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Volatile organic compounds (VOCs) in food processing and their impact on microbial communities

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Abstract

Food preservation and supply is more and more becoming a big issue in times of increasing population. The main objective of this study was to identify sustainable and highly efficient decontamination strategies to overcome modern food processing issues. By combining cultivation-dependent methods, RT qPCR, confocal laser scanning microcopy, and HiSeq Illumina amplicon sequencing highly active volatile organic compounds (VOCs), diazine derivatives, were evaluated for their antimicrobial potential. Product treatment strategies were developed to analyze the stabilizing efficiency of novel formulations in combination with bakery goods and hatching eggs. Moreover, bioinformatic tools were used to observe diazine-induced structural changes in bacterial communities. Following several optimization steps, the spoilage-reducing application on toast bread using highly active diazine derivative formulations has successfully been shown. The shelf life could be extended by up to one week. Further experiments confirmed that the volatile activity of the VOCs led to an effective decontamination of usually inaccessible surfaces. VOCs treatment of hatching eggs was evaluated as a new strategy to prevent bacterial contaminations influencing a safe chicken development. In terms of decontamination efficiency, reduction rates up to 99.6% confirmed the applicability of the innovative approach. Specific operational taxonomic unit (OTU) enrichment was found after the decontamination and suboptimal process management. Surface treatments with inappropriate amounts of volatile diazines showed increasing proportions of *Pseudomonadacae* and *Clostridiales*, both taxonomic groups including potential pathogens. These findings facilitated further process optimization and provided new insights into the mode of action of diazines. Taken together, valuable information was gained for newly developed preservation and decontamination technologies. Highly efficient antimicrobial diazine compounds showed a successful applicability in the tested fields and gave a promising outlook for future applications.

Kurzfassung

Neue Konservierungsstrategien werden immer wichtiger in Zeiten steigender Bevölkerungszahlen und Versorgungsengpässen. Diese Arbeit beschäftigte sich mit der Erforschung nachhaltiger und höchst effektiver Konservierungsstrategien um Probleme in der Lebensmittelindustrie zu bewältigen. Mit Hilfe Wachstums abhängiger Methoden, RT qPCR, Konfokaler LASER-Raster-Mikroskopie und HiSeq Amplicon Sequenzierung wurden hochaktive flüchtige organische Verbindungen, Diazin Derivate, auf ihr antimikrobielles Potenzial untersucht. Mit speziellen Produktbehandlungsstrategien wurde die Haltbarkeitsverbesserung von Backwaren unter Anwendung von entwickelten Formulierungen und Dekontaminationsprozesse von Bruteiern untersucht. Durch Bioinformatik unterstütze Auswertungsmethoden wurden, durch niedrige Konzentrationen induzierte, strukturelle Artenvielfaltsveränderungen beobachtet, was neuen Einblick in die Wirksamkeit der Diazine gewährte. Speziell entwickelte Diazin Formulierungen für den Einsatz auf bestimmten Nahrungsmitteln wurden mit Stress tests auf ihre Wirksamkeit geprüft. Haltbarkeitsanwendungen auf Toastbrot unter Einsatz hoch aktiver Diazin Derivat Formulierungen waren, nach mehreren Optimierungsschritten, erfolgreich. Die Lagerbeständigkeit konnte um bis zu einer Woche verlängert werden. Nachfolgende Experimente zeigten, dass durch die Flüchtigkeit dieser hoch aktiven natürlichen organischen Verbindungen auch Dekontaminationsprozesse schwer erreichbarer Oberflächen, über die Gasphase, möglich sind. Bruteierbehandlungsstrategien wurden entwickelt für die sichere keimfreie Entwicklung von Küken während des Brütens. Keimreduzierungen von bis zu 99,6% zeigten reproduzierbaren Behandlungserfolg. Ineffiziente Prozessführung kann jedoch zur Anreicherung bestimmter OTUs führen. Niedrige Diazin Konzentrationen führten zu erhöhtem Auftreten von *Pseudomonadacae* und *Clostridiales*, beides Gruppen potentieller Pathogene. Diese Ergebnisse erleichterten zukünftige Prozessoptimierung und gaben mehr Einblick in den Wirkungsmechanismus von Diazinen. Zusammenfassend wurden wichtige Erfahrungswerte für die neu entwickelten Behandlungs- und Konservierungsstrategien generiert. Hoch aktive Diazin Verbindungen zeigten erfolgreich ihre Anwendbarkeit in den getesteten Bereichen und gaben vielversprechenden Ausblick auf weitere, zukünftige Anwendungen.

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1. Introduction

1.1 Food preservation

There is a long history of food preservation. Ancient man survived by things found in nature. Simple preservation strategies, such as drying food in the heat of the sun or freezing meat in cold environments, were soon used to become independent from successful hunts or harvests on a daily basis. Food spoilage starts from the moment of harvest. To survive, human beings had to develop more advanced technologies of food preservation. Drying, freezing, fermenting, pickling or curling were used for thousands of years [1]. The invention of canning by a French chemist named Nicolas Appert in the 19th century was a turning point in food preservation. Food heated in closed containers was stable until the container was opened or the seal was damaged. This was fifty years before Louis Pasteur discovered the relationship between food spoilage and microorganisms. In the following years more and more food preservation techniques were developed using new food modification or packaging methods [2].

1.1.1 The necessity and requirements for novel preservatives

Food preservation is essential for extending the time food is safe to eat and nutritionally viable. Vegetables, fresh fruits and animal products tend to spoil quickly without preservation, refrigeration or dehydration. Effective preservation techniques, including sugar, salt, vinegar or brine, make an inhospitable environment for mold, bacteria and other harmful organisms and prevent them from growing in and on the food [3,4]. However, modern food technology cannot be imagined without chemical preservation ingredients such as benzoates (e.g. sodium benzoate), nitrites (e.g. sodium nitrite) or sulphites (e.g. sulphur dioxide) [5]. Even if food preservation techniques seem to be advanced, one-third to one-half of all food produced ends as food waste due to numerous causes during production, processing, trading and consumption [6,7]. Consumer requirements change constantly. In recent years needs for longer storable food is changing to more natural, fresher, healthier food products. At the same time, food quality and safety to prevent food poisoning are still key requirements in food production [8]. Currently used food preservation techniques are listed in Table 1.

Table 1: Existing technologies for food preservation.

Way of preventing microorganisms or spoilage	Preservation techniques	Reference
Restriction of access	Packaging	[8,9]
Inactivation of microorganisms	Heat pasteurization Radiation Addition of enzymes High pressure Electric shock treatment	[8]
Prevention or slowing down of growth	Freezing Acidification Fermentation Drying Vacuum Addition of preservatives	[8]

To assure food quality and prevent risks of food poisoning researchers work constantly on improving preservation techniques. However, the consumers need for more natural and healthier food products limits the preservation techniques and new technologies are required [8].

1.1.2 Natural preservatives

Natural food preservatives such as rosmarinic acid, carnosic acid, polyphenols, parabens, essential oils and many more are an upcoming trend in the food industry [10,11]. The antimicrobial effect of rosmarinic acid, bee propolis or grapefruit seed extract is applied in medical applications and cosmetics [12]. Essential oils are tested in the preservation of cheese products [13]. Grapefruit seed extract is used as preservative by many handcrafters in water containing products [12]. However, to guarantee food safety and quality of a product, also with usage of naturally derived preservatives, highly active molecules are needed. Pyrazine derivatives, naturally found in many vegetables, have recently been discovered to have not only pharmacological activities but also antimicrobial potential for the food industry [14–16]. During a period of intense fouling of Styrian oil seed pumpkins *Paenibacillus* spp., producing pyrazine derived volatile organic compounds (VOCs), were isolated from healthy oil pumpkins. Those pyrazines were found to have a strong antimicrobial

efficacy on many microorganisms including *E. coli*, *S. aureus*, *C. albicans* and *Penicillium* sp. [17,18]. These findings lead to the development of biocontrol agents using *Paenibacillus polymyxa* and other bacterial isolates [17,18]. Other plant-associated VOC-producing bacterial isolates such as *Serratia plymuthica*, *Stenotrophomonas rhizophila*, *Bacillus subtilis* or *Pseudomonas fluorescens* also often showed antifungal activity [19,20]. Many identified VOCs have great potential not only as biocontrol agents but also for a variety of other industrial applications, e.g. food preservation [14,15,21].

1.2 Pyrazine derivatives

1.2.1 Chemical properties of pyrazines

Pyrazines are heteroatomic aromatic organic compounds with two nitrogen atoms within the ring structure. In pyrazine derivatives the two nitrogen atoms are in para-orientation (1,4-diazine) [22]. All four other ring-carbon atoms are open for substitution with alkyl or alkoxy residues (Fig. 1). Alkylated pyrazine derivatives are the most abundant of all different pyrazine derivative subcategories (Table 2) [23]. Due to the fact pyrazines evaporate at a relatively low temperature and their low molecular weight, they are classified as volatile organic compounds (VOCs) [24]. Pyrazine derivatives often tend to have a very distinguished odor and are used in food industry as flavoring additives due to their low flavor thresholds [22,23,25]. Moreover, they are also found naturally in various vegetables and heated food as aromatic components [26,27].

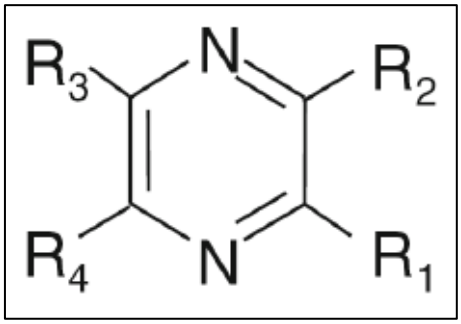
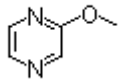
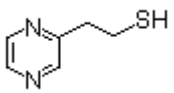


Figure 1: Structural formula of the pyrazine molecule. Common substitutes in nature are alkyl and alkoxy residues [2].

Table 2: Pyrazine derivative classification into subcategories depends on its substitutes. Examples for each group is shown by name and structural formula [22].

Group	Substitutes	Example	Reference
1	No	 Pyrazine	[28]
2	hydrocarbon substituent (alkyl, alicyclic or alkylaryl)	 2-Methylpyrazine	[28]
3	oxygenated functional groups and aliphatic side chains (alkoxy or acetyl)	 Methoxypyrazine	[28]
4	thiol or sulphide functional groups in the aliphatic side chain	 2-Pyrazinylethane thiol	[28]

Pyrazines were examined by the FEMA (Flavor and Extract Manufacturers Association) expert panel in 2002. The LD_{50} was determined in a range from 500 to 2500 mg/kg in rats, which indicates a low toxicity level. Further toxicity studies showed no histopathologic changes, no genotoxicity, no carcinogenicity and no hence effects. It was concluded that the human consumption of low amounts as used in flavoring industry is safe, due to the fact some vegetables contain even higher amounts naturally [22].

1.2.2 Pyrazine derivative occurrences in nature

Pyrazines are widely spread in nature. Plants serve as natural source for pyrazines, while many bacteria are used for the industrial production of pyrazine molecules [29]. The occurrence in animals, plants and bacteria suggests a broad variety of functions.

Pyrazine derivatives are found naturally in different types of vegetables. Due to their typical odor they give those vegetables a characteristic smell. 3-isobutyl-2-methoxypyrazine and 3-sec-butyl-2-methoxypyrazine are for instance found in beetroots, silver beets, carrots, broccoli, cucumber, asparagus and lettuce and are their main aromatic compound or contribute to their flavor among other compounds [26,30]. 3-isobutyl-2-methoxypyrazine plays also an important role as an flavor

component in wines, giving them a typical grassy aroma, which is associated with grapes and wine [31–34].

Pyrazine derivatives are also produced naturally during the Maillard reaction in cooking processes. During this heat induced, non-enzymatic reaction, an amino acid reacts with α-dicarbonyl compounds (sugars) and leads to the synthesis of an aroma compound (Strecker degradation). Most pyrazines are formed at temperatures between 120°C and 150°C. The pyrazine derivative formed depends on the reacting amino acid [23]. 2-acetylpyrazine, giving popcorn its typical aroma, is only one example of the many pyrazine derivatives produced during a Maillard reaction in food processing [35,36].

Occurrence of pyrazines has also been reported in some animals and plants. Thereby they can be involved in several processes. such as serving as alarm molecules in chemical communication or being trail pheromones, as attractants or deterrents without having a beneficial or harmful effect [37]. The maintenance of colony integrity and cohesiveness at social insects depends on volatile secretory products such as pyrazines [38]. Pyrazines were already identified in several species of ants, wasps, flies, bees, aposematic beetles, butterflies and plant bugs in 1990 [39,40].

Plant volatile formation is widely spread among different species. Thereby they can either be involved in sexual deception or expulse possible enemies such as herbivores [41]. In vertebrates, such as rabbits and cattle, a pyrazine binding protein was found in the nasal cavity, which supports the hypothesis of pyrazine being an alerting molecule in these animals [42]. Furthermore, smoke of burning wood contains several pyrazine molecules, which indicates pyrazine-smell could also be used as a fire warning system [37]. Fear inducing reactions were also observed in vertebrates such as cattle or rats when they are triggered with wolf urine containing a pyrazine-mixture [43].

Volatile organic compounds are found to be emitted from various bacterial and fungi species. Pyrazines are hypothesized to play an important role for bacteria, as they are often released in mixtures of various molecules [29,44]. Tetramethylpyrazine was found to be produced by *Bacillus subtilis* and gives fermented soybeans their characteristic aroma [45]. Pyrazine mixtures are known to be released by *Bacillus cereus* and, the plant-growth promoting bacteria, *Paenibacillus polymyxa* [46–48]. Furthermore, *Pseudomonas* species *perolens* and *taetrolens* have been found to be responsible for a potato-like odor in beef, lamb and pork meat, due to the production

of 2-methoxy-3-isopropylpyrazine [49]. Several other bacterial species, including *Corynebacterium glutamicum, Chondromyces crocatus* and the marine bacteria *Sulfitobacter pontiacus* were reported to be pyrazine producers [44,50].

Hydroxylated pyrazines are typical metabolites of several fungi [29]. Aspergillic acid (2-hydroxy-3-isobutyl-6-sec-butylpyrazine-1-oxide), produced by *Aspergillus flavus* is commonly known for its antibiotic potential [51]. Other pyrazine derived, antimicrobial molecules such as neohydroxyaspergillic acid, neoaspergillic acid and pulcherriminic acid have found to be produced by *A. sclerotiorum* and *Candida pulcherrima* [52,53].

The role of pyrazine compounds for microbes is not well studied so far. There are several possible reasons why bacteria and fungi produce pyrazine derivatives [54]:

- As communication signals for inter- and intraspecific communication
- As quorum sensing molecules (cell-to-cell communication)
- As carbon outlet
- As growth promotion or inhibition molecules

Pyrazine derivatives are also responsible for some undesired flavors in food products [55]. Bacterial released alkylmethoxypyrazines are, for instance, effecting aroma defects in coffee, moldy off-flavor in eggs, fish and dairy products [56,57].

1.2.3 Application of pyrazines

Pyrazines are used for several applications. The flavoring industry benefits from their odor characteristics, as well as the pharmaceutical industry which found some beneficial medication applications.

In the food industry, pyrazine derivatives are widely used as flavoring additives to imitate the flavors of different natural products. Pyrazines are known for the broad variety of different aromas and their low odor threshold values. There are also no allergies affected by most pyrazine compounds known, due to the fact they naturally occur in most vegetables [23,25].

A few molecules, such as 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine and acetylpyrazine make almost two thirds of the total production volume of pyrazine derivatives annually [22]. Microorganisms such as *Bacillus subtilis*, *Corynebacterium glutamaticum* and *Lactococcus lactis* are commonly used for this industrial process [23,58]. Acetylpyrazine and Trimethylpyrazine are due to a popcorn- and bread crust-

like flavor and a baked potato or roasted nut aroma respectively used as food additives to enrich the flavor [59].

Pyrazines are also used to revive flavors that are lost during cooking processes or to give food products new tastes and scents [60]. Pyrazines as food additives are commonly used in baked goods, cereals, candies, chewing gum, beverages, soups, jams, cocoa, coffee, chocolate, gingerbread, popcorn, tacos, pizza and chips as well as milk, eggs, fish and meat products [61].

Due to the strong antimicrobial effects and their volatile properties different pyrazine derivatives also have a broad field of application in the pharmaceutical industry. Pyrazine derivatives show a wide range of biological activities including antimicrobial, anticancer, anti-tubercular, antiviral, antiepileptic and antiinflammatory activities. For this reasons, pyrazine and its derivatives are also interesting for the pharmaceutical industry [62]. Furthermore, the herbicidal, nematicide or insecticide characteristics of pyrazine derivatives is applicable in agricultural chemicals [29,63].

Pyrazinamide (PZA) is only one of the pyrazine derivatives used in the pharmaceutical industry. PZA is an important tuberculosis drug. It affects only slow growing *Mycobacterium tuberculosis* colonies by entering the cells via passive diffusion. A nicotinamidase converts PZA into pyrazinoic acid (POA), which when again excreted is protonated (HPOA). By reentering the cells the cytoplasm gets acidic and leads to inhibition of membrane transport [64]. There are several targets reported for PZA in literature, such as fatty acid synthetase I and II, ribosomal protein S1 (RpsA) or PanD, but the actual mode of action is not clear yet [65–67].

1.3 Objectives of this study

The focus of this study was the observation of possible application fields in food industry and the determination of pyrazine derivative impact on whole bacterial communities using microbiome analysis. For this purpose two food products were selected and observed using different technologies.

1.3.1 Preservation of bakery products

Toast bread is a food product found in almost every household. The main problem for the food industry is to provide the consumer with a fresh, good tasting product, even two weeks after production. The issue they are facing is mainly mold forming spores

inside of the packages. Even if the industry is using cleanrooms for the production, molds, known for producing several mycotoxins, is still a big problem. Today, there are several possibilities for a product treatment which guarantees a certain shelf life [68–70]. However, not all methods are allowed in all countries without specific package labeling. Pyrazine organic compounds could not only be used to treat the toast breads but also enhance the taste of the product at the same time. Since they are widely spread in nature, no allergies are known and they are allowed as flavoring additives, no specific labeling as stabilization compound is needed [22,25]. Therefore, in this study pyrazine mixtures with needed odor characteristics and efficacies were developed and tested not only on several bacteria but also on *Penicillium sp*. spores, which are the main cause for a mold development and contamination on toast [68]. Special developed toast stress tests using whole toast packages were used to determine the increased shelf life of treated samples.

1.3.2 Decontamination of hatching eggs

The hatching industry is facing serious problems due to microbial contaminations. Losses up to 20% can be caused by bacteria affecting the healthy development of new born chicken [71,72]. Typical contaminations are caused by *Salmonella, Pseudomonas, Micrococcus* or *Escherichia* [73]. Today the industry uses a relatively harsh method to treat the eggs before incubation. Formaldehyde fumigation is used to reduce the bacterial contaminations on the egg shell surface, to guarantee no problems during the chicken development [74]. Since formaldehyde is a potential carcinogenic hazard for humans and the European Union is going to ban the method, hatcheries are searching for environmental friendly alternatives [75,76].

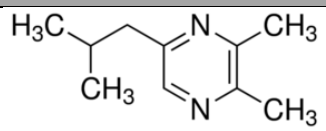
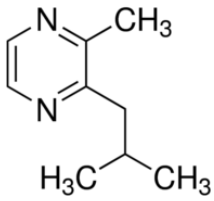
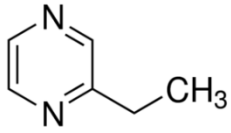
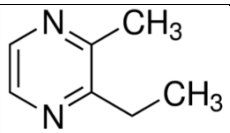
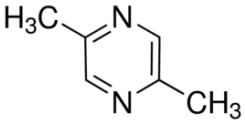
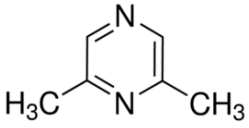
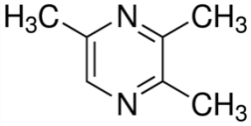
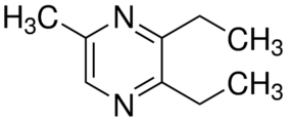
In this study the potential of pyrazine derivatives as hatching egg treatment was observed by developing simple treatment strategies and evaluating those using conventional plating tests and real-time qPCR. Moreover the effect of pyrazines on whole microbial communities was analyzed using CLSM microscopy and amplicon sequencing.

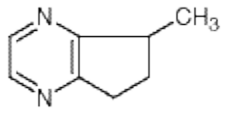
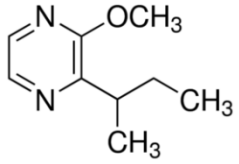
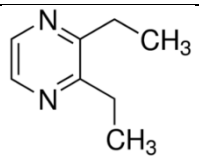
2. Material and methods

2.1 Alkylated pyrazine derivatives

Pyrazine derivatives and their abbreviations used in this study can be found in Table 3.

Table 3: List of utilized pyrazines, their used abbreviation in this thesis and structure found in literature.

