Kaunas University of Technology
Faculty of Mechanical Engineering and Design

Development of Antifungal Coating Containing Combination of Synergetic Essential Oils for Bread Paper Packaging

Master's Final Degree Project

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Project author

Lect. Laura Gegeckienė
Supervisor

Kaunas, 2022

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Industrial Engineering and Management (6211EX018)

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Task of the Master's final degree project

Given to the student – Dovilė Rožėnaitė

1. Title of the project

Development of Antifungal Coating Containing Combination of Synergetic Essential Oils for Bread Paper Packaging

(In English)

Priešgrybelinės dangos su sinergetinių eterinių aliejų mišiniu popierinei duonos pakuotei kūrimas

(In Lithuanian)

2. Hypothesis

The coating of the packaging from the mixture of essential oils extends the shelf life of the bread

3. Aim and tasks of the project

Aim: develop an antifungal properties containing coating made of mixture of synergetic essential oils for bread's paper packaging to extend it's shelf life.

Tasks:

1. To analyze which synergetic combinations of essential oils work best against bread mold fungicides and compare them;
2. To develop a methodology for impregnation of paper packages with an essential oil coating;
3. To investigate the effectiveness of the developed packaging in order to prolong the time before the appearance of mold.

4. Initial data of the project

N/A

5. Main requirements and conditions

Environmental conditions: room temperature (18-24°C); materials: MMK cardboard, multi-seeded bread, essential oils, wax; specific equipment: texture analyzer, scales, pipette, roller.

Project author	Dovilė Rožėnaitė		
	(Name, Surname)	*(Signature)*	*(Date)*
Supervisor	Laura Gegeckienė		
	(Name, Surname)	*(Signature)*	*(Date)*
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Rožėnaitė Dovilė. Development of Antifungal Coating Containing Combination of Synergetic Essential Oils for Bread Paper Packaging. Master's Final Degree Project, supervisor lect. Laura Gegeckienė; Faculty of Mechanical Engineering and Design, Kaunas University of Technology.

Study field and area (study field group): Production and Manufacturing Engineering (E10), Engineering Sciences (E).

Keywords: bread, paper packaging, essential oil, antifungal

Kaunas, 2022. 51 p.

Summary

The purpose of the study was to create the coating with the synergetic essential oils mix for bread to replace the usual plastic package with an environmentally friendly and mold resistant option. Different mixtures efficiency was analyzed and compared. 3 pairs of essential oils containing citral-eugenol, citral-thymol and carvacrol-thymol were selected for the experiment part. 3 types of bread were compared and the most susceptible to fungi was selected for further studies. 3 coatings were made of divergent essential oils blend, containing the required concentration of constituent, and soy wax. These coatings were applied to the carton’s box inner surface for antifungal properties and reduced air permeability. Samples of bread were kept in 4 packages: an original plastic bag, paper box with one wall coating, all inner surface coating and without any coating. These packages were compared to each other by lost weight amount and textural parameters before and after storing. This was done to find out that the coating helps to retain moisture in the product. 2 layers of the coating were applied for the last part of the experiment where citral-thymol and carvacrol-thymol coatings were effective to inhibit mold activity. Coating without bending edges covered, accurate synergistic concentrations and sensory analysis performance should be considered as an opportunity for future research.

Rožėnaitė Dovilė. Priešgrybelinės dangos su sinergetinių eterinių aliejų mišiniu popierinei duonos pakuotei kūrimas. Magistro baigiamasis projektas, vadovė lekt. Laura Gegeckienė; Kauno technologijos universitetas, Mechanikos inžinerijos ir dizaino fakultetas.

Studijų kryptis ir sritis (studijų krypčių grupė): Gamybos inžinerija (E10), Inžinerijos mokslai (E).

Reikšminiai žodžiai: duona, popierinė pakuotė, eterinis aliejus, priešgrybelinis

Kaunas, 2022. 51 p.

Santrauka

Tyrimo tikslas – sukurti dangą su sinergetiniu eterinių aliejų mišiniu duonai, kuri pakeistų įprastą plastikinę pakuotę ekologiška ir atsparią pelėsiams. Išanalizuotas ir palygintas skirtingų mišinių efektyvumas. Eksperimentinei daliai buvo atrinktos trys poros eterinių aliejų, kurių sudėtyje yra citralio-eugenolio, citral-timolio ir karvakrolio-timolio. Tarpusavyje buvo palygintos trys duonos rūšys ir labiausiai imli pelėsiams atrinkta tolesniems tyrimams. Trys dangos buvo pagamintos iš skirtingų eterinių aliejų mišinio, turinčio reikiamos koncentracijos sudedamųjų dalių, ir sojų vaško. Dangos buvo užteptos ant kartoninės dėžutės vidinio paviršiaus, siekiant suteikti priešpelėsinių savybių ir sumažinti oro pralaidumą. Duonos mėginiai buvo laikyti keturiose pakuotėse: originaliame pastiko maišelyje, popierinėje dėžutėje, padengtoje danga ant vienos sienos, vidinio paviršiaus ir be dangos. Šios pakuotės buvo palygintos tarpusavyje pagal prarastą svorį ir tekstūros parametrus prieš ir po laikymo. Buvo išsiaiškinta, kad danga padeda išlaikyti drėgmę gaminyje. Paskutinėje tyrimo dalyje dėžutės buvo padengtos dviem sluoksniais, kuriame citralio-timolio ir karvakrolio-timolio dangos veiksmingai slopino pelėsių aktyvumą. Dangos padengimas išvengiant lenkimo briaunų, tikslių sinergetinių koncentracijų užtikrinimas ir juslinės analizės atlikimas turėtų būti apsvarstyti metodai kaip ateities tyrimų galimybėmis.

