

Isolation and Inhibition of Psychrotrophic Fungi in Dairy Products

Ashley Cheng¹, Isha Medikonduru², Leela Mouli^{3*},
Akshaya Ramakrishnan⁴, Nardeen Mikhail Hanna⁵

¹Aragon High School, San Mateo, CA, USA, ²American High School, Fremont, CA, USA, ³Irvington High School, Fremont, CA, USA, ⁴Mission San Jose School, Fremont, CA, USA, ⁵ASDRP, Fremont, CA, USA

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Abstract

The growth of fungi is inevitable in dairy products. They are common contaminants and are often responsible for differences in the color and flavor of a dairy product. The control of fungal spoilage is also a major concern for scientists and food producers as the growth of fungi results in great food waste and economic losses. Though most fungi are harmless, certain fungi produce mycotoxins which can also contribute to various health problems and illnesses. Therefore, using food preservatives is essential in our foods to avoid contamination. However, the chemical preservatives have proven to be harmful for human health, thus the need for using safe, natural preservatives has increased. Essential oils of aromatic plants have been recently used as antimicrobials and food preservatives. This study was conducted to isolate and identify some of the psychrotrophic fungi that contaminate dairy products in our refrigerators using morphological and molecular tools. In order to control the fungal growth, we studied the effect of essential oils: eugenol and isoeugenol, as well as the temperature effect on two of the isolated fungal species using a radial growth method. Our experiments revealed that isoeugenol had a better inhibition effect on the two tested fungi: *Penicillium paneum* and *Penicillium verrucosum*. The essential oils as well as lower temperature, 15°C were good parameters to inhibit/reduce the fungal growth.

Keywords: Psychrotrophic Fungi, Penicillium, Essential Oils, Mycotoxins, Refrigerators

1. Introduction

Food spoilage during storage is a major environmental problem and is a great concern for food industries. There is about 1.3 billion tons of food that is wasted every year from initial agricultural production down to the consumer (Gustavsson, 2011). Fungi are common contaminants of dairy products stored in refrigerators. They are responsible for visible or non-visible defects. It can lead to a significant food waste as well as important economic losses (Garnier, et al., 2017). The group of fungi that is capable of growing at low temperatures is called Psychrotrophs (Basavabharati and Prabha,

2015). Those fungi have the ability to use many substrates including carbohydrates, organic acids, proteins, and lipids, which are present in milk and its products in order to grow (Veld, 1996).

The predominant psychrotrophic mold species isolated from refrigerated foods were *Penicillium* (49%) and *Aspergillus* (38%) (Torrey and Marth, 1977). The psychrotrophic mold species of *Penicillium* were also predominant in fermented dairy products (Bullerman, 1981). The refrigerated storage and pH of traditional dairy products helps the growth of psychrotrophic molds which may have harmful effects on humans and animals, as well as cause defects in products (Basavabharati and Prabha, 2015).

* Corresponding Author
leelamouli03@gmail.com

Advisor: Nardeen Mikhail Hanna
nardeen.mikhail@asdrp.org

Most of the psychrotrophic molds produce proteinases and lipases that change the composition of milk constituents leading to defects that in turn cause economic loss to the producers.

Some of these molds are mycotoxin producers. Mycotoxins are secondary metabolites that are produced by fungi (Del Palacio, et al., 2016). Most mycotoxins are chemically stable and survive food processing, which is a concern to human health. In cheese, the most hazardous mycotoxins are ochratoxin A and Aflatoxin M1. They are produced by fungal species either via direct cheese contamination or indirect contamination (where the milk used to make the cheese was contaminated) (Hymery, et al., 2014). A case has been reported of a man with a history of poorly controlled diabetes, acute myelogenous leukemia, and prolonged neutropenia who presented a unilateral headache, fever, nausea, and vomiting. A sinus culture yielded *Mucor circinelloides* and the suspected source of infection was contaminated Greek yogurt, which he consumed several days before developing these symptoms (Lazar, et al., 2014).

