applied sciences

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Antimicrobial and Odour Qualities of Alkylpyrazines Occurring in Chocolate and Cocoa Products

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Abstract: A total of 40 pyrazine compounds of cocoa and chocolate were discovered through a literature analysis. Data on the alkylpyrazines' antibacterial properties were gathered. It was discovered that 12 substances were effective against bacteria, fungus and oomycetes. Following calculations using PASS and AntiBac-Pred, 15 substances were identified as potentially having the ability to inhibit the growth of the *Picornavirus*, *Yersinia pestis*, Resistant *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG (Bacillus Calmette-Guerin), *Micrococcus luteus* and *Corynebacterium jeikeium*. The study of odour qualities led to the identification of the most potent odourants among alkylpyrazines as well as the aroma composition of cocoa and chocolate. Pyrazines have been given GRAS (generally recognized as safe) status by the Flavor and Extract Manufacturers Association (FEMA) for use as flavouring additives in food, demonstrating that this class of chemicals is a secure substitute for managing and combating microbial contamination, which also provides beneficial odour properties to the recipient. A statistical evaluation of the correlation between the odour threshold and the probability of antibacterial activity was performed. A more in-depth investigation of the antimicrobial and olfactory activities of alkylpyrazines is required in the future.

Keywords: alkylpyrazine; pyrazine; heterocycles; antimicrobial activity; odour threshold; odour activity value; cocoa; chocolate

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Citation: Cherniienko, A.; Pawełczyk, A.; Zaprutko, L. Antimicrobial and Odour Qualities of Alkylpyrazines Occurring in Chocolate and Cocoa Products. *Appl. Sci.* **2022**, *12*, 11361. https://doi.org/10.3390/app122211361

Academic Editor: Lidia Feliu

Received: 24 October 2022
Accepted: 7 November 2022
Published: 9 November 2022

1. Introduction

The Maya people, who were the first to produce and use the cacao plant, are credited with the invention of chocolate around 400 AD. The dried cocoa beans were then combined with water, cinnamon and pepper to make a drink [1]. It is assumed that the Spanish were the first ones to bring cocoa beans to Europe from their trip to South America in the 16th century as a present to Prince Philip. In the 17th century, it gained popularity in Spain and was drunk as a beverage just as the Maya people did [2]. Until the 19th century, when the manufacturing of pulverized chocolate and defatted cocoa was developed and they were widely distributed around Europe as a food commodity, cocoa was regarded as a luxury good [3]. *Theobroma cacao* L., a member of the *Malvaceae* family, is the cacao tree which produces the cocoa beans used to make chocolate in modern times [4]. For thousands of years, people have valued chocolate for its rich flavour. The process of turning raw cocoa seeds into cocoa powder produces the distinctive aroma of chocolate. Fermentation, drying, roasting, grinding cocoa beans, combining all components, conching and tempering are the phases of cocoa manufacturing [5]. The largest per capita consumption of chocolate in 2016 was found in Western Europe: 11 kg per person in Great Britain, 9.6 kg in Ireland, 9.5 kg in Switzerland and around 7.5 kg each in Austria and Germany. In the United States of America, about 4.6 kg per person has been eaten annually [6]. In 2006, the annual yield of cocoa beans was approximately 3 million metric tons [7], 4 million tons in 2011–2012 [8] and 4.8 million tons in 2018 [9]. Cocoa's trade value increased by roughly 7% every year between 2014 and 2018 and between 2019 and 2025, and the average annual increase in

the value of cocoa beans is estimated to be 7% as well, amounting to a USD 16 billion commodity circulation [9], which points at the increase in the demand for cocoa and chocolate. Chocolate has gained in popularity also due to its physiological and possible health benefits, including blood pressure management, insulin levels, vascular functions, oxidation processes, prebiotic effects, glucose homeostasis and lipid metabolism [4]. The fermentation, drying and roasting of cocoa beans, and the conching of chocolate masses all involve significant chemical reactions that produce flavour and odour [5]. Those qualities are brought on by a variety of chemical compounds, including alcohols, phenols, pyrazines, esters, aldehydes and ketones. We chose to concentrate on pyrazine derivatives, which were abundant in great quantities in cocoa and goods made from cocoa, including chocolate, in this paper. Pyrazines belong to the group of heterocyclic diazines. They consist of an aromatic ring with two atoms of nitrogen situated in a 1,4-orientation [10]. The relevance of pyrazines in the food business is predicted to expand in the next years due to an increased demand for convenience goods, as well as rising public knowledge about the components and origins of their daily meals. Depending on the substitution and concentration of the roasty, nutty and earthy aroma is characteristic of pyrazine use, and as a result, they have attracted attention from the food sector as key components in raw and roasted meals. Pyrazines are also used as an ingredient in herbicides, insecticides, cosmetics and medicinal substances [11]. The most intriguing feature of pyrazine as a potential therapeutic molecule is the ease with which the hydrogen atoms in its aromatic rings can be substituted to produce various derivatives. While such structures exhibit a wide variety of characteristics and biological functions, we focused on the odour and antimicrobial capabilities of alkylpyrazines.

2. Pyrazines in Chocolate and Cocoa

2.1. The Way of Formation of Pyrazine in Cocoa and Cocoa Products

The odour of a wide variety of goods, including cocoa, coffee, cooked meat, mushrooms, bread, sesame oil and seeds, roasted nuts (macadamia, peanuts, hazelnut), almonds, popcorn, tobacco, potato chips, rum, whiskey, soy sauce and vegetables (beans, spinach, sweet corn, green bell peppers, red peppers, cucumbers, green peas, lettuce, tomatoes, cabbage, carrots and pumpkin) is affected majorly by pyrazines. Scientists began investigating the widespread occurrence of alkylpyrazines in natural goods in the middle of 1960, and as a result, knowledge of pyrazines and their olfactory properties began to expand. During that time, the process of creating alkylpyrazines during the production of cocoa was identified [12]. The genetic background of the seed, harvesting, fermenting, drying and roasting all play an essential role in creating the aroma of cocoa. Raw cocoa beans taste and smell like vinegar, and different flaws such as the processing of unripe or overripe fruit, inadequate aeration, the absence of mixing of the fruit, infection with microbial pathogens and/or smoke damage as a consequence of inappropriate drying may impair the distinctive bitter and astringent taste and the residual sweetness of fermented beans [3,13]. Following the application of a crude fermentation process to the fresh seeds, accompanied by drying and roasting, the characteristic aroma of cocoa is created [7].

2.1.1. Fermentation Stage of Cocoa Manufacturing

Raw cocoa beans have an unappealing acidic (vinegar-like) and astringent flavour and must ferment before being dried and roasted for the cocoa odour to fully develop. The enzymatic hydrolysis of polysaccharides and protein fractions results in the release of Maillard precursors, such as free amino acids and reducing sugars, which are then transformed into the essential volatile components of cocoa—many of which are pyrazines—during the roasting of cocoa beans via the Maillard and Strecker reactions [12,14]. Fresh cocoa beans undergo the rapid metabolization of sugars from the mucilaginous pulp of the seeds to create volatile and nonvolatile organic acids; the degradation of proteins to produce peptides and free amino acids; the oxidation of polyphenols to create insoluble compounds (o-quinones); and the hydrolysis of glycosides (mainly anthocyanins). Firstly, a variety

of yeasts and bacteria operate synergistically to ferment the pulp sugar, converting it to alcohol and CO_2. Polysaccharides are degraded by enzymes and other glycosidases. This is demonstrated by the fruit pulp turning into a liquid and draining away, which enhances aeration and causes acetic acid bacteria to convert alcohol to acetic acid. The pH of cocoa beans is lowered by acetic acid to between 4.5 and 5.5; the temperature rises to 45–50 °C and the cell walls of the cacao seed become permeable; and an oxidative process takes control of the entire mass, triggering the growth of lactic bacteria, allowing for enzymatic reactions and subsequent fermentation [15,16]. Then, phenolic compound condensation and oxidation processes become the focus. The initially bitter and astringent cocoa flavour is softened by a decrease in the number of soluble phenols. Drying the seeds to a water content of less than 8% finally puts an end to the oxidation reactions. The precursors of aroma compounds (reducing sugars and nitrogenous intermediates) are produced by the breakdown of proteins and peptides during fermentation along with the free amino acids. Additionally, it has been discovered that the amount and timing of the cocoa beans' acidification affect how some fragrance precursors are produced. Tetramethylpyrazine, 2-ethyl-3,5-dimethyl- and 2,3-diethyl-5-methylpyrazine are significant flavour-active substances created during fermentation. Additionally, roasting yields of Maillard intermediates were found to have more pyrazines in well-fermented beans than in little or unfermented beans [3,8,10,13,17].

2.1.2. Roasting Stage of Cocoa Manufacturing

The oxidation reactions, which started during fermentation, continue at a dry heat processing stage. Incompetent drying may lead to an inability to produce compounds that are important for the odour of cocoa, such as pyrazines, and high levels of off-odour carbonyl compounds [8,10]. It is known that varying the temperature and roasting duration have a major impact on the overall aroma produced [7,10,18]. The Maillard process, also known as the nonenzymatic browning reaction, is primarily a reaction between the reducing sugars and free amino groups of amino acids. Leucine, alanine, phenylalanine and tyrosine, which are hydrophobic amino acids produced by proteinase reactions during fermentation, as well as the reducing sugars fructose and glucose obtained from sucrose hydrolysis, were observed as contributing significantly to the Maillard process. The roasting procedure typically results in the formation of heterocyclic aromatic compounds such as pyrazines (with the exception of tetramethylpyrazine, which is biosynthesized during cocoa fermentation), the efficient inhibition of polyphenol oxidase activity and a decrease in free acetic acidity, and thereby eliminates some unpleasant volatile constituents of cocoa. A considerably higher pH is required for the Maillard process to produce maltol as well as several nitrogen-containing molecules like pyrazines, the concentration of which in fermented cocoa beans may be reduced by a low pH and depends on the roasting treatment's temperature and duration. The majority of pyrazine formation occurs once amino ketones react, frequently with the addition of a Strecker aldehyde as a substituent on the pyrazine ring. The breakdown of glucose, which is initiated through an amino acid mostly in the Maillard reaction, results in the production of highly reactive dicarbonyl molecules such glyoxal and methylglyoxal, which are converted into amino ketones. They could be made only from glucose and glycine, and in this case, the main byproducts are pyrazine and methylpyrazine. Nonetheless, side chains could also be added to the pyrazine ring by the inclusion of an aldehyde that can also react with a dihydropyrazine in a process known as the "a + a + b" mechanism, where "a" is the dicarbonyl or hydroxycarbonyl and "b" is an aldehyde. By incorporating valine into a glucose/glycine structure, the Strecker aldehyde 2-methylpropanal will be produced, which when added to the pyrazine molecule will result in pyrazines with a 2-methylpropyl substituent. This aldehyde can be produced by the Maillard reaction from the Strecker degradation of the specific amino acid. Additionally, the relationship between the alkylpyrazines 2,3-dimethylpyrazine, tetramethylpyrazine and trimethylpyrazine is utilized to gauge the extent of cocoa roasting [13,14,16,17,19–21]. 2,5-diketopiperazines, another type of heterocyclic molecule that contributes to the choco-

late flavour by adding pleasant bitterness, were discovered to be possible identifiers of cocoa bean variety as well as indications of postharvest heat processing [22]. Additionally, a crucial function for alanine and/or its Strecker aldehyde, acetaldehyde, in the creation of chocolate odour is suggested by the presence of an ethyl group in several pyrazine compounds present in cocoa and chocolate [20]. It was determined that the side groups on dioxo-compounds control the precise pyrazine structure. For instance, pyruvaldehyde and valine produce the nutty-tasting 2-methyl-propanal and 2,5-dimethyl pyrazine as their final products [13]. It was discovered that temperatures over 130 °C and a roasting time over 25 min had a positive impact on the concentration of all methylpyrazines [17,23]. The rapid drying of cocoa beans, on the other hand, traps volatile acids inside the beans, which is undesirable because the high acid concentration can result in an off-putting aroma of both cocoa and chocolate [15].