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Abbreviations:

EU – European Union;

ADA – azodicarbonamide;

Spp. – species;

LAB – lactic acid bacteria;

EO – essential oil;

MIC – minimum inhibitory concentration;

FIC – Fractional inhibitory concentration;

MRSA – methicillin resistant *Staphylococcus aureus*;

PDA – potato-dextrose agar;

MMK – Mayr-Melnhof Karton;

TPA – texture profile analysis.

Terms:

Minimum inhibitory concentration – the smallest quantity of a chemical that precludes observable bacterial growth.

Fractional inhibitory concentration – the sum of the FICs of each drug tested when used in combination.

Introduction

Bread is the staple food known centuries ago and up to this time used by many consumers on the daily basis. Its simplicity determined the longevity of this product. It is essential in the human diet as it provides a proportionate amount of various nutrients and micronutrients.

Bread can be made of only 4 vital ingredients: flour, water, yeast and salt. Nevertheless, the technology of production in the industry varies from the simplest and quickest to more time and space consuming recipes. But the main manufacturers concern is to give the consumer what is needed. Even this traditional food must be altered to fit under trends and requirements nowadays people have.

One of the main concerns in the world is health. The expansion of the food industry has also led to an increase in human morbidity. Therefore, nutrients, especially food additives, started to be more closely controlled after the emergence of scientifically proven threats. Even though these substances are strictly observed to ensure appropriate quantities and materials are used, consumer opinion is still negative towards them.

Another crucial problem is the environment. Plastic waste generated in greater vast each year is accumulating and taking more and more space not only on land but also in water and even air. This is because plastic is cheap, strong and quickly made material leading to being the main type of packaging nowadays and creating this loophole situation.

A variety of studies are made on diverging sustainable materials. But their usual disadvantage is expensive and lengthy production processes leading back to using plastic. Another commonly used packaging type is paper. Due to absorption and permeability, paper packages are not suitable alone to be suitable for some types of foodstuff and plastic is incorporated, creating the packaging not recyclable.

By combining naturally occurring substances, new packaging can be created to have the needed qualities. In this case, provide antifungal properties to sustain the product fresh for the same length or even longer. Also, keep the product fresh for as long or longer than the current packaging by giving the packaging the properties it requires for the specific product.

Aim: develop an antifungal properties containing coating made of mixture of synergetic essential oils for bread’s paper packaging to extend it’s shelf life.

Tasks:

1. To analyze which synergetic combinations of essential oils work best against bread mold fungicides and compare them;
2. To develop a methodology for impregnation of paper packages with an essential oil coating;
3. To investigate the effectiveness of the developed packaging in order to prolong the time before the appearance of mold.

1. Analytical part

1.1. Relevance and novelty

People in European Union (EU) tend to care about their environment. According to research by Eurobarometer [1] conducted in 2017, a major part of the population of the EU is concerned about protecting their habitat. Lithuanians identify that the main problem is the amount of waste generated. Over 70% of people are anxious about plastic effect on their health and nature. Wherefore about 30% of the residents avoid plastics, which are used only once. The main solutions to this issue, according to humans of Europe, are given in Fig. 1.

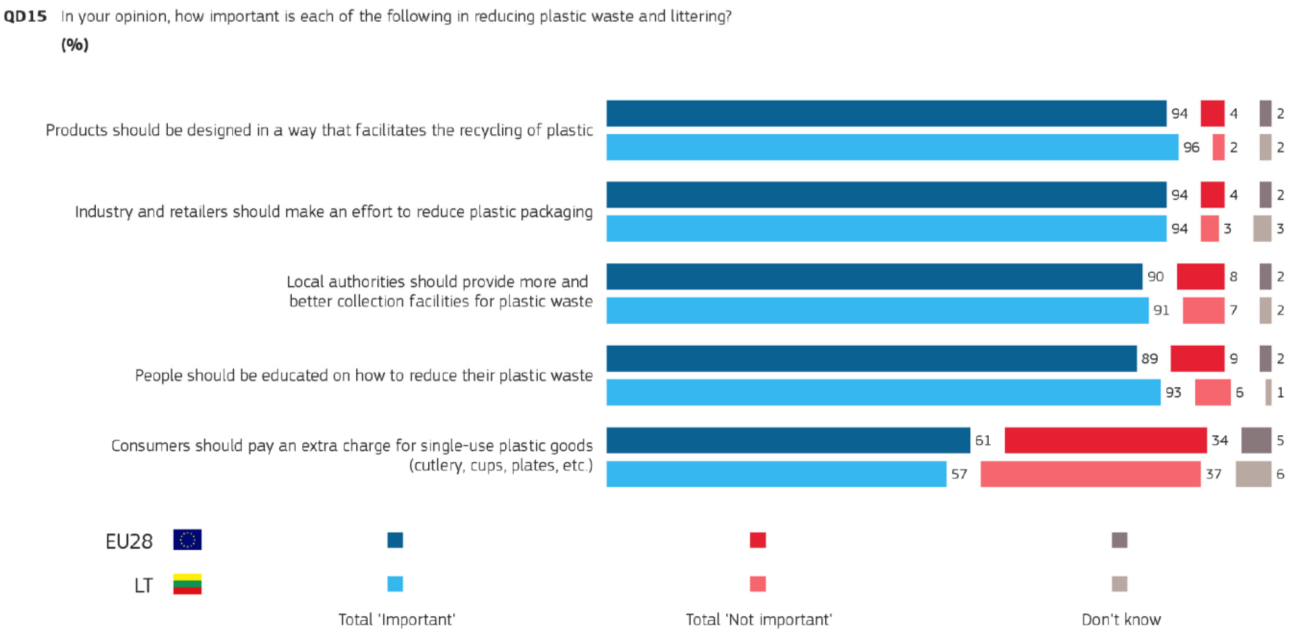

Fig. 1. Opinions of the people of the European Union on ways to reduce plastic waste [1]

Both Europeans and Lithuanians share the same idea. The core is to find alternatives and reduce the consumption of this material by replacing it with more environmentally friendly packaging. But in the current day, there are not many options in the market. Recently applied plastic taxation is the least attractive idea. The consumer would like such products to disappear altogether and all available options to be environmentally friendly.