Pyrazine	Abbreviation	Structure 1: [77] 2: [78]
5-isobutyl-2,3-dimethylpyrazine	5IB23MP	 1
2-isobutyl-3-methylpyrazine	2IB3MP	 1
2-Ethylpyrazine	2EP	 1
2-Ethyl-3-Methylpyrszine	2E3MP	 1
2,5-Dimethylpyrazine	25DMP	 1
2,6-Dimethylpyrazine	26DMP	 1
2,3,5-Trimethylpyrazine	235TMP	 1
2,3-Diethyl-5-Methylpyrazine	23DE5MP	 1

5H-5Methyl-6,7-dihydrocyclopentapyrazine	5H5M67DHP	 2
2-Methoxy-3-(1-methylpropyl)pyrazine	2MOX3MP	 1
2,3-Diethylpyrazine	23DEP	 1

2.2 Growth media and solutions

If not specifically mentioned, all chemicals, culture media and hardware were obtained from the following companies: Eppendorf (Hamburg, Germany), Fluka (Buchs, Switzerland), Greiner Bio-one (Kremsmünster, Austria), Carl Roth (Karlsruhe, Germany), Sarstedt (Nümbrecht, Germany), Sigma-Aldrich (St. Louis, USA), Biowest (Nuaillé, France), Fermentas (St.Leon-Rot, Germany), Merck (Darmstadt, Germany) and Invitrogen (Lofer, Austria).

If not explicitly stated, all used media and solutions were autoclaved at 121°C for 15 min to ensure sterility.

Instant media such as NA (Nutrient agar), NB (Nutrient broth medium) and Potato dextrose agar (PDA) were prepared according the manufacturer's instructions (Sifin, Germany; Carl Roth, Germany).

For the preparation of nutritive agar plates, 18 g/L Agar-Agar were used.

Phosphate-buffered saline (PBS) Buffer (1X):

- NaCl 8 g
- KCl 0.2 g
- KH_2PO_4 0.24 g
- $Na_2HPO_4*2H_2O$ 1.44 g
- pH adjustment with HCl to 7.4

NaCl solution (0.85%):

- NaCl 8.5 g/L

2.3 Evaluations of alkylated pyrazines

2.3.1 Antibacterial and antifungal activity test

To test antimicrobial efficiencies of different pyrazine derivatives a standard protocol was developed and performed with three different microorganisms from the strain collection of the institute of environmental biotechnology (Graz University of Technology):

- *Escherichia coli* K12
- *Staphylococcus aureus* 25923
- *Candida albicans* H5

The antibacterial and antifungal efficacy of pyrazine derivatives was determined in standard 1.5 mL reaction tubes. A 1 mL solution with an OD_{600} of 0.002 was prepared by diluting ONCs with PBS and mixed with pyrazine to a total concentration of 0.3%. As a control no pyrazine was added to solutions with the same cell density. The typical incubation time was four hours with constant shaking of 330 rpm on 30 °C. After incubation, 100 µL of the solution were plated out (not diluted and 10^{-2}) on NA agar (*E. coli* and *S. aureus*) and PDA agar (*C. albicans*). Colony forming units were counted after incubating the plates over night at 30 °C. Reduction rates were calculated for each pyrazine derivative with the colony forming unit difference between treated and negative control sample.

2.3.2 Sensory evaluation of pyrazine mixtures

After determination of the efficacy of single pyrazine derivatives, the development of pyrazine mixtures was the next step for a successful application on natural products. Due to the specific smell of different pyrazine compounds they are more or less suitable for different applications. The combination of different pyrazine derivatives could not only lead to a reduction of microbial contamination but also enhance the taste of a product. Based on observed qualities and odor characteristics of the different pyrazine molecules including reduction rate and odor perception, pyrazine mixtures were developed for a possible application on toast bread.

2.3.3 Reduction assays with Penicillium sp. spores

The developed pyrazine mixtures were repeatedly tested using the antimicrobial and antifungal efficacy test. They were developed for the application on toast bread and

therefore the antifungal test was not only performed using *C. albicans*, but also *Penicillium* sp. spores, directly isolated from molding toast bread. The *Penicillium* sp. spores were diluted in 0.85% NaCl solution to get an estimated spore count of 30 spores per mL using the thoma cell counting method. 1 mL aliquots of this dilution were incubated with 3 µL of the pyrazine mixtures overnight. For a control, 1 mL of spore solution was incubated without adding pyrazine solution. After the incubation 100 µL of the incubated spore solutions were plated out on PDA agar. Colony forming units were counted after incubating the plates 3 days at room temperature.

2.3.4 Volatile activity test with Penicillium sp. spores

Pyrazine derivatives are volatile organic compounds, which is an advantage in comparison to other antimicrobial molecules. The efficacy of pyrazines is not due to direct contact with the compound, but can also bypass longer distances. Using dual culture petri dishes the volatile activity of pyrazines on *Penicillium* sp. spores was tested. One chamber of the plates was filled with PDA agar and ~100 spores were plated out onto the agar. Into the second chamber a pyrazine solution was put on a thin glass cover slip (140 µL of TB2 V3). The cover slip was used to hold the pyrazine drop into place and prevent the pyrazine from reacting with the material of the agar plate. The mold growth on the plates was observed daily for a week. The control plate contained no pyrazine.

2.3.5 Shelf-life extension of bakery products

For the efficacy test of the application on toast bread, whole packages of toast bread were inoculated using *Penicillium* sp. spores and several formulations as a treatment were applied. Around 15-30 spores were applied into every toast bread package by counting the spore density under the microscope using a thoma cell counting chamber. The treatments and spore solution was mixed shortly before addition. A total volume of 330 µL (0.3% pyrazine content) of spore-treatment mixture was added to the empty package and spread on the inside before putting the toast bread into it. This way, 54 packages for each treatment were repackaged and inoculated with spores and treatments. For a negative control no treatment was added to the packages. Instead of treatments oil or water was used to add the same volume. The used treatments are listed in Table 4. All the different packages were stored for two weeks and looked through daily to observe mold formation. After all the negative controls showed mold formation the experiment was stopped.

Table 4: Different treatments used in the toast stress test. Different carriers were used and pyrazine formulation was added for a total concentration of 0.3%.

Treatment	Carrier	Pyrazine formulation
Control 1	Water	-
Control 2	Rape seed oil	-
Treatment 1	Rape seed oil	TB2 V3
Treatment 2	Triacetin	TB2 V3

Triacetin used in the Treatment 2 is an organic component widely used in the food industry as a carrier for aroma components [79].

2.4 VOCs treatment of chicken eggs

Egg samples for this experiment were obtained from four different producers. Two different brands from the supermarket, eggs from the farmers market and eggs directly from a styrian farmer were analyzed during this study. Table 5 shows all specifications from the observed egg samples.

Table 5: Origin of egg samples and observed in this study.

Producer	Abbreviation	Origin	Chicken keeping	Approval number
Tonis Freilandeier	TF	Supermarket	Free range	AT61436EG
Da komm' ich her	DB	Supermarket	Deep litter system	AT61297EG
Rudolf Pirstinger	PR	Farmers market	Free range, biological	AT61528EG
Arztenbauernhof	AZ	Farmer	Free range	-

2.4.1 Passive treatment

For the passive treatment 12 eggs from four different producers were incubated in a closed plastic container (Figure 2). A total of 10 mL of 5IB23MP was placed in a petridish underneath the eggs. During a 6 h incubation time the pyrazine evaporated into the headspace surrounding the eggs. As a control, eggs were incubated using the same plastic containers without addition of pyrazine. After treatment samples from three eggs for each sample were prepared using the method described in 2.4.3.

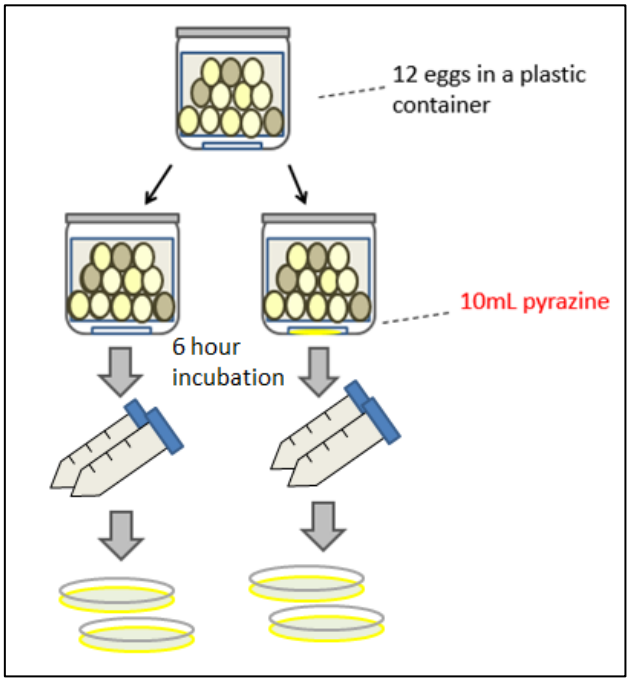


Figure 2: Schematic flowchart of the passive treatment in sealed plastic containers with sample preparation.

2.4.2 Thermic treatment

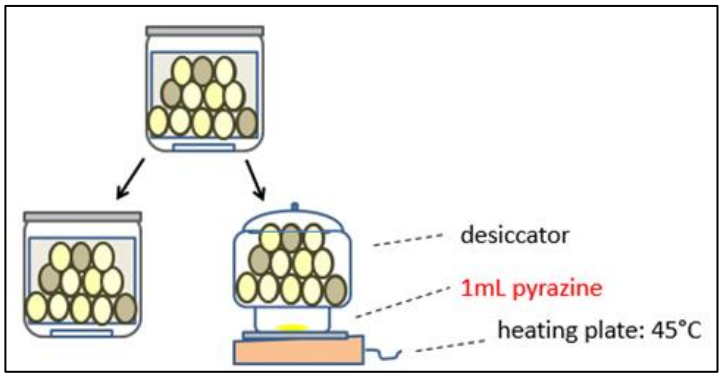


Figure 3: Schematic flowchart of the thermic treatment. A sealed desiccator was placed on a heating plate for the volatile treatment.

The thermic treatment was performed in a desiccator. A total of 1 mL of 5IB23MP was evaporated underneath the eggs using a heating plate set on 45 °C. The temperature had no influence on the eggs, due to the distance between heating plate and eggs. All the pyrazine was evaporated in a short time period and condensing pyrazine was re-evaporated immediately. The eggs were incubated for 6 h. As a control, eggs were incubated in a plastic container for the same time without addition of pyrazine (Figure 3). After incubation, the samples were prepared as described in the following chapter.

2.5 Specific preparation of egg samples

Egg samples were prepared with standardized protocols to analyze contamination levels on the treated samples compared to the control samples. Cultivation dependent methods, as well as real-time qPCR, were performed using the same samples. Additionally, structural community changes during the treatments were analyzed using amplicon sequencing.

2.5.1 Microbial treatment efficacy evaluation

After passive, thermic or no treatment (=control) the CFU on the eggshells were analyzed. For the sample preparation the shells from 3 eggs were washed for 15 min on 400 rpm with 20 mL of PBS in 50 ml reaction tubes. Thereafter, 100 µL of the not diluted solution, a 10^{-1} and a 10^{-2} dilution was plated on NA agar to analyze the CFU (colony forming units). After incubating the agar plates over night at 30 °C, the colony forming units (CFU) were counted. Reduction of colony forming units indicated a reduced contamination level on the treated eggs.

2.5.2 Propidium monoazide treatment of egg shells

A total of 1 mL of the previously prepared egg shell solution was treated with 20 µL PMA (propidium monoazide) to reduce the free DNA or DNA from dead bacteria in the sample [80]. After adding the PMA the samples were incubated for 50 min in the dark with slight shaking every 10 min in 1.5 ml reaction tubes. To start the PMA reaction after incubation in the dark, the samples were placed under blue LED light (465-475 nm) for 10 min with opened lid. After PMA treatment a pellet was formed using 16000 rpm for 20 min and 4 °C. The pellet was further used for DNA extraction and 16S rDNA analysis.

2.5.3 Screening of bacteria isolated from egg shells

From passive treated sample agar plates as well as their correspondent controls a total of 66 different bacterial isolates were analyzed using Sanger sequencing and pyrazine screening. DNA from 34 treated and 32 untreated (control) isolates was isolated using the technique described in 2.5.4. Using the universal 27f/1492r primerset the 16S rRNA gene was amplified and sent for Sanger sequencing. Sequencing results were analyzed using Blast search and species were determined

up to the genus level. The Isolates were also characterized using the antibacterial activity pyrazine test described in 2.3.1. Bacterial solutions of an OD_{600} of 0.002 were incubated for 4 hours with a pyrazine (5IB23MP) concentration of 0.3% as well as without pyrazine as a control. 100 µL of the incubated solution was plated onto NA agar plates (Undiluted and 10^{-2} dilution) to analyze the sensitivity of the isolates. The isolates were further characterized as sensitive or not sensitive to pyrazine depending on the found CFU on the agar plates. Significant reduction from 50-100% (visible reduction) was classified as sensitive, whereas not visible reduction was classified as not sensitive to pyrazine.

2.5.4 Community DNA extraction

The genomic DNA was extracted using the MP FastDNA® Kit for Soil. In the first step the previously obtained pellet was resuspended in 978 µL Sodium Phospate Buffer. By further following the protocol from the kit the DNA was extracted and was ready for further analysis.

2.6 HiSeq Illumina amplicon study

2.6.1 Barcoded primer 16S rDNA PCR

The extracted DNA was used as a template for PCR amplification. Barcoded PCR was performed using the universal bacterial primer set 515f/806r to target the 16S rDNA hypervariable region 4 [81]. Different barcodes for each sample were needed to reassemble the sequencing data later on. The PCR for each sample was performed 4 times and after performing a control gel to identify the correct PCR fragment length, the amplified DNA was purified and subsequently pooled using the Promega Wizard® SVGel and PCR Clean Up System Kit. The temperature program for the PCRs is shown in Table 6. Barcoded samples were pooled equimolarly and sent for paired-end HiSeq Illumina sequencing (MWG Eurofins, Germany).

Table 6: Temperature program for the 16S rDNA PCR. The temperature profile (step 2-4) was repeated for 30 cycles.

Temperature	Time
95 °C	5 min
96 °C	60 sec
54 °C	60 sec

74 °C	60 sec
74 °C	Hold 10 min
4 °C	∞

2.6.2 Data evaluation using the QIIME 1.9.0 pipeline

After HiSeq Illumina sequencing of the barcoded PCR fragments the data was analyzed using the pipeline QIIME 1.9.0. The QIIME workbench is an complex, open source bioinformatics tool to analyze Illumina or 454 sequencing data for microbiome studies [82]. Initially, the raw Illumina HiSeq forward and reverse reads were joined (default method: fastq-join) for all of the 64 samples. The fastq sequence data demultiplexing was performed with QIIME default setting. Additionally, the sequences were quality-checked for chimeric sequences (usearch7) [83]. Subsequently, the OTU table was generated with the script “pick_open_reference_otus.py” using default settings and reference database. Single- and doubletons were filtered from the dataset. The greengenes database (release 13_5) was employed for reference sequences and taxonomy assignment. OTU clustering was performed with a sequence similarity threshold of 97% representing theoretical taxonomic units at species level. The “core_diversity_analyses.py” script was used to generate all summarize taxa through plots, alpha- and beta-diversity plots as well as statistical evaluations plugging several scripts together. Sampling depth was rarefied to 21000 based on the biome summarize-table outcome and the lowest number of counts. Comparative analysis on OTU frequencies to identify statistically significant differences between OTU abundances in the three different treatments was performed with the QIIME script “group_significance.py” using the Kruskal-Wallis test based on the rarified OTU table. Based on the comparative analysis a pie-charted network was generated using Cytoscape 3.4.0 to visualize the significant differences between the treatments. The 100 first results with the lowest p-value were used as the dataset.

2.7 16S rRNA real time qPCR

The DNA extracts from the egg samples were further analyzed using qPCR. The qPCR was performed using real-time quantitative method. 16S rDNA was amplified using a Unibac II 515f and 927r primerpair. The quantification was performed in a

Corbett Research thermocycler and Sybr Green was added for quantification. The PCR mixture and the temperature profile for the qPCR are shown in Table 7 and 8.

Table 7: PCR mixture for a 7 µL preparation.

Solution	Amount in µL
2x KAPA Sybr Green	5
BSA	0.2
Unibac II 927r	0.12
Unibac II 515f	0.12
ddH_2O	0.8
DNA	0.8

Table 8: Temperature program for the qPCR. The temperature profile (step 2-4) was repeated for 40 cycles.

Temperature	Time
95°C	5 min
95°C	20 sec
54°C	15 sec
72°C	30 sec
72°C	10 min
4°C	∞

The standard curve (Figure 4) was performed using a standardized *Bacillus cereus* 16S rDNA gene fragment and further 1:10 dilutions to cover the full range of measurement. This way gene copy numbers from 0 to $1.67*10^7$ could be measured.

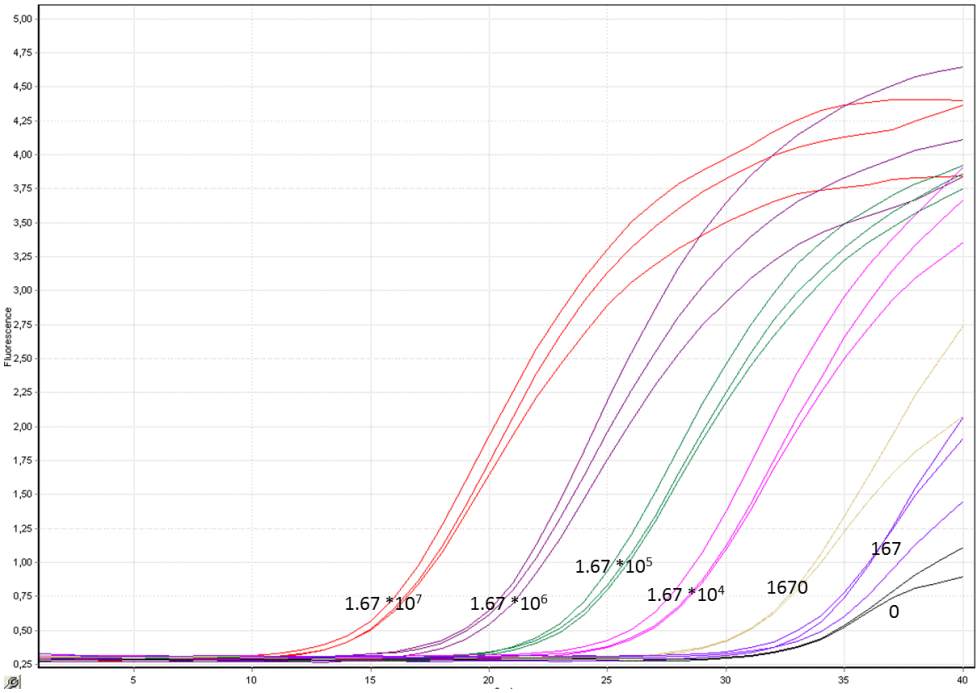


Figure 4: RT qPCR standard curves obtained with dilutions of a *Bacillus cereus* 16S rDNA gene fragment (gene copy numbers indicated). The standard was used to calculate the gene copy numbers in the samples.

Three replicates of each standard dilution were made to generate a mean value. The standard values were employed later on to determine the gene copy numbers in the analyzed samples.

Negative controls (pure dH_2O was added to the DNA extraction kit) were also analyzed and further subtracted from the measured sample values to reduce the method derived inaccuracies. All measurements were repeated three times and mean values were generated.

2.8 Confocal laser scanning microscopy of the egg surface

For the visualization of 5IB23MP thermic treated eggs the eggshells of thermic treated eggs and not treated eggs were analyzed using the LIVE/DEAD® BacLightTM Bacterial Viability Kit (L7012, Molecular Probes, Life Technologies). Micrographs were made using a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany). The two compounds assay contains the SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain propidium iodide (PI). The excitation/emission wavelengths are at 485/530 nm for the green fluorescence and at 485/630 nm for the red fluorescence.

Shells from overnight thermic treated eggs and not treated eggs were observed. The thermic treatment was performed using 1 mL of 5IB23MP and the eggs containing desiccator was placed on a heating plate set on 45 °C to evaporate the pyrazine into the eggs headspace. After incubation the thermic treated and not treated eggshells were cracked into small pieces and placed in 1.5 mL reaction tubes with 1 mL of NaCl. A 3 µL 1:1 solution of LIVE/DEAD mixture (1.5 µL SYTO 9 and 1.5 µL PI) was added and incubated for 15 min. After incubation the eggshell pieces could be analyzed immediately using the confocal laser-scanning microscope.

2.9 Egg shell penetration test using GC/MS

For a further analysis of the sterilization process during the utilized pyrazine treatment, a GC/MS experiment was developed analyzing whether the pyrazine is penetrating the egg shells or not. A penetration could indicate that further investigations are needed to analyze the affections of pyrazine on the chicken development, due to the possible direct contact of the developing chicken with pyrazine molecules. For the experiment three thermic treated eggs were compared to not-treated eggs. The egg surfaces were washed using dish washing soap to get rid of pyrazine on the egg shell and not to contaminate the samples. This is an established method to prevent contamination of food ingredient analysis samples with exterior factors [84]. After washing the eggs, the egg white and yolk was placed into a small erlenmeyer flask and 4 mL of hexane were added. The flasks were placed on a shaker for 30 min and 250 rpm. Using a microseparator the hexane phase was extracted and placed into a GC vial. Additionally to the treated and not treated eggs, pyrazine was added to the egg white and yolk of not treated eggs (0.1 µL and 1 µL) and pyrazine was extracted using the same method. With this method it was possible to analyze the amount of recovered pyrazine using the extraction method.