Scientists are looking for efficient solutions to prevent and/or limit fungal spoilage in dairy products. Different traditional technologies are used to control contaminants such as air treatment, cleaning and disinfection procedures, heat treatment, and water activity reduction during refrigeration (Altunatmaz, 2012). Moreover, chemical preservatives such as benzoate and sorbate are used to avoid fungal spoilage (Silva and Ladon, 2016). However, adverse side effects have been reported as a result of using these chemical preservatives. Benzoates have been suspected to cause allergies, asthma, and skin rashes. Also, sorbates were reported to cause urticaria and contact dermatitis (Kinderlerer and Hatton, 1990).

Therefore, using natural safe preservatives such as essential oils to prevent food contamination is being considered in the food industry (Nasery, et al., 2016). Aromatic and medicinal plants produce essential oils in the form of secondary metabolites (Pandey, et al., 2017). These compounds have been tested to as antibacterial and antifungal agents against some microorganisms. Eugenol is a phenylpropanoid and is extracted from different natural sources such as clove

trees (*Syzygium Aromaticum*). It has antimicrobial activity against microorganisms such as bacteria and fungi. Eugenol and its derivatives were studied on *Botrytis cinerea* and showed a good inhibitory effect on this fungus (Olea et al., 2019). However, these oils have not been studied on *Penicillium paneum* and *Penicillium verrucosum*.

The information provided in the previous background explains how important it is to prevent fungal contamination to ensure a healthy food delivered to the consumers. Besides, there is a high demand to use natural compounds rather than using synthetic ones. Therefore, in this study, different fungal species were isolated from various dairy products. *Penicillium paneum* and *Penicillium verrucosum* isolated from Mozzarella and Havarti cheese, were selected to study the effect of the essential oil, eugenol, and its derivative, isoeugenol as well as the effect of temperature on their radial growth.

2. Methods

Ten dairy samples were collected from home refrigerators including milk, sour cream, havarti cheese, yogurt, pepper jack cheese, sharp cheddar cheese, medium cheddar cheese, roomy cheese, and butter. All samples had already shown a significant fungal growth.

Czapek-Dox agar media was used as a general culture media that allowed the growth of most fungi. Rose Bengal dye and the antibiotic (chloramphenicol) were added to the media to suppress bacterial growth. Chilvers et al. (1999) proved that the combination of Chloramphenicol and Rose Bengal Dye has been scientifically proven to inhibit bacterial growth along with the presence of light. Chloramphenicol itself has also been deemed as a durable antibiotic due to its ability to withstand heat, which allows for it to be added to any medium before sterilization (King, et al., 1979). In order to prepare food homogenates, 5 grams of each rotten dairy sample was taken, and mixed with 10 ml of deionized water. The samples were then crushed with a mortar and pestle and transferred into an Eppendorf tube which was then vortexed. 200 µl of the fungi mixture was added to each set petri dish and

incubated at 20 degrees Celsius for eight days. Duplicate plates were prepared from each isolate. Afterward, an additional direct inoculation of the contaminated food samples was done for each dairy sample. So, three plates from each food sample were prepared.

On the ninth day of inoculation, fungi were taken from each petri dish and re-inoculated into a new set of petri dishes, all without Rose Bengal dye and chloramphenicol. The new set of petri dishes were then incubated at 20°C for ten days. The reinoculation process was repeated a total of 3 times to be ready for the identification.

2.1 Fungal Isolates Identification

Both macroscopic and microscopic examination of the isolated fungi were done using the identification books “Identifying fungi: A clinical laboratory handbook” (St-Germain and Summerbell, 2011), and “Fungi and Food Spoilage” (Pitt and Hocking, 2009). We also did molecular identification to confirm our isolated species.

2.2 Molecular Method

Mycelia was scraped from each of the eight samples’ petri dishes and mixed with liquid nitrogen until it became a fine powder. The genomic DNA was then extracted from each powder using the procedure outlined in the Zymo Quick-DNA™ Fungal/Bacterial Miniprep Kit (Catalog No. D6005). The DNA concentration of each sample was measured using a fluorometer.