Paper usage is constantly growing as it is an easily accessible, cheap and strong material, which can be fully recycled. If not, it completely decomposes naturally in 2-6 weeks [2]. Meanwhile, plastic waste takes years and decomposes into small parts – microplastics – of which some types can never fully degrade.

However, paper is not a perfect choice either - it is not suitable for some types of food due to its permeability to air, being hygroscopic to water and oil. Overcoming these issues can make the paper excellent packaging material for most goods. Not a lot of environmentally friendly decisions are available to fix this situation.

Statista [3] demonstrates, that the growth of paper packaging in food product packaging raised by 15% in 4 years, but the problem of sustainability remains as ordinarily there is a combination of a few different materials. In that way, they cannot or are not separated and are not recycled. Consequently, entirely natural packaging is needed to be developed to overcome this trouble.

The majority of commodities in the market come with packaging. They all have different requirements to fulfil to be able to be used as packaging for diverse types of goods. To take food products into consideration, they have even greater regulations because their safety depends on their package. As a result, the quality of a product also is preserved and safety may not only be maintained but also increased. In that way, the short shelf life of microbiologically susceptible edibles can be prolonged.

Sales of baked goods are forecasted to grow continuously, which means that the quantity of the produced goods rises [4]. The demand for healthier products is growing particularly fast as consumers are more and more concerned about their wellbeing. But it is not that easy make a switch to a completely new type of product. The only way to retain and attract new customers is to follow the trends. All biggest companies have some of these in the assortment together with other healthier choices to traditional bread. Therefore, the bread with added seeds or grains is gaining a lot of popularity

With the need for green packaging, the need for natural products also has risen. People tend to care about their health more. German Federal Institute for Risk Assessment conducted a survey [5] in 2021 to assess current consumer attitudes towards food additives. The respondents showed their deep understanding of the distribution, functions and impact of E numbers on the user. The main functions of additives, according to responders, are to affect flavor and shelf life. One of the questions asked was about food preferences in the grocery store. A visual representation of the response is shown in Fig. 2.

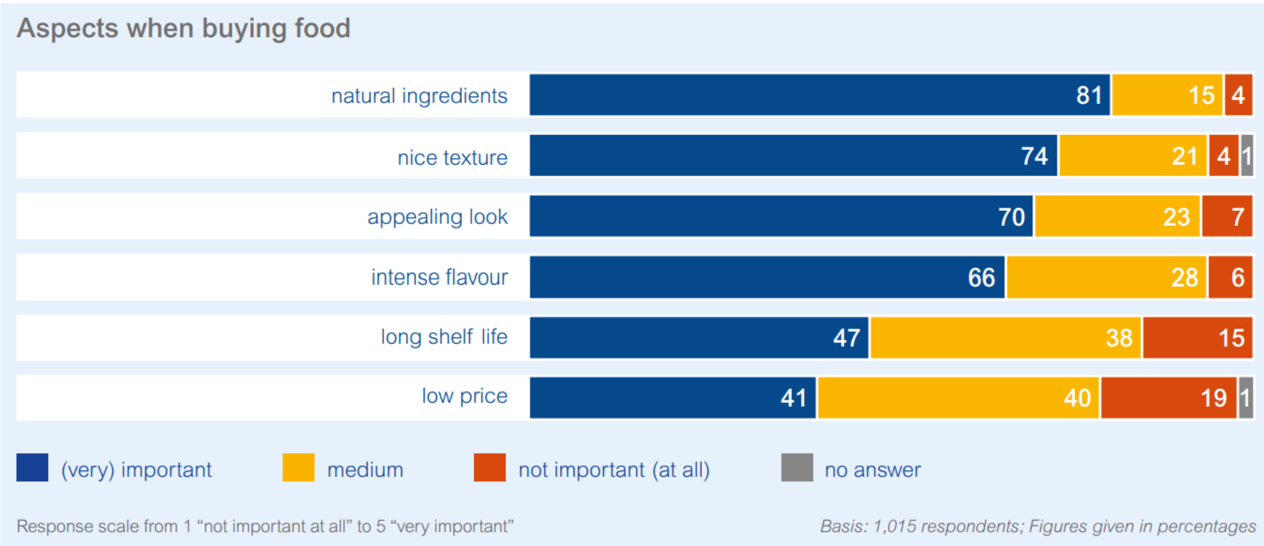

Fig. 2. The most vital aspects in choosing food for Germans [5]

Even knowing the benefits of enhancing the products with additives, the majority still choose simple and understandable composition over better foodstuff qualities. Even cost is the least place as the most important influencer, meaning people tend to spend more money buying a quality product. The most important trait of foodstuff people are buying is simplicity and natural components. The definition of the pure product is mainly defined by food additives – 55% of the German population avoids them [5].

Since the beginning of the use of artificial food additives, both consumers and manufacturers received what they wanted – better food in every way from the quality side: enhanced flavor, texture, appearance and shelf-life. That seized the chance to make better products even cheaper than before. The advantages were worshipped until the disadvantages became clear. Various health problems are related to separate additives. Due to this, there are strict regulations that meter the quantity or even ban them from usage nowadays.