2.1.3. Conching Stage of Chocolate Manufacturing

Conching is the most significant final process in the creation of chocolate from a technological standpoint, influencing both its texture and aroma, which typically causes a change in colour as a result of tannin oxidation and emulsification. The flavour profile is enhanced while the content of free acids and some other aromatic cocoa byproducts is decreased throughout this process [13,15]. It involves mixing hot liquids for at least eight hours in conch-style tanks. The effect of conching on the concentration of volatile chemicals during chocolate manufacture is still up for debate, but comparisons between before and after conching show that, despite the heating method not producing any new important odourants, levels of branched pyrazines increase while the majority of Strecker aldehydes are lost through evaporation [20,21,24]. There is general agreement that following conching, chocolates exhibit noticeably less overall off flavour from polyphenols and other byproducts. Heterocycles only saw an increase in concentrations of the least volatile substances, including tri- or tetramethylpyrazine, isobutyl and isopentylpyrazines [13].

2.2. Alkylpyrazines Detected in Cocoa and Chocolate

Over the years, numerous scientists have studied the unique combination of the components inside cocoa and chocolate. In Bonvehí et al.'s research [17], the pyrazine-related molecules made up just over 40% of the cocoa powder essence. In total, 40 pyrazines which are present in cocoa and chocolate among the literature sources were identified. In Figure 1, their formulas are displayed.

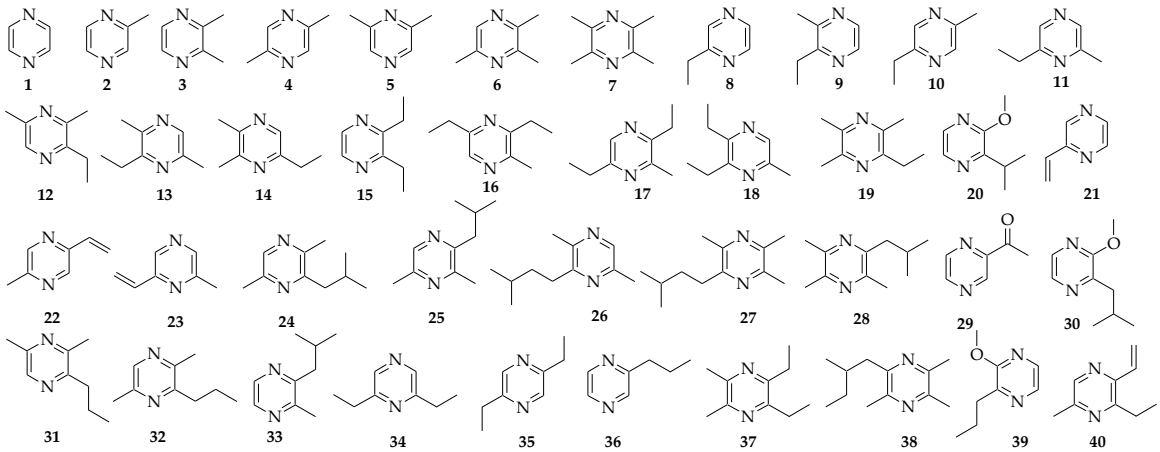

Figure 1. Pyrazines, which were detected in samples of cocoa and chocolate.

The "odour fingerprint" of each alkylpyrazine present in cocoa and chocolate is distinctive. We gathered information on their olfactory profiles from literature sources and from the Good Scents Company Information System (TGSCIS) [25], and we compiled the information in Table 1.

Table 1. The aroma profile of the pyrazines found in cocoa and chocolate.

CIN	Compound name	LSOP in Cocoa	LSOP in DC	LSOP in MC	LS of Odour Description	Odour Description from Literature	Odour Description from The Good Scent Informational System
1	pyrazine	[17]	[13,20]		[12,13,17,26]	pungent, sweet, corn-like, nutty, chocolate, hazelnut, green	pungent, sweet, corn, roasted, hazelnut, barley
2	2-methylpyrazine	[14,17,23]	[13,15,20,21,24]		[12,17,20,21,24,26]	nutty, hazelnut, chocolate, cocoa, roasted, green, sweet, meat, fruity	nutty, cocoa, roasted, chocolate, peanut, green, earthy
3	2,3-dimethylpyrazine	[14,17,22,27]	[13,15,20,21,24]		[12,17,18,21,24,28]	caramel, cocoa, hazelnut, nutty, roasted, bitter, green, coffee, potato, baked	nutty, cocoa, peanut butter, coffee, caramel, roasted potato, musty
4	2,5-dimethylpyrazine	[14,17,19,23,27]	[13,15,20,21,24,29]		[12,13,17–19,21,24,28,29]	cocoa, roasted nuts, rum, fried potato, popcorn, chocolate, chemical, ether, green, roasted barley, butter	cocoa, roasted, nutty, beef, woody, grassy, medicinal, earthy
5	2,6-dimethylpyrazine	[14,17,23]	[21]		[12,17,18,21,28]	nutty, peanut, coffee, green, ether, chocolate, fruity, sweet, roasted, potato	ether, cocoa, nutty, roasted, beefy, coffee, buttermilk
6	trimethylpyrazine	[7,10,14,16,17,19,23,27,28,30]	[13,15,20,21,24,29]	[13]	[3,7,10,12,13,16,17,19–21,24,28–30]	earthy, cocoa, roasted, nutty, peanut, baked, fried potato, fruity, ether, pungent, green, vegetable, sweet, beans	nutty, earthy, powdery, cocoa, potato, roasted
7	tetramethylpyrazine	[10,19,23,27]	[13,15,20,21,24]		[10,12,13,19–21,24]	candy, chocolate, sweet, milky, nutty, peanut, grassy, green, mocha coffee, sour, roasted, beans	nutty, musty, chocolate, coffee, cocoa, burnt, musty, vanilla
8	2-ethylpyrazine	[14,17]	[13,20,21,29]		[13,17,20,21,26,29]	peanut, peanut butter, nutty, roasted, rum, ether, cereal, musty, green, sweet	peanut butter, nutty, woody, roasted, cocoa, coffee, meaty
9	2-ethyl-3-methylpyrazine	[14,17,23]	[13,20,21]		[13,17,21,28]	hazelnut, roasted, raw potato	nutty, musty, corn, raw, earthy, bready
10	2-ethyl-5-methylpyrazine	[14,17]	[13,20,21]		[13,17,18,21]	nutty, raw potato, roasted, grassy, green, cocoa	coffee, beans, nutty, grassy, roasted
11	2-ethyl-6-methylpyrazine	[14,19,27]	[13,15,20,21]		[13,18,19,21]	cocoa, roasted, potato, green, nutty, popcorn, sweet	roasted, potato
12	2-ethyl-3,5-dimethylpyrazine	[7,13,16,30]	[13,15,20,21,29,31]	[13]	[3,7,13,16,20,28–31]	earthy, chocolate, roasted, sweet, woody, potato chips, smoky, praline, rum, vegetable	burnt, coffee, nutty, roasted, woody, potato-chip-like,
13	2-ethyl-3,6-dimethylpyrazine	[7,14,16,19,23,27,30]	[13,20,21,29,31]	[13]	[3,7,13,19,20,28–31]	praline, rum, nutty, earthy, potato, popcorn, roasted, smoky, vegetable	burnt, coffee, nutty, roasted, woody
14	2-ethyl-5,6-dimethylpyrazine	[10,14]	[21,29]		[10,21,28,29,32]	deep roasted, cocoa, chocolate, baked potato, earthy, nutty	burnt, popcorn, roasted, cocoa
15	2,3-diethylpyrazine	[14,17]			[17,28]	nutty, hazelnut, cereal, meaty, earthy	raw, nutty, pepper, bell pepper
16	3,5-diethyl-2-methylpyrazine	[14,19,23,27]	[13,20]		[13,19,20,28]	cocoa, chocolate, rum, roasted, nutty, green, bell pepper, popcorn, sweet	nutty, meaty, vegetable

Table 1. *Cont.*

CIN	Compound name	LSOP in Cocoa	LSOP in DC	LSOP in MC	LS of Odour Description	Odour Description from Literature	Odour Description from The Good Scent Informational System
17	2,5-diethyl-3-methylpyrazine	[14,28]	[13,20]		[13,20]	cocoa, chocolate, rum, roasted, meaty, sweet	hazelnut, roasted, meaty
18	2,3-diethyl-5-methylpyrazine	[7,13,14,16,23,30]	[13,20,30]	[13]	[3,13,16,28,30,31,33,34]	nutty, cocoa, roasted, vegetable, earthy, chocolate, potato chips	musty, nutty, earthy, roasted, potato, dusty, vegetable, green, meaty
19	2,3,5-trimethyl-6-ethylpyrazine	[10,14]	[15,21,24]		[21,24]	chocolate, cocoa, coffee, sweet, hazelnut, roasted	-
20	2-methoxy-3-isopropylpyrazine	[13,30]	[13,20,31]	[13]	[3,13,20,28,30,31,33]	bell pepper, earthy, green pea, beans, hazelnut,	vegetable, earthy, potato
21	2-ethenylpyrazine		[13,20]			nutty	nutty, hazelnut
22	2-ethenyl-5-methylpyrazine	[29]	[29]		[29]	vegetal, earthy, roasted, coffee	coffee
23	2-ethenyl-6-methylpyrazine	[27]	[13,20,21,29]		[13,20,21,29]	roasted, smoked, praline, rum, nutty, hazelnut, vegetable, earthy	nutty
24	2,5-dimethyl-3-isobutylpyrazine		[13,20,29]		[13,20,29]	cocoa, hazelnut, musty, earthy, roasted, nutty, vegetable, pepper	cocoa, hazelnut, musty, earthy, roasted, nutty
25	2,6-dimethyl-3-isobutylpyrazine		[13,20]		[13,20]	cocoa, hazelnut, musty, earthy, roasted, nutty, vegetable, pepper	cocoa, hazelnut, musty, earthy, roasted, nutty
26	2,5-dimethyl-3-isopenthylpyrazine		[13,20]		[13,20]	roasted, sweet, green	fruity
27	2,3,5-trimethyl-6-isopenthylpyrazine	[14]	[29]		[29]	floral, anise, minty	-
28	2,3,5-trimethyl-6-isobutylpyrazine	[29]	[29]		[29]	vegetal, cucumber	-
29	2-acetylpyrazine		[29,31]		[3,12,29]	nutty, popcorn, roasted corn, dirt, burnt, sweet	popcorn, nutty, corn, bread crust, chocolate, hazelnut, coffee
30	2-methoxy-3-isobutylpyrazine	[16]	[31]		[3,12,16,28,31,33]	green bell pepper, green pea, vegetable, hot paprika	pea, green bell pepper
31	2,6-dimethyl-3-propylpyrazine	[23]			[28]	earthy	nutty, hazelnut, roasted
32	2,5-dimethyl-3-propylpyrazine	[23]				-	nutty, hazelnut, roasted
33	2-isobutyl-3-methylpyrazine	[14]				-	herbal, green, sweet
34	2,6-diethylpyrazine	[14,29]	[29]		[29]	vegetable	nutty, hazelnut
35	2,5-diethylpyrazine	[14]				-	nutty, hazelnut
36	2-propylpyrazine	[14]			[25]	nutty, green, vegetable	green, vegetable, nutty, hazelnut, barley, roasted, corn
37	2,3-diethyl-5,6-dimethylpyrazine	[14]				-	-
38	2,3,5-trimethyl-6-(2-methylbutyl)pyrazine	[14]			[32]	coffee	-
39	2-*sec*-butyl-3-methoxypyrazine		[31]		[31]	pea, roasted	musty, green pea, bell pepper, green, vegetable, nutty, potato
40	3-ethyl-5-methyl-2-ethenylpyrazine		[31]		[31]	roasted, popcorn	earthy

CIN—compound identification number; LSOP—literature sources of presence; LS—literature source; DC—dark chocolate; MC—milk chocolate.