The internal transcribed spacer region “ITS” were amplified. The ITS region is the most recommended universal fungal barcode sequence that is used because of its highest probability of successful identification of fungi. The DreamTaq Hot Start PCR Master Mix (2X) with primers ITS1F and ITS4 (forward and reverse primers) were used (White, et al., 1990). 25µl of PCR mixture was mixed with 2.5 µl of each of the two primers: ITS1F and ITS4, 10µl of our sample’s DNA, and 10µl of nuclease free water. The eight samples were placed in Perkin Elmer Cetus 480 Variable Temperature DNA Thermal Cycler at fixed intervals: 95° for 5 minutes, then a

repetition of 35 cycles for 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 1 minute, followed by 72°C for 8 minutes, and then a 5-minute hold at 4°C.

Detection of PCR-amplified products was performed by electrophoresis on 1 % ethidium bromide-stained agarose gel. Agar gel was made with 25 mL of water, 0.25 g of biology-grade agar powder and 12.5 µl of ethidium bromide. The water and agar powder were microwaved on high for approximately 75 seconds on before mixed with the ethidium bromide and set to dry in a mold. The PCR products had left to cool, then 8.3µl of loading dye was added to each 10 µl of dyed sample (including a DNA ladder) which were then put into each well. The machine ran at 110 volts for around 20 minutes, until the gel traversed across the plate. The agar plate was removed and was put on top of a UV light, where the bands could be seen. The base pairs for each sample came to 500 base pairs for pepper jack, sharp cheddar, butter, milk, and Havarti cheese, and 850 base pairs for sour cream and roomy cheese.

PCR products were purified using the procedure provided with the kit “DNA Clean & Concentrator™-5”. 10µl of each DNA sample was taken and added to 50 µl of DNA binding buffer as a 5:1 ratio had to be maintained. The mixture was put into a column with a collection tube underneath and centrifuged. 200 µl of DNA wash buffer was added and followed by centrifuging. 15 µl of DNA elution buffer was then added and stored in an Eppendorf tube. The concentrations of each sample were taken again using fluorometer. The purified PCR products were sent to Elim Biopharmaceuticals to do Sanger sequencing. The consensus sequences of the ITS region were submitted for a BLAST search using the NCBI GenBank database to obtain species-level information.

2.3 Essential Oils and Temperature Effect

The antifungal activity of Eugenol and Isoeugenol was tested on two of the isolated fungal species: *Penicillium paneum* and *Penicillium verrucosum* which were isolated from mozzarella cheese and Havarti cheese respectively. Eugenol (concentrations applied were: 250, 500, and 750 ppm) and isoeugenol (concentrations applied were: 250, 500 and 750 ppm) were dissolved in 5% of Dimethyl Sulfoxide solution (DMSO). CDA media was used as a growth medium

for radial growth measurements to test the inhibitory effect of eugenol and isoeugenol essential oils at three different temperatures: 15°C, 20°C, and 25°C. They were added to the medium after sterilization and daily measurements were taken for 7 days. Three readings were recorded daily for each treatment (n=3).

3. Results

Out of the ten collected dairy samples, eight different species of *Penicillium* were identified. They were as follows: Yogurt - *Penicillium decumbens*, Butter - *Penicillium brevicompactum*, Cheddar cheese (Sharp + Medium) - *Penicillium commune*, Roomy cheese and pepper jack - *Penicillium crustosum*, Sour cream - *Penicillium solitum*, Milk - *Penicillium chrysogenum*, Havarti cheese - *Penicillium verrucosum*, Mozzarella - *Penicillium paneum*. Most of the *Penicillium* spp. are known as mycotoxin-producers, which are hazardous to humans.