The main categories of food additives, used in breadmaking are antioxidants, emulsifiers and preservatives. The main representative of the first class is ascorbic acid better known as vitamin C. It is one of the most common additional ingredients in bread products because of its benefits and safety for the consumer. Ascorbic acid improves the rheological properties of the dough – it retains more air causing bigger volume. Dough's strength and elasticity increase leading to better overall textural qualities. Non-European countries use Azodicarbonamide (ADA) for the same features. However, ADA is banned in Europe because of being cancerogenic. Secondly, emulsifiers have two main functions: to soften a crumb and strengthen gluten. Mono- and diglycerides are frequently used emulsifiers as their employment is about 70% compared with over. Monoglycerides help to retain the initial textural properties of bread over the life span and prevent moisture loss also known as stalling. Lastly, the final type is preservatives, which are most needed because of their ability to inhibit bacterial and fungal activity. Most popular in use are salts of propionic acid (or propionates) altogether with sorbic acid (sorbates) [6].

The use of food additives is defined by regulation (EC) NO 1333/2008 of the European Parliament and of the Council of the EU. The permitted levels for those additives are set out in Table 1.

Table 1. Levels of use of most common bread food additives [7]

Additive group number	Additive group name	Maximum level (mg/kg or mg/ml)
E 200-203	Sorbic acid	2000
E 280-283	Propionic acid	From 1000 to 3000
E 300	Ascorbic acid	Unlimited
E 471	Mono- and diglycerides of fatty acids	Unlimited

It can be seen, that the only limited additive group is preservatives since they are the only ones with a negative impact on human health if overused. Propionates stimulate glucagon and norepinephrine hormones and endogenous glucose production which eventually disrupt metabolism [8]. Sorbates are not hazardous to human health but have the potential to be mutagenic [9]. Even the slightest risk is important when most or even all the food, that is eaten, contains potentially harmful substances. Therefore, to attain the same result without preservatives, new technologies and development must be applied.

Even though bread is baked in an oven at a high temperature, making the conditions impossible to survive for microorganisms, secondary contamination is inevitable. Freshly baked bakeries must be cooled down before packing into the primary package to prevent water condensation. Even with the attentive care for the cleanliness of the surfaces, microorganisms in the air often get on the product.

Afterwards, bread is stored under the recommended conditions, which are also favorable for the growth of mold. Warm conditions, moisture and a porous structure providing a place where to spread lead to these microorganisms' activity.

Bread spoilage is mostly caused by *Aspergillus* and *Penicillium* species (spp.). Their visualization is provided in Fig. 3.

Fig. 3. *Penicillium* (on right) and *Aspergilus* spp. (on left) growth on bread [10,11]

As it can be seen, *Penicillium* spp. caused mold is in white and green color, responsible for about 90% cases why wheat products spoil. *Aspergillus* is known for its black color. These microorganisms give the product an unpleasant odor, appearance and at the same time produce dangerous substances - mycotoxins - which are dangerous for human consumption [12].

All major microbiological spoilage pathogens and their internals are listed in Fig. 4.

Spoilage Agents		Properties of Colony
Moulds	*Penicillium* spp.	Blue/green, flat, spread rather slowly
	Aspergillus niger	Black, fluffy, spreading with sporeheads often clearly visible
	Aspergillus flavus	Olive green
	Aspergillus candidus	Cream
	Aspergillus glaucus	Pale green
	Cladosporium spp.	Dark olive green, flat, spread slowly
	Neurospora stophila	Salmon pink, fluffy and fast spreading
	Rhizopus nigricans	Grey/black, very fluffy and fast spreading
	Mucor spp.	Grey
Bacteria	*Bacillus subtilis* or *Bacillus licheniformis*	Irregular shape, white and dull colour
Yeasts	*Hyphopichia burtonii*	Slow growth on bread surface, low, white, spreading colonies
	Pichia anomala	
	Scopsisfi buligera	
	Pichia burtonii	Very fast growth on bread
	Zygosaccharomyces bailii	Smooth, round, convex and white to cream coloured
	Torulaspora delbrueckii	
	Pichia membranifaciens	
	Candida parapsilosis	

Fig. 4. The main microorganisms causing bread spoilage and their attribute [13]

1.2. Similar research of achieving the clean label

Clean label, a statement which expresses that the product is chemical additives free, is nowadays markets aspiration. This term represents a product, which ingredient list is simpler and contains fewer elements. There are no E numbers, all components are written by their name and are known by customers. It also states that the product is free from genetically modified organisms, pesticides and other controversially treated artificial items. Even though there is no scientific definition of this phrase, it first appeared about 40 years ago when customers began to reject food labels which contain E-numbers because they were purportedly linked to bad health effects. However, the use of this term skyrocketed only 10 years ago because it was used much more in a variety of sources, clearly reflecting the term's expanding prominence [14].

Scientists are looking for ways to fulfil clean label requirements and reach the same or even better results by implementing different ingredients, techniques and technologies. The overview of how to exchange the largely used unnatural additives that change the properties of the dough and preservatives in bread is submitted in Fig. 5.