2.9.1 GC/MS analysis

Compound separation and detection was performed on a system combining a GC 7890A with a quadrupol MS 5975C (Agilent Technologies, Waldbronn, Germany). Liquid injected samples were run through a (5%-phenyl)methylpolysiloxane column, 60 m × 0.25 mm i.d., 0.25 µm film thickness (DB-5MS; Agilent Technologies, Waldbronn, Germany), followed by electron ionization (EI; 70 eV) and detection (mass range 25-350). The inlet temperature was adjusted to 270 °C. For the temperature

gradient the GC column was kept at 70 °C for 1.5 min, raised to 200 °C at a rate of 16 °C/min and finally maintained at 200 °C for 0.5 min. The helium flow rate was set to 1.2 mL/min. Obtained spectra were compared with NIST Mass Spectral Database 14 entries to identify the right pyrazine component.

3. Results

3.1 Antimicrobial activity of pyrazine derivatives

Antibacterial and antifungal activity tests showed different pyrazine efficacies on different microorganisms. Highly active compounds were identified for the application on natural products.

3.1.1 Antibacterial activity

The antibacterial activity of the pyrazine derivatives used in this study was observed by sequential analysis of single pyrazines in 0.3% pyrazine solution using 1.5 mL tubes, during an incubation of 4 h. Initial cell densities were set to an OD_{600} of 0.002 in a 1 mL reaction volume. Each pyrazine was tested on *E. coli* (gram$^-$) and *S. aureus* (gram$^+$). After incubating the microorganisms in 0.3% pyrazine derivative solution at 30°C and 330 rpm for 4 h, 100µL of not diluted and 10^{-2} solution was plated on NA agar to analyze the CFU. Figure 5-8 show the results of the plated agar plates. For non-active pyrazine derivatives the 10^{-2} dilution plates are shown. Highly active pyrazine derivatives are shown on not diluted plates. The results indicate a low activity for 2E3MP, 25DMP, 26DMP, 2EP and 235TMP whereas pyrazines such as 2IB3MP, 23DEP, 2MOX3MP, 5H5M67DHP and 2DE5MP showed a high efficacy on the tested bacteria. This indicates that not all pyrazine derivatives have the same reduction rates and are not equally suitable for an application in stabilization processes.

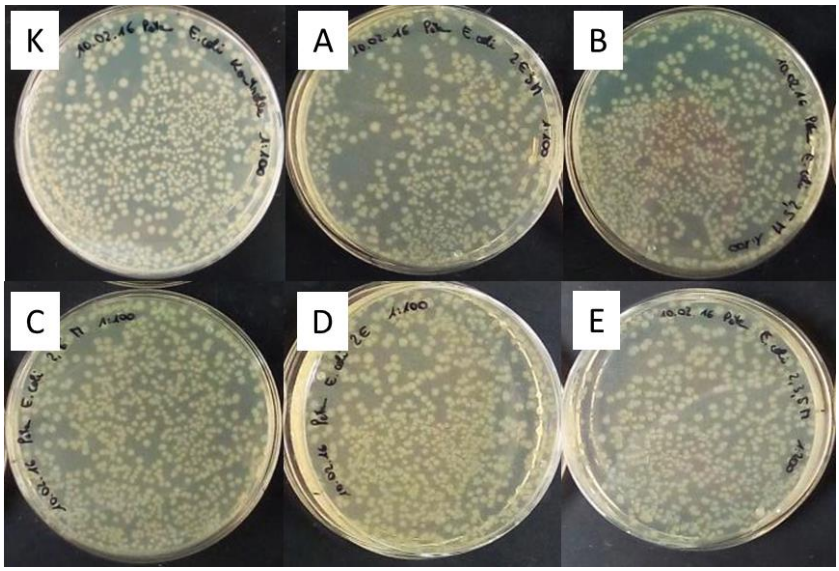


Figure 5: *E.coli* incubated with non-active pyrazines with low reduction rates (0.3%, 4h). K: control; A: 2E3MP; B: 25DMP; C: 26DMP; D: 2EP; E: 235TMP. Plates show the 10^{-2} solution plated on NA.

Non-active pyrazine derivatives show no or very little visible effect on the incubated *E.coli* solutions when compared to the control in the upper left corner (Figure 5). The highly effective pyrazines incubated using the same techniques showed a very strong effect on the *E. coli* cells. Cell forming units were vastly reduced and reductions rates observed were up to 100% (Figure 6, Table 9).

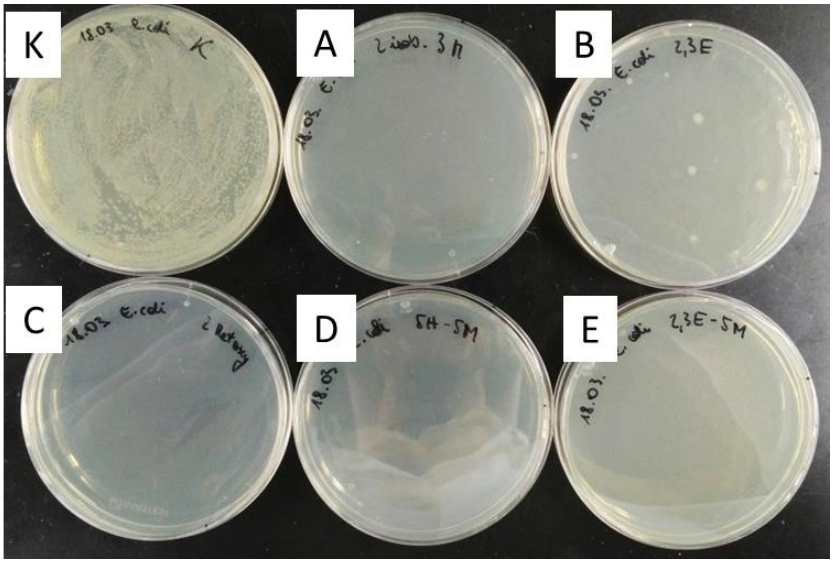


Figure 6: *E.coli* incubated with highly active pyrazines (0.3%, 4h). K: control; A: 2IB3MP; B: 23DEP; C: 2MOX3MP; D: 5H5M67DHP; E: 2DE5MP

The CFU/mL were calculated from the plate tests and the reduction rates of the single pyrazine derivatives analyzed by comparing the CFU/mL from treated and not treated (control) samples. The results can be found in Table 9 below.

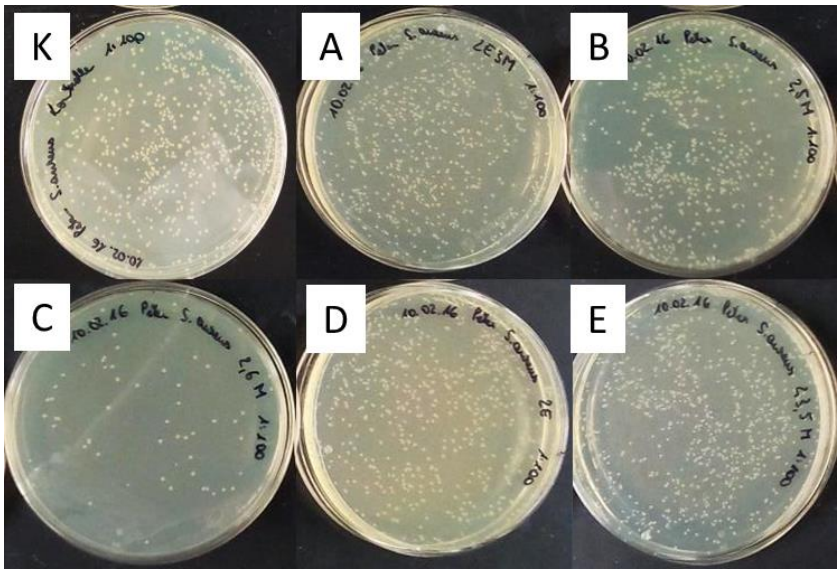


Figure 7: *Staphylococcus aureus* incubated with non-active pyrazines with low reduction rates (0.3%, 4h). K: control; A: 2E3MP; B: 25DMP; C: 26DMP; D: 2EP; E: 235TMP

Similar to the *E. coli* tests before, non-active pyrazine derivatives show no or very little visible effect on the incubated *S. aureus* solutions when compared to the control in the upper left corner (Figure 7). The highly effective pyrazines incubated using the same techniques showed a very strong effect on the *S. aureus* cells. Cell forming units were significantly reduced and reductions rates observed were up to 100% (Figure 8, Table 9).

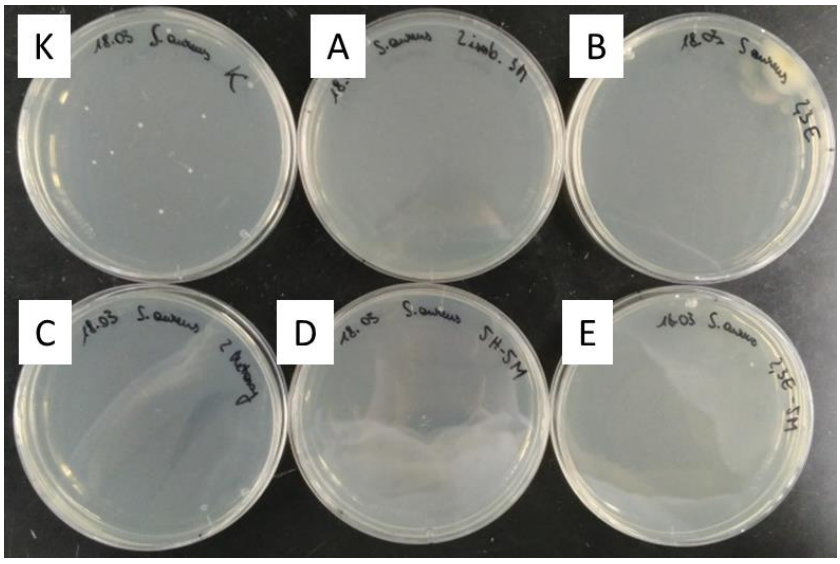


Figure 8: *Staphylococcus aureus* incubated with highly active pyrazines (0.3%, 4h). K: control; A: 2IB3MP; B: 23DEP; C: 2MOX3MP; D: 5H5M67DHP; E: 2DE5MP

The CFU/mL were calculated from the plate tests and the reduction rates of the single pyrazine derivatives analyzed by comparing the CFU/mL from treated and not treated (control) samples. The results can be found in Table 9.

3.1.2 Antifungal activity

Antifungal activity of the pyrazine derivatives used in this study was analyzed using *Candida albicans* as a model organism. The procedure was analogue to the antibacterial activity test performed on *E. coli* and *S. aureus*. An OD_{600} solution of 0.002 was incubated with a 0.3% pyrazine concentration in 1.5 mL tubes at a total volume of 1 mL. After incubation on 30°C for 4 hours and 330 rpm, 100 µL of the solution was plated not diluted and in a 10^{-2} dilution on PDA plates to analyze the CFU/mL. Figure 9-10 show the plates from treated samples and controls. The results indicate a low activity for 2E3MP, 25DMP, 26DMP, 2EP and 235TMP whereas pyrazines such as 2IB3MP, 2MOX3MP and 2DE5MP showed a high efficacy on *C. albicans*. These findings are similar to the efficacy tests on *E. coli* and *S. aureus* in the previous experiment, but not all highly effective pyrazines showed a strong reduction on *C. albicans*.

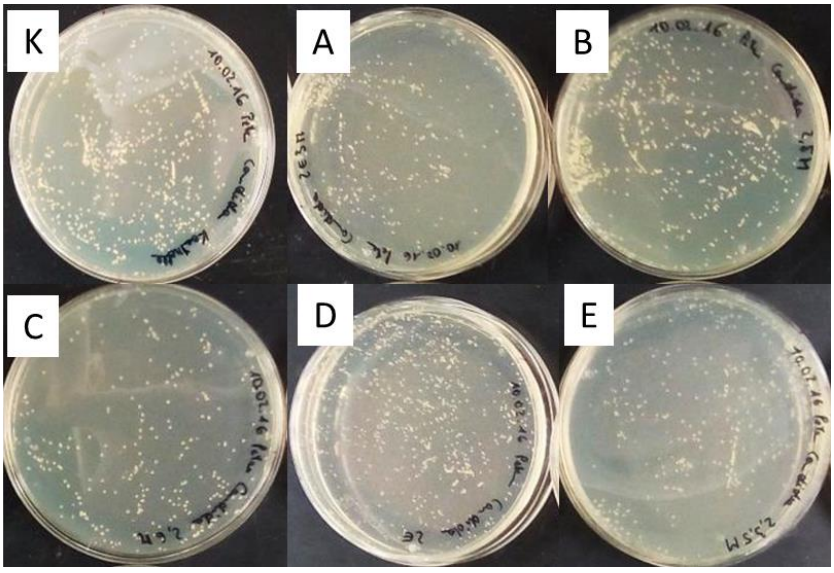


Figure 9: *Candida albicans* incubated with non-active pyrazines with low reduction rates (0.3%, 4h). K: control; A: 2E3MP; B: 25DMP; C: 26DMP; D: 2EP; E: 235TMP

Similar to the antibacterial tests before, non-active pyrazine derivatives show no or very little visible effect on the incubated *C. albicans* solutions when compared to the control in the upper left corner (Figure 9). Not all highly effective pyrazines, incubated using the same techniques, showed a very strong effect on the *C. albicans* cells. Cell

forming units were significantly reduced in some cases and reductions rates observed were up to 100% (Figure 10, Table 9). Nevertheless, 5H5M67DHP and 23DEP showed less strong effect on *C. albicans* while being very effective on *E. coli* and *S. aureus*.

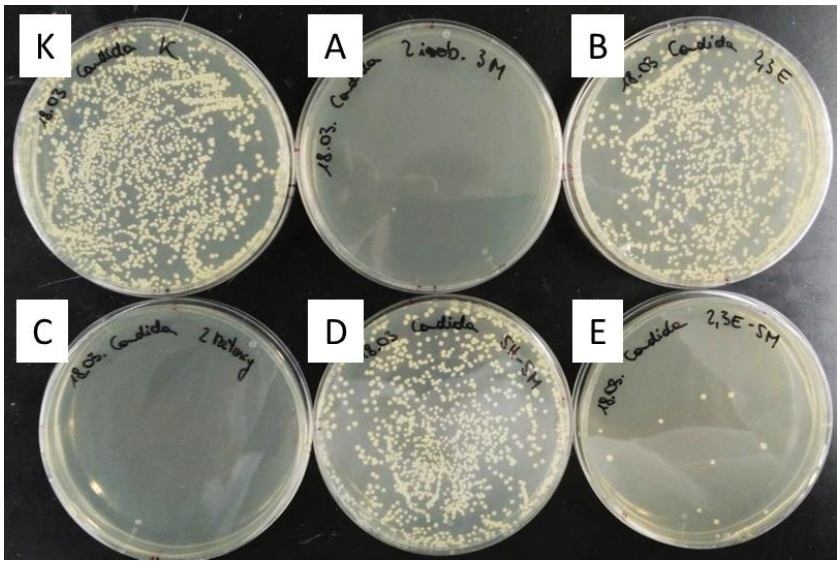


Figure 10: *Candida albicans* incubated with highly active pyrazines (0.3%, 4h). K: control; A: 2IB3MP; B: 23DEP; C: 2MOX3MP; D: 5H5M67DHP; E: 2DE5MP

The CFU/mL were calculated from the plate tests and the reduction rates of the single pyrazine derivatives analyzed by comparing the CFU/mL from treated and not treated (= control) samples. The results can be found in Table 9.

Most pyrazine derivatives had very similar effects on all three microorganisms tested, but slight differences can also be seen. This indicates a dependency of the reduction rate from the pyrazine compound used and its characteristics.

Table 9: Reduction rates of different pyrazines calculated after treatment of 3 different microorganisms. *E. coli*, *S. aureus* and *C. albicans* were incubated for 4 h with 0.3% pyrazine concentrations and CFUs were examined using cultivation dependent methods.

Pyrazine	Reduction of *E. coli*	Reduction of *S. aureus*	Reduction of *C. albicans*	Reduction mean
26DMP	3%	10%	33%	15%
25DMP	0%	0%	0%	0%
2EP	0%	0%	0%	0%
235TMP	12%	0%	37%	16%
2E3MP	23%	0%	26%	16%
5H5M67DHP	100%	100%	75%	92%
23DEP	99%	100%	59%	86%
23DE5MP	89%	90%	98%	92%
2IB3MP	100%	100%	100%	100%
5IB23MP	100%	100%	100%	100%
2MOX3MP	100%	100%	100%	100%

3.1.3 The correlation between log P value and reduction rate

During the antimicrobial activity tests pyrazines with a high log P value showed a higher reduction rate while lower log P values led to lower reduction rates. The log P value is a partition coefficient of a compound between 1-octanol and water [85]. In Figure 11 the log P values of the pyrazine derivatives used in this study are shown as bar chart, whereas the calculated reduction rates of the different microorganisms (*E. coli*, *S. aureus* and *C. albicans*) are shown as lines. Pyrazine derivatives with lower

log P values showed also lower reduction rates, whereas pyrazines with higher log P values showed up to 100% reduction of the tested microorganisms. This indicates a correlation between log P value and reduction rate.

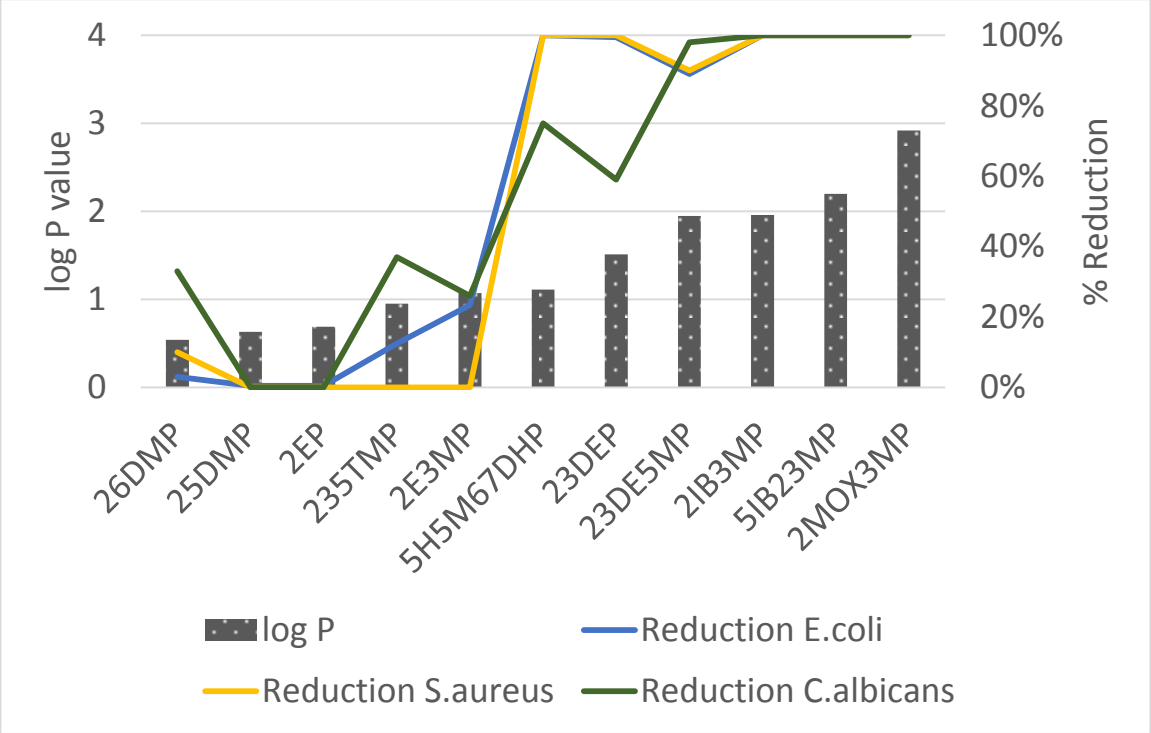


Figure 11: Correlation between log P value (columns) and reduction of *E. coli, S. aureus* and *C. albicans* (lines) based on CFU counts after a 4 hour incubation time.

Figure 11 also indicates some differences between the three different microorganisms tested. While *E. coli* and *S. aureus* reacted very similar to the pyrazine derivatives tested, *C. albicans* showed some differences. As mentioned before, 5H5M67DHP and 23DEP were less effective on *C. albicans*, while 235TMP and 26DMP showed stronger effect compared to the other microorganisms tested. Nevertheless, the correlation between log P value and reduction rate is strong in all results.

3.1.4 Odor characteristics of pyrazine compounds

Pyrazine derivatives are known for their distinguished smell and are therefore widely used in the flavoring industry. Odor characteristics are also of great importance when it comes to stabilization applications on food products. During this study odor characteristics of the different pyrazine derivatives were examined and extracted from literature. Table 10 shows the odors found for the different pyrazine derivatives. Odor perspectives were found in literature and classified as intensive for a very strong smell or weak when the smell was less notable. Pyrazine derivatives with a weaker smell

are more interesting for an application in food industry, so the taste is not highly affected by the pyrazine added.

Table 10: List of used pyrazines and their characteristics. Odor and log P values found in literature [86]. Used abbreviations in this thesis.