The orthonasal profiles of cocoa and dark chocolate (expressed as the number of pyrazine compounds with specific aroma characteristics found in cocoa and dark chocolate) are presented in Figure 2. Despite having fairly comparable characteristics, dark chocolate samples had somewhat higher levels of pyrazine chemicals along with nutty, roasted, cocoa, grainy, potato, meat, earthy and green odour characteristics, proving the assumption that the conching stage produces an even deeper scent profile of cocoa goods.

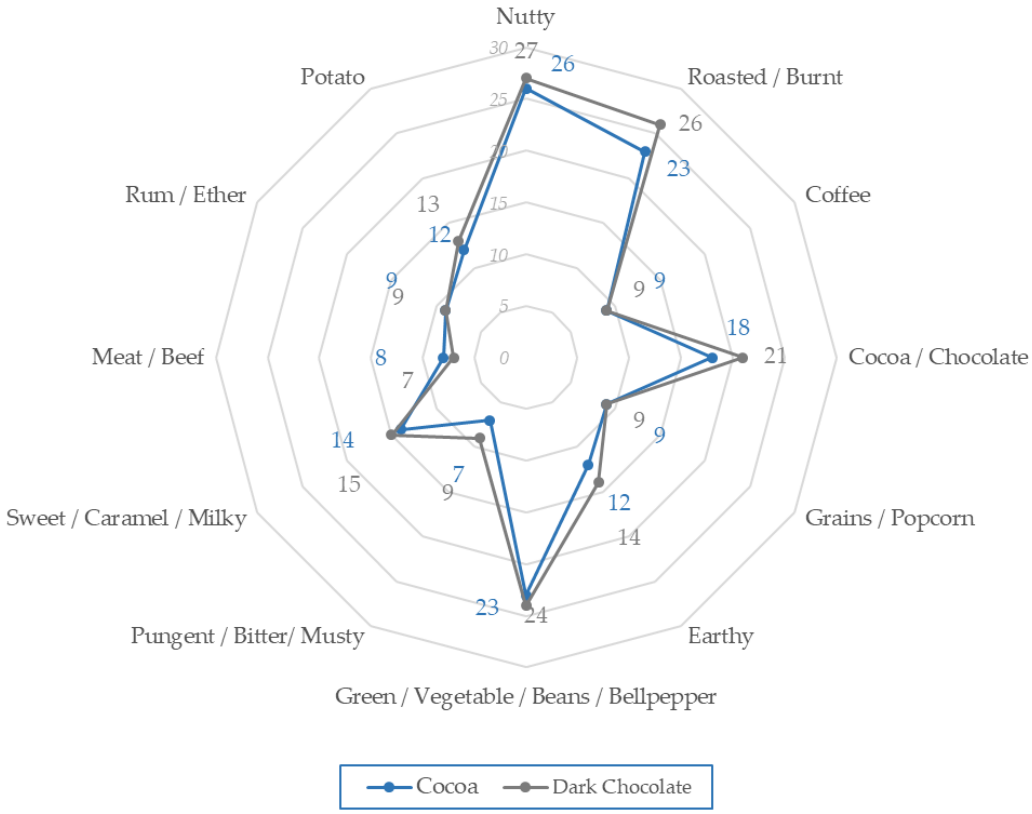

Figure 2. Alkylpyrazines orthonasal profile of cocoa and dark chocolate.

3. Antimicrobial Activity of Pyrazines

Chocolate and cocoa products are well known for their distinguished aroma qualities. One of the most important odour-active groups of compounds present in chocolate is pyrazines. We decided to evaluate the useful biological activities of those good-smelling structures, namely their antibacterial, antioomycete and fungicidal activities.

Pyrazines are widely spread in nature. In addition to being found in vegetables, fruits, herbs and heated food—in which they play a key role in the flavour—they also appear frequently in animal and plant life processes: insects produce pyrazines as semiochemicals for inter- and intraspecific communications [35]; 2,5-dimethylpyrazine inhibits the effect of estradiol and plays a volatile pheromone role in rats; pyrazine, methylpyrazine, ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, trimethylpyrazine and 2-methoxy-3-methylpyrazine diminish vascularization and the development of embryonic tissue, and reduce oviduct function in mammals [36]. The widespread presence of methoxypyrazines in insects has given rise to the hypothesis that they act as all-purpose warning signals of toxic insects [37].

The alkylpyrazines present in cocoa are broadly applied in healthcare. Tetramethylpyrazine, a key ingredient in ligustrazine (an extract of Ligustricum wallichii), is commonly used in Chinese traditional medicine to treat cardiovascular diseases or to enhance blood flow during an ischemic attack in coronary vessels [38]. It was proved that tetramethylpyrazine influences ATP-dependent K^+ channels, tetrodotoxin-resistant Na^+ channels and voltage-dependent Ca^{2+} channels. Additionally, it is known to stimulate the

α_1-adrenoreceptors of smooth muscles [36,39]. By lowering NADPH-oxidase activity in the rat kidney epithelial cell-like cell line NRK-52E, tetramethylpyrazine has antioxidative and anti-inflammatory actions [40]. 2,5-Dimethylpyrazine was found to lengthen the period of therapeutic sleep in mice induced by phenobarbital, as well as to promote GABAergic activity in the mouse's central nervous system. Additionally, rat cognitive abilities are considerably enhanced by tetramethylpyrazine [39]. Due to the recently identified interaction between the corresponding carboxylic acid metabolite and the G-protein coupled receptor GPR109A, 2,5-dimethylpyrazine therapy decreased the plasma levels of nonesterified fatty acids in rats [40].

Derivatives of pyrazine are widely used as medicinally important compounds. The pyrazine ring is a basic setting structure in drug design. There is currently a massive range of therapeutic pyrazine derivatives utilized in the medical field: antimycobacterial (Pyrazinamide, Morinamide, Sulfametopyrazine), antiviral (Favipiravir), anticancer (Bortezomib, Zibotentan), diuretic (Amiloride, Benzamil), anti-insomnia (Zopiclone, Eszopiclone), antidiabetic (Glipizide), treatment for Alzheimer's disease (Elenbecestat), insecticide and nematicide (Thionazine), antidandruff (2-Mercaptopyrazine), hypolipidemic (Acipimox), antitumour (Oltipraz), antihepatitis C (Telaprevir), curing smoking addiction (Varenicline), antidepressant (WAY-208,466), μ-opioid agonist (Mirfentanil) for pulmonary arterial hypertension as a PGI-2 receptor agonist (Selexipag) and other therapeutic activities are still being researched [41,42].

Pyrazines have been given GRAS (generally recognized as safe) status by the Flavor and Extract Manufacturers Association (FEMA) for use as flavouring additives in food [43,44]; they are as well included in the European Union's list of authorized flavouring agents [18], demonstrating that this class of chemicals is a secure substitute for managing and combating microbial contamination. Moreover, around 700 kg of alkylpyrazines are used as flavouring agents annually in the United States, resulting in an estimated average intake of about 37 mg/kg per person [40]. The possible antimicrobial activities of the pyrazine compounds found in cocoa products were the focus of this paper.

3.1. Antibacterial Activity

Tetramethylpyrazine (TMP), as an illustrative example, revealed itself in studies as an active antibacterial agent for various strains of bacteria. TMP, also known as ligustrazine, is a bioactive compound that has been known for its antibacterial properties in Ancient Chinese medicine [45,46]. Liu et al. [47] studied the TMP effect on gut opportunistic bacteria such as Clostridium perfringens, Escherichia coli, gram-negative bacteria and Salmonella of broilers with necrotic enteritis (NE). The populations of gut opportunistic bacteria expanded in infected broilers ($p < 0.05$), which was then diminished by the introduction of TMP ($p < 0.05$) into the feeding scheme. Compelling linear and quadratic trends of the implication of TMP on the growth of Salmonella ($p = 0.001$) and gram-negative bacteria ($p = 0.000$) were spotted. Although the level of bacterial contamination was below the control, TMP also weakened the enterotoxin markers, including the level of endotoxin and diamine oxidase, which further indicated its potent antibacterial properties. They additionally detected the antioxidative and anti-inflammatory properties of TMP by reducing inflammatory factors such as tumour necrosis factor-α (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6); reducing oxidative products such as malondialdehyde (MDA) and protein carbonyl (PCO); increasing the level of antioxidation factors; and reducing glutathione (r-GSH), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), which could serve as an additional beneficial impact on contaminated organisms. Liu et al. [48] studied the influence of TMP addition to those infected with Salmonella Typhimurium. They found a decreasing effect on Salmonella load in the ileum, spleen, mesenteric lymphoid follicles and skin, compared with the negative control ($p < 0.05$), and S. Typhimurium was challenged ($p < 0.01$) by 11.4% to 23.6% and 13.8% to 45.8%. TMP was found to be active against Campylobacter jejuni load in rabbits [46]. C. jejuni is a fast-spreading bacteria, which could infect humans by consuming contaminated food, particularly poultry. Treatments with different doses of

TMP showed a partial inhibition of C. jejuni in rabbit caecal content, skin, liver and spleen. An increase in the dose of TMP provoked a linear ($p < 0.001$) and quadratic ($p \leq 0.026$) reduction in C. jejuni loads in the liver and spleen, which may be interpreted as affecting the influence of TMP on bacterial transmission. It was discovered that a dietary addition of TMP also activates the production of endogenous antimicrobial peptides in the caecum (NP4 = defencing neutrophil peptide 4, MCP2 = macrophage cationic peptide 2, LGALS3 = galectin 3, CAMP = cathelicidin antimicrobial peptide) and skin (SEM = standard error of the mean, NP4, MCP2, LGALS3). The best results presented CAMP with a linear trend ($p < 0.001$) and reached the control level along with LGALS3 with a linear trend ($p < 0.001$) in the caecum. It was suggested by Zou et al. [45] that TMP and other pyrazine compounds provide an antibacterial effect by purifying reactive oxygen species, which act as a suppressor of bacterial growth and inflammation.

Pyrazine compounds could be exploited as antimicrobial agents against plant infections in addition to being antibacterial agents for humans and animals. Recently, volatile organic compounds (VOCs) produced by microorganisms have been evaluated as a new way of fighting plant microbial contamination [49]. 2,3-Dimethylpyrazine and 2,5-dimethylpyrazine antibacterial efficacy was tested on 10^3 *E. coli* cells with 0.3%, 0.6% and 1.2% dosages incubated for 2, 4 and 6 h. Even though 2,3-dimethyl-pyrazine caused no reduction in bacterial colonies, 2,5-dimethylpyrazine showed a great decrease in *E. coli* cell concentration. Higher doses applied and a longer incubation time boosted the effect. The bactericidal effect appeared at a 1.2% concentration regardless of the period of incubation and at 0.6% starting from the fourth hour of incubation [35]. Pyrazine derivatives were discovered to be the predominant class of chemical constituents in Black pepper root entophytic Bacillus megaterium BP17 volatiles after a dynamic headspace study. Pyrazine and 2,5-dimethylpyrazine were among those with the highest amounts. Ethylpyrazine, methylpyrazine and 2-ethyl-3-methylpyrazine were also found. The antibacterial compounds 2-ethyl-3-methylpyrazine (growth inhibition 72.89% at 335 μg/mL, 91.65% at 504 μg/mL and 95.9% at 672 μg/mL) and 2,5-dimethylpyrazine (growth inhibition 69.75% at 504 μg/mL and 79.87% at 672 μg/mL) exhibited impressive activity against the soil-borne plant pathogenic bacterium Ralstonia solanacearum, which frequently annihilates Irish potatoes, tomatoes, peppers and eggplants [50].