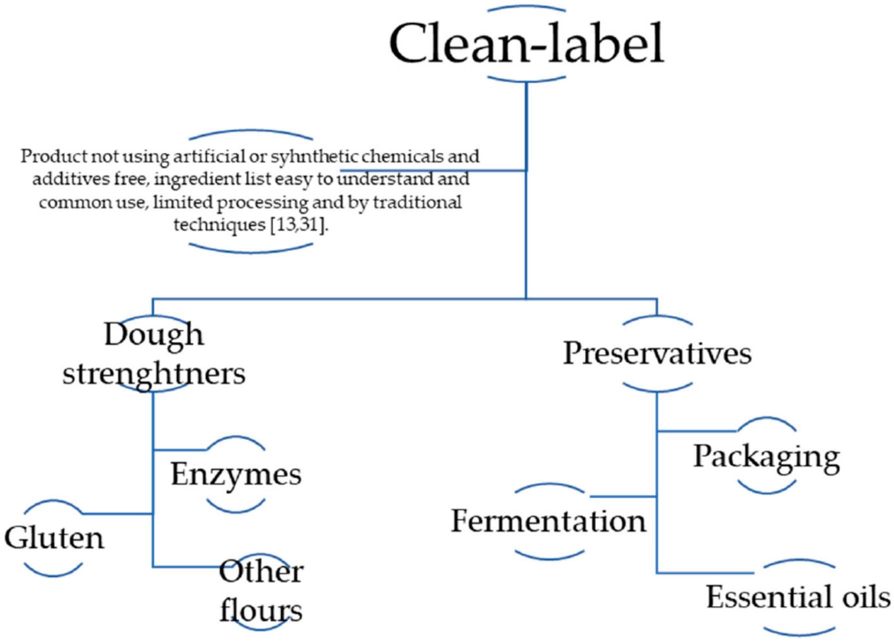

Fig. 5. Ways to replace food additives in bread products [15]

1.2.1. Dough strengtheners

Regarding the dough strength, it for the most part depends on flour. If the characteristics of the flour used do not meet the requirements, additives are used to achieve the same result as while using the quality ingredient.

The main requirements for good flour are:

- Protein that is sufficient in quantity and creates gluten that is suitable in terms of elasticity, strength, and stability when hydrated;
- amylase activity and damaged starch levels should be sufficient to generate enough sugars to support yeast enzyme activity and enough gas is released throughout fermentation and proofing;
- the moisture content should not exceed 14% to ensure safe storage [16].

Flour meeting such conditions is considered to be strong. The flour influence on final product texture using the same technique exhibited in Fig. 6.

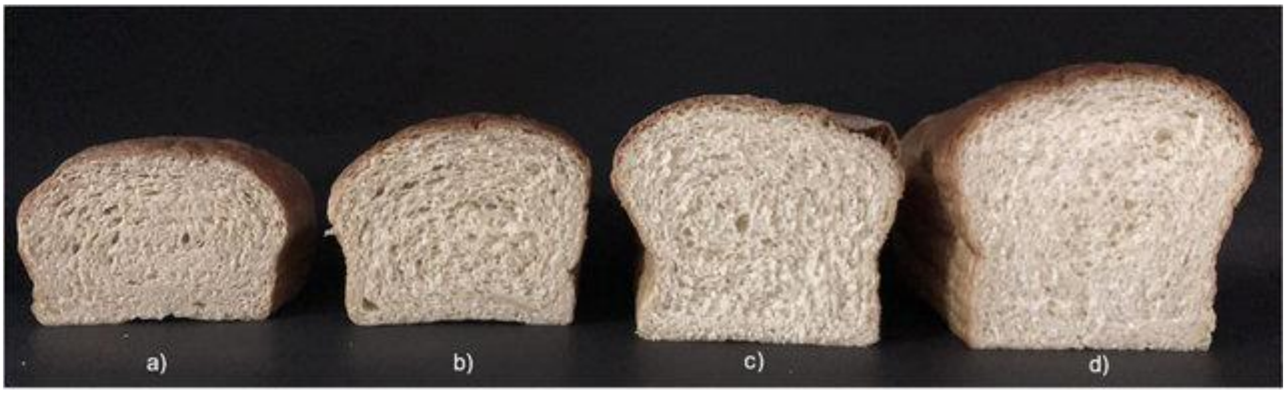

Fig. 6. Difference of bread with a) 20% b) 13,2% c) 5% and d) 0% substituent of wheat flour [17]

The volume of bread is the biggest using only flour. That results in moist and soft bread because flour was able to absorb enough water, create a strong gluten network and maintain the gasses inside. If the flour is weak and is not available to form proper texture, more natural ways to substitute artificial matter could be an addition of extra enzymes or gluten, depending on what used flour lacks or simply using a divergent kind of it.

1.2.2. Preservatives

In terms of extension of lifespan, there is no such substitute used to replace commonly used preservatives. But this topic is widely discussed exploring a wide range of ways to alternate them. Three groups, singled out mostly, are fermentation, essential oils and alternative packaging.

Fermentation is a natural process known and used for a long time since ancient days. It is used in a variety of foodstuff including milk, meat, vegetables and others even nowadays. The fermentation by microorganisms not only helps to prevent spoilage but also improves product texture and flavor qualities. The main disadvantage of this process during bread making is the time length it is occurring – a transformation of starch into carbon dioxide, alcohol, organic acids and other flavor formers takes up to 2,5 hours. Only smaller or dedicated traditional style bakeries manage to maintain this technology due to its duration, specific technological process that must be maintained and demands for a lot of premises [18].

Leavened bread can be achieved by a couple of methods. The most popular is using soda and natural using yeast and/or lactic acid bacteria (LAB). Longest, but best result achieving one is making a sourdough – using a bread starter which includes yeast and LAB. Its metabolic products especially enhance the quality of bread not only by already mentioned benefits, but likewise, it maintains anticarcinogenic and cholesterol-reducing abilities [18]. The ability of yeast and LAB to preserve the bread is proven by many studies. Some of them are exhibited in Table 2.