Pyrazine	Odor	Log P value	Abbreviation
5-isobutyl-2,3-dimethylpyrazine	weak; green	2.2	5IB23MP
2-isobutyl-3-methylpyrazine	weak; green	1.96	2IB3MP
2-Ethylpyrazine	weak; nutty	0.69	2EP
2-Ethyl-3-Methylpyrszine	intensive; hazelnut, earthy, potato	1.07	2E3MP
2,5-Dimethylpyrazine	weak; meaty, medicinal, nutty, woody	0.63	25DMP
2,6-Dimethylpyrazine	-	0.54	26DMP
2,3,5-Trimethylpyrazine	-	0.95	235TMP
2,3-Diethyl-5-Methylpyrazine	intensive; hazelnut, meaty, vegetable	1.95	23DE5MP
5H-5Methyl-6,7-dihydrocyclopentapyrazine	intensive; earthy, peanut	1.11	5H5M67DHP
2-Methoxy-3-(1-methylpropyl)pyrazine	intensive; green, vegetable, pepper	2.92	2MOX3MP
2,3-Diethylpyrazine	weak; -	1.51	23DEP

3.1.5 Development of pyrazine mixtures

Odor characteristics and reduction efficiency are the most important properties of pyrazine compounds for a stabilizing application. The focus of this study was also on the development of pyrazine mixtures to prevent possible upcoming resistances and to use the pyrazine characteristics to enhance the flavor of the product. In this study possible active mixtures for the application on toast bread were developed. Based on the odor properties and the antimicrobial potencies pyrazines were chosen and mixed in different compositions found in Table 11.

Table 11: Different pyrazine mixtures. Four pyrazines were used in different combinations.

Pyrazine/ Treatment	TB2 V1 *	TB2 V2 *	TB2 V3 *
25DMP	4	4	4
2E3MP	1.5	1.5	1
23DEP	1.5	2.25	2
2IB3MP	3	2.25	3

*volumes in µL for a total volume of 10 µL

3.2 Stabilizing application on bakery products

3.2.1 Activity tests of pyrazine mixtures on Penicillium sp. spores

Mold formation is the biggest problem of the stability of toast bread. *Penicillium* sp. was isolated from molding toast bread to work with a representative isolate in all experiments. *Penicillium* sp. is a spore forming ascomycetous fungus [87]. Using the microscope and a thoma chamber spores can be counted and by diluting the spores/mL set to a wanted number. In this test a spore number of ~30 spores/mL were incubated with different pyrazine mixtures (Table 11) in 1.5 mL reaction tubes overnight (30°C, 330 rpm). As a negative control no pyrazine was added into one tube. After incubation 100 µL of not diluted solution was plated on PDA agar plates (Figure 12).

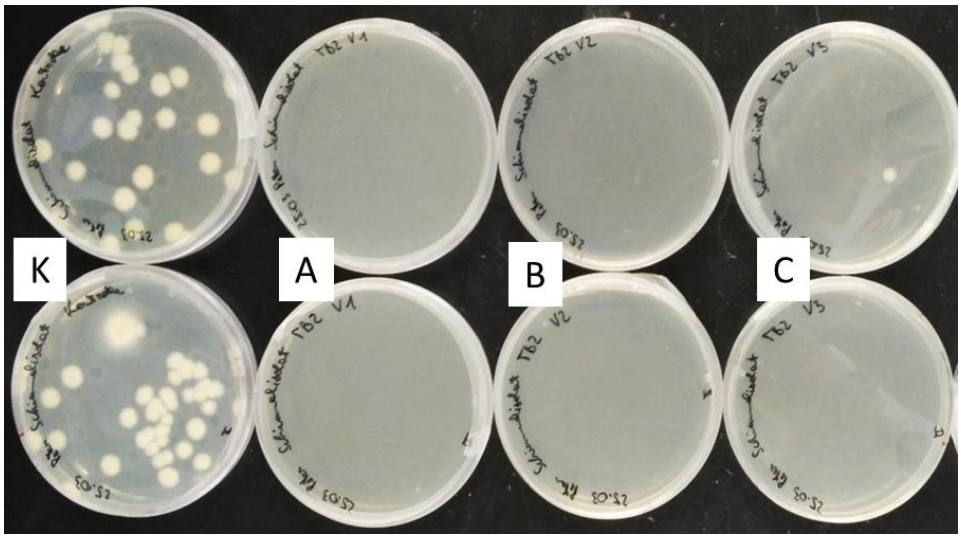


Figure 12: Isolated *Penicilium* sp. spores from toast bread incubated over night with different pyrazine formulations. K: control; A: TB2 V1; B: TB2 V2; C: TB2 V3

All three pyrazine formulations showed a very strong effect on the *Penicillium* sp. spores. The reduction rates of the spores were 100% for all formulations and both replicates. On one plate for TB2 V3 (Figure 12, C) a bacterial contamination was found after incubating the PDA plates. Nevertheless, this experiment showed the strong effect pyrazine derivatives have on *Penicillium* spores.

3.2.2 Volatile activity on dual culture plates

In the antimicrobial efficacy tests and activity test on *Penicillium* sp. spores, pyrazine was in direct contact to the microorganisms over a liquid phase. The next step was to test the activity of pyrazine compounds when there is no direct contact between the pyrazine and the place of action. Pyrazine, being a volatile organic compound, is easily going into the gas phase and can, this way, have an impact on microorganisms or fungi even through long distances. Using dual culture plates the volatile activity of pyrazines on *Penicillium* sp. spores was tested. One chamber of the plates was filled with PDA agar and ~100 spores were plated out onto the agar. A pyrazine solution was put on a glass cover slip into the second chamber of the plate (140 µL TB2V3). The cover slip was used to hold the pyrazine drop in place and prevent the pyrazine reacting with the material of the agar plate. This was noticed during previous experiments and can also be seen on the plate covers during this experiment (Figure 13, Day 3-6). The plates were observed daily for a week. The negative control contained no pyrazine. Pyrazine mixtures had a noticeable impact on the spore development also over the headspace, as can be seen in Figure 13. Overall, no

reduction of the spores was noticeable, due to the very high density of the spores, but a delay in spore growth can be noticed. The blurring of the petri dish is due to the reaction of the pyrazines with the petri dish polymer.

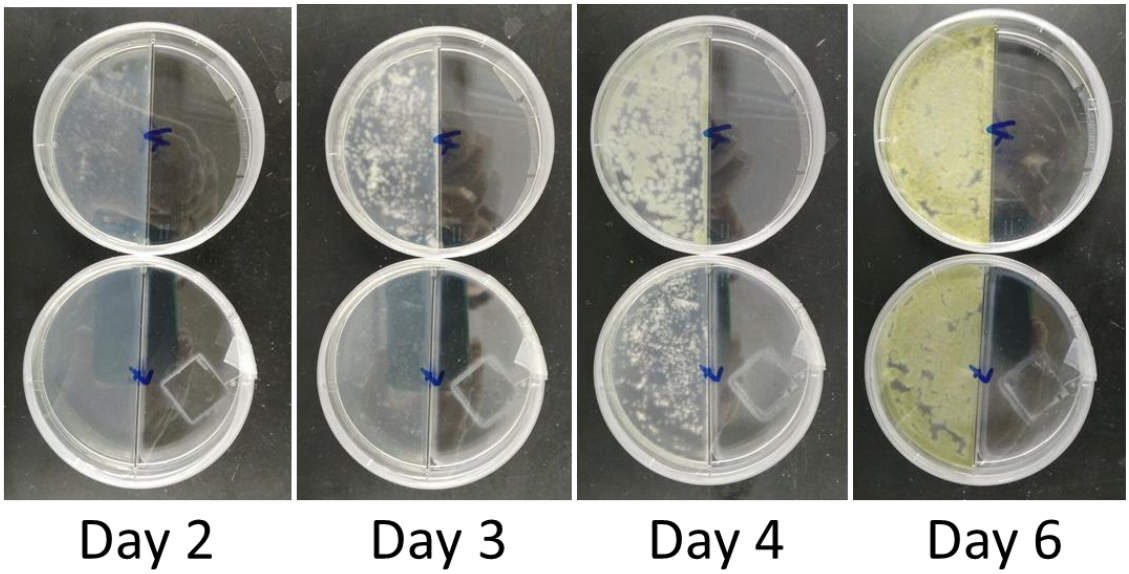


Figure 13: Spore growth of ~100 *Penicillium* sp. spores plated on PDA dual agar plates. Upper row: control (no pyrazine), lower row: pyrazine treated (140 µL TB2 V3) over the headspace

3.2.3 Shelf-life extension of bakery products

The efficacy of pyrazine compounds on *Penicillium* sp. causing mold on toast bread has been shown in the previous experiments. The final experiment for a successful application on toast bread was testing the formulations directly on toast bread. For this purpose, whole toast bread packages were inoculated with *Penicillium* sp. spores and different treatments were added (Table 4). A total of 54 toast bread packages were inoculated for each treatment. As a negative control oil or water was used instead of pyrazine formulation. The development of mold formation on the incubated toast breads was analyzed by visual inspection daily and can be seen in Figure 14.

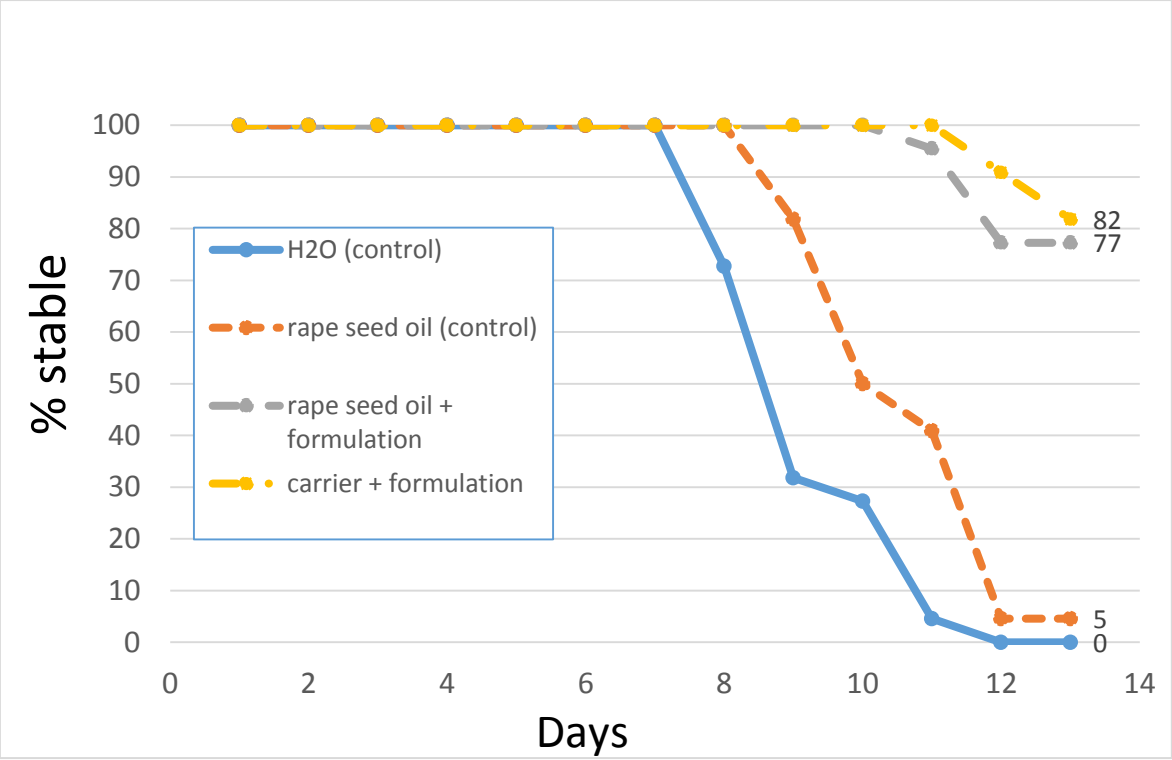


Figure 14: Extension of shelf life of toast bread after inoculation with *Penicilium sp.* spores and treatment with different formulations and corresponding controls. The toast breads were incubated for 13 days and visually inspected for mold growth.

The mold formation was progressive, due to the fact these toast breads were inoculated with spores. However, the effect of treatments on the mold formation was evident. Treatment of the toast breads increased the shelf-life of the toast by up to a week. After 12 days almost all controls were showing mold formation, whereas only 10 % of the treated samples were molding at that time.

3.3 Hatching egg treatment

This experiment was performed to investigate the effect of pyrazine derivatives on total microbial communities. For this purpose, egg samples were treated using two different treating methods to decontaminate the egg surface. The microbial reduction was investigated using cultivation dependent methods, microscopy, qPCR and amplicon sequencing.

3.3.1 Cultivation dependent methods

Egg samples from four different producers (Table 5) were treated using two different methods. Passive treatment is based on the natural evaporation of the pyrazine compound whereas thermic treatment increased the evaporation using low heat. Treatment efficiency was analyzed using growth dependent methods and CFU counts. After sample preparation PBS solution was plated on NA agar plates. Colony forming units (CFU) were counted and recalculated on the gram eggshell used. Figure 15 and 16 show the found CFU for the four different producers for the control and treated samples.

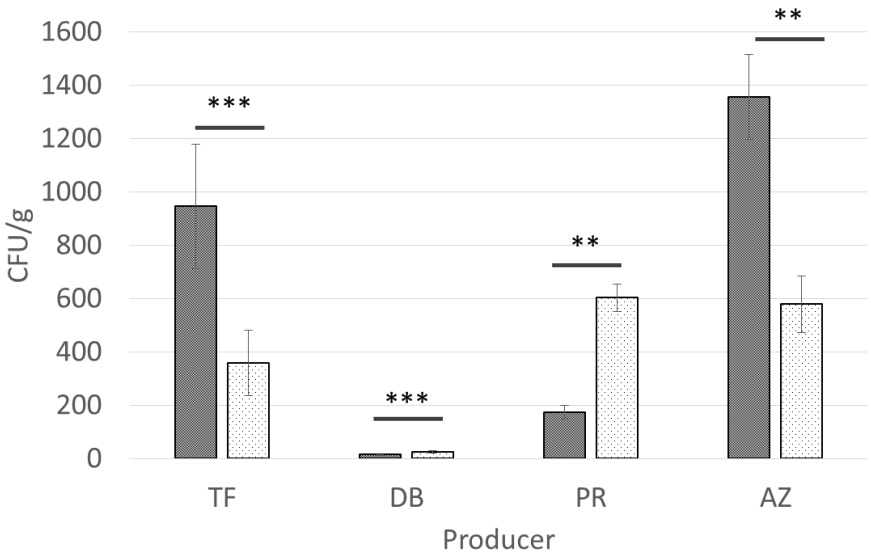


Figure 15: Colony forming units found on passive treated samples from different egg producers. Gray: control; white: passive treated sample

Significance: ***: p > 0.05; **: p < 0.05; *: p < 0.01 (Paired t-Test)

Colony forming units decreased in some cases (TF and AZ), while increased in others during the passive treatment (DB and PR). Overall the significance of the observed values was relatively low due to the high standard deviations (Figure 15). Observed contamination levels were higher on samples from TF and AZ compared to DB and PR.

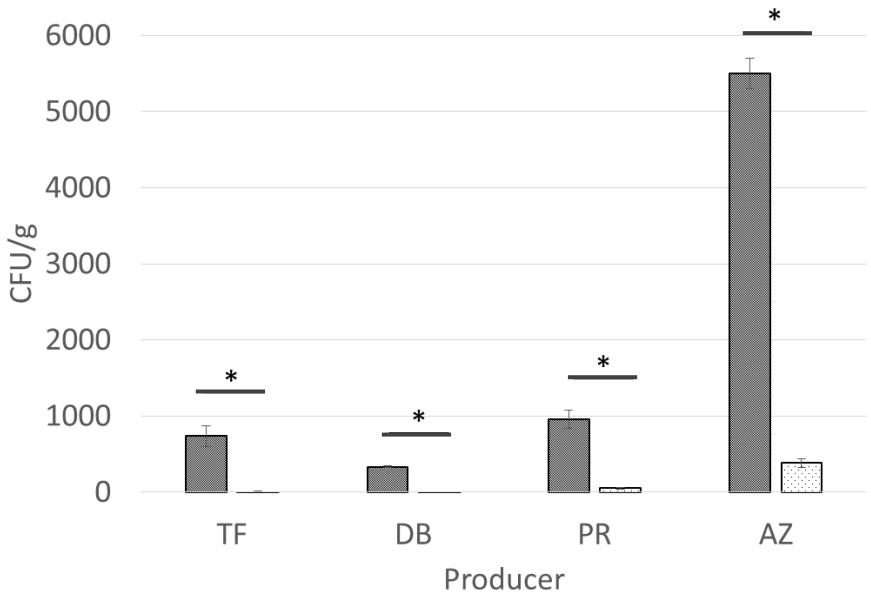


Figure 16: Colony forming units found on thermic treated samples for different producers. Gray: control; white: thermic treated sample

Significance: ***: p > 0.05; **: p < 0.05; *: p < 0.01 (Paired t-Test)

Thermic treated samples showed reduced CFU counts compared to the corresponding control samples (Figure 16). Reduction was highly significant throughout all samples even though, contamination levels on the different samples were significantly different. Table 12 summarizes the calculated reduction rates on the different analyzed samples for the two decontamination methods.

Table 12: Reduction rates calculated from CFU counts of growth dependent methods.

	Treatment	
Producer	**Passive treatment**	**Thermic treatment**
TF	-62.2%	-98.2%
DB	+77.8%	-99.6%
PR	+249.6%	-94.5%
AZ	-57.4%	-93.1%

Passive treatment showed decreasing effects form -57% and increasing effects up to +250%, whereas thermic treatment showed decreasing effects from -93% up to -99.6%.

3.3.2 Activity screenings with egg shell-associated bacteria

Isolates from passive treatment agar plates as well as their correspondent controls were analyzed using Sanger sequencing and pyrazine sensitivity tests. Genus as well as pyrazine (5IB23MP) sensitivity of each isolate were determined. Sanger sequencing results as well as the found sensitivity of the isolates is listed in Table 13.

Table 13: Characteristics of isolated bacteria. CFUs from passive treated samples and controls were isolated and sequenced. Additionally their pyrazine tolerance was analyzed using pyrazine treatment. Genera found less than 2 times were listed as "other".

	Treated		Untreated	
Isolate identification at genus level	**Isolated species**	**Thereof sensitive**	**Isolated species**	**Thereof sensitive**
Bacillus spp.	5	2	5	3
Staphylococcus spp.	10	8	9	6
Pseudomonas spp.	1	1	4	4
Acinetobacter spp.	4	4	0	0
Psychrobacter spp.	4	4	4	3
Stenotrophomonas spp.	3	3	3	3
Unidentified sp.	2	2	2	2
Other	5	4	5	5
Total	**34**	**28**	**32**	**26**

Passive treated (34) as well as not treated isolates (32) were analyzed. From the 66 isolates 54 were found to be sensitive to the tested pyrazine derivative. A total of 12

different genera were identified in which *Bacilli* and *Staphylococci* were found to be the most prominent. *Baccilus spp*. were the most resistant species to pyrazine. All species except for *Actinetobacter spp.* were isolated from both treated and untreated samples.

3.3.3 Confocal laser scanning microscopy

LIVE/DEAD-stained treated and untreated egg shells were analyzed using confocal laser scanning microscopy. Micrographs of living cells stained green and dead cells stained red are shown in Figure 17. Picture A to C show untreated egg shells, whereas picture D to F show treated egg shells.

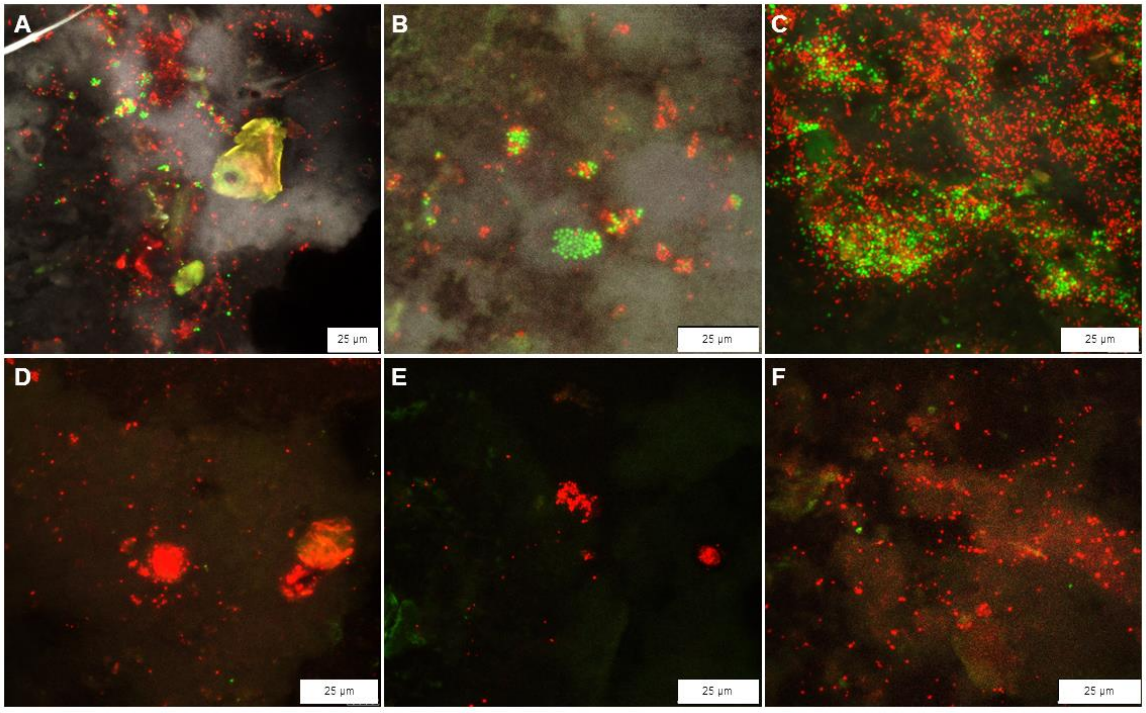


Figure 17: Confocal laser scanning microscopy following 5IB23MP treatment of egg samples using LIVE/DEAD staining of the egg shells. A-C: control, D-F: shells from thermic treated eggs.