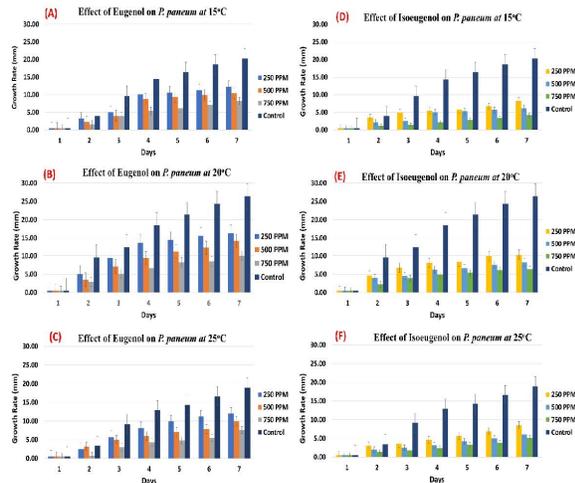


Figure 1: Growth Rate of *P. paneum* when in contact with (A) Eugenol at 15°C, (B) Eugenol at 20°C, (C) Eugenol at 25°C, (D) Isoeugenol at 15°C, (E) Isoeugenol at 20°C, and (F) Isoeugenol at 25°C, compared with the control for 7 days. Legend (top to bottom) for subgraphs A, B, and C: Eugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). For subgraphs D, E, and F: Isoeugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). The data are expressed as the mean ± SEM (n = 3)

Our experiment with the essential oils and the temperature effect showed that isoeugenol was better than eugenol in terms of inhibiting the radial growth of the selected fungi, *Penicillium paneum* and *Penicillium verrucosum* (Figure 1 & Figure 2). Moreover, 20°C was the optimum growth temperature for both fungal species. In the figures 1 and 2, our control samples (no oils added) showed that both *P. paneum* and *P. verrucosum* grew optimally at this temperature, while at the other two temperatures, their growth was better at 15°C than at 25°C. The percentage of inhibition for each treatment was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Growth rate of the control} - \text{Growth of the treated fungus with oil}}{\text{Growth rate of the control}} \times 100$$

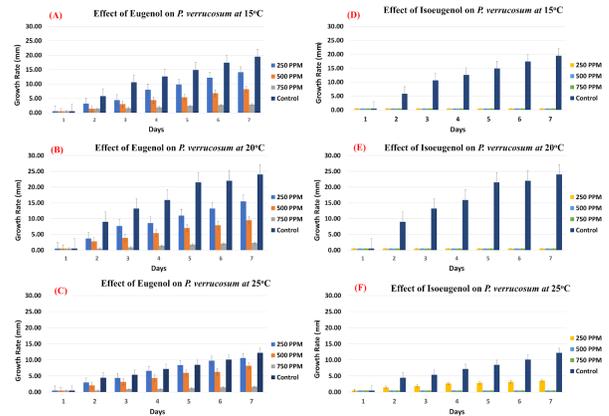


Figure 2: Growth Rate of *P. verrucosum* when in contact with (A) Eugenol at 15°C, (B) Eugenol at 20°C, (C) Eugenol at 25°C, (D) Isoeugenol at 15°C, (E) Isoeugenol at 20°C, and (F) Isoeugenol at 25°C, compared with the control for 7 days. Legend (top to bottom) for subgraphs A, B, and C: Eugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). For subgraphs D, E, and F: Isoeugenol concentrations of 250 ppm, 500 ppm, 750 ppm and control plates (no oil). The data are expressed as the mean ± SEM (n = 3)

The experiment revealed that at concentration 750 ppm, the highest inhibition percentage of isoeugenol on *P. paneum* was 79.3% at 15°C (Figure 3). At the same temperature, the eugenol also appeared to inhibit the *P. paneum* growth by a maximum inhibition 59.6% at 750 ppm (Figure 3). The radial

growth decreased as the concentration of the essential oils increased (Figure 3 & Figure 4).

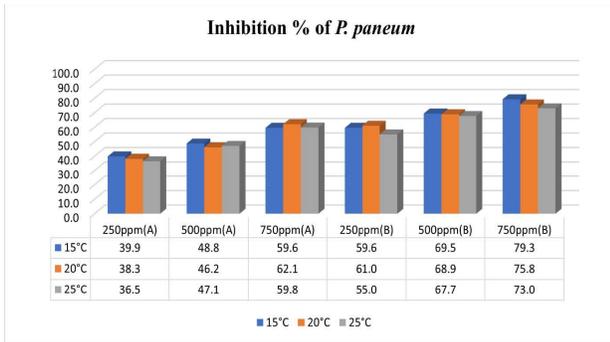


Figure 3: Percent inhibition of *P. paneum* when exposed to 250 ppm (A), 500 ppm (A), and 750 ppm (A) of eugenol at 15°C, 20°C, and 25°C and 250 ppm (B), 500 ppm (B), and 750 ppm (B) of isoeugenol at 15°C, 20°C, and 25°C.