Table 2. Lactic acid bacteria and yeast ability to extend shelf life [19]

Microbes	Product	Starter Culture Used, Compounds	Shelf Life/Fungal Inhibition
Lactic acid bacteria	Pan bread	*Lactobacillus plantarum*	7 days after baking, *A. niger* growth was lower
	Quinoa and rice bread	*Lactobacillus reuteri*, *Lactobacillus brevis.*	2 days extended shelf life
	Bread	*Lactobacillus plantarum*	>14 days extended shelf life
	Gluten-free breads	*Lactobacillus amylovorus*	4 days extended shelf life
	Bread	*P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, and KTU05-10	8 days fungal growth inhibition
	Bread	*Lactobacillus hammesii*	6 days extended mold-free shelf life.
	Bread	*Lactobacillus plantarum* 1A7	Up to 28 days fungal inhibition
Yeast	Pan bread	*Penicillium anomala* SKM-T	Overall storage life is 6–8 days, when appearing with fewer fungi count
	Wheat sourdough	*W. anomalus* LCF1695	Up to 14 days shelf life

Even diverge types of products are positively affected: the shelf life can be extended from 2 to 14 days and fungal inhibition can proceed up to 28 days. Even with these astonishing benefits bakeries are shifting away from this process and searching for another way to attain the same result.

The most researched and promising type to preserve bread and other food products is active packaging. It improves food quality and safety by adding active ingredients directly to the packaging material rather than the food.

One type of solution can be active packaging, which means that there is an active agent incorporated in the package. For antimicrobial control in foodstuff, one of the agents preventing the activity of fungicides is an essential oil (EO). It also exhibits flavoring qualities resulting in changing the original taste or aroma if a greater amount is used. But another feature EO excels is the synergism between its different components. Although the main components, which are responsible for the most of composition, are responsible for these main features, the other compound in small percentages often contributes to the enhancement of the wanted effect. Combining two diverse EO or their parts could lead to an even greater outcome. Thus, money is saved and a lower portion of the material is needed denoting that it may not change the natural characteristics of affected item [20].

EO are already studied to be applied in the bakery products industry with known benefits. Micro capsulated rosemary oil in fresh dough and nano emulsions of clove bud and oregano EO on sliced bread maintained to keep bread mold-free longer. Mustard EO implemented in modified atmosphere packaging is the strongest inhibitor of fungi causing microorganisms compared with cinnamon, garlic, clove, oregano and vanilla EO [21]. Edible films from methylcellulose and nano emulsions of clove bud and oregano EO were established by Otoni et al. [22] to determine that all of them are effective, but the incorporation of clove bud shows the best results.

These examples demonstrate that it is feasible to integrate these diverge methods together to achieve an even better result. The purpose of this work is also to incorporate the EO and active packaging. Three mixtures of synergetic EO will be selected after studying their effect on bread mold causing funguses.

1.3. Selection of research / engineering methods that are used for solving of scientific / engineering problems

1.3.1. Analysis of synergy between EO components

The comparison of inhibitory effect to *Penicillium roqueforti* and *Aspergillus niger* between cinnamaldehyde, citral, eugenol, carvacrol, salicylic acid, thymol, perillaldehyde, oligochitosan, natamycin, Ɛ-polylysine and their combination was performed by J. Ju, et al. [23]. The materials were chosen according to literature analysis information about their interaction with other substances. The study consists of 2 parts: experiment in vitro and on the product.

The first fragment of investigation consists of a comparison to inhibit fungus growth with different concentrations of fungicides, inhibition determination of minimal inhibitory concentration (MIC) of both fungi for each fungicide and fractional inhibitory concentration (FIC) for a mix of fungicides. MIC was identified by comparison of different concentration solutions of each fungicide. These solutions were prepared by a two-fold serial dilution method as it is shown in Fig. 7.

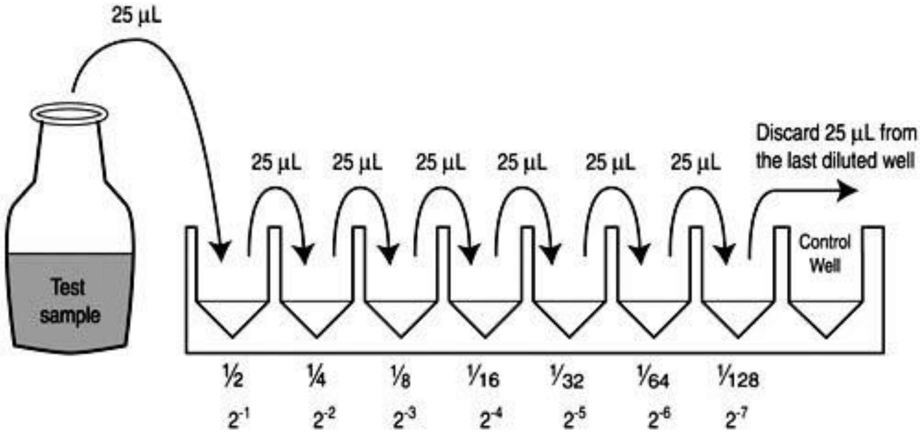

Fig. 7. Two-fold dilution method [24]

The same amount of spore suspension was added to each well of a plate with the set concentration of fungistat and potato dextrose broth and the plate was cultured for a day to determine the minimum concentration of fungicide needed to inhibit the growth.

Once the MIC values were found out, the FIC were established. Once more the dilution was used to obtain concentrations: 4, 2, 1, 1/2, 1/4 ,1/8 and 1/16 x MIC. Merged MIC was discovered by the checkboard method as it is demonstrated in Fig. 8.