The idea of applying pyrazines to food is a good concept for the food industry since they have antibacterial and smell- and taste-improving characteristics. For example, 2-isobutyl-3-methylpyrazine was used for the first time as a potential preservative for chicken meat strips by Schöck et al. [44]. Studies have shown that coupling maltodextrin with 2-isobutyl-3-methylpyrazine may reduce the microbial contamination of processed meat. Additionally, using this alkylpyrazine may enhance the flavour and aroma of food by introducing roasted undertones. The testing of 2-isobutyl-3-methylpyrazine on *Escherichia coli* and *Staphylococcus aureus* showed strong bactericidal properties (*E. coli* minimum inhibition concentration (MIC)—3 mg/mL, minimum bactericidal concentration (MBC)—3 mg/mL; *S. aureus* MIC—5 mg/mL, MBC—6 mg/mL), with the same MIC and MBC towards *S. aureus* as 5-isobutyl-2,3-dimethylpyrazine, which was found by Lange [35] as a strong antibacterial and antifungal agent. An additional comparison of the impact of 2-isobutyl-3-methylpyrazine, 2-isopropyl-5-methylpyrazine, 2-isobutylpyrazine and 2-isopropylpyrazine on *E. coli* strains (10^5–10^7 CFU/mL) with 0.3% doses showed 2-isobutyl-3-methylpyrazine to be bactericidal to 10^6 and 10^7 CFU/mL, while other pyrazine derivatives showed to be lethal only on a 10^5 CFU/mL invasion level. The findings demonstrate that incubation time has less of an impact on the application efficiency of pyrazine derivatives than chemical concentration. The study of Haidar et al. [51] provides additional support for this theory, showing that the addition of concentrations of 100 and 200 μL of the bacterial volatile product 2,5-dimethylpyrazine, produced by Bacillus pumillus, significantly lowered Phaeomoniella chlamydospora mycelial growth by 55 and 67%, respectively.

The study by Fabio et al. [52] proposed a completely novel method of visualizing the action of the Pyrazinamide (PA, pyrazine-2-carboxamide) on Mycobacterium tuberculosis

depending on the pH. PA is a crucial element of medical protocols for treating tuberculosis for approximately 65 years. It was shown that PA functions as a prodrug by converting into pyrazinoate through an enzymatic process and existing in tubercular cells in equilibrium with pyrazinoic acid, with a correlation between pH and the relative amount of both forms. The graphic image of this process is presented in Figure 3. Over a variety of pH values, the impacts of total pyrazinoic acid (pyrazinoic acid + pyrazinoate) on an M. tuberculosis culture were evaluated. In neutral pH environments, M. tuberculosis showed resistance to PA and required high dosages to have antibacterial activity; however, in acidic pH environments, the concentration of medication required to destroy bacterial colonies was lower. The total amount of growth inhibition caused by pyrazinoic acid rose as the pH of the culture medium was lowered from 7.3 to 6.4. Total pyrazinoic acid, when added to bacterial suspensions, enhanced either the acidification of the bacterial cytoplasm and loss of membrane potential when the ambient pH decreased, which is consistent with growth inhibition. According to the findings, pyrazinoic acid, which is thought to be a protonophore and operates as a proton uncoupler, is the drug's active form.

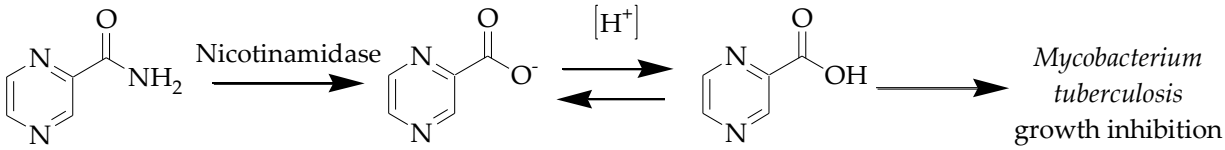

Figure 3. Mechanism of antitubercular activity of Pyrazinamide.

This suggests a molecular explanation for why the drug's activity is pH dependent. The metabolism of alkylpyrazines in the human body was the subject of study by Kremer et al. [40]. It was revealed in which way 2-methylpyrazine, which is typically present in chocolate and cocoa in high concentrations [17], can be metabolized. Following the consumption of 20–31.2 µg/kg of 2-methylpyrazine, pyrazine-2-carboxylic acid and 5-hydroxypyrazine-2-carboxylic acid were detected in the urine of experimental individuals in proportions of 64% and 26%, respectively.

By merging those two studies, it may be assumed that 2-methylpyrazine intake, whose metabolite is pyrazine-2-carboxylic acid, may adversely affect the use of tubercular medicine PA, whose active form is the same molecule.

3.2. Antifungal and Antioomycete Activity

Multiple studies revealed how pyrazine compounds had potent antifungal and antioomycete properties. The antagonism of those substances against oomycetes and funguses that are harmful to plants is particularly prevalent. Agisha et al. [43] discovered the properties of pyrazine derivatives produced by plant endophyte Pseudomonas putida BP25—2: 2,5-dimethylpyrazine; 2-methylpyrazine; 2-ethyl-5-methylpyrazine; and 2-ethyl-3,6-dimethylpyrazine. Those volatile organic compounds (VOCs) performed compelling inhibitory activity against oomycete pathogens, Phytophthora capsici and Pythium myriotylum; fungal pathogens, Rhizoctonia solani, Colletotrichum gloeosporioides, Athelia rolfsii, Gibberella moniliformis and Magnaporthe oryzae; bacterial pathogen, Ralstonia pseudosolanacearum and plant parasitic nematode, Radopholus similis. 2-Ethyl-3,6-dimethylpyrazine was the compound with the most impressive performance against the majority of the pathogens and its EC50 (half maximal effective concentration) values varied between 13 and 145.8 µg/mL. 2-Ethyl-5-methylpyrazine and 2-ethyl-3,6-dimethylpyrazine fully restrained bacterial growth on a CPG agar at 339 and 509 µg/mL, respectively. Against Phytophthora rot on Black pepper shoot cuttings 2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine and 2-ethyl-3,6-dimethyl-pyrazine performed a contraction of contamination at 21 µg/mL, and 2-methylpyrazine at 42 µg/mL, with no signals of toxicity.

Pyrazine derivatives including 2,5-dimethylpyrazine, 2-ethyl-3-methylpyrazine, 2-ethylpyrazine and 2-methyl pyrazine were shown by Munjal et al. [50] in their study on

Black pepper root entophytic Bacillus megaterium BP17 volatiles as effective antifungal and antioomycete chemicals. At concentrations of 504 μg/mL for 2,5-dimethylpyrazine, 2-ethyl-3-methylpyrazine and 2-ethylpyrazine and 672 μg/mL for 2-methylpyrazine, trials on the oomycete Phytophthora capsici revealed the full suppression of mycelial growth. The same potent inhibition of the fungus Magnaporthe oryzae's mycelial growth was seen: total inhibition for 2-ethyl-3-methylpyrazine at 168 μg/mL, total inhibition for 2-ethylpyrazine and 2-methylpyrazine at 672 μg/mL and 73.68% inhibition for 2,5-dimethylpyrazine at 672 μg/mL.

2-Methylpyrazine was found to be a major volatile product synthesized by four bacterial strains of Bacillus spp. and performed a strong inhibition of the Colletotrichum gloeosporioides in the concentration of 100 μL/L, inhibiting mycelial growth from 8.59 cm to 1.45 cm. C. gloeosporioides is one of the most common Colletotrichum fungal plant pathogens which provokes plant disease anthracnose [49]. It was found that 2,5-dimethylpyrazine, trimethylpyrazine and tetramethylpyrazine as one of the products of the metabolism of cyanogenic Pseudomonas strains are active against oomycete Phytophthora infestans, which is considered one of the harshest and widespread pathogens of Solanum tuberosum [53].

Chuankum et al. [54] found that despite not showing a decrease in the germination of fungal spores, pyrazine and 2,5-dimethylpyrazine are formed in fungistatic soils and may contribute to the process of soil volatile fungistatication through synergistic actions. Pyrazine derivatives were identified as the primary components of bacterial metabolism in the gas chromatography–mass spectrometry (GC-MS) study of the volatiles of two bacterial strains, Bacillus pumilus and *Paenibacillus* species. Bacillus pumilus mostly produced 2,5-dimethylpyrazine, with lower amounts of 2-ethyl-3,5-dimethylpyrazine. The *Paenibacillus* species metabolic products contained the highest quantities of not recognized pyrazine derivative and 2,6-bis(2-methylpropyl)-pyrazine. The presence of pyrazine compounds was at least 90% in those strains; in addition, they had high in vitro mycelial growth inhibition activity against Phaeomoniella chlamydospora, the most common fungus responsible for Esca disease, which majorly impacts the world wine industry by infecting grapes (inhibition rates of 71 and 74.3%, respectively, by *B. pumilus* and *Paenibacillus* sp.), highlighting the crucial function of pyrazine derivatives in fungal antagonism [51].

In research by Mülner et al. [55], mass spectrometric identification was conducted on microbial volatiles of strains associated with antagonistic effects on *Sclerotinia sclerotiorum* and *Rhizoctonia Solani*. Out of 41 microbial volatiles, seven were pyrazine derivatives: 2-methyl-5-(1-methylethyl)-pyrazine, 2-isobutyl-3-methylpyrazine, 2-methoxy-3-(2-methylpropyl)-pyrazine, 2,6-dimethyl-3-sec-butylpyrazine, 2,3-dimethyl-5-(2-methylpropyl)-pyrazine, 2,5-dimethyl-3-(2-methylpropyl)-pyrazine and 2-ethylpyrazine, produced mainly by *Bacillus cereus* (RS-MS53) and Bacillus aerius (RS-So365). 2-Ethylpyrazine and 2,3-dimethyl-5-isobutylpyrazine applied separately or combined were found to significantly lower the germination level of sclerotia of Sclerotinia sclerotiorum after 96 h of incubation on potato-dextrose agar plates.

The testing of 2-isobutyl-3-methylpyrazine applications for the treatment of the fungus *Candida albicans* H5 showed the inhibition (MIC) and then total destruction (MFC) of fungal colonies on a 4 mg/mL concentration application. Slightly better results out of all the tested pyrazines showed 5-isobutyl-2,3-dimethylpyazine, with MIC = 3 mg/mL and MFC = 4 mg/mL [35].

VOCs bioactivity, which was produced by *Lysobacter capsici* AZ78, was evaluated against soil-borne fungal plant pathogens, such as *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia minor* [56]. The most consistent compounds found in the PDA medium of the volatile profile were 2,5-dimethylpyrazine, 2-isopropyl-3-methoxypyrazine and 2-ethyl-3-methoxypyrazine, out of which the first and second are present in chocolate. The MIC was measured on three strains of previously mentioned pathogens. To three different pathogen strains, all three compounds exhibited antibacterial activity. The most vulnerable to the action of pyrazine derivatives was *S. minor*, as it was inhibited by the addition of 7.5 mg of 2-ethyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine for more than

80% and by the addition of 7.5 mg of 2,5-dimethylpyrazine for more than 50%. R. solani turned out to be the most stable: a maximum of more than 50% of growth was inhibited by relatively high doses of 2,5-dimethypyrazine (10 mg) and was inhibited for more than 10% by 2-ethyl-3-methoxypyrazine (7.5 mg).