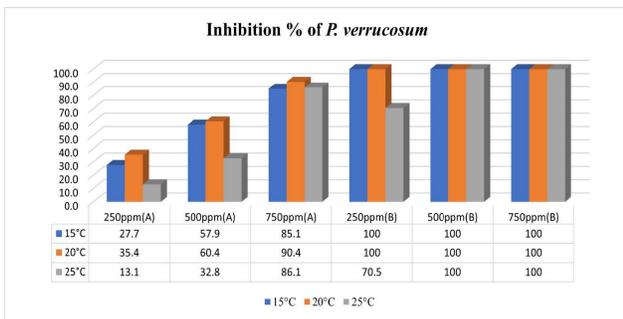


Figure 4: Percent inhibition of *P. verrucosum* when exposed to 250 ppm (A), 500 ppm (A), and 750 ppm (A) of eugenol at 15°C, 20°C, and 25°C and 250 ppm (B), 500 ppm (B), and 750 ppm (B) of isoeugenol at 15°C, 20°C, and 25°C

However, the inhibition percentage of eugenol on *P. verrucosum* was higher at 20°C (Figure 4). The isoeugenol at the tested concentrations was able to inhibit the growth by 100% for *P. verrucosum* except at 25°C at 250 ppm concentration as shown in Figure 2-D, E & F and the growth was still inhibited by 70.5% (Figure 4). However, isoeugenol could not completely stop the growth of *P. paneum* as depicted in figure 1-D, E & F. The inhibition percentage was 61%, 69.5%, and 79.3% at 250 ppm, 500 ppm, and 750 ppm respectively on the growth rate regardless of the temperature (Figure 3). Although the growth of *P. paneum* did not stop at all concentrations of eugenol and isoeugenol, the fungal growth rate decreased as

the concentration increased (Figure 1). Our work also revealed that both oils had greater inhibitory effect on *P. verrucosum* than their effects on *P. paneum*. The *P. verrucosum* growth was inhibited by a maximum of 90.4% at 750 ppm eugenol (Figure4); but for *P. paneum*, the inhibition rate was only 62.1% at the same concentration (Figure 3). The highest percentage of inhibition by the added essential oils were observed at temperatures 15°C and 20°C. Although both fungi did not grow well at 25°C without any oil added, and the growth rate of the control at 25°C was less compared to the growth at the other two temperatures, the oils inhibitory effect was less at this temperature.

4. Discussion

Fungi are common contaminants of dairy products, which provide a favorable environment for their growth as they are responsible for visible or non-visible defects, such as off odor and flavor (Garnier et al., 2017). Although temperature is one of the major controlling factors of food quality and safety because of its influence on microbial growth rates, fungal spoilage is still an issue for dairy manufacturers. The reason behind this fact is that psychrotrophic microorganisms have the ability to grow at normal refrigeration temperatures (Altunatmaz, 2012). Furthermore, fungi may originate from milk or may be introduced during cheesemaking either from the environment or are deliberately inoculated using commercial ripening cultures (Hymery, et al., 2014).

Eight different *Penicillium spp.* were identified in all our isolates using morphological and molecular methods. *Penicillium commune* was isolated from sharp and medium cheddar cheese. This fungus belongs to the Ascomycota phylum, which has been often isolated from hard or semi-hard cheeses (Garnier, et al., 2017). It is also well known for being one of the most common fungal spoilage molds of cheese, which produces two neurotoxins, penitrem A and roquefortine, and many mycotoxins, such as cyclopiazonic acid and regulovaline A and B (Gqalen, et al., 2001). Garnier et al. (2017) reported that *Penicillium commune*, along with many other fungi like *Penicillium solitum*, *Penicillium*

crustosum, *Penicillium verrucosum*, *P. chrysogenum*, *Penicillium nalgiovense*, and *Penicillium griseofulvum* were isolated from hard and semi-hard cheeses but were also found in many other milk products such as butter, yogurt, and milk.