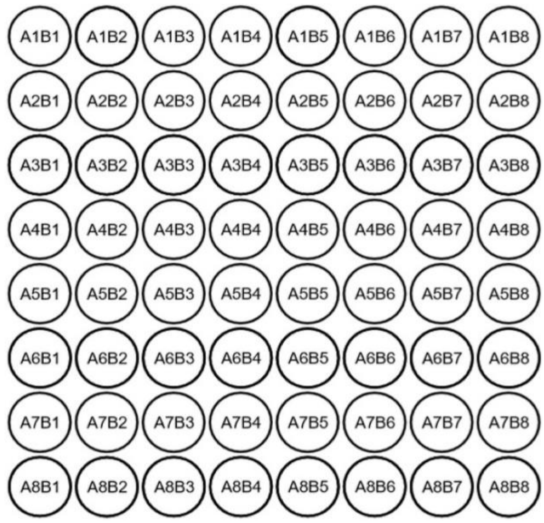

Fig. 8. The checkboard method used to determine FIC [23]

Both antifungal agents were put into 96-well plate: A was filled horizontally beginning with the highest concentration in the first row and B – vertically – with the greatest concentration in the first column. After the first test, all 45 combinations were tested against *Penicillium* genus and 28, after rejecting 3 agents with no effect on stopping growth, against *Aspergillus* genus. FIC was calculated by the formula:

$$FIC = \frac{MIC\ of\ A\ in\ combination}{MIC\ of\ A} + \frac{MIC\ of\ B\ in\ combination}{MIC\ of\ B} \quad (1)$$

Synergism was credited if the sum of Eq. 1 was equal to or greater than 0,5.

The results are demonstrated in Table 3.

Table 3. Synergetic antifungal compounds pairs against [23]

Fungistat		**MIC of single fungistat (mg/ml)**		**MIC of both combined fungistats (mg/ml)**		**FIC value**
A	B	A	B	A	B	
Penicillium roqueforti						
Citral	**Eugenol**	0.25	0.25	0.06	0.06	**0.48**
	Oligochitosan		1.30	0.06	0.33	0.49
	Ɛ-polylysine		0.25	0.08	0.03	0.44
	Thymol		0.67	0.08	0.08	**0.37**
Eugenol	Carvacrol	0.25	0.25	0.06	0.06	0.48
	Ɛ-polylysine		0.25	0.06	0.06	0.48
Carvacrol	Perillaldehyde	0.25	0.67	0.06	0.17	0.49
	Thymol		0.67	0.06	0.17	0.49
Salicylic acid	Thymol	1.30	0.67	0.33	0.08	0.37
Oligochitosan	Perrilaldehyde	1.30	0.67	0.33	0.17	0.50
Namamycin	Thymol	0.08	0.67	0.02	0.17	0.50
Perrilaldehyde	Thymol	0.67	0.67	0.17	0.17	0.50
Aspergillus niger						
Thymol	**Citral**	0.67	0.67	0.17	0.17	**0.50**
	Cinnamaldehyde		0.25	0.17	0.06	0.49
Salicylic acid	Citral	1.30	0.67	0.33	0.17	0.50
	Carvacrol		0.67	0.33	0.17	0.50
Namamycin	Citral	0.16	0.67	0.04	0.17	0.50
Carvacrol	Citral	0.67	0.67	0.17	0.17	0.50
Eugenol	**Citral**	0.25	0.67	0.06	0.17	**0.49**

12 of the total 73 groups were synergistic. 26% were effective to *Penicillium roqueforti* and 25% to *Aspergillus niger*. Only 2 combinations of tested sets work against both fungi: citral-thyme and citral-eugenol duo. Both groups have citral incorporated. It can be seen that this element is a strong inhibitor for both spp. because it is in 33% of sets in inhibiting *Penicillium* and in 71% in *Aspergillus*. Synergetic effect lowered the MIC of citral but for *Penicillium* genus still proportionally lower concentration was needed to achieve the synergy. Another component is eugenol. It is a slightly lower antifungal effect having component as just this 1 combination was calculated for *Aspergillus* and 3 for *Penicillium.* Both MIC of eugenol for both genus alone and combined were the same meaning the antifungal ability for fungus is the same. The last element of EO is thymol and it is more functional against *Penicillium roqueforti.*

The ratios of these binary mixtures are the most vital aspect to make it work. 3 of discussed duos are required to have the same proportions. Nonetheless, eugenol and citral mix against *Aspergillus niger* are divergent. 0.06 mg/ml and 0.17 mg/ml respectively are required to reach MIC making the ratio almost 1:3. For the combination to be efficient against the pair of tested fungi, upper values should be selected for each compound taking into consideration both MIC. The concentration of citral and eugenol would be 0.17 mg/ml and 0.06 mg/ml, of citral and thymol – 0.17 mg/ml and 0.17 mg/ml proportionately.

1.3.2. Citral and eugenol

In citral, geranial (α-citral) and neral (β -citral) occur as a combination of the two isomers with a slightly bigger part of α isomer. Because of the strong citrus smell, it is used in the industries of cosmetics, food and beverages. Citral is the main element of lemongrass EO, owning over a 70 percent and it has been demonstrated to have anti-inflammatory and anti-corrosive properties against a variety of microbial spp. in several experimental and clinical trials, and there is growing evidence that citral operates as a fungicidal and bactericidal agent [25]. Both components' formulas are presented in Fig. 9.

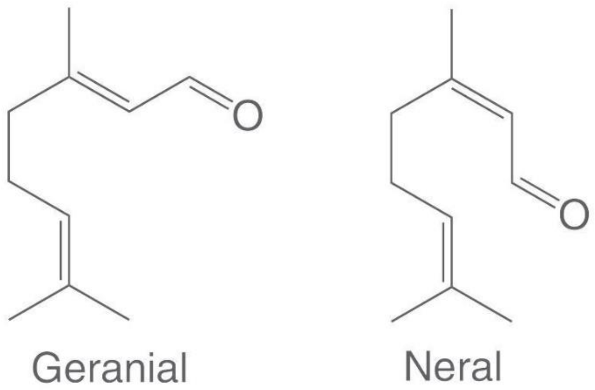

Fig. 9. Formulas of citral isomers geranial and neral [26]

Clove essential oil contains eugenol in the amount of 70-90%, a volatile phenolic component and is in charge of the clove scent. Eugenol is a phenylpropanoid that has a spicy scent and is pale yellow. This component can as well be produced from nutmeg, cinnamon, basil, and bay leaf [27]. The structure of the eugenol is previewed in Fig. 10.