3.3. Structure–Antimicrobial Activity Relationship of Alkylpyrazines

Lange [35], based on her research, hypothesized that differences in the antibacterial activity of alkylpyrazines would result from the substance's hydrophobicity. These conclusions were reached by comparing the MIC and MBC of a series of pyrazine derivatives with the corresponding structures' Log *P* values (partition coefficient, which is the ratio of concentrations of a compound in a mixture of two immiscible solvents at equilibrium). First, the Log *P* coefficient values and antibacterial activity against *E. coli* and *S. aureus* were ranked. Starting with the strongest antibacterial properties of 5-isobutyl-2,3-dimethylpyrazine (Log *P* = 2.32, MIC *E. coli* = 3, MIC *S. aureus* = 4), following 2-isobutyl-3-methylpyrazine (Log *P* = 1.96, MIC *E. coli* = 3, MIC *S. aureus* = 5), 2-isobutylpyrazine (Log *P* = 1.715, MIC *E. coli* = 4, MIC *S. aureus* = 8), 2-isopropyl-5-methylpyrazine (Log *P* = 1.626, MIC *E. coli* = 5, MIC *S. aureus* = 10) and ending with 2-isopropylpyrazine (Log *P* = 1.281, MIC *E. coli* = 7, MIC *S. aureus* =13) being the weakest. The more hydrophobic structures appear to achieve a limited suppression of microbial cells at lower doses, according to the correlation between those values. This theory was further supported by a comparison of 2,3-dimethylpyrazine's lower Log *P* and a lack of antibacterial capabilities to 2,5-dimethylpyrazine's stronger hydrophobic and antibacterial qualities. The notion that the action of pyrazines on the microbial membrane is what causes them to be germ destroying may then follow. It demonstrates that treating gram-positive *S. aureus*, which has a larger and stronger cell membrane, required higher amounts of the chemical compared with treating gram-negative *E. coli*. Wide hydrophobic surfaces are present in pyrazine compounds, and they vary depending on the substitution position in the molecule. Tetramethylpyrazine may successfully cross the brain's hematoencephalic barrier, according to Tsai et al. [38]. Moreover, tetramethylpyrazine was transported via the transcellular pathway via a partition mechanism in an experiment by Chen et al. [57], as was infiltrated buccal mucosa by a passive diffusion process. These results validate the idea that pyrazine chemicals readily dissolve in the lipid bilayers of biological membranes. Nie et al. [58] compared substituents of tetramethylpyrazine, triethylpyrazine and tetraethylpyrazine and how they grow more complicated structurally and more lipophilic; these medications will partition into the lipid membrane and modify the fluidity of the membrane, which may result in a quantitative change in their biological activity, in addition to their interactions with membrane proteins. In a conducted study [36], 2-methylpyrazine, 2,5-dimethylpyrazine, 2,3-dimethylpyrazine and tetramethylpyrazine were positioned on various levels of the membrane depending on how hydrophobic they were. In the deepest area of the bilayer centre, the most hydrophobic tetramethylpyrazine was discovered. As a result of this, it may be assumed that the number and length of the substitutes of the pyrazine ring and the free volume distribution in the bilayer have an impact on pyrazine derivatives' lateral diffusion and the point of accumulation. By easily entering into the bilayer of microbial cell on different depths, due to high hydrophobicity and relatively tiny molecule dimensions, some alkylpyrazines may directly interact with membrane proteins, for example, G-protein, and cause membrane deformation through the modification of their lipid environment. Those actions may be damaging or lethal to bacteria cell [35,36].

Results from a literature analysis of the antimicrobial properties of alkylpyrazines from chocolate are summarized in Tables 2 and 3 below.

Table 2. Antibacterial and antinematode action of alkylpyrazines found in literature sources.

CIN	Pyrazine Compound	*Campylobacter jejuni*	*Clostridium perfringens*	*Escherichia coli*	*Ralstonia pseudosolanacearum*	*Ralstonia solanacearum*	*Salmonella*	*Staphylococcus aureus*	*Plant parasitic nematode Radopholus similis*
2	methylpyrazine				[43]				[43]
4	2,5-dimethylpyrazine			[50]	[43]	[50]			[43]
7	tetramethylpyrazine	[46]	[47]	[47]			[47,48]		
9	2-ethyl-3-metylpyrazine					[50]			
10	2-ethyl-5-methylpyrazine				[43]				[43]
13	2-ethyl-3,6-dimethylpyrazine				[43]				[43]
33	2-isobutyl-3-methylpyrazine			[35]				[35]	

Table 3. Antifungal and antioomycete action of alkylpyrazines found in literature sources.

CIN	Pyrazine Compound	*Athelia rolfsii*	*Candida albicans*	*Colletotrichum gloeosporioides*	*Gibberella moniliformis*	*Magnaporthe oryzae*	*Phaeomoniella chlamydospora*	*Phytophthora capsici*	*Phytophthora infestans*	*Phytophthora rot*	*Pythium myriotylum*	*Pythium ultimum*	*Rhizoctonia solani*	*Sclerotinia minor*	*Sclerotinia sclerotiorum*
2	methylpyrazine	[43]		[43,49]	[43]	[43]		[43,50]			[43]		[43]		
4	2,5-dimethylpyrazine	[43]		[43]	[43]	[43,50]	[51]	[43,50]	[53]	[43]	[43]	[56]	[43,56]	[56]	
6	trimethylpyrazine								[53]						
7	tetramethylpyrazine								[53]						
8	ethylpyrazine					[50]		[50]							[55]
9	2-ethyl-3-metylpyrazine					[50]		[50]							
10	2-ethyl-5-methylpyrazine	[43]		[43]	[43]	[43]		[43]		[43]	[43]		[43]		
12	2-ethyl-3,5-dimethylpyrazine						[51]								
13	2-ethyl-3,6-dimethylpyrazine	[43]		[43]	[43]	[43]		[43]		[43]	[43]		[43]		
20	2-methoxy-3-isopropyprazine											[56]	[56]	[56]	
33	2-isobutyl-3-methylpyrazine		[35]												

3.4. AntiBac-Pred and PASS Testing

We decided to undertake the prediction of biological activity against microbes methods on the pyrazine compounds found in cocoa and chocolate as a part of our independent research. With AntiBac-Pred [59], we tested some relevant pyrazine structures. This online tool aims to assist users in making informed decisions about the chemical compounds to be utilized in antibacterial experimentation. Data on antibacterial activity that are accessible in ChEMBL (a database of bioactive molecules with drug-like properties) were used to construct this application. It gives users the option to categorize chemical structures according to whether they inhibit or do not inhibit the growth of 353 distinct bacterial strains,

including resistant and nonresistant strains [60]. The AntiBac-Pred assessment of pyrazine structures revealed antibacterial activity towards Corynebacterium jeikeium, Resistant Mycobacterium tuberculosis and Yersinia pestis. A total of eight representatives were discovered to be potentially active against Yersinia pestis, with high possibility values (Pa > 0.7) for pyrazine, tetramethylpyrazine, 2,3-dimethylpyrazine and 2,5-dimethylpyrazine, and higher medium possibility values (Pa > 0.6) for 2,6-dimethylpyrazine, 2-methylpyrazine and trimethylpyrazine. 2,5-Dimethylpyrazine and 2-methylpyrazine had medium odds (Pa > 0.5) of inhibiting Resistant Mycobacterium tuberculosis. Moreover, it may solidify our hypothesis regarding 2-methylpyrazine having a potential beneficial impact on Pyrazinamide antitubercular activity, by metabolizing to its active form of pyrazine-2-carboxylic acid. Tetramethylpyrazine, which was not included in Table 2, also had a Pa value of 0.5628 against Mycobacterium bovis BCG, and 3-ethyl-5-methyl-2-ethenylpyrazine also had a Pa value of 0.6276 against Micrococcus luteus. Samples, including 2-isobutyl-3-methoxypyrazine, 2,3,5-trimethyl-6-isopenthylpyrazine, 2-isobutyl-3-methylpyrazine and 2-isobutyl-3,5,6-trimethylpyrazine, displayed moderate Pa values (Pa > 0.5) against Corynebacterium jeikeium.

The highly credible predictions for more than 500 biological activities are provided by the PASS program (Version 2.0) [61], which is based on a regression approach applied to noncongeneric chemical series. In leave-one-out cross-validation, the average prediction accuracy is over 85%. The PASS method is based on the study of SARs for the training set, which presently consists of around 46,000 medicines, drug candidates and lead compounds with experimentally determined biological activity. In PASS chemical descriptors, the so-called Multilevel Neighbourhoods of Atoms (MNA) are employed. The structural formula given in the MOL-file (SDF-file) form serves as the foundation for the creation of the collection of MNA descriptors [62,63]. The testing in PASS against Picornavirus, which can cause a number of diseases such as the common cold, poliomyelitis, meningitis, hepatitis and paralysis [64], revealed excellent findings in terms of biological activity from a wide range of pyrazine derivatives. Pyrazine, 2,3-dimethylpyrazine, tetramethylpyrazine and 2-methylpyrazine showed the most likely antiviral (Picornavirus) efficacy. When evaluating the compounds' hypothesized antibacterial activity, results with a Pa greater than 0.5 were deemed relevant and are presented in Table 4.

Table 4. The probability (Pa) for the compounds to produce biological activity related to antibacterial action as predicted by the AntiBac-Pred application, and the probability to be active (Pa) for the compounds to produce biological activity related to antiviral (Picornavirus) action as predicted by the PASS application.

CIN	Compound Name	AntiBac-Pred			PASS
		Yersinia pestis	Resistant *Mycobacterium tuberculosis*	*Corynebacterium jeikeium*	*Picornavirus*
1	pyrazine	0.7689			0.674
7	tetramethylpyrazine	0.7431			0.632
3	2,3-dimethylpyrazine	0.7010			0.635
4	2,5-dimethylpyrazine	0.7010	0.6459		0.591
5	2,6-dimethylpyrazine	0.6560			0.587
2	2-methylpyrazine	0.6050	0.5963		0.601
6	trimethylpyrazine	0.6050			0.522
15	2,3-diethylpyrazine	0.5018			0.564
9	2-ethyl-3-methylpyrazine				0.539
19	2,3,5-trimethyl-6-ethylpyrazine				0.536
30	2-isobutyl-3-methoxypyrazine			0.6632	
28	2,3,5-trimethyl-6-isobutylpyrazine			0.5910	0.532
27	2,3,5-trimethyl-6-isopentylpyrazine			0.5910	
33	2-isobutyl-3-methylpyrazine			0.5242	0.535
8	2-ethylpyrazine				0.523

Summarizing all of the above, the alkylpyrazines present in chocolate and cocoa may be used as effective antibacterial agents in food, for plants and perhaps even as a supplement to current treatments for humans with microbial diseases. Their widespread dispersion in nature and extensive use over a long period of time all across the world determine the safety of their use. There should be more investigations into their antibacterial capabilities.

4. The Quantification of the Impact of Alkylpyrazine Compounds on the Aroma of Cocoa and Chocolate

4.1. Odour Values of Pyrazine Compounds in Cocoa

There are currently close to 10,000 chemicals known thanks to numerous research on the composition of volatile organic compounds (VOCs) of food conducted over the past 50 years. This number is still rising as a result of research efforts in the field of fragrance research, which are concentrated on using GC-MS (gas chromatography–mass spectrometry) to identify all VOCs. It is believed that each VOC that can get to these receptor proteins in the nasal cavity can interact with the human odourant receptors. It is also well known that a specific dose (quantity) of a particular VOC is required to elicit a receptor response. In order to form the overall fragrance image in the brain, only the pattern of reactions to those odourants which can cause a signal at the receptor level ought to do so. The absolute concentration of a volatile required to interact at the human receptor level is currently impossible to directly determine. As a result, it has long been established that odour thresholds (OTs) are a helpful tool for assessing the odour activity of VOCs. The minimum concentration at which an odour may be sensed is known as the OT. A relation between the concentration of a given volatile in the food and the odourant's OT has been presented as a useful technique to evaluate its smell contribution within the set of food volatiles because OT data are frequently provided as concentration per litre. Assigned as an aroma value or odour activity value (OAVs) is this concentration to the OT ratio. To identify odour-active constituents in complex mixtures of odourless volatile constituents, methods using GC-O (gas chromatography–olfactometry) in tandem with dilution to OT methods, such as AEDA (Aroma-Extraction Dilution Analysis) or CHARM (Combined Hedonic Aroma Response Measurement) analysis, as well as other GC-O-related techniques, such as olfactometry global analysis and OSME (the finger span matching method), are all helpful tools [3,7,33]. Following an analysis of the literature sources, pyrazines present in cocoa and chocolate were identified together with their concentrations, flavour dilution factors (FD), odour thresholds (OTs), and odour activity values (OAV). Those values varied significantly in certain situations, based on the origin of the cocoa and the method used to roast and ferment the beans to determine the concentration, as well as the measuring method and environment to determine the OT and FD.