Thermic treated egg shells in comparison to control egg shells showed reduced bacterial numbers. Big living colonies were found on control egg shells, whereas no living cells were found on treated samples.

3.3.4 RT qPCR (Real-time quantitative PCR)

Real time qPCR was performed as a quantitative measurement of gene copy numbers found in the DNA extracts from the different egg samples. Results generated using growth depending methods do not picture the whole bacterial community on the analyzed samples. Figure 18 and 19 show the results from the real-time qPCR of the DNA extracts from all samples. Untreated samples (control) are shown in white and corresponding treated samples in black. Significance was analyzed using a t-test.

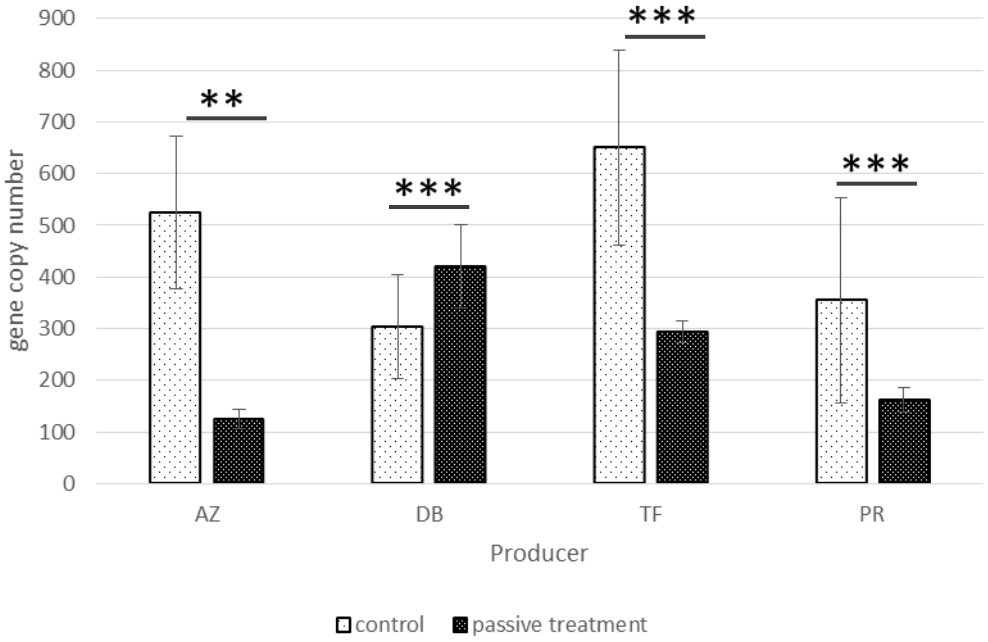


Figure 18: gene copy numbers of 5IB23MP-passive-treated samples and corresponding controls analyzed using RT qPCR. Eggs obtained from four different producers.

Significance: ***: p > 0.05; **: p < 0.05; *: p < 0.01 (Paired t-Test)

Analyzed gene copy numbers from the different producers were found to be reduced after treatment in most cases. The passive treatment showed decreasing gene copy numbers for all producers except for DB. However, measured reduction p-values showed low significance (Figure 18). In contrast, the thermic treatment showed reduced gene copy numbers in all treated samples compared to their corresponding controls. The variance, however, of the measured values was very high resulting in low significances except for producer AZ (Figure 19).

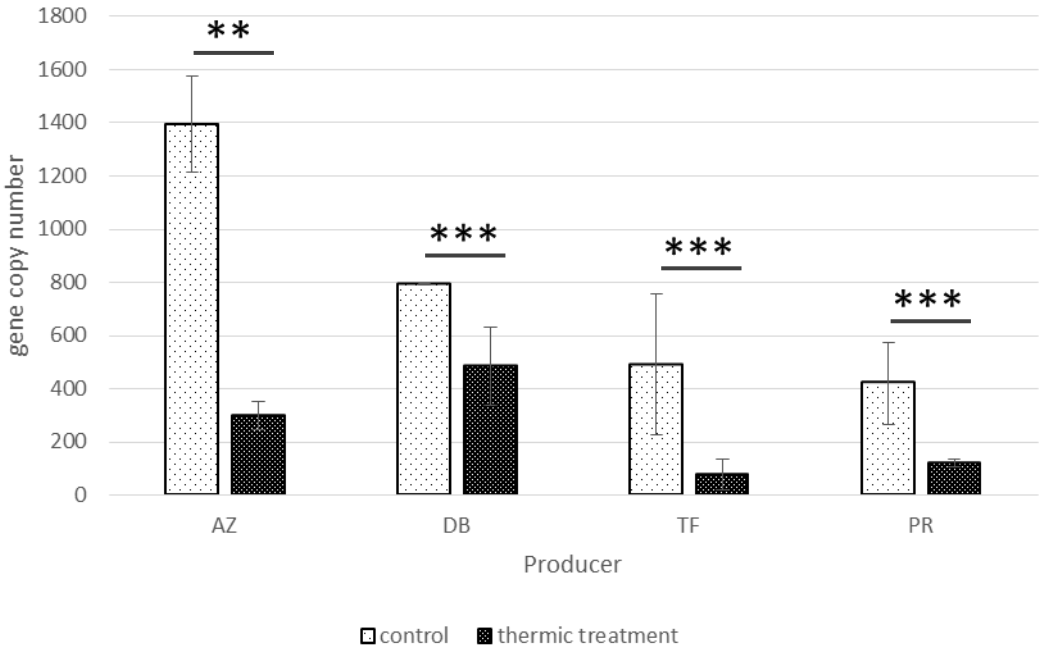


Figure 19: gene copy numbers of 5IB23MP-thermic-treated samples and corresponding controls analyzed using RT qPCR. Eggs obtained from four different producers.

Significance: ***: p > 0.05; **: p < 0.05; *: p < 0.01 (Paired t-Test)

Gene copy numbers of all tested samples were in an equal range (~100 to 800) except for the thermic-treatment control AZ. The gene copy numbers found in this sample were nearly twice as high as the other controls.

3.3.5 Amplicon sequencing

Barcoded PCR samples were analyzed using a QIIME 1.9.0 pipeline. Diversity on order level is shown in Figure 20. The diversity was very high in all samples. Samples from the same producers showed similar compositions on order level. The control (samples from DNA extracts from dH_2O) however showed very similar order compositions to the samples.

Pseudomonadales (4-52%), *Enterobacteriales* (1-10%), *Sphingomonadales* (2-17%), *Streptophyta* (1-11%), *Burkholderiales* (5-12%), *Actinomycetales* (1-8%), *Xanthomonadales* (1-9%), *Rhizobiales* (1-16%), *Bacillales* (2-14%), *Clostridiales* (3-52%), *Lactobacillales* (1-9%) and *Flavobacteriales* (1-4%) were the most prominent orders in all samples (all mean values). Outstanding is the high amount of *Clostridiales* in samples from DB (17-52%) as well as *Pseudomonadales* in samples from AZ (15-36%) and PR (12-52%).

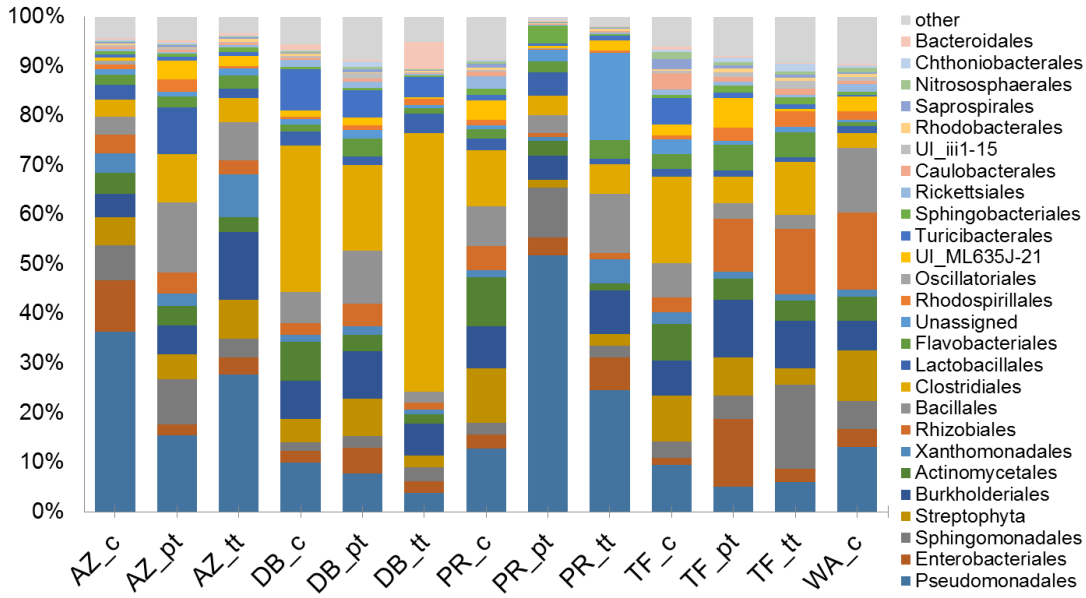


Figure 20: Observed diversity in all samples (order level). The bacterial diversity was very high between the samples due to varying residues on the eggs. c: control; pt: passive treatment; tt: thermic treatment

Treatment efficiency was analyzed by clustering all samples to their respective treatment. Figure 21 shows the community compositions on phylum level. Not treated samples and thermic treated samples seem to be very similar whereas passive treatment showed an increasing *Proteobacteria* fraction (from 43% to 57%). *Actinobacteria* are reduced during both treatments (from 8% in the control to ~4%). The fraction of unassigned OTUs increased during thermic treatment (from 2% in the control up to 8%). Detailed mean values can be found in Table 17 in the appendix.

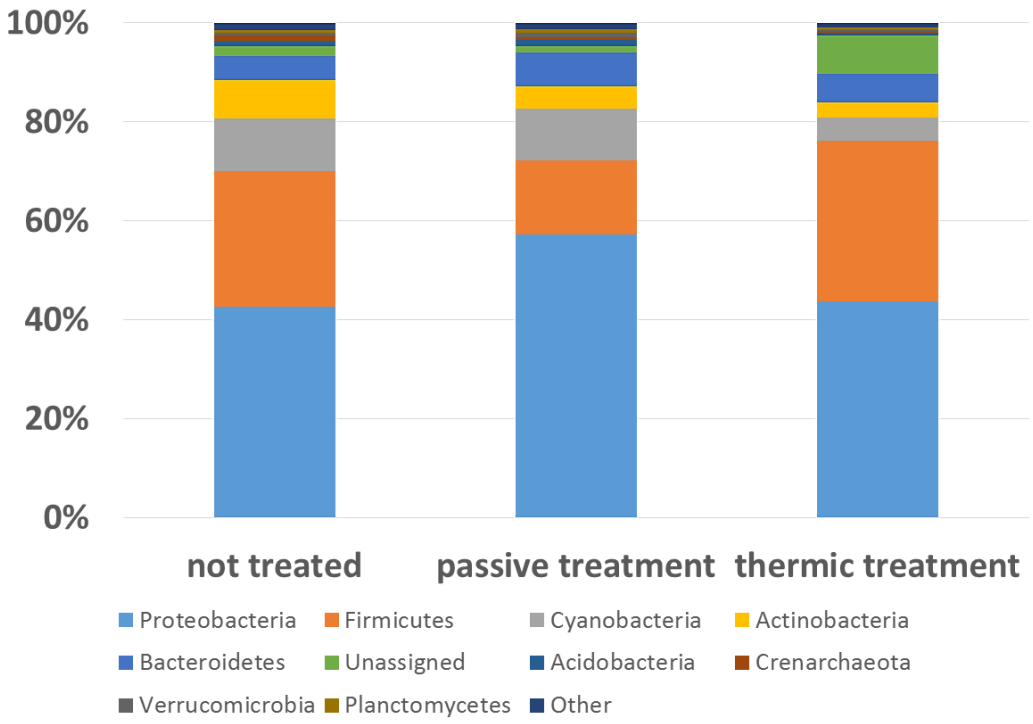


Figure 21: Community shift after treatment of egg samples. Identified OTUs are showed on phylum level.

Alpha rarefaction diversity (Figure 22) showed no significant differences between the treatments and the controls. The most observed OTUs however were found in the passive treatment, while the control samples had the least OTUs.

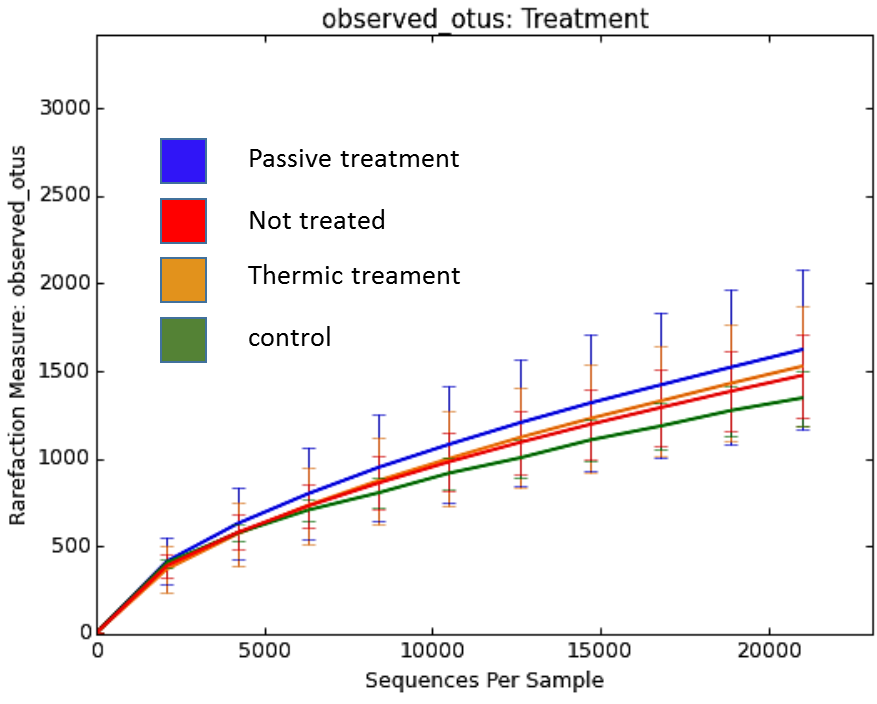


Figure 22: Alpha diversity found in the samples. Observed OTUs per treatment are indicated.

Beta diversity (Figure 23) showed varying diversities between the different producers. No specific grouping of the different treatments was observed due to the high variation between the different dots. Control samples (untreated) seemed to be the most similar throughout all producers.

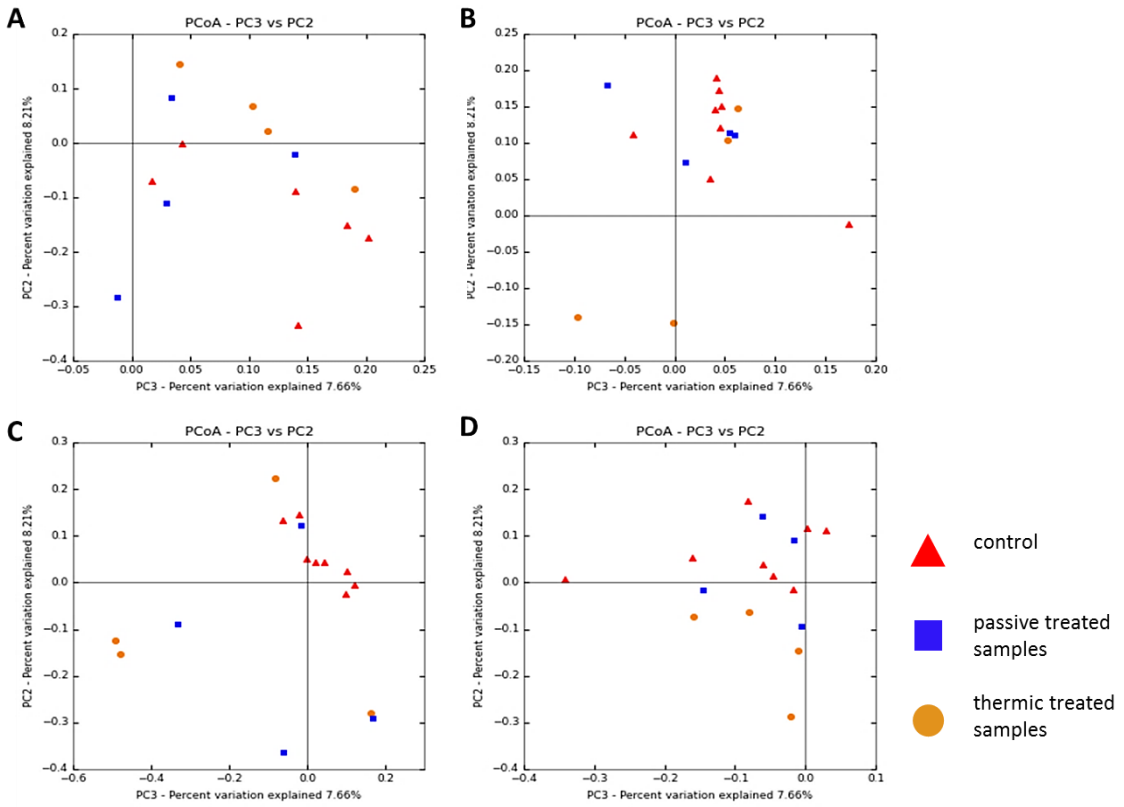


Figure 23: 2D plots of beta diversity of treated and control eggs analyzed in the amplicon study. A: AZ; B: DB; C: PR; D: TF

Group significance evaluations found significant differences between the treatments. Using Cytoscape, the 100 first results with lowest p-value were plotted analyzing the total abundance of the OTUs and their appearance in the treated samples. Figure 24 shows color coded pie charts showing the abundance of specific OTUs in the three different treatments. *Micrococcus* was the most abundant OTU found, with a total 3160 counts, while the other shown OTU counts range from 10-1000. The total abundance is indicated by the node size.

Connections between treatments nodes and OTU nodes show the presence of the OTU in the treatment. The most OTUs were found in both passive and thermic treatments, while only a few OTUs were found only in one treatment. The pie charts in the nodes indicate the fraction of the OTU found in the specific samples.

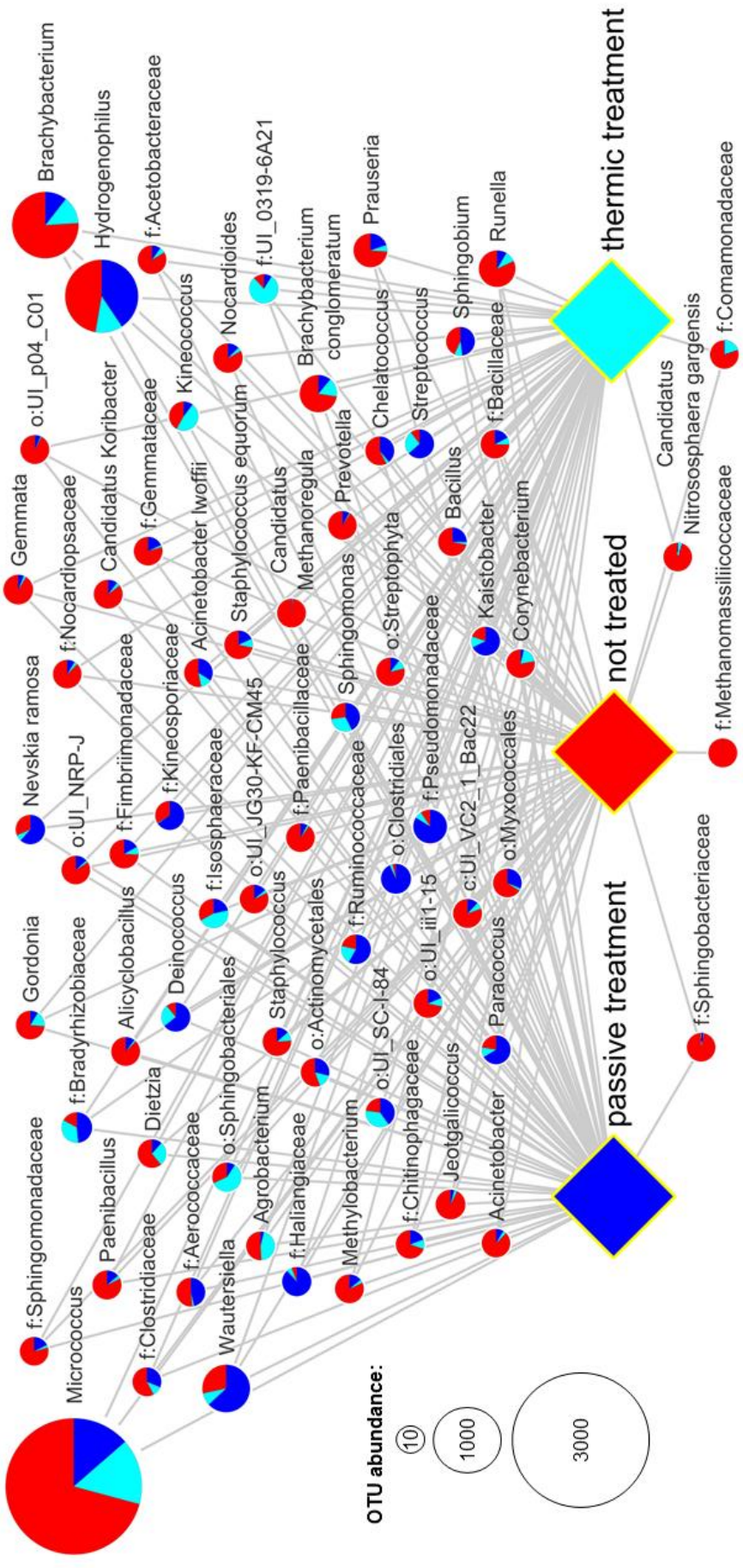


Figure 24: Abundance of OTUs on treated and untreated samples. The node size correlates to the total abundance. Pie charts indicate the fractions found in distinct samples.