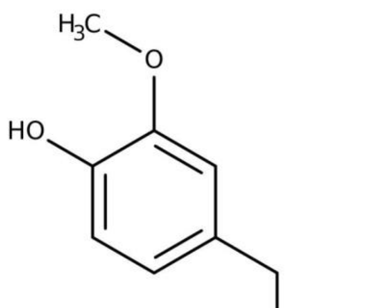

Fig. 10. The formula of eugenol [28]

Eugenol has a wide range of bioactivities and is employed in a variety of industries. The pharmacy field benefits from antiseptics with antibacterial qualities, the cosmetology industry benefits from intense scent with antimicrobial capabilities, and the food industry benefits from flavor. Apart from its flavor, eugenol can be effective in the preservation of edibles since it has antifungal properties against a variety of spp., including those that are commonly responsible for bread contamination [27].

Citral and eugenol combination effectiveness on the product was tested in another study by J. Ju, et al. [29]. Microcapsules were made by combining the corn starch and citral and eugenol by a ratio of 1:1. After their formation, they were sealed with the matter to form EO containing sachets which were used for further investigation. To determine the sachet influence on bread‘s quality, they were tested while storing the packaged bread kept at a controlled temperature of 25°C and 35 °C. A control sample was only packed bakery good to collate the fungi growth, which was done daily. To inspect the EO effect on flavor of the bread, the sensory evaluation was held between 16 persons and the results were evaluated on 9-point hedonic scale.

The results of the first part of the experiment are visualized in Fig. 11.

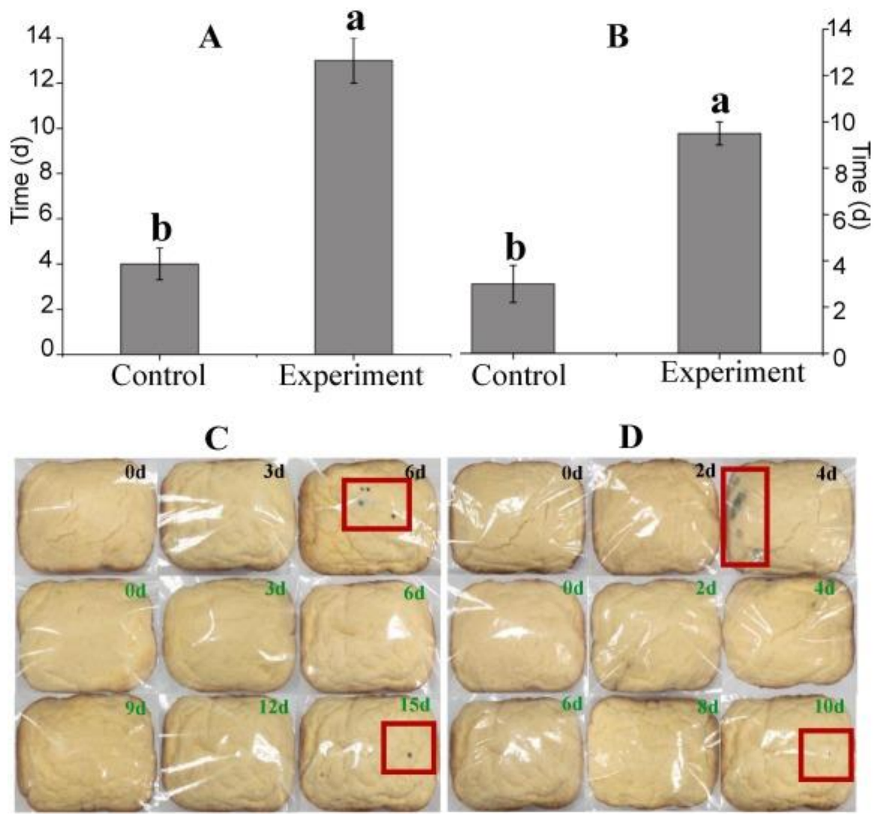

Fig. 11. Shelf of life determination while storing at 25°C (A and C) and 35°C (B and D) [29]

The results show that sachets containing EO were able to prolong the validity of bread by more than 50%. Although EO affected bread got mold, it was significantly smaller in comparison with control. Even storing samples in the temperature which is favorable for the multiplication of fungi - 35°C - the growth was inhibited for 6 more days using EO supplement, but 5 days less than storing at ambient temperature.

The outcome of the sensory analysis is displayed in Table 4.

Table 4. Sensory analysis results of bread containing EO sachets [29]

Storage time (d)	Sensory score	
	25 °C	35 °C
0	9	9
9	-	6.2
12	7.0	-

The outcome of this evaluation is that EO sachets do not affect the taste or aroma of bread. Score after storing naturally went down as the product was not that fresh anymore. Although the sensory score after 12 storage days at room temperature was not that bad – just 2 points lower than an initial starting point. As research participants have not noticed anything unusual it can be pointed out that EO sachets do not transmit their sensory values to the product and even supports existing features.

1.3.3. Citral and thymol

Thymol is a colorless crystalline monoterpene phenol found in plants belonging to the *Thymus* genus. It has been used in traditional medicine for generations and has been proven to have antioxidant, antibacterial, antifungal and many more qualities. It is also utilized in the domains of cosmetics and dentistry [30]. The formula of thymol can be seen in Fig. 12.

