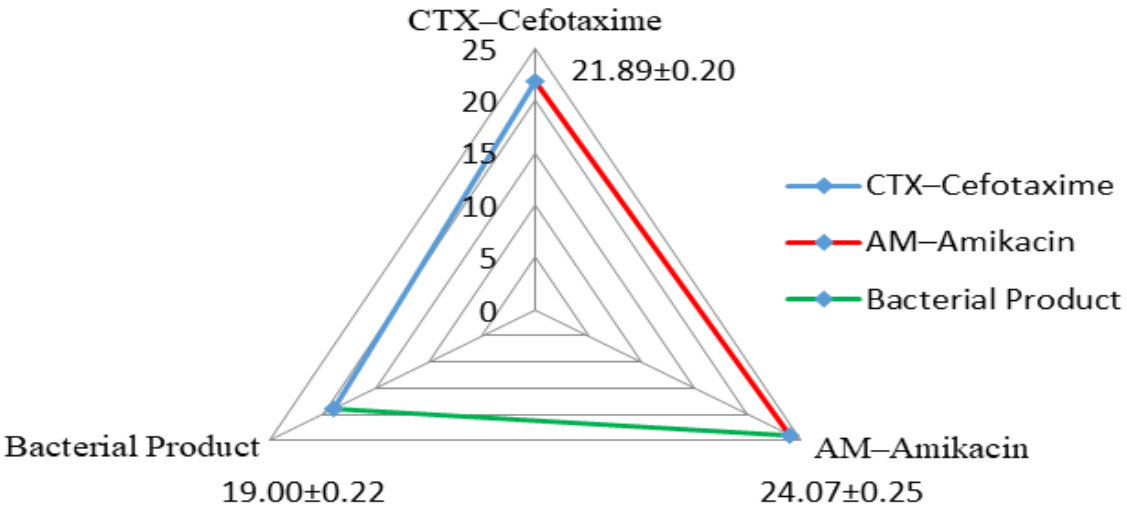


Figure 5. Bioactive secondary metabolites of *Streptococcus pyogenes* and standard antibiotics AM–Amikacin, and CTX–Cefotaxime against *Staphylococcus epidermidis*

In the ambulatory clinical context, sore throats are among the most frequently reported ailments. At the point of care, it would be extremely helpful to have rapid, culture-independent diagnostic methods that do not require pharyngeal swabs to direct antibiotic treatment for outpatients. There has been little progress in detecting volatiles during oropharyngeal infection, despite the potential of this strategy. The most frequent bacterium that causes bacterial pharyngitis, *Streptococcus pyogenes*, produces a distinct pattern of volatile chemicals, as our study shows. Notably, we isolate volatile compounds that set this species apart from the usual suspects when it comes to oropharyngeal colonisation by humans [12-15]. Our findings pave the way for further clinical research into breath volatile detection to differentiate S. pyogenes pharyngeal infections. For diagnostic purposes, the ideal volatile biomarker for Staphylococcus pyogenes would be produced solely by this organism and would only be released when the infection is active, not during colonisation. Understanding the metabolic origin of volatiles produced by bacteria is important for predicting whether candidate biomarkers will have the sensitivity and specificity needed for ongoing diagnostic development, as clinical studies of individuals with pharyngitis symptoms are time-consuming and expensive [16, 17].

Nonetheless, only twelve volatile chemicals exceeding quantitation limits were considered in this focused research. Researchers in another study looked at the volatile headspace composition above implanted Detroit cells with S. pyogenes and influenza A virus. The scientists observed notable variations in the amounts of volatile organic compounds (VOCs) released by infected and non-infected cells; however, they did not examine the VOC profile of *S. pyogenes* in cells on its own [18, 19]. Similarly, cultivated Streptococcus pneumoniae volatiles have been reported before, but no direct comparisons to oropharyngeal bacterial species-specific volatiles have been made. In this research, we make use of recent technical developments that allow for a more comprehensive and objective method of identifying discriminating volatile biomarkers [20-23].

Similarly, the breakdown of amino acids—a key source of energy in TH media—probably leads to the generation of branch aldehyde. As an example, 3-methylbutanal can be converted to 3-methyl-1-butanol by means of alcohol dehydrogenases, while aldehyde dehydrogenases can be used to oxidise aldehydes to carboxylic acid. All of the

streptococcal species that were tested emitted 3-methylbutanal and 3-methyl-1-butanol. Based on our data, it seems that the underlying pattern of volatile organic compound production by these bacteria is due to amino acid breakdown [24-27], not byproducts of fatty acid metabolism. Ehrlich pathway enzymatic conversion of branched-chain amino acids (e.g., leucine and isoleucine) likely produces microbially derived short-chain-branched alcohols such 3-methyl-1-butanol.

Conclusion:

In conclusion, our results support the need for further research into volatile biomarkers for oropharyngeal streptococcal species discrimination. Notably, the headspace of *Streptococcus pyogenes* cultures contains a number of the discriminant chemicals, including pyrazine compounds, which are typically not detected in the exhaled breath of healthy humans. Since these substances are seldom found, it is reasonable to assume that some of these potential biomarkers are not normally generated by other microbes in the oropharyngeal environment. It follows that volatile biomarkers of acute streptococcal pharyngitis, such as those specific to *S. pyogenes*, hold great potential. To confirm our potential biomarkers in individuals with and without acute bacterial pharyngitis that occurs naturally, further research is needed. In the context of a severe illness, bacterial volatile production is likely to change according to a number of circumstances, including the availability of metabolites and the host's interaction with the bacteria through inflammatory reactions. An innovative and non-invasive method that can confirm the identification of pathogenic *S. pyogenes* infection in the airways is breath analysis. This will assist guide the timely and appropriate use of antibiotics upon infection confirmation. The metabolites of *Streptococcus pyogenes* exhibited outstanding activity against *Escherichia coli*, with an average value of 17.37±0.23, according to the findings of the antibacterial activity test .

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