Within our study *Penicillium brevicompactum* was isolated from butter. This fungus was previously isolated from fruits and can grow between -2°C and 30°C with an optimum near 23°C (Pitt and Hocking, 2009). In the same study in which *P. commune* was isolated from cheese (Garnier, et al., 2017), *P. brevicompactum* was similarly isolated from various hard and semi-hard cheeses. *P. brevicompactum* produces the weak mycotoxin mycophenolic acid which is not a concern in foods. *Penicillium solitum* was isolated from sour cream, but Yin et al. (2016) asserted that this fungus is often found in fruits and is usually responsible for the decay of apples and pears (pome fruits). Furthermore, *P. Solitum* was the most dominant species isolated from Italian hard cheese (Decontardi, et al., 2017). *P. solitum* is one of the less important species of fungal organisms responsible for food decay.

In the mozzarella cheese, *Penicillium paneum* was identified. According to the article from American Society for Microbiology, *P. paneum* is a common contaminant found in cereal grains (Chitarra, et al., 2004). This fungus can grow at low temperatures, low pH, high levels of carbon dioxide, and in acidic conditions. However, in this research, it was isolated from a sample of a dairy product. So, as a result *P. paneum* can grow both in wheat products as well as dairy products in many different conditions. *P. paneum* is known to produce patulin, which is mutagenic, immunotoxic, and neurotoxic (Cole and Cox, 1981).

Penicillium Crustosum was isolated from roomy cheese and pepper jack cheese in our experiment. This fungus is usually reported causing blue mold on pome fruits but is also found on cheese and nuts. It produces many mycotoxins such as roquefortine C, terrestric acid, and penitrem A. According to Vico et al. (2014) *Penicillium Crustosum* was found on apples in Serbia However, in this research, *Penicillium Crustosum* was detected in dairy products, rather than fruits.

Another species of fungi obtained from yogurt

was *Penicillium decumbens*. Liu et al. (2013) found this fungus in decayed straw-covered soil in China. In their experiment, it was used to produce industrial-scaled cellulase. However, Vadillo et al. (1987) reported *Penicillium spp.* in pasteurized milk, which is consistent with our results.

Penicillium verrucosum was isolated from Havarti cheese. This fungus is known to grow between 0°C and 31°C with an optimum temperature around 20°C (Pitt and Hocking, 2009). Kure and Skare (2019) reported that *P. verrucosum* was found in hard, semi-hard and semi-soft cheeses from Greece, Denmark, and Spain. It produces intoxicating mycotoxins called ochratoxin A (OTA). This mycotoxin is an immunosuppressive and teratogenic (Pitt and Hocking, 2009). It has also been classified as genotoxic and a possible human carcinogen (Pfohl-Leskowicz and Manderville, 2007). OTA production increases when temperatures are between 10°C and 21°C. Therefore, the presence of this fungus in our cheese is a huge concern for human health.

Penicillium chrysogenum was found in milk. This *Penicillium spp.* has been frequently observed in cheese from Denmark, USA, and Spain (Kure and Skare, 2019). *Penicillium chrysogenum* is rarely pathogenic, but it was reported to cause health problems to people with weak immune systems (Adrian, et al., 2005). Moreover, *Penicillium chrysogenum*, *P. citrinum*, *P. commune*, *P. decumbens*, and *P. roqueforti* were also reported to be isolated from cheese surfaces stored at refrigeration temperature (6± 2°C) (Makki, 2019).