In Table 5 we present information from the literature sources regarding concentration, OT, FD factors and OAVs of cocoa, dark and milk chocolate samples.

Table 5. Concentrations and Odour Parameters of Alkylpyrazines Present in Cocoa and Chocolate.

CIN	Odour Threshold, µg/L	Flavour Dilution Factor			Odour Activity Values	Concentration, mg/kg		
		Cocoa	Dark Chocolate	Milk Chocolate		Cocoa	Dark Chocolate	Milk Chocolate
1	175,000 [a] [12,65], 180,000 [a] [17], 300,000 [a] [26], 500,000 [a] [12]				0.00024 [pw] [17]	0–0.067(0.043) [pw] [17]	0.144 [20]	
2	60 [a] [17], >2000 [b] [28], 27,000 [a] [24], 30,000 [a] [26], 60,000 [a] [12,65], 100,000 [a] [12]		2 [20]		<1 [dc] [24], 4.83 [pw] [17]	0.11–0.37 (0.29) [pw] [17], 0.316–0.364 [pw] [14]	0.009–0.099 [24], 1.329–2.544 [20], 0.8 [15]	
3	400 [a] [12], 800 [a,d] [18,66], 880 [b] [28], 2500 [a] [12,17,65]		256, >4096 [20]		0.084 [pw] [17]	0.01–0.44 (0.21) [pw] [17], 0.097–0.107 [pw] [14], 0.27–3.5 [rcb] [27]	0.034–0.059 [24], 0.59–0.802 [20], 2.74–15.11 1.99–10.18 [15]	
4	7.9 [e] [67], 1700 [a] [17], 1800 [a] [12,65], 1820 [b] [28], 2600 [a] [24]	27 [lq] [19]	81 [19]	27 [19]	0.65 [pw] [17], <1 [dc] [24]	0.204–0.24 [lq] [19], 0.23–1.69 (1.10) [pw] [17], 0.25–2.47 [rcb] [27], 0.474–0.59 [pw] [14]	0.014–0.078 [24], 0.34–0.376 [19], 0.094–1.434 [20], 1.99–10.18 [15]	0.051–0.065 [19]
5	400 [a,d] [18,66], 1500 [a] [12,65], 1720 [b] [28], 9000 [a] [17]				0.027 [pw] [17]	0.11–0.39 (0.24) [pw] [17], 0.109–0.275 [pw] [14]		
6	0.087 [e] [67], 50 [b] [28], 90 [a] [3], 180 [c] [31], 290 [a] [24], 400 [a] [12], 290 [7,16], 1800 [a] [17]	27 [lq] [19], 32 [pw] [30], 128 [pw] [7], 256 [rcb] [16]	243 [19], 256 [20]	32 [30], 81 [19]	0.46 [pw] [17], <1 [pw] [7], <1 [dc] [24], 1 [dc] [31], 3.2 [rcb] [16]	0.073–0.093 [lq] [19], 0.21–1.71 (0.82) [pw] [17], 0.2 [pw] [7], 0.303–0.749 [pw] [14], 0.38–5.39 [rcb] [27], 0.92 [rcb] [16]	0.053–0.307 [24], 0.23–0.245 [31], 0.241–0.283 [19], 1.702–2.359 [20], 15.01–81.39 [15]	0.095–0.129 [19]
7	1000 [a] [12], >2000 [b] [28], 10,000 [a] [17], 38,000 [a] [24]	243 [lq] [19]	81 [19], 2048, >4096 [20]	9 [19]	<1 [dc] [24]	0.13–2.68 [rcb] [27], 1.636–1.714 [lq] [19]	0.128–2.543 [24], 1.064–1.222 [19], 6.135–7.983 [20], 60.31–285.74 [15]	0.223–0.269 [19]
8	>2000 [b] [28], 4000 [a] [26], 6000 [a] [12,17,65]		32 [20]		0.05 [pw] [17]	0.15–0.41 (0.30) [pw] [17], 0.132–0.322 [pw] [14]	0.336–0.539 [20]	
9	0.55 [e] [67], 35 [b] [28], 130 [a] [17,65], 500 [a] [66]				1.62 [pw] [17]	0.13–0.42 (0.21) [pw] [17], 0.015–0.021 [pw] [14]	0.342–0.345 [20]	
10	16 [a,d] [18,66], 100 [a] [17,65]				2.8 [pw] [17]	0.12–0.47 (0.28) [pw] [17], 0.099–0.129 [pw] [14]		
11	40 [a,d] [18,66]	9 [lq] [19]	81 [19]	27 [19]		0.068–0.096 [lq] [19], 0.115–0.133 [pw] [14], 0.29–7.27 [rcb] [27]	0.144–0.164 [19], 0.47–15.23 [15]	0.057–0.087 [19]

Table 5. *Cont.*

CIN	Odour Threshold, µg/L	Flavour Dilution Factor			Odour Activity Values	Concentration, mg/kg		
		Cocoa	Dark Chocolate	Milk Chocolate		Cocoa	Dark Chocolate	Milk Chocolate
12	0.00186 [e] [67], 0.011 [b] [28], 0.04 [a] [3], 0.4 [a] [65], 1.7 [c] [31], 2 [a] [68], 2.2 [c] [3,7,16]	256 [pw, rcb] [13,16,30], 2048 [pw] [7]	32, 256 [20], 512, 1024 [32]	1024 [30]	7.6 [rcb] [16], 14 [pw] [7], 16 [dc], 24 [dc] [31]	0.017 [rcb] [16], 0.031 [pw] [3,7], 0.043–0.055 [lq] [19], 0.14–2.95 [rcb] [27]	0.0273–0.0401 [31], 0.452–0.546 [19], 0.728–1.177 [20], 0.98—6.77 [15]	0.065–0.095 [19]
13	0.00186 [e] [67], 3.6 [b] [28], 8.6 [a] [33], 9 [a] [3], 57 [c] [3,7,16], 76 [c] [31]	27 [lq] [19], 32 [pw] [30], 64 [rcb] [16], 256 [pw] [7]	2, 4 [31], 32, 256 [20], 729 [19]	27 [19], 512 [30]	<1 [dc] [31], 1 [rcb] [16], 1.2 [pw] [7]	0.07 [pw] [3,7], 0.024–0.026 [pw] [14], 0.056 [rcb] [16], 0.23–1.83 [rcb] [27]	0.0556–0.0572 [31], 0.728–1.177 [20]	
14	200 [b] [28], 530 [a] [32,34]					0.235–0.261 [pw] [14]		
15	6.6 [b] [28]					0.11–0.27 [pw] (0.16) [17], 0.057–0.079 [pw] [14]		
16	0.9 [b] [28]	27 [lq] [19]	243 [19], >4096 [20]	9 [19],		0.082–0.122 [lq] [19], 0.18–1.31 [rcb] [27], 1.209–1.291 [pw] [14]	0.152–0.172 [20], 0.214–0.262 [19]	0.045–0.057 [19]
17	>170 [b] [28]		>4096 [20]				0.152–0.172 [20]	
18	0.0002 [e] [67], 0.014 [b] [28], 0.031 [a] [33], 0.09 [a] [3], 0.5 [c] [7,16], 7.2 [c] [31]	256 [pw, rcb] [7,13,16,30]	512, 2048 [31]	512 [30]	<1 [dc], 2 [dc] [31], 6.6 [rcb] [16], 16 [pw] [7]	0.01–0.014 [pw] [14] 0.0033 [rcb] [16], 0.0082 [pw] [3,7]	0.00286–0.0113 [31], 0.8–5.07 [15]	
19						0.047–0.061 [pw] [14]	0.013–0.267 [24]	
20	0.002 [a] [3,12], 0.002 [b] [28] 0.0039 [a] [33], 0.004–0.024 [a] [33], 0.01 [c] [31], 0.024 [a] [66], 0.001–0.1 [a] [67]	512 [pw] [13,30]	2 [31], 128, 512 [20]	64 [30]	1 [dc], 8 [dc] [31]		0.00001–0.00008 [31]	
21	>2000 [b] [28]						0.027–0.042 [20]	
23			8, 16 [20]			0.17–1 [rcb] [27]		
24	>2000 [b] [28]		2, 128 [20]				0.146–0.191 [20]	
25	>2000 [b] [28]		2, 128 [20]				0.146–0.191 [20]	
26			32 [20]				0.392–0.435 [20]	
27						0.044–0.046 [pw] [14]		
29	48 [c] [31], 62 [a] [3]		128 [31]		<1 [dc] [31]		0.0033–0.00535 [31]	
30	0.002 [a] [3,12,65], 0.003 [b] [28] 0.0062 [a] [33], 0.002–0.045 [a] [33], 0.04 [c] [31], 0.045 [a] [66], 0.8 [c] [3,7,16], 0.002–0.1 [a] [69]	128 [rcb] [16]	2 [31]		1.2 [rcb] [16], 6 [dc], 9 [dc] [31], 12 [pw] [7]	0.00085 [pw] [3,7], 0.00094 [rcb] [16]	0.00024–0.00036 [31]	

Table 5. *Cont.*

CIN	Odour Threshold, µg/L	Flavour Dilution Factor			Odour Activity Values	Concentration, mg/kg		
		Cocoa	Dark Chocolate	Milk Chocolate		Cocoa	Dark Chocolate	Milk Chocolate
31	24 [b] [28]							
32	>2000 [b] [28]							
33	35 [65]							
34	6 [65]					0.234–0.276 [pw] [14]		
35	20 [65]					0.135–0.151 [pw] [14]		
36	300 [a] [26]					0.01–0.014 [pw] [14]		
37						0.033–0.043 [pw] [14]		
38	1120 [a] [32]					0.053–0.069 [pw] [14]		
39	0.46 [c] [31]		512 [31]		1 [dc] [31]		0.00046–0.00058 [31]	
40			8, 32 [31]					

CIN—compound identification number; [a]—odour threshold was determined in water, [b]—odour threshold was determined in diethyl ether; [c]—odour threshold was determined in sunflower oil; [d]—odour threshold measured in 9:1 solution of dichloromethane: cyclopentanone; [e]—odour threshold measured in triethylcitrate; [lq]—in cocoa liquor; [pw]—in cocoa powder; [rcb]—in roasted cocoa beans; [dc]—in dark chocolate at reference [20] presented FD factors and concentrations of two samples of dark chocolate after conching (final product); at reference [15] were evaluated concentrations of alkylpyrazines in three dark chocolates from blend cocoa mass and three single-origin dark chocolates. During their research, Bonvehí et al. [17] found the OAVs for the majority of the substances present in cocoa samples. This ratio would show which compounds, for foods like cocoa, are present well beyond their OT (concentration-to-OT ratio higher than 1) and are capable of contributing to the odour, and which compounds are present well below their OT (concentration–OT ratio under 1) and are not capable of doing so. This ratio may be used to estimate the likelihood that a chemical contributes to the cocoa powder's scent. According to Frauendorfer and Schieberle's research [7], Strecker aldehydes and pyrazines play a significant role in the aroma of cocoa powder. With OAVs greater than 1, 2,3-diethyl-5-methylpyrazine, 2,5-diethyl-3,5-dimethylpyrazine, 2-isobutyl-3-methoxypyrazine and 2,6-diethyl-3,6-dimethylpyrazine were considered to be significant aroma-valuable components of cocoa powder.