Most of the nodes (80) show a high fraction for not treated samples, while some of the nodes indicate an increasing fraction of the OTU induced by a specific treatment. A total of 6 OTUs were increased by thermic treatment and 14 OTUs by passive treatment. *Clostridiales* as well as *Pseudomonadaceae* are some examples highly increased by passive treatment. *Kineococcus* and *Isosphaeraceae* were found to be increased by thermic treatment.

3.3.6 Egg shell penetration test using GC/MS

To gain insight if pyrazines penetrate the egg shells, thermic treated eggs were observed using GC/MS evaluation. Treated eggs as well as controls were first washed using dish washing soap to avoid the transfer of pyrazine from the shells into the egg white and yolk. The eggs were cracked and pyrazine was extracted using hexane as an organic solvent. As a control pyrazine was added to untreated eggs to determine if pyrazine can be recovered by the method (spiked samples). GC standards were made by diluting pyrazine solutions in hexane. The obtained standard is shown in Figure 25.

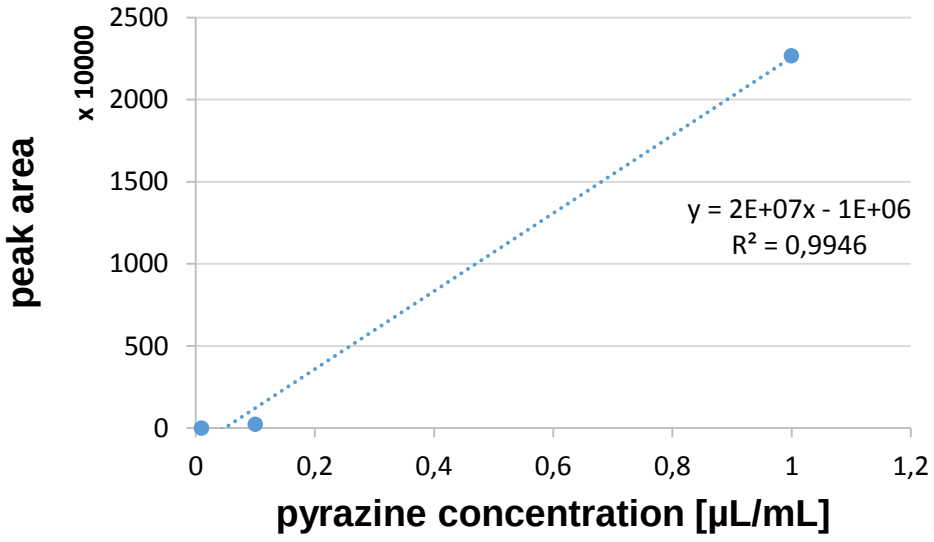


Figure 25: GCMS standard curve for the calculation of sample concentrations.

The concentration of pyrazine found in the samples was calculated using the GC standard. Only sample 3 contained any pyrazine (Figure 26), while no pyrazine was detected in the two other thermic treated eggs.

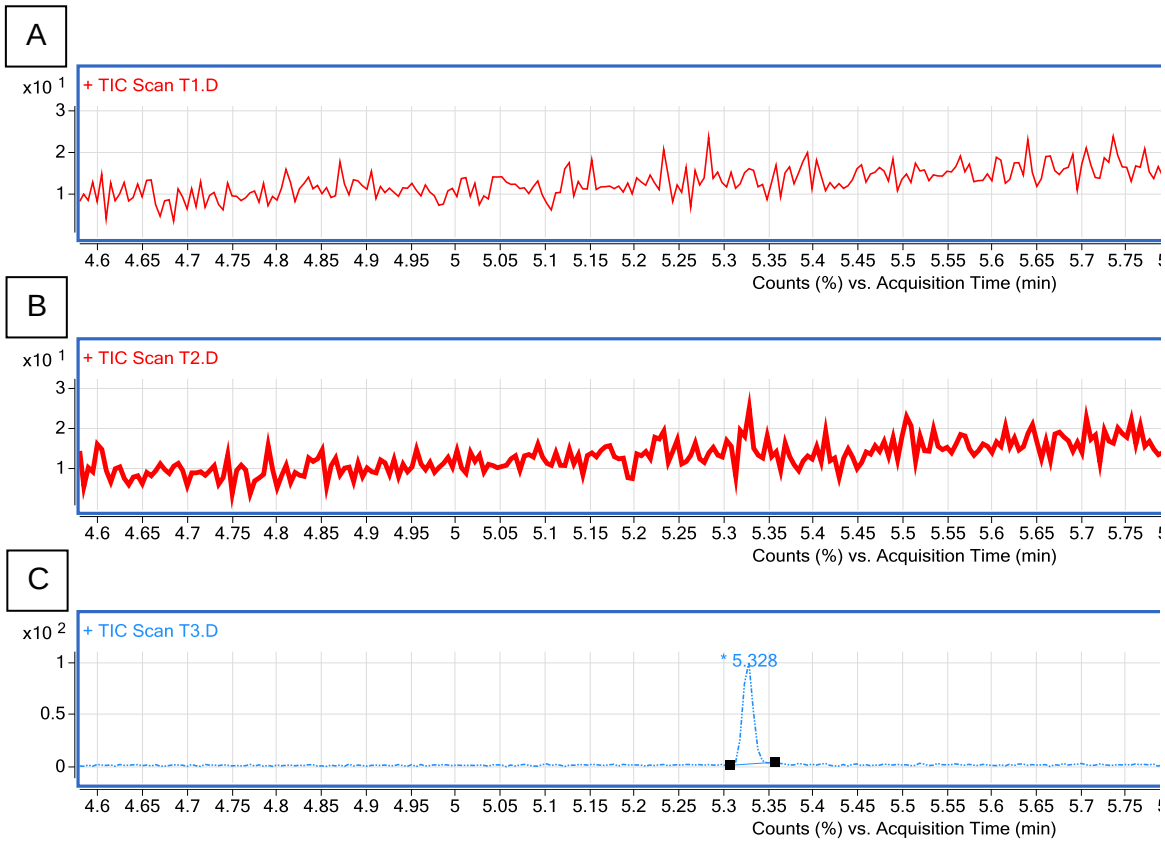


Figure 26: GCMS peak of tested samples after thermic treatment. A: Sample 1; B: Sample 2; C: Samples 3

The obtained peaks of the spiked samples are shown in Figure 27. The measured concentration from spiked samples was used to determine the concentration in the original sample 3.

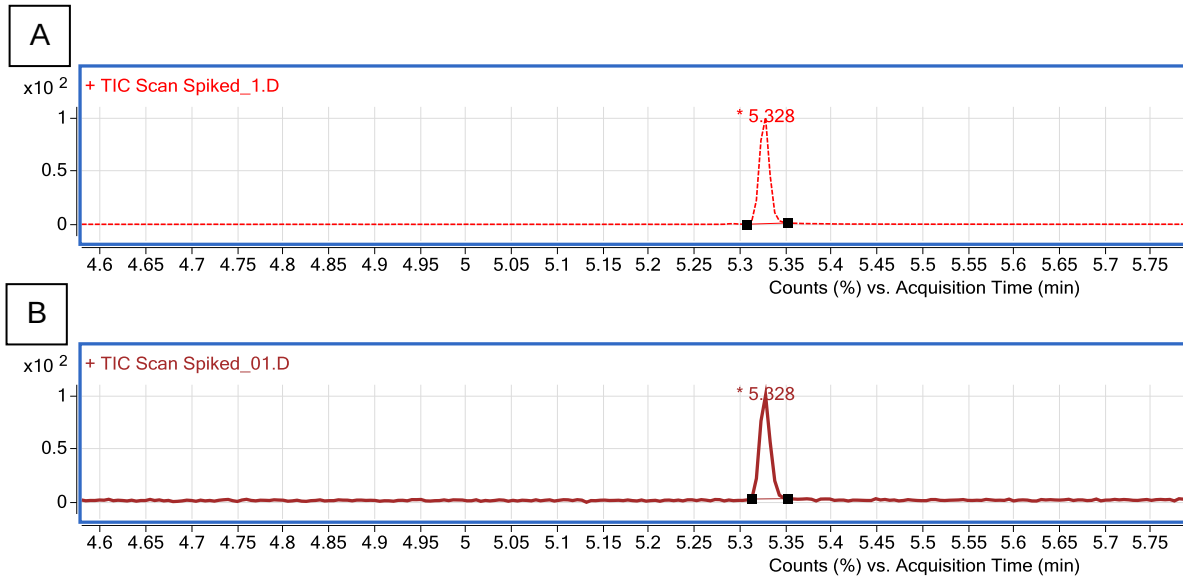


Figure 27: GCMS peak of spiked samples. A: Spiked sample (+1 µL); B: Spiked sample (+0.1 µL)

The spiked samples showed pyrazine was recovered from the obtained samples by the extraction method and pyrazine amount in the samples was determined (Table 14). As shown previous (Figure 26) pyrazine was only found in one sample. Measured concentration in the sample matched with the concentration in spiked sample 0.1.

Table 14: Calculated pyrazine concentrations in the analyzed samples

Sample	Concentration [µL/mL]	Pyrazine in sample
Spiked sample (+0.1 µL)	0.051	0.1 µL
Spiked sample (+1µL)	0.08	1 µL
Sample 1	0	0 µL
Sample 2	0	0 µL
Sample 3	0.051	**0.1 µL**

4. Discussion

Food preservation is becoming more and more important. While up to one third of the global food production is ending as waste, the industry is facing consumers' needs for quality and food safety [6,7]. The food industry is lacking new preservation methods to fulfill the demand for long time storable food and natural, organic taste at the same time [8]. Natural preservative compounds are an upcoming trend in food preservation [11]. However, highly active compounds are needed to prevent food poisoning. Pyrazine derivatives were recently found to have antimicrobial effects on several microorganisms, including *Escherichia coli, Salmonella typhimurium* and *Vibrio cholerae* [16]. Alkylated pyrazine molecules are naturally widely spread in food products such as vegetables. They contribute also to the flavor of heated food. Food products are the main source of pyrazine for humans [23,26,27]. Pyrazine derivatives are also found in social insects and terrestrial vertebrates where they occur as communication molecules for intra- and interspecific signaling [37]. Due to their odor and low olfactory threshold pyrazine derivatives have a broad field of application in food processing and flavoring [23,61]. However, very little is known about the antimicrobial effects of pyrazine compounds [63]. The focus of this thesis was to determining the antimicrobial potential of different pyrazine molecules and mixtures. Pyrazine molecules were picked from a whole set of different compounds (Table 3) due to their antimicrobial reduction rates and their olfactory properties and composed to pyrazine mixtures for an application on toast bread. Antimicrobial characteristics were observed using standardized efficacy tests on *E. coli*, *S. aureus* and *C. albicans*. Further investigations on the impact of pyrazine molecules on whole microbial communities were done using amplicon sequencing and egg samples.

4.1 Novel insights in pyrazine antimicrobial activity

The first goal of this study was to determine the antimicrobial potential of different pyrazines (Table 3) on *E. coli*, *S. aureus* and *C. albicans*. Low concentrations of 0.3 % pyrazine derivatives were applied to defined cell concentrations to evaluate the antimicrobial activity. Different pyrazine derivatives showed varying efficacies. Antimicrobial tests showed very similar effects of the different pyrazine compounds on all three tested microorganisms. Gram$^-$ *E. coli*, gram$^+$ *S. aureus* as well as the yeast *C. albicans* seemed to be equally affected by pyrazines. This study revealed the high efficacy of compounds such as 2IB3MP, 23DEP, 2MOX3MP, 5H5M67DHP and

2DE5MP, while others seemed to have very little effects. Some slight differences of the efficacies were observed comparing bacteria and yeast reduction rates (Table 9).

However, reducing microbial effects are stronger for some pyrazines, characterized through stronger impacts on bacteria, while other pyrazines seem to have no impact at all. This is highly determined by the chemical properties of the pyrazine molecules. The pyrazine derivatives used in this study differ in substituents on the ring carbon atoms. Depending on the substituents and therefor caused changes in the chemical properties of the pyrazine molecule the reduction rate is affected. In general, longer side chains such as isobutyl or propyl- chains lead to stronger antimicrobial effects. This is also seen when we look at the log P values of the different pyrazines. The log P value is very different throughout the broad number of pyrazine molecules used in this study (Table 10, Figure 11). It ranges from 0.5 (26DMP) to 3 (2MOX3MP).

Interestingly, a longer alkyl side chain of pyrazine molecules such as 5IB23MP leads to a relatively high log P value. The log P value indicates hydrophilic or hydrophobic characteristics of a molecule. Higher log P values correlates more hydrophobic properties of a compound [85]. From the experiments in this study we can see, that higher log P values of a pyrazine derivative most likely indicates a higher reduction rate of the compound (Figure 11). This leads to speculations about the so far unknown mode of action. A molecule with a higher log P value could experience enhanced intercalation into the cellular membrane or alleviated penetration of the membrane, due to the similar chemical properties to phospholipids.

Knowing the correlations between log P values and efficacy of pyrazine molecules is the first step in developing highly efficiency pyrazine mixtures for an application on food products. However, there are more important characteristics of pyrazine derivatives. Odor is the most important factor after the efficacy. Table 10 gives a look into the broad variety of odors of the different pyrazine derivatives used in this study. Intensive odors such as 2MOX3MP (intensive green, vegetable, pepper) can lead to problems when applied to certain food products, due to the big impact on the taste of the product. For a well-balanced pyrazine mixture not only efficacy but also odor has to be taken into account. Of course there are a lot of possible pyrazine mixtures, but in this thesis the main focus was the development of a formulation that is suitable for the application on toast bread (Table 11).

4.2 Shelf life extension with pyrazine application on bakery products

The first highly efficient stabilization application of pyrazine formulations on food products was realized on toast bread. For this study a whole set of experiments were performed. First of all, the activity of pyrazine compounds on *Penicillium* sp., causing the mold on toast bread, had to be observed. Even if the toast is packaged in clean rooms, mold forming spores find their way into toast packages and therefore mold is causing big problems in the industry [69]. No antifungal compound is allowed to be used facing this problem without special labeling. Pyrazine derivatives are classified as flavoring additives and need therefore no special labeling as stabilization compound. Pyrazine derivatives can be used as flavoring additives, while competing also against the common mold formation on toast.

The experiment was performed using three different strategies. At first, the activity test of the previous developed pyrazine mixtures was performed analog to the antimicrobial activity tests. Spore solutions were incubated with added formulations and antifungal activity was observed on PDA agar plates. Secondly, the volatile activity of pyrazine compounds was examined with dual culture plates without direct contact of the spores and the pyrazine molecules. The only contact was possible over the headspace. Thirdly, the application on toast bread was performed using a stress test method. By incubating toast breads, inoculated with *Penicillium* sp. spores, with different treatments mold formation was observed over two weeks. With these three experiments the feasibility of the application of pyrazine formulations on toast bread was examined.

Firstly, the antifungal activity was tested analog to the antimicrobial activity tests. Spore solution was incubated with different pyrazine formulations and treatment efficacy was evaluated using plate tests (Figure 12). The high efficiency of pyrazine derivatives on spores was demonstrated in this experiment. The reduction of mold growth was shown for all formulations after incubating the spores in liquid pyrazine formulations for all three developed mixtures. This result demonstrates the high efficacy of pyrazine derivatives on spores having direct contact with the formulations.

Secondly, the efficacy of pyrazine derivatives without direct contact was demonstrated in the next experiment. Using dual petri dishes with two separated chambers the volatile activity of pyrazines and their effect on mold growth was shown (Figure 13). No countable reduction of mold formation was noticed, due to the high number of spores used in the experiment. The high number of spores was due to inaccuracies

during the spore counting with the thoma cell counting chamber. Nevertheless, a strong effect of the pyrazine treated samples was demonstrated. Mold growth was slower with pyrazine treatment compared to the negative control. Even on perfect growth conditions, such as on PDA media, pyrazine treatment was able to reduce the mold formation. This experiment indicates a strong volatile efficacy of pyrazine derivatives over the gas phase. This can be a valuable characteristic for the application on food products as well as the decontamination of hardly accessible surfaces.

The final experiment to show a successful application of pyrazine formulations as stabilization compounds on toast bread was shown using a stress test. Whole toast packages were inoculated with *Penicillium* sp. spores to simulated contaminated toast. Treatments were added directly to the empty packages before packaging the toast. The efficacy of pyrazine treatment was successfully shown during the two weeks of incubation time. All negative controls showed mold formation after 12 days whereas only 10-20 % of the treated samples were molding at that time. The shelf life of the treated toast breads was expanded by up to a week, even in artificially highly contaminated toast packages (Figure 14). Pyrazine formulations were able to reduce the mold growth by their antimicrobial and volatile activity. These findings indicate a suitable effect for the application in the bakery industry. Shelf-life extension by up to a week could reduce the amount of toast bread and other bakery products ending as waste. Implementation of the treatment into packaging process could be realized by high performance spraying equipment applying a certain amount of formulation to every toast package.

4.3 New decontamination strategies for hatching eggs

In this study new highly efficient decontamination strategies were developed to overcome food processing issues in hatcheries. Hatching eggs are affected by microbial contaminations and are nowadays treated with formaldehyde to guarantee a safe chicken development [73,74]. Typical contaminations are caused by *Salmonella, Pseudomonas, Micrococcus* or *Escherichia* [73]. Since formaldehyde is a dangerous compound and affects human health, hatcheries are looking for new environmental friendly decontamination methods [75]. This experiment was performed to investigate the effect of pyrazine derivatives on total microbial communities, as well as developing new treatment strategies for round and hard-to-reach surfaces. For this purpose, egg

samples were treated using two different treating methods to decontaminate the egg surface. The microbial reduction was investigated using cultivation-dependent methods and RT qPCR, while deeper insights on the microbial communities were generated using confocal laser scanning microscopy and amplicon sequencing.

4.3.1 Cultivation dependent treatment comparison

Using cultivation-dependent methods, colony forming units of treated and control samples were compared and treatment efficacy was able to be measured. Two different treatment strategies were applied on the eggs. The volatile characteristic of pyrazine compounds was used in both strategies. In a so called passive treatment pyrazine compounds were placed underneath the egg samples in a closed container and natural evaporation took place during the incubation time. This way, pyrazine molecules filled the headspace of the treated eggs and managed to decontaminate the samples. In the second strategy, evaporation was further induced using heat. This way all applied pyrazine was transferred into the gas phase and higher pyrazine concentrations in the headspace were achieved. Egg samples were treated for 6 hours using both treatment strategies and 3 eggs were used for the preparation of each sample. Four samples were prepared for each producer and treatment. This way, a sufficient number of replicates were used to avoid stable-derived contamination biased results. Egg shells were standardized washed with PBS buffer to wash off the bacterial contaminations and the solution was plated out on NA agar plates for CFU analysis. Calculated CFUs per gram egg shell (Figure 15 and 16) show the achieved reduction. Not all treatments were as effective as others. Over all, the thermic treatment was highly effective while passive treated samples in some cases showed increased CFU counts. The increased CFU counts were due to stable-derived high contaminations on the treated samples. Nevertheless, thermic treatment showed better results in all cases. Reduction rates calculated from the CFU counts show, higher applied pyrazine concentrations were able to reduce the CFU on the egg shell surface by up to 99.6% (Table 12).

LIVE/DEAD-staining micrographs confirmed the high efficiency of thermic treatment (Figure 17). High numbers of living cells forming big colonies were found on control egg shells. However, thermic treated egg shells showed no visible living cells as well as a reduced number of bacteria. During the 6 hours of incubation time the bacterial populations on the egg shells were able to grow on the control egg shells resulting in high numbers of living bacteria. In contrast, thermic treatment highly affected the

growth of bacteria on the egg shells. No living cells were found on the egg shells after treatment. This confirms the results using cultivation dependent methods. Untreated, control eggs showed high numbers of bacterial CFUs while thermic treated samples had reduced CFU numbers. This experiment highlighted this effect successfully using LIVE/DEAD staining.