Three concentrations of the selected essential oils were used ranging from 250 ppm to 750 ppm of eugenol and its analogue, isoeugenol, because the US Food and Drug Administration (FDA) recommends using less than 1500 ppm of essential oil concentrations extracted from cloves. Although it has been approved for use in food as a safe food preservative, studies showed that clove oil is toxic to human cells if ingested in high concentrations. It has been shown to cause life-threatening complications such as acute respiratory distress syndrome and fulminant hepatic (liver) Failure (Kegley, et al., 2010). In our experiment, isoeugenol has shown better inhibitory effect on the two selected species,

Penicillium verrucosum, and *Penicillium paneum* (Figure1 & Figure 2). It completely suppressed the growth of *Penicillium verrucosum* at the three concentrations except at 250 ppm at 25°C (Figure2). For *Penicillium paneum* isoeugenol had a significantly greater effect than eugenol, but did not suppress the growth completely, or as much as it suppressed *Penicillium verrucosum* (Figure1). Isoeugenol and eugenol have been reported to have the ability to slow or stop the growth of fungi (Torrey and Marth, 1977). According to Hamini-Kadar et al. (2014), the eugenol has stopped the growth of *Fusarium redolens* and *Fusarium commune* at 500 ppm. However, in this research, the eugenol did not stop the *P. verrucosum* nor *P. Paneum* growth. 750 ppm inhibited the growth of *P. verrucosum* by a maximum of 90.4% (Figure4), while *P. paneum* growth was suppressed by 62.1% (Figure3). Šimović et al. (2014) used 250 ppm and 750 ppm of eugenol and carvacrol, which showed significant inhibitory effects against fungal pathogens, specifically *Aspergillus carbonarius* and *Penicillium roqueforti*. They incubated them at 15°C and 25°C and measured the growth rate for six days. They asserted that the eugenol had a synergy effect on the watermelon, because the inhibitory effect was greatest at 15°C. This is consistent with this study as the percentage of inhibition of eugenol and isoeugenol was the highest at 15°C by 59.6% and 79.3% respectively on *P. paneum* (Figure3). However, the greatest inhibition of eugenol on *P. verrucosum* was 90.4% at 20°C (Figure4). Our experiments suggest that isoeugenol is the best essential oil that can be used as an antifungal agent against the tested fungal species since it inhibited *P. verrucosum* by 100% and *P. paneum* by 79.3% at 750 ppm respectively. At lower temperature 15°C and 20°C, the percent of inhibition for both fungi were closer to or higher than that at 25°C (control temperature outside fridge). Therefore, using isoeugenol as a food additive for dairy products stored at refrigerators is recommended.

The antifungal mode of action of eugenol and its analogs needs further investigation, but it is known to affect cell proliferation. Using eugenol as an antimicrobial agent altered cell membrane and cell wall structures of proliferating *Saccharomyces cerevisiae* cells resulting in the release of cellular

content (Bennis, et al., 200; Hyldgaard, et al., 2012; Maia da Silva, et al., 2018). One study evaluated the isoeugenol's antifungal action against *Candida spp.*, and stated that isoeugenol inhibits H⁺-ATPase, which triggers intracellular acidification and cell membrane breakages (Bhatia, et al., 2012).

5. Conclusion

The purpose of our research was to identify different psychrotrophic fungi from dairy products. Eight different *Penicillium spp.* were isolated from different dairy products stored in refrigerators. The inhibitory effect of eugenol and its analogue, isoeugenol, was studied at different temperatures on the radial growth rate of *P. paneum*, and *P. verrucosum*, which were isolated from Mozzarella and Havarti cheese respectively. Isoeugenol had a greater effect of suppressing the fungal growth in comparison to eugenol at all the selected temperatures. *P. Paneum*, and *P. verrucosum* grew optimally at 20°C as they are considered psychrotrophic fungi. The essential oils were successfully able to lower the fungal growth at lower temperature 15°C and 20°C. In conclusion, isoeugenol is the best essential oil that can be used as an antifungal agent against the tested fungal species. Treating food with essential oils could be a viable solution to the spoilage problem of our dairy products, however further work is still needed to study a wider range of eugenol analogs on more fungal species. Food decay is an ever-growing issue in the food industry. Spoilage of our food with mycotoxin-producing fungi can cause serious health problems in humans. Therefore, using preservatives to avoid contamination and the eventual production of mycotoxins in our food is essential. Since synthetic preservatives have appeared to be harmful to human health and the environment, the use of natural and safer preservatives is currently recommended. Using essential oils of aromatic plants as preservatives is suggested to help prevent the deterioration of food.

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