Summarizing the information from Table 5, the main contributors to cocoa aroma are: 2-methylpyrazine (OAV = 4.83 [17]), trimethylpyrazine (OAV = 3.2 [16]), tetramethylpyrazine (relatively high concentrations covering high OT), 2-ethyl-3-methylpyrazine (OAV = 1.62 [17]), 2-ethyl-5-methylpyrazine (OAV = 2.8 [17]), 2-ethyl-3,5-dimethylpyrazine (7.6 [16], 14 [7]), 2-ethyl-3,6-dimethylpyrazine (OAV = 1 [16], 1.2 [7]), 2,3-diethyl-5-methylpyrazine (OAV = 6.6 [16], 16 [7]), 2-methoxy-3-isopropylpyrazine (very low OT = 0.002 μg/L [3,12], 0.002 μg/L [28], 0.0039 μg/L [33], 0.004–0.024 μg/L [33], 0.01 μg/L [31], 0.024 μg/L [66], 0.001–0.1 μg/L [69]), 2-methoxy-3-isobutylpyrazine (OAV = 1.2 [16], 12 [7]). The main contributors to dark chocolate aroma are trimethylpyrazine (OAV = 1 [31]), tetramethylpyrazine (very high concentration (mg/kg)—6.135–7.983 [20], 60.31–285.74 [15]), 2-ethyl-3,5-dimethylpyrazine (OAV = 16, 24 [31]), 2,3-diethyl-5-methylpyrazine (OAV = 2 [31]), 2-methoxy-3-isopropylpyrazine (OAV = 1, 8 [31]), 2-methoxy-3-isobutylpyrazine (OAV = 6, 9 [31]) and 2-sec-butyl-3-methoxypyrazine (OAV = 1 [31]).

Schnermann and Schieberle [30] were the first researchers to identify the main odour-active components in cocoa mass and milk chocolate by using GC-O in conjunction with an AEDA. Two pyrazine compounds were found to have the highest flavour dilution (FD) factors: 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine both in cocoa mass and in milk chocolate. The findings showed that the roasted cocoa mass is probably where these milk chocolate scent components come from. Obviously, however, the most prevalent volatiles in the samples are usually not the odourants with a low OT and high OAVs, as is typical in GC-O analyses. The high concentration here seems to be crucial and makes up for the lack of strong odour characteristics. In Deucscher et al.'s investigation [29], trimethylpyrazine and tetramethylpyrazine were the two pyrazines which were present in the highest concentrations across all of the dark chocolate samples and played a key role in chocolate odour.

4.2. Pyrazine Content Differences in Cocoa, Dark and Milk Chocolate

It is common practice to combine cacao beans from various sources while making chocolate. The origin of chocolate is distinguished by a distinct flavour and scent that come from the cocoa beans used, which are primarily influenced by the location of the plantation. Flavour volatiles are one of the most significant factors influencing the acceptability of cocoa beans and chocolate, which is essential to the popularity of cocoa beans and cocoa-based goods like chocolate.

According to the study conducted by Godočiková et al. [15], single-origin and blend chocolate with various quantities of the cocoa mass showed substantial variances in the overall level of alkylpyrazines. These findings imply that the final odour potential, which was not specified for these products, is greatly influenced by the properties of the raw materials utilized as their origin. Comparing chocolates of different origins, samples from different blends revealed variations in the content of fragrance components. For example, the content of tetramethylpyrazine varied from 60.31 mg/kg in the blend sample and 285.74 mg/kg in the Madagascar single-origin sample, which was found to be the highest concentration of alkylpyrazines, namely tetramethylpyrazine. Additionally, trimethylpyrazine (15.01–81.39 mg/kg), 2,5-dimethylpyrazine (1.99–10.18 mg/kg), 2,3-dimethylpyrazine (2.74–15.11 mg/kg) and 2,6-dimethyl-3-ethylpyrazine (0.98–6.77 mg/kg) were spotted at high concentrations.

Pyrazines are a sign of a healthy fermentation process and can be used to evaluate the quality of dried fermented beans. In the research by Schnermann and Schieberle [30], which were comparing pyrazine content in cocoa mass and milk chocolate, some compounds demonstrated very noticeable variations in their odour activity, especially 2-methoxy-3-isopropylpyrazine (FD = 64—milk chocolate, FD = 512—cocoa mass) and 2-ethyl-3,6-dimethylpyrazine (FD = 32—cocoa mass, FD = 512—milk chocolate), which may be explained by the theory that these fragrance molecules may partially evaporate during the conching process.

Chocolates, dark and milk, have been classed depending on their formula, and one of the most crucial ingredients in the production of milk chocolate, milk powder, can significantly alter the physical and organoleptic qualities of chocolate. Although the aroma of milk chocolate may be enhanced by the addition of dairy products, the presence of milk fat caused a notable reduction in the release of volatile substances. In a study by Afoakwa et al. [21], it was proven that the release of alkylpyrazines is considerably reduced by chocolate's enhanced particle size distribution and fat content.

By using GC-MS, Liu et al. [19] evaluated the amount of the key aroma-active chemicals in cocoa liquid, dark and milk chocolate, and compared them using GC-O-MS (gas chromatography–olfactometry–mass spectrometry), AEDA and sensory assessment. Strecker aldehydes, pyrazines, pyrroles and carboxylic acid made up 76% of the entire value of odourants with FD factors 81 in the dark chocolate, but milk chocolate only contained about 37.5% of this indication. Dark chocolate had substantially higher concentrations of all six pyrazines (2.654 mg/kg) than milk chocolate (0.619 mg/kg) and slightly more than cocoa liquid (2.2213 mg/kg). Numerous pyrazines, including 2,5-dimethylpyrazine, 2-ethyl-6-methylpyrazine, trimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine and 3,5-diethyl, 2-methylpyrazine increased in comparison with the cocoa liquid during the manufacturing process. This was consistent with the fact that during conching, low volatile pyrazine concentrations considerably increase. The significant difference in these chemicals between the two kinds of chocolate also suggested that the milk chocolate's lower pyrazine concentration was due in part to its distinct conching conditions.

4.3. Challenges in Odour Values Measurement of Alkylpyrazines

Nonetheless, as these data frequently differ greatly in the literature, determining the OT is certainly not without its challenges. The use of reference odourants which were tainted with other odour-active substances or that were created during the storage of the reference chemical, as well as the use of a panel of assessors with an inadequate number of members to accurately determine the OT, may be the sources of these discrepancies. Even tiny quantities of trace impurities may have an impact on the target compound's OT since they are odour active. The OT may be significantly raised by interactions with other odour-producing chemicals. For instance, it was discovered that the OT for 2-ethyl-3,6-dimethylpyrazine is 0.8 μg/L of water. Later, in the synthesis, they found trace quantities of the more odour-active 2-ethyl-3,5-dimethylpyrazine. After purification and another look at the tests, a ten-fold higher OT for 2-ethyl-3,6-dimethylpyrazine (8.6 μg/L) was reported. It demonstrates that multiple precautions must be followed in order for OT evaluations to produce valid results, and it is advised that all compounds undergo a GC-O purity check before OT evaluations because reference substances may be compromised by odour-active trace compounds [3,33]. It was not feasible to distinguish 3-ethyl-2,5-dimethylpyrazine from 2-ethyl-3,5-dimethylpyrazine in Deucscher et al.'s research [29] since both compounds had identical mass spectra, the same LRIs on both DB-FFAP and DB-5 columns and identical odour descriptors.

4.4. Structure–Odour Relationship in Alkylpyrazines

Multiple studies of the structure–activity connection of the odour properties of pyrazine derivative products point to the possibility that differences in odour activities depend on the chemical structure of such molecules, specifically on the kind of substituent groups and the placement of the substituents tied to the pyrazine ring.

The structural correlations Schiffman et al. [12] observed were as follows: the incorporation of a highly polar group among those presented in cocoa alkylpyrazines (such as methoxy) tends to lower OT values; the incorporation of a bulky alkyl side chain tends to decrease the OT; the introduction of the strong electron inductive group (-OR) as an osmophoric group has a massive impact on both the relative OT and olfactory perception. Additionally, pyrazine and 2-methylpyrazine had a higher OT than other more substituted pyrazines in their study (OT methylpyrazine = 60,000 μg/L > OT ethylpyrazine = 6000 μg/L > OT

isobutylpyrazine = 400 μg/L > OT 2-methoxy-3-methylpyrazine = 4 μg/L > OT 2-methoxy-3-isobutylpyrazine = 0.002 μg/L).

Yoshii et al. [70], using comparative molecular field analysis and 3D modelling of pyrazine structures with the green odour, also discovered that adding a substructure or a negatively charged group, such as a functional group that contains an oxygen atom, is preferred to boost the odour intensity. This was also proved by Schimazaki et al.'s QSAR analysis [67], which evaluated the significance of electronic characteristics based on the influence of the polar substituents in the pyrazine rings, which can have a major impact on the odour intensity. The more odour-active pyrazine compounds have a greater lone-pair orbital energy, according to correlation studies between olfactory activities and orbital energies. These findings imply that polar substituents at position 3 in the pyrazine ring may have a slight electrical impact that helps to produce odour activity. However, it is logical to assume that steric factors are the primary causes of pyrazine's odour activity and that the lesser electronic effects are overshadowed by the dominating steric contribution.

Teranishi et al. [65] examined the OT in the water of a number of methoxypyrazines connected to 2-methoxy-3-isobutylpyrazine. The OT rises when methyl groups are added to the ring, indicating a significant drop in odour strength, while the aroma typically stays that of green peppers. When compared with 2-methoxy-3-isobutylpyrazine, the OT is raised by 105 times when the methoxy group is removed. It is also clear that the OT of 2-methoxy-3-alkylpyrazines is significantly lowered or that the odour intensity is increased as the chain length rises. As the side chain's carbon atom count decreases, the rise becomes steadier. Regarding the methoxypyrazines, the OT gradually diminishes or the smell intensity increases as the side chain length increases. However, unlike the methoxypyrazines, the OT was lowered rather than raised by the inclusion of additional groups to the pyrazine ring. However, methoxypyrazines typically have thresholds that are substantially lower than those of alkylpyrazines, suggesting that the methoxy group has a crucial role in relation to the OT.

By using gas chromatography–olfactometry, Wagner et al. [28] measured the OT of 80 alkylpyrazines and developed some hypotheses regarding the relationship between odour and structure. Amongst mono-, di-, tri- and tetramethylpyrazine, trimethylpyrazine had the lowest OT. In the case of comparing 2,6-dimethyl- and trimethylpyrazine, the OT was reduced by a factor of roughly 34 when a third methyl group took the place of a hydrogen atom in 2,6-dimethylpyrazine. When this substitution took place in position 3, comparing 2,5-dimethylpyrazine and trimethylpyrazine, there was almost the same decrease, whereas in position 5, comparing 2,3-dimethylpyrazine and trimethylpyrazine, there was an 18-fold reduction in the OT. In every instance, the aroma transitioned from nutty to roasted. Trimethylpyrazine was converted by swapping out the methyl group in position 2 to 2-ethyl-3,5-dimethylpyrazine, which has a 4500-fold lower OT than trimethylpyrazine. The pyrazine molecule's ethyl groups in positions 3 or 5 were less successful in lowering the OT than those in position 2. The OT rose when a propyl and a butyl group took the place of an ethyl group in position 2 of 2-ethyl-3,5-dimethylpyrazine. When the propyl group was switched out for an isopropyl group in 3,5-dimethyl-2-propylpyrazine, the OT rose even higher. Their pea-like odour and extremely low OT, which supported earlier findings, showed that 3-alkyl-2-methoxypyrazines belong to a unique class of odourants. The pleasant odour was unaffected even though the OT rose when the 3-isopropyl-2-methoxypyrazine methoxy substituent was replaced by methyl and an ethyl group. Inferring that either their OT is substantially lower than those of the majority of alkylpyrazines or their quantities in foods are significantly higher, only a limited subset of alkylpyrazines have been evaluated by AEDA as potent odourants. For instance, although their OT values are rather high, alkylpyrazine trimethylpyrazine, tetramethylpyrazine and 3-ethyl-2,5-dimethylpyrazine have been found in cocoa mass and are generated in quantities that make up for their mild odour. Additionally, these findings were then used to specify the geometry of a hypothetical receptor. By superimposing the reduced structures of pyrazines with a low OT, the geometrical shape of a hypothetical receptor was obtained (Chem-X

force-field minimization). By superimposing pyrazines with a high OT, they discovered sterically prohibited regions in the model [28].