Results generated with methods, which rely on bacterial growth on cultivation media, do not picture the whole bacterial community found in the analyzed samples. The analysis of the extracted DNA from the samples also includes uncultivable species. However, PMA treatment, to reduce free DNA from dead bacteria, is a crucial step not to produce false results.

The effect seen from the growth dependent methods was confirmed by the RT qPCR analysis. Passive treatment led to reduction of the gene copy numbers in all samples except from the producer DB (Figure 18). This increasing effect can be caused by stable derived dirt residues on the samples or unsuccessful PMA treatment for those samples. In contrast, thermic treatment was effectively reducing the gene copy numbers in all samples (Figure 19). However, the measured significances of the reductions in gene copy numbers were low due to the broad variance between the measured values. Nevertheless, the results using growth dependent methods were confirmed by RT qPCR analyzing the gene copy numbers in the samples. The found contamination levels on the analyzed samples were similar to the found gene copy numbers in their DNA extracts. The AZ control for the thermic treatment for instance, was nearly twice as high as the other treatments. This is confirmed also by the calculated CFU in the growth dependent method (Figure 16).The contamination on the TF control for the passive treatment was also quite high in the growth depending method as well as in the qPCR results. This shows a strong connection between found CFU on the samples and gene copy numbers in the DNA extracts from the same sample. The significance however was lower for the RT PCR compared to the observed reductions in CFU using cultivation-dependent methods.

The experiment shows how two different methods can have complementary outcomes depending on the observed characteristic. Analyzing CFU on agar plates is limited by bacteria that grow on the specific agar plates used. In contrast, RT qPCR is more sensitive in analyzing all DNA derived from the samples, but samples preparations can have a big impact on the results. The combination, however, of the two methods gives a good insight into the real mechanisms in the samples during treatment.

Additionally, a total of 66 isolates retrieved from passive treatment agar plates and their correspondent controls were analyzed using Sanger sequencing and pyrazine sensitivity tests. The isolates were characterized as well as their sensitivity to 5IB23MP to determine what kind of bacteria grow on treated or untreated egg shells. The pyrazine tolerance was tested to identify pyrazine sensitive as well as resistant bacterial strains (Table 13). A total of 12 different bacterial genera were identified within the 66 isolates. All of the genera were found in both treated and untreated samples except for *Acinetobacter* spp. which was only found in treated samples. *Acinetobacter* belongs to the gram$^-$ *Gammaproteobacteria* and known for nosocomial and other infections [88]. However, all tested *Acinetobacter* isolates were sensitive to pyrazine and highly affected by elevated pyrazine concentration. As seen in the growth dependent method analysis before, passive treatment was less effective compared to thermic treatment. This is also why all genera found in the control were also found in treated samples. However, only a few bacterial strains were found to be tolerant to pyrazine. The most tolerant stains were found to be *Bacillus* spp. who are reported to be pyrazine producers [89]. *Bacillus* spp. are also spore forming gram$^+$ bacteria, which makes them more tolerant to starvation and external influences [90]. The bacterial strains isolated from passive treatment agar plates showed mostly high sensitivity to 5IB23MP and their growth can therefore be avoided by higher pyrazine concentrations. Due to the low CFU numbers on thermic treatment agar plates, no isolates were taken from there.

This experiment highlights the broad diversity of genera found on the egg shell surface. Using growth dependent methods a high number of different bacterial strains were isolated and characterized. Less effective passive egg treatment was shown to spare various pyrazine sensitive bacterial strains on the egg shells. More effective treatments (thermic treatment) can overcome this effect resulting in better decontamination of the egg shells.

4.3.2 Structural community changes during hatching egg treatment

Community changes during pyrazine treatment were observed using HiSeq Illumina amplicon sequencing. Using the QIIME 1.9.0 pipeline and core diversity analysis the construction of different plots was possible. Diversity between the samples on order level (Figure 20) was very high. Some orders, such as *Pseudomonadales* and *Clostridiales* were popular in most of the samples. The corresponding samples demonstrated some similarities. However, obtaining the changes in the bacterial

community form this plot was not possible. The clustering of the samples by their treatment was easier to interpret (Figure 21). On phylum level the composition of the untreated samples and the thermic treated samples were very similar. The passive treatment in contrast was different and showed an increase in *Proteobacteria*. This indicates a community change during passive treatment induced by lower pyrazine concentrations. Passive treatment as shown in the growth dependent methods was not able to decontaminate the high contaminations on the egg surfaces. The isolation of the bacterial colonies growing on passive treatment agar plates showed, most of the bacterial strains found after passive treatment are highly affected by pyrazine derivatives. The increase of *Proteobacteria* in Figure 21 indicates a similar effect. While thermic treatment affects all bacterial strains in a similar fashion passive treatment leads to a shift in the bacterial community which is shown by an increase of some phyla.

However, the alpha rarefaction OTU plots show no significant increase in OTUs during a specific treatment (Figure 22). The control (DNA extracts from dH_2O) however showed a similar number of OTUs as the analyzed samples. As already seen in Figure 20 the control samples contained similar order compositions as the observed samples.

In the beta diversity plots no specific clustering of the treatment groups were found (Figure 23). PR and DB showed a sight clustering of the not treated samples, which seemed to be quite similar. The samples were throughout very diverse and showed high variances.

Group significance analysis of the first 100 results with the lowest p-value showed an interesting development of the OTU composition in the different treatments (Figure 24). *Micrococcus* was found to be the most prominent species in the dataset having 3160 total mean read counts. It is also confirmed by literature that Micrococcus among a broad number of *Enterobacteriacea* is widely found on egg samples [73]. All other OTUs analyzed had OTU counts from 10 to 1000. The OTU counts are indicated as the node area. The pie charts indicate the fraction of the OTUs found in a specific treatment. While most of the nodes showed the biggest fraction of the OTU in the not treated samples some community changes during passive treatment were indicated by OTUs such as *Clostridiales* or *Pseudomonadaceae*. This indicates an increase of specific OTUs during passive treatment, due to the differences of pyrazine efficiency on different microorganisms. The graphic, however, shows only a fraction of the

complete dataset but gives a valuable insight into the mechanism of community shifts during the treatments. The enrichment of specific OTUs during the treatment can influence the composition of the bacteria growing on the hatching eggs during the incubation and, this way, also on the development of the chicken.

4.3.3 Pyrazine penetrating the egg shell

Decontamination compounds penetrating the egg shells can have invasive effects on the chicken development [91]. This experiment was performed to determine whether pyrazine molecules can penetrate the egg shells during treatment or not. In the experiment three treated eggs were analyzed as well as spiked eggs with added pyrazine. Only one of the three eggs contained any pyrazine (Figure 26). Pyrazine recovery of the method was demonstrated using the spiked samples with added pyrazine. The theoretical amount of pyrazine in sample 3 was 0.1 µL (Table 14).

This experiment showed pyrazine molecules can potentially penetrate the egg shell during treatment. Of course, pyrazine found in sample 3 can also be due inefficient washing procedure of the egg surface. However, the quite high amount found in the sample is more likely penetration derived. More investigations in this field are needed to determine if the penetration can have an impact on the chicken development or the use of pyrazine for the hatching egg decontamination is not only efficient but also safe.

4.4 Further prospective

This study successfully showed two different application strategies for pyrazine derivatives in food processing. The utilization of high efficient formulations with complementary designed odor characteristics was shown on toast bread. This strategy is applicable in many different other food products due to the broad variety of highly active pyrazine molecules and their wide odor range. For instance, other bakery products are easily treatable with slight altered mixtures, as used in this experiment. Volatile efficacy of the pyrazine compounds was successfully shown not only by slowing down mold growth on agar plates but also by treating egg samples. This shows, normally inaccessible or hard to treat surfaces can be easily treated using volatile organic compounds. This property opens a broad field of application not only in food processing but also in facility cleaning processes.

The right concentration during the treatment leads to full decontamination processes. Microbiome analysis showed almost all microorganisms are affected by pyrazine compounds in an equal fashion. Slight community changes were detected in this study only when lower concentrations were used. This ubiquitous efficacy is highly favored in any decontamination application or process.

5. Conclusion

Daily needs for consumers are causing more and more problems for the food industry. Organic, long lasting food is needed, while preventing food poisoning is still a big issue. Conservation methods are invasive to the product's taste and freshness while consumers tend to spend more money on organic products without artificial preservatives. Highly active natural preservatives are needed to provide the market with fresh, organic, natural food products.

In this study molecules of high efficacy against microbial contaminations were identified and application strategies were developed to treat occurring problems in food industry. Highly active pyrazine compounds were tested against different microorganisms to determine their antimicrobial potential and analyze differences between the pyrazine derivatives. Characteristics such as log P value and odor were shown to be highly valuable to decide which component is suitable for applications in specific fields. Mixtures of active compounds were shown to have high efficacy on microorganisms and fungi. Volatile activity of pyrazine derivatives is valuable for decontamination processes without direct contact of the compounds and the microorganisms. Application trials on toast bread successfully showed the high potential of preservation techniques using pyrazine derivatives. Hatching egg decontamination was also successfully demonstrated using different strategies. Pyrazine induced community changes were observed in passive treated samples using lower pyrazine concentrations. Simple pyrazine activity tests showed varying effects on different microorganisms and was confirmed using HiSeq Illumina sequencing strategies to determine the differences in OTU compositions of different treated samples. Nevertheless, the ubiquitous efficacy on almost all microorganisms was shown during thermic treatment. Using higher pyrazine concentrations led to equal reduction of almost all microorganisms resulting in desired decontamination. Egg treatment application was successfully shown, but pyrazine penetrating the egg shells can still be an issue in future application. Pyrazine molecules could have an impact on the chicken development and is further to be investigated.

Nevertheless, the study showed the high applicability of high efficient pyrazine derivatives in food processing and opens a broad field of other possible applications in the future.

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7. Appendix

7.1 List of abbreviations

%	percentage
µ	micro
BSA	bovine serum albumin
CFU	colony-forming unit
dH_2O	distilled water
e.g.	exempli gratia
etc.	et cetera
g	gram
h	hour
HCl	hydrochloride
KH_2PO_4	potassium dihydrogen phosphate
l	liter
LB	Luria-Bertani broth
log P	partition coefficient in 1-octanol/water of a substance
m	milli
M	molar
$MgSO_4$	magnesium sulphate
min	minute
n	nano
NA	nutrient agar
Na2HPO4*2H2O	di-sodium hydrogen phosphate dihydrate
NaCl	sodium chloride
NB	Nutrient broth
OD	optical density
ONC	overnight culture
OTU	operational taxonomic unit
PBS	phosphate-buffered saline
PDA	Potato dextrose agar
qPCR	quantitative polymerase chain reaction
rcf	relative centrifugal force
rpm	rounds per minute
RT	room temperature or real time
VOCs	volatile organic compounds

7.2 Additional tables

Table 15: OTU composition on order level in the different samples. c: control, pt: passive treatment, tt: thermic treatment. Dark gray: values used for Figure 20, white: summarized as "OTHER". All values rounded.

ORDER	AZ_c	AZ_pt	AZ_tt	DB_c	DB_pt	DB_tt	PR_c	PR_pt	PR_tt	TF_c	TF_pt	TF_tt	WA_c
Pseudomonadales	36.26%	15.35%	27.65%	10.01%	7.69%	3.76%	12.84%	51.59%	24.51%	9.54%	5.08%	6.02%	13.09%
Enterobacteriales	10.37%	2.32%	3.40%	2.36%	5.22%	2.39%	2.82%	3.71%	6.65%	1.28%	13.70%	2.66%	3.55%
Sphingomonadales	7.12%	8.99%	3.81%	1.70%	2.31%	2.89%	2.29%	10.02%	2.34%	3.30%	4.59%	16.90%	5.76%
Streptophyta	5.59%	5.08%	7.91%	4.69%	7.53%	2.27%	11.06%	1.48%	2.30%	9.31%	7.69%	3.41%	10.09%
Burkholderiales	4.73%	5.73%	13.61%	7.71%	9.55%	6.43%	8.49%	4.95%	8.76%	7.09%	11.66%	9.55%	6.08%
Actinomycetales	4.30%	4.03%	3.04%	7.85%	3.42%	1.84%	9.92%	2.98%	1.47%	7.33%	4.29%	4.14%	4.82%
Xanthomonadales	3.92%	2.48%	8.51%	1.39%	1.72%	1.07%	1.41%	0.73%	4.82%	2.39%	1.34%	1.18%	1.38%
Rhizobiales	3.72%	4.28%	2.91%	2.29%	4.56%	1.40%	4.80%	0.82%	1.31%	2.98%	10.72%	13.13%	15.64%
Bacillales	3.61%	14.08%	7.70%	6.30%	10.53%	2.19%	8.05%	3.51%	11.85%	6.89%	3.09%	2.89%	12.89%
Clostridiales	3.55%	9.81%	4.84%	29.58%	17.31%	51.98%	11.34%	3.92%	5.94%	17.34%	5.41%	10.75%	3.04%
Lactobacillales	2.90%	9.38%	1.84%	2.69%	1.80%	4.01%	2.35%	4.74%	1.10%	1.71%	1.22%	0.84%	1.49%
Flavobacteriales	2.12%	2.10%	2.70%	1.43%	3.57%	0.98%	1.77%	2.20%	3.79%	2.84%	5.18%	5.03%	0.77%
Unassigned	1.01%	1.07%	1.43%	1.09%	1.72%	0.71%	0.79%	2.41%	17.61%	3.05%	0.84%	1.05%	0.48%
Rhodospirillales	1.00%	2.38%	0.41%	0.53%	0.84%	1.19%	1.16%	0.19%	0.41%	0.72%	2.56%	3.21%	1.70%
Oscillatoriales	0.84%	0.06%	0.04%	0.05%	0.10%	0.03%	0.04%	0.06%	0.04%	0.04%	0.03%	0.04%	0.03%
Ul_ML635J-21	0.59%	3.72%	2.02%	1.25%	1.60%	0.42%	3.95%	0.54%	2.02%	2.20%	5.94%	0.38%	2.88%
Turicibacterales	0.58%	0.77%	0.81%	8.29%	5.37%	4.03%	1.02%	0.39%	1.04%	5.36%	1.12%	0.96%	0.45%
Sphingobacteriales	0.58%	0.63%	0.98%	0.45%	0.61%	0.26%	1.23%	3.61%	0.31%	0.60%	1.49%	1.36%	0.56%
Rickettsiales	0.53%	0.31%	0.38%	1.44%	1.11%	0.27%	2.60%	0.14%	0.20%	1.06%	0.77%	0.55%	1.55%
Caulobacterales	0.44%	0.49%	0.51%	0.45%	0.62%	0.28%	0.83%	0.20%	0.30%	3.40%	0.88%	1.22%	0.63%
Ul_iii1-15	0.33%	0.33%	0.17%	0.39%	1.17%	0.17%	0.47%	0.10%	0.12%	0.39%	0.87%	1.55%	0.85%

Rhodobacterales	0.31%	0.20%	0.57%	0.37%	0.24%	0.18%	0.41%	0.10%	0.40%	0.40%	0.84%	0.64%	0.56%
Saprospirales	0.29%	0.39%	0.32%	0.23%	0.63%	0.23%	0.74%	0.18%	0.24%	1.99%	0.67%	0.71%	0.40%
Nitrososphaerales	0.27%	0.19%	0.22%	0.19%	0.36%	0.10%	0.48%	0.06%	0.07%	1.46%	0.65%	0.65%	0.82%
Chthoniobacterales	0.26%	0.31%	0.11%	0.27%	1.10%	0.13%	0.34%	0.07%	0.12%	0.39%	0.86%	1.54%	0.58%
Bacteroidales	0.26%	0.51%	0.47%	1.21%	0.31%	5.37%	0.13%	0.10%	0.17%	0.60%	0.32%	0.13%	0.21%
Halobacteriales	0.24%	0.00%	0.01%	0.00%	0.00%	0.00%	0.14%	0.00%	0.01%	0.14%	0.00%	0.00%	0.00%
Aeromonadales	0.23%	0.02%	0.18%	0.02%	0.05%	0.02%	0.07%	0.03%	0.03%	0.03%	0.04%	0.02%	0.00%
RB41	0.22%	0.20%	0.06%	0.45%	0.24%	0.05%	0.11%	0.04%	0.08%	0.43%	0.43%	0.39%	0.47%
Myxococcales	0.19%	0.18%	0.19%	0.35%	0.56%	0.33%	0.18%	0.07%	0.13%	0.36%	0.49%	0.34%	0.50%
Gaiellales	0.15%	0.24%	0.07%	0.07%	0.65%	0.07%	0.48%	0.04%	0.09%	0.20%	0.35%	0.82%	0.03%
Pasteurellales	0.14%	0.08%	0.07%	0.06%	0.08%	0.07%	0.06%	0.03%	0.04%	0.06%	0.19%	0.19%	0.27%
Planctomycetales	0.13%	0.00%	0.02%	0.09%	0.01%	0.02%	0.08%	0.00%	0.00%	0.03%	0.02%	0.16%	0.24%
Gemmatales	0.13%	0.21%	0.16%	0.08%	0.23%	0.03%	0.20%	0.02%	0.03%	0.19%	0.32%	0.50%	0.14%
NRP-J	0.11%	0.02%	0.02%	0.06%	0.03%	0.00%	0.50%	0.01%	0.05%	0.32%	0.04%	0.01%	0.00%
Verrucomicrobiales	0.11%	0.06%	0.02%	0.06%	0.07%	0.05%	0.02%	0.00%	0.03%	0.09%	0.15%	0.06%	0.00%
Methylophilales	0.10%	0.07%	0.06%	0.09%	0.08%	0.06%	0.44%	0.03%	0.12%	0.16%	0.22%	0.26%	0.06%
Rhodocyclales	0.09%	0.88%	0.52%	0.29%	0.16%	0.51%	0.55%	0.06%	0.08%	0.15%	0.11%	0.39%	0.17%
Acidobacteriales	0.09%	0.02%	0.04%	0.02%	0.23%	0.01%	0.38%	0.01%	0.02%	0.07%	0.10%	0.13%	0.01%
Deinococcales	0.09%	0.02%	0.04%	0.18%	0.12%	0.01%	0.14%	0.01%	0.02%	0.04%	0.06%	0.02%	0.15%
Pedosphaerales	0.09%	0.08%	0.05%	0.04%	0.14%	0.02%	0.09%	0.01%	0.02%	0.12%	0.12%	0.15%	0.09%
C:Ellin6529	0.09%	0.05%	0.03%	0.06%	0.14%	0.02%	0.04%	0.02%	0.03%	0.09%	0.17%	0.21%	0.24%
C:ZB2	0.09%	0.02%	0.07%	0.09%	0.25%	0.06%	0.09%	0.00%	0.02%	0.05%	0.07%	0.07%	0.01%
SBR1031	0.08%	0.04%	0.01%	0.13%	0.16%	0.07%	0.08%	0.01%	0.02%	0.11%	0.10%	0.06%	0.08%
Oceanospirillales	0.08%	0.02%	0.05%	0.05%	0.04%	0.11%	0.02%	0.03%	0.01%	0.01%	0.04%	0.06%	0.00%
Cytophagales	0.08%	0.12%	0.13%	0.34%	0.12%	0.10%	0.29%	0.04%	0.08%	0.31%	0.15%	0.14%	0.46%
Pirellulales	0.07%	0.03%	0.02%	0.02%	0.05%	0.01%	0.08%	0.00%	0.00%	0.07%	0.38%	0.08%	0.41%
Opitutales	0.07%	0.04%	0.07%	0.07%	0.39%	0.12%	0.04%	0.02%	0.04%	0.10%	0.22%	0.18%	0.01%
Solirubrobacterales	0.07%	0.10%	0.08%	0.13%	0.26%	0.07%	0.07%	0.02%	0.04%	0.13%	0.16%	0.23%	0.16%

32-20	0.07%	0.04%	0.00%	0.01%	0.16%	0.03%	0.00%	0.00%	0.01%	0.04%	0.04%	0.10%	0.00%
Legionellales	0.07%	0.05%	0.10%	0.12%	0.07%	0.11%	0.06%	0.05%	0.04%	0.12%	0.21%	0.10%	0.15%
Alteromonadales	0.06%	0.11%	0.09%	0.05%	0.21%	0.04%	0.41%	0.03%	0.09%	0.07%	0.06%	0.08%	0.01%
Chlorophyta	0.06%	0.03%	0.03%	0.13%	0.02%	0.01%	0.34%	0.01%	0.01%	0.04%	0.11%	0.11%	0.14%
Fusobacteriales	0.06%	0.01%	0.03%	0.09%	0.00%	0.18%	0.02%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%
Chlamydiales	0.06%	0.04%	0.07%	0.06%	0.21%	0.03%	0.08%	0.02%	0.02%	0.07%	0.13%	0.02%	0.01%
Neisseriales	0.05%	0.03%	0.06%	0.07%	0.01%	0.08%	0.02%	0.02%	0.04%	0.09%	0.17%	0.05%	0.19%
JG30-KF-CM45	0.05%	0.02%	0.02%	0.02%	0.06%	0.07%	0.11%	0.00%	0.01%	0.07%	0.08%	0.08%	0.00%
Ellin6513	0.05%	0.04%	0.05%	0.29%	0.07%	0.08%	0.08%	0.04%	0.02%	0.07%	0.22%	0.03%	0.01%
Gemellales	0.05%	0.01%	0.02%	0.05%	0.00%	0.03%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.02%
Coriobacteriales	0.04%	0.10%	0.04%	0.20%	0.12%	1.13%	0.10%	0.03%	0.04%	0.11%	0.05%	0.03%	0.02%
DS-18	0.04%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%
SC-I-84	0.03%	0.07%	0.04%	0.02%	0.14%	0.01%	0.02%	0.01%	0.02%	0.04%	0.15%	0.22%	0.19%
MLE1-12	0.03%	0.09%	0.07%	0.09%	0.41%	0.02%	0.24%	0.01%	0.03%	0.09%	0.22%	0.02%	0.01%
C:Alphaproteobacteria	0.03%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.03%	0.03%	0.00%	0.00%
Acidimicrobiales	0.03%	0.04%	0.04%	0.05%	0.31%	0.06%	0.04%	0.01%	0.04%	0.11%	0.16%	0.17%	0.01%
C:Gemm-1	0.03%	0.04%	0.01%	0.01%	0.03%	0.02%	0.00%	0.00%	0.00%	0.03%	0.06%	0.09%	0.41%
MND1	0.03%	0.00%	0.00%	0.12%	0.03%	0.00%	0.03%	0.00%	0.00%	0.03%	0.00%	0.01%	0.29%
Nostocales	0.03%	0.00%	0.03%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.11%	0.02%	0.32%	0.02%
C:Anaerolineae	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
Bifidobacteriales	0.03%	0.07%	0.08%	0.21%	0.05%	0.47%	0.04%	0.01%	0.03%	0.06%	0.02%	0.02%	0.01%
Anaeroplasmatales	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.16%
Rhodothermales	0.03%	0.00%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
H39	0.03%	0.00%	0.01%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.22%
N1423WL	0.03%	0.04%	0.01%	0.01%	0.19%	0.01%	0.11%	0.03%	0.01%	0.08%	0.16%	0.25%	0.00%
C:BD7-11	0.03%	0.00%	0.00%	0.02%	0.06%	0.00%	0.17%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
Gemmatimonadales	0.02%	0.08%	0.01%	0.03%	0.02%	0.04%	0.05%	0.00%	0.01%	0.05%	0.09%	0.10%	0.31%
Nitrospirales	0.02%	0.02%	0.04%	0.08%	0.22%	0.07%	0.04%	0.00%	0.04%	0.04%	0.07%	0.05%	0.66%

C:Deltaproteobacteria	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.10%
Solibacterales	0.02%	0.02%	0.05%	0.03%	0.11%	0.00%	0.00%	0.00%	0.01%	0.06%	0.09%	0.11%	0.12%
C:Gemm-3	0.02%	0.00%	0.00%	0.03%	0.01%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%
Thermales	0.02%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Entotheonellales	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Betaproteobacteria	0.02%	0.01%	0.05%	0.01%	0.11%	0.00%	0.02%	0.00%	0.01%	0.07%	0.07%	0.08%	0.05%
0319-7L14	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.03%	0.04%	0.03%
Roseiflexales	0.02%	0.10%	0.02%	0.04%	0.02%	0.01%	0.03%	0.01%	0.02%	0.12%	0.17%	0.30%	0.30%
Sediment-1	0.02%	0.02%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%	0.04%	0.20%
P:FBP	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:0319-6E2	0.02%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.05%	0.00%
Sva0725	0.02%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.04%	0.01%	0.00%
C:PRR-11	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.02%	0.00%
WD2101	0.02%	0.17%	0.02%	0.04%	0.19%	0.01%	0.21%	0.01%	0.02%	0.11%	0.22%	0.27%	0.69%
HTCC2188	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
C:028H05-P-BN-P5	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Ktedonobacterales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%
P:WPS-2	0.01%	0.02%	0.01%	0.00%	0.00%	0.00%	0.03%	0.00%	0.02%	0.05%	0.01%	0.00%	0.00%
Ellin5290	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.04%	0.03%	0.00%
P:Verrucomicrobia	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Ellin6067	0.01%	0.03%	0.04%	0.04%	0.03%	0.01%	0.10%	0.02%	0.09%	0.03%	0.11%	0.20%	0.24%
OPB54	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
Chloroflexales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
CL500-15	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
Stramenopiles	0.01%	0.04%	0.00%	0.02%	0.00%	0.00%	0.03%	0.00%	0.00%	0.01%	0.07%	0.14%	0.11%
Cardiobacteriales	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Gitt-GS-136	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.04%	0.00%
Ellin329	0.01%	0.05%	0.02%	0.00%	0.15%	0.00%	0.09%	0.00%	0.02%	0.14%	0.03%	0.07%	0.00%

C:S035	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A21b	0.00%	0.05%	0.00%	0.00%	0.04%	0.00%	0.02%	0.00%	0.00%	0.01%	0.08%	0.19%	0.00%
Acholeplasmatales	0.00%	0.02%	0.00%	0.03%	0.02%	0.33%	0.02%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%
Erysipelotrichales	0.00%	0.03%	0.00%	0.06%	0.09%	0.07%	0.15%	0.02%	0.00%	0.03%	0.03%	0.00%	0.00%
Caldilineales	0.00%	0.02%	0.00%	0.00%	0.15%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.30%	0.00%
Procabacteriales	0.00%	0.01%	0.00%	0.05%	0.01%	0.00%	0.04%	0.01%	0.02%	0.02%	0.05%	0.00%	0.00%
C:Acidobacteria-5	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.02%	0.03%	0.00%
C:C0119	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.02%	0.03%	0.00%
RF39	0.00%	0.00%	0.00%	0.06%	0.02%	0.05%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%
AKYG885	0.00%	0.02%	0.03%	0.04%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.04%	0.01%	0.00%
Chthonomonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.02%	0.01%	0.04%	0.00%
Holophagales	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.06%	0.00%
Desulfuromonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%	0.01%	0.00%
P:Chloroflexi	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
CPla-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%
AKYG1722	0.00%	0.00%	0.01%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%
Thermotogales	0.00%	0.26%	0.00%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Nitrosomonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.04%	0.00%	0.00%
Phycisphaerales	0.00%	0.00%	0.00%	0.00%	0.09%	0.02%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%
11-24	0.00%	0.01%	0.00%	0.00%	0.07%	0.00%	0.02%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
B07_WMSP1	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.03%	0.02%	0.04%	0.00%
Chromatiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.02%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%
C:Betaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:VHS-B5-50	0.00%	0.00%	0.00%	0.00%	0.12%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Gammaproteobacteria	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
Spirobacillales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DRC31	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylacidiphilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%

C:OM190	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
WCHB1-50	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.04%	0.00%
P:Acidobacteria	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
PK29	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
Bdellovibrionales	0.00%	0.01%	0.00%	0.03%	0.14%	0.01%	0.34%	0.00%	0.03%	0.03%	0.10%	0.01%	0.00%
C:TM7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
Synergistales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%
Vibrionales	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
SM1D11	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:VC2_1_Bac22	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
C:Alphaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Leptospirales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%
C:SJA-4	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
A31	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Elusimicrobiales	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pseudanabaenales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Anaerolineales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.07%	0.00%
BD7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%
SJA-22	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.04%	0.00%
PHOS-HD29	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
Ellin6537	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
Thermogemmatisporales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
Herpetosiphonales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%	0.01%	0.00%
FAC87	0.00%	0.00%	0.00%	0.06%	0.05%	0.00%	0.02%	0.00%	0.02%	0.00%	0.02%	0.00%	0.00%
C:Clostridia	0.00%	0.13%	0.00%	0.04%	0.00%	0.03%	0.01%	0.00%	0.00%	0.01%	0.01%	0.03%	0.00%
C:TK10	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%
P:Chlorobi	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%
C:Bacilli	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

E2	0.00%	0.07%	0.00%	0.00%	0.00%	0.00%	0.13%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%
Thermoanaerobacterales	0.00%	0.02%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
RF32	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Sphaerobacterales	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Campylobacterales	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Rubrobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Anaerolineae	0.00%	0.00%	0.00%	0.00%	0.17%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Pla3	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
IS-44	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
IIb	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Gammaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Synechococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
AF420338	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
FW68	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Gallionellales	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:FCPU426	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
C:OPB41	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
agg27	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
mle1-48	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
RB046	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
KD8-87	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.01%	0.00%
C:SC3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Acidobacteria-6	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Dictyoglomales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
NB1-j	0.00%	0.00%	0.00%	0.00%	0.14%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.17%
C:MB-A2-108	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.15%
P:Planctomycetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.14%
CCU21	0.00%	0.00%	0.00%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.02%	0.01%	0.00%	0.14%

Aquificales	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.51%	0.00%	0.00%	0.02%	0.00%	0.00%	0.14%
C:Gemmatimonadetes	0.00%	0.02%	0.00%	0.00%	0.04%	0.00%	0.08%	0.00%	0.00%	0.00%	0.05%	0.08%	0.12%
Thiotrichales	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.10%
ML615J-28	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.08%
Fimbriimonadales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.01%	0.00%	0.00%	0.07%
MIZ46	0.00%	0.00%	0.02%	0.00%	0.00%	0.04%	0.00%	0.00%	0.00%	0.02%	0.02%	0.00%	0.07%
C:S085	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.06%
Syntrophobacterales	0.00%	0.01%	0.00%	0.01%	0.16%	0.00%	0.03%	0.00%	0.00%	0.06%	0.04%	0.03%	0.06%
C:BPC102	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
Chroococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.01%
Hydrogenophilales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
LD1-PA13	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
AKIW781	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%
C:PAUC37f	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:koll11	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.06%	0.00%	0.03%	0.00%	0.01%	0.00%	0.00%
SHA-26	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Gemm-5	0.00%	0.00%	0.00%	0.01%	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:GN07	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%
C:Thermomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
B97	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Spirochaetales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
p04_C01	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:OD1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%
SJA-36	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Stigonematales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
MVS-40	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
MWH-UniP1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanobacteriales	0.00%	0.03%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

C:OPB50	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:Proteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:WS4	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
MBA08	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
DH61	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
Ignavibacteriales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
SBla14	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
GCA004	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
CFB-26	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Elev-1554	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
YS2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:TM7-1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thiobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Cerasicoccales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
FAC88	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
SHA-20	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
CW040	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
ASSO-13	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfovibrionales	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Pla4	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:SM2F11	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
TG3-1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:SM1A07	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:Proteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanomicrobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%
C:Gemmatimonadetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
SHA-98	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:OPB56	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

SHUX583	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%
Cenarchaeales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:SAR202	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
S0208	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:WS1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:ABS-6	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:Gemmatimonadetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
SJA-15	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
envOPS12	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%
258ds10	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%	0.00%
JG30-KF-AS9	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%
C:TK17	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%
C:GKS2-174	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfobacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:SHA-109	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
K:Bacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:EC1113	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
EW055	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:SHA-37	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:ABY1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:TM7-3	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:BD1-5	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:Actinobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Victivallales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Armatimonadales	0.00%	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Halanaerobiales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Pla1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Deferribacterales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

WCHB1-41	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Deltaproteobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
NKB15	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Haloplasmatales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
GMD14H09	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Desulfarculales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
S-70	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Chloracidobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
BSA2B-08	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Chloroplast	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Opitutae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methylococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:Firmicutes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Methanosarcinales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
I025	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Micrococcales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
S-BQ2-57	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Ardenscatenales	0.00%	0.00%	0.00%	0.00%	0.05%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:TM7	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thiohalorhabdales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Mollicutes;Other	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
pGrfC26	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:RB25	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:TM7	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
P:Verrucomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Salinisphaerales	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:Clostridia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:P2-11E	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Appendix

C:4C0d-2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
CV90	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
C:PBS-25	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
other	4.34%	4.76%	3.35%	5.54%	8.70%	5.12%	8.55%	0.88%	1.80%	6.06%	7.91%	9.30%	9.51%

Table 16: Diversity on phylum level. Dark gray: values used for Figure 21, white: values summarized to "OTHER". All values are rounded.

PHYLUM	not treated	passive treatment	thermic treatment
Proteobacteria	42.53%	57.16%	43.69%
Firmicutes	27.51%	15.00%	32.50%
Cyanobacteria	10.60%	10.45%	4.60%
Actinobacteria	7.74%	4.52%	3.07%
Bacteroidetes	4.90%	6.80%	5.75%
Unassigned	1.91%	1.26%	7.77%
Acidobacteria	1.11%	1.33%	0.64%
Crenarchaeota	1.10%	0.47%	0.21%
Verrucomicrobia	0.60%	0.95%	0.50%
Planctomycetes	0.49%	0.70%	0.24%
Chloroflexi	0.44%	0.57%	0.37%
Gemmatimonadetes	0.23%	0.30%	0.14%
Euryarchaeota	0.20%	0.02%	0.01%
Aquificae	0.10%	0.00%	0.01%
[Thermi]	0.09%	0.04%	0.02%
OD1	0.07%	0.08%	0.05%
Chlamydiae	0.07%	0.09%	0.03%
Tenericutes	0.05%	0.01%	0.14%
Fusobacteria	0.05%	0.00%	0.07%
Armatimonadetes	0.05%	0.04%	0.03%
Nitrospirae	0.04%	0.05%	0.05%
WPS-2	0.03%	0.01%	0.01%
WS3	0.02%	0.02%	0.01%
OP3	0.02%	0.00%	0.01%
TM6	0.01%	0.00%	0.00%
BRC1	0.01%	0.00%	0.00%
k__Bacteria;Other	0.00%	0.00%	0.00%
AD3	0.00%	0.00%	0.00%
Chlorobi	0.00%	0.02%	0.00%
Deferribacteres	0.00%	0.00%	0.00%
Dictyoglomi	0.00%	0.00%	0.00%
Elusimicrobia	0.00%	0.00%	0.00%
FBP	0.00%	0.00%	0.00%
FCPU426	0.00%	0.00%	0.00%
Fibrobacteres	0.00%	0.00%	0.00%
GN02	0.00%	0.00%	0.02%
Lentisphaerae	0.00%	0.00%	0.00%
SBR1093	0.00%	0.00%	0.00%
Spirochaetes	0.00%	0.00%	0.00%
Synergistetes	0.00%	0.00%	0.00%

TM7	0.00%	0.02%	0.00%
Thermotogae	0.00%	0.04%	0.01%
WS1	0.00%	0.00%	0.00%
WS2	0.00%	0.00%	0.00%
WS4	0.00%	0.00%	0.00%
Other	1.48%	1.31%	0.99%

Table 17: Mean OTU counts of the 100 OTUs with lowest p-value from group significance evaluation. Values indicate the abundances in the specific treatments. Total values were applied on Figure 24 to determine the node size.

GENUS	thermic treatment_mean	not treated_mean	passive treatment_mean	Total
Micrococcus	492.56	2237.63	429.81	3160.01
Dietzia	198.25	918.60	76.38	1193.23
Brachybacterium	150.13	846.30	117.31	1113.74
Hydrogenophilus	158.06	617.77	529.88	1305.70
f:Nocardiopsaceae	23.13	359.03	72.81	454.97
Corynebacterium	78.81	354.00	228.94	661.75
Paracoccus	99.38	240.83	268.56	608.77
Runella	22.75	194.13	20.06	236.95
Brachybacterium conglomeratum	42.56	187.97	28.69	259.22
Wautersiella	48.25	159.13	353.69	561.07
Prauseria	9.81	114.80	30.81	155.43
Paracoccus	33.00	78.63	120.69	232.32
Jeotgalicoccus	1.19	33.60	1.44	36.23
Gemmata	0.94	32.20	2.13	35.26
Nocardioides	1.31	24.23	3.63	29.17
f:Bacillaceae	1.31	20.40	3.38	25.09
o:Myxococcales	0.63	16.17	1.56	18.35
f:Gemmataceae	0.50	15.03	1.31	16.85
o:Streptophyta	1.44	14.73	1.31	17.48
f:Pseudomonadaceae	10.69	14.40	129.44	154.53
Paenibacillus	0.63	13.43	2.25	16.31
f:Fimbriimonadaceae	1.50	12.70	2.88	17.08
f:Methanomassiliicoccaceae	0.00	12.47	0.00	12.47
c:UI_VC2_1_Bac22	1.06	11.37	1.69	14.12
o:UI_p04_C01	0.13	11.33	0.81	12.27
o:Streptophyta	5.50	11.23	2.94	19.67
Alicyclobacillus	0.25	10.63	1.25	12.13
f:Bradyrhizobiaceae	21.44	10.60	30.06	62.10
Candidatus Koribacter	0.50	9.80	1.19	11.49
Kineococcus	11.00	9.60	2.38	22.98

o:Ul_JG30-KF-CM45	1.00	9.43	1.31	11.75
Nevskia ramosa	1.94	8.93	17.44	28.31
o:Ul_NRP-J	0.06	8.90	0.56	9.53
Candidatus Methanoregula	0.06	8.77	0.06	8.89
f:Clostridiaceae	0.38	8.63	1.06	10.07
f:Isosphaeraceae	11.69	8.50	5.56	25.75
o:Ul_iii1-15	1.13	8.40	2.19	11.71
Methylobacterium	0.38	7.63	1.31	9.32
f:Ruminococcaceae	7.00	7.33	19.88	34.21
f:Aerococcaceae	1.13	7.10	1.31	9.54
o:Streptophyta	2.69	7.03	0.31	10.03
o:Streptophyta	3.75	6.30	2.56	12.61
Corynebacterium	1.19	6.30	1.25	8.74
Chelatococcus	0.38	6.30	4.25	10.93
o:Sphingobacteriales	11.50	6.20	1.81	19.51
f:Kineosporiaceae	0.13	5.93	11.38	17.43
o:Ul_JG30-KF-CM45	0.25	5.57	0.94	6.75
Sphingomonas	1.56	5.13	2.19	8.88
Sphingobium	0.88	5.07	1.63	7.57
f:Paenibacillaceae	0.13	5.03	0.44	5.60
o:Streptophyta	3.06	4.87	0.81	8.74
o:Myxococcales	0.06	4.87	0.63	5.55
f:Bacillaceae	0.88	4.83	2.81	8.52
f:Acetobacteraceae	0.38	4.77	0.56	5.70
Gordonia	1.13	4.73	0.56	6.42
Prevotella	0.06	4.37	0.38	4.80
o:Ul_SC-I-84	6.88	4.27	7.44	18.58
f:Gemmataceae	0.13	4.20	0.94	5.26
f:Bacillaceae	0.38	3.50	0.81	4.69
Agrobacterium	3.00	3.37	0.25	6.62
Corynebacterium	0.75	3.33	0.38	4.46
f:Sphingomonadaceae	0.13	3.27	0.88	4.27
f:Sphingomonadaceae	0.13	3.10	0.69	3.91
o:Streptophyta	19.06	3.00	3.31	25.38
Bacillus	0.13	3.00	1.06	4.19
Staphylococcus	1.00	2.97	1.31	5.28
o:Streptophyta	2.19	2.93	1.50	6.62
Paracoccus	1.44	2.93	8.69	13.06
f:Chitinophagaceae	52.69	2.90	4.44	60.03
Dietzia	1.19	2.63	0.50	4.32
Candidatus Nitrososphaera gargensis	0.13	2.40	0.00	2.53
f:Ul_0319-6A21	15.56	2.30	1.75	19.61
Staphylococcus equorum	0.31	2.23	0.56	3.11
Kaistobacter	1.38	2.17	7.44	10.98
f:Sphingobacteriaceae	0.00	2.13	0.06	2.20

Corynebacterium	0.50	2.03	0.06	2.60
f:Chitinophagaceae	0.31	2.03	0.56	2.91
o:Ul_NRP-J	0.00	1.97	0.31	2.28
f:Clostridiaceae	0.00	1.97	1.31	3.28
Deinococcus	4.06	1.77	10.50	16.33
Corynebacterium	0.31	1.73	0.81	2.86
o:Streptophyta	0.31	1.73	0.19	2.23
o:Clostridiales	1.38	1.70	41.31	44.39
f:Nocardiopsaceae	0.00	1.67	0.38	2.04
o:Actinomycetales	0.44	1.57	0.81	2.82
f:Comamonadaceae	0.38	1.53	0.00	1.91
Sphingobium	0.31	1.47	4.50	6.28
f:Aerococcaceae	0.06	1.47	0.00	1.53
Streptococcus	3.63	1.43	8.75	13.81
o:Streptophyta	0.19	1.43	0.19	1.81
Staphylococcus	0.19	1.40	0.25	1.84
Sphingomonas	0.00	1.40	0.00	1.40
Acinetobacter	0.06	1.37	0.25	1.68
Acinetobacter	0.13	1.33	0.13	1.58
f:Nocardiopsaceae	0.06	1.33	0.13	1.52
o:Myxococcales	0.00	1.33	0.00	1.33
Corynebacterium	0.44	1.30	0.00	1.74
f:Haliangiaceae	1.31	1.27	19.50	22.08
Acinetobacter lwoffii	0.31	1.27	0.81	2.39

Table 18: GC standard peak areas and concentrations. Measured concentrations of the spiked samples were used to calculate the concentration in the analyzed samples.

Peak	Area [mAU]	Concentration [µl/mL]
Standard1	22671070	1
Standard2	233313	0.1
Standard3	4938	0.01
Spiked 01	22338	0.051
Spiked_1	607470	0.08
Sample 3	18241	0.051

7.3 List of figures

7.4 List of tables