By using QSAR analysis, Buchbauer et al. [69] confirmed that there are favourable steric locations 2 and 3 and that the inclusion of bulky groups in these regions will intensify the odour (lower the OT). The bulkiness, on the other hand, is unfavourable at positions 5 and 6 for the low OT. Additionally, a low Log P value of the substituent in position 2 was favourable for a low OT. The decreasing order of the OT for the dialkylpyrazines, as noted by Mihara and Masuda [66], is as follows: 2,5-, 2,6- and 2,3-positions. The order of the decreasing OT is inverted for methylpyrazines that contain a polar group, such as an ethoxy-, methoxy-, methylthio- or acetyl: 2,3-position, 2,6-position or 2,5-position. Additionally, it seemed that the reduction in the lipophilicity of the pyrazines caused the OT to drop.

Out of all those findings, we can make a summary of the structure features of pyrazine that enhance great odour intensity:

1. Positions 3 and 5 were not acceptable for an ethyl group; only position 2 was. In addition, a propyl, butyl, pentyl, isobutyl or hexyl group in position 2 was too bulky, and the OT was high in these compounds compared with 2,3-dimethylpyrazine, which was not substituted by them. However, positions 2 and 3 are more suitable for bulky groups than positions 5 and 6 regarding the OT value;
2. Replacing the H atom in position 2 with a methyl group reduced the OT, and the lengthening of the side chain to ethylpyrazine decreased the OT even further;
3. For compounds with a low OT, the only group allowed in position 5 is the methyl group;
4. For a low OT, position 6 should be unsubstituted;
5. For the dialkylpyrazines, the substitution position affects the OT by lowering the OT at 2,5-dialkyl-pyrazine, then 2,6-dialkyl-pyrazine and then 2,3-dialkyl-pyrazine.
6. The incorporation of a highly polar group among those presented in cocoa alkylpyrazines (such as methoxy) tends to lower OT values. As an example, 3-alkyl-2-methoxypyrazines belong to a unique class of odourants with very low OT values.

5. Relationship between Odour and Antimicrobial Activities of Pyrazines

In scientific debates on the sense of smell, it has become common knowledge that olfaction and emotion are tightly related. Because they excite olfactory receptors and relate to emotionally intense events, alkylpyrazines have powerful odour-beneficial effects. Human sensitivities to chemosensory and olfactory effects can become constitutional through neuroplastic changes in the olfactory pathways to the limbic system and other parts of the brain related to hedonic feeling. Such situations can increase the intake of pyrazine-containing foods and pharmaceuticals through associative learning. According to certain theories, smells may affect mood: arouse strong feelings of pleasure or repulsion, lead to relaxation or bring back emotional memories [71,72]. That is why we decided to focus on pyrazines, which are known for having distinctive fragrance properties. These structures have also lately been discovered to possess potential antibacterial properties. The issue of patients disliking drugs due to their unpleasant flavour may one day be resolved by combining those two features of pyrazines.

We made the decision to investigate the correlation between aroma and the antibacterial capabilities of alkylpyrazines. It was chosen to use the odour threshold (OT) and probability (Pa) values of the antibacterial activity generated by AntiBac-Pred as a comparison value. The decision to count the arithmetic medium of the OT measured in water was made in light of the fact that the values of OT vary considerably among the literature sources we examined. A statistical hypothesis test, or as it is called, a *t*-test, was conducted with PQStat software. Because testing the association between raw OT value and Pa yielded insufficient data, it was decided to use log10 of the OT as a correlation value. The data that were analysed are presented in Table 6. The relationship between the odour activity values (OAVs) and Pa was not statistically significant and is not included in the paper.

Table 6. Comparison values for the correlation analysis.

Compound Name	Pa of Antibacterial Activity from AntiBac-Pred			Log_{10} of Odour Threshold
	Yersinia pestis	*Mycobacterium tuberculosis*	*Corynebacterium jeikeium*	
pyrazine	0.7689			5.460521
tetramethylpyrazine	0.7431			4.213065
2,3-dimethylpyrazine	0.7010			3.091079
2,5-dimethylpyrazine	0.7010	0.6459		3.308208
2,6-dimethylpyrazine	0.6560			3.560305
2-methylpyrazine	0.6050	0.5963		4.637609
trimethylpyrazine	0.6050			2.758911
2,3-diethylpyrazine	0.5018			0.8195439
2-isobutyl-3-methoxypyrazine			0.6632	−1.540607
2-isobutyl-3-methylpyrazine			0.5242	1.544068

The results of the independent groups' *t*-test showed that the p-value was 0.000371 at significance level α = 0.05. This number indicates evidence of a strong correlation between the OT and antibacterial activity probability. The Spearman's rank–order correlation test showed a *p*-value of 0.224445, which points at some predisposition of correlation as well. The results of the tests are presented in Table 7 and Figure 4.

Table 7. Results of *t*-test and Spearman's rank–order correlation between log_{10} of odour threshold and probability of antibacterial activity from AntiBac-Pred of pyrazine compounds.

***t*-Test of Independent Groups**		**Spearman's Rank–Order Correlation Test**	
Analysed variables	2	Analysed variables	2
Significance level, α	0,05	Significance level, α	0.05
Correction for different variances	No	Number of pairs	12
Difference of the means	−2.340587	r	0.37895
−95% CI for the difference	−3.496677	Std. err. of r	0.292643
+95% CI for the difference	−1.184498	−95% CI for r coefficient	−0.267219
Standard error of the difference	0.557454	+95% CI for r coefficient	0.790038
Pooled standard deviation	1.365478	t-statistic for r	1.294923
t-statistic	−4.198709	Degrees of freedom	10
Degrees of freedom	22	Two-sided *p*-value	0.224445
Two-sided *p*-value (*t*-test)	0.000371		

The fact that pyrazines are potent antibacterial agents with pleasant odour effects on the items they are employed on cannot be disregarded and should be investigated on a deeper level. A great example of this combined proficiency application of pyrazines was demonstrated by Schöck et al. [44]. 2-Isobutyl-3-methylpyrazine showed itself as a promising preservative for chicken meat strips and added roasted undertones to the flavour and scent of the food. The textile business is another industry that integrates the antibacterial and aroma properties of chemicals. Fard et al. [73] looked into the connection between the scent intensity and antibacterial properties of encapsulated thyme essential oil on cotton fabrics in their study. The correlation coefficient r = 0.988 between the odour intensity and antibacterial activity of samples was found to be strong. The success of this initiative may benefit textile and clothing consumers tremendously because the antibacterial activity of materials can be simply determined by analysing the scent assessment via the e-nose system. This also demonstrates the importance of research into the links between odour and the antibacterial capabilities of odourants, such as pyrazines.

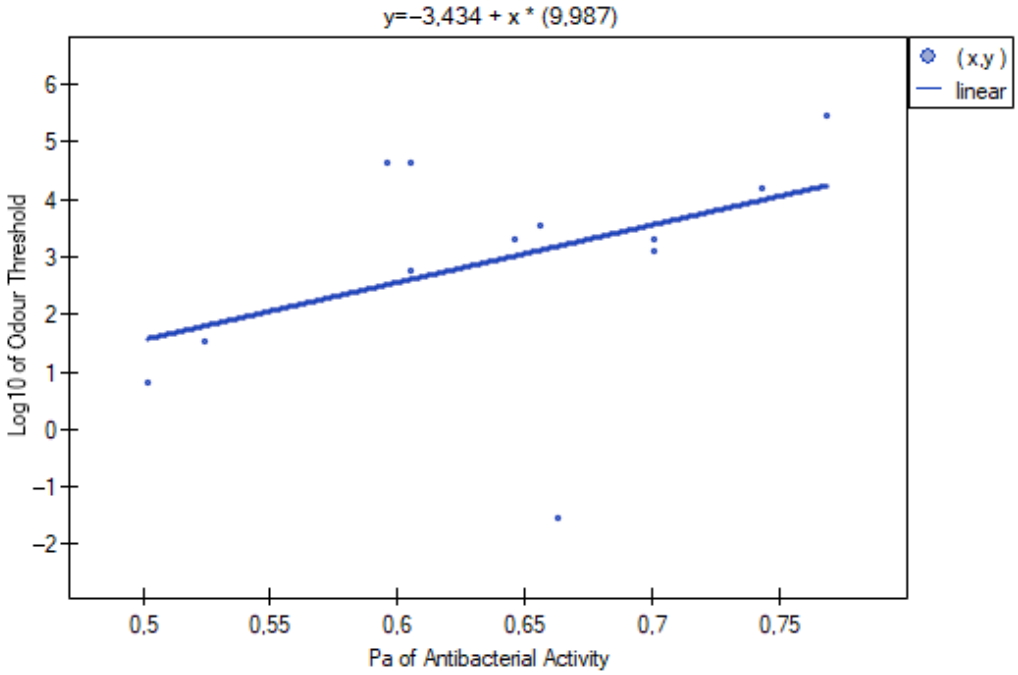

Figure 4. Scatter plot of odour threshold (OT) and Pa values of the antibacterial activity of pyrazines. A sign "*" - means multiply.

6. Conclusions

Following a thorough analysis of the literature data gathered for this research, it can be concluded that the alkylpyrazines found in cocoa and chocolate may be potential antibacterial, antifungal and antioomycete agents for plants, food and possibly even as an additional therapy for *Mycobacteria*-caused tuberculosis. Alkylpyrazines also have strong odour-beneficial effects since they stimulate olfactory receptors and link to emotionally compelling experiences. Through neuroplastic alterations in the olfactory pathways to the limbic system and other regions of the brain related to hedonic sensation, human reactions to chemosensory and olfactory effects can become constitutional. Through associative learning, such occurrences can encourage the consumption of pyrazine-containing goods and medications. That can also serve as cues for the heightened hedonistic valence of stimuli, increasing the desire of consumption. Therefore, integrating odour-beneficial, hedonistic and antibacterial properties with one another may allow scholars to address several pressing issues in the food and medical industries. Firstly, the Flavor and Extract Manufacturers Association (FEMA) and the European Union's list of approved flavouring agents have both recognized the use of pyrazines in food and plants for human consumption to be safe food. The flavour profile of products will be significantly improved by adding alkylpyrazines to produced food and still-growing plants, as well as protection from bacterial, fungal and oomycete-induced illnesses. Secondly, by inducing pleasant olfactory system signals, adding pyrazines as an extra therapy to medication therapy may positively influence the course of the disease and also address the issue of patients' antipathy to drugs and refusal to take them. The *t*-test demonstrated a substantial correlation between the odour threshold and the probability (Pa) of antibacterial activity. It is necessary to further examine alkylpyrazines' antimicrobial and odour activities due to the many promising benefits they own that have already been presented in this paper.

Author Contributions: Conceptualization, L.Z. and A.P.; methodology, A.P.; software, A.C.; validation, L.Z., A.P. and A.C.; formal analysis, A.P.; investigation, A.C.; resources, L.Z.; data curation, L.Z.; writing—original draft preparation, A.C.; writing—review and editing, A.P. and L.Z.; visualization, A.C.; supervision, L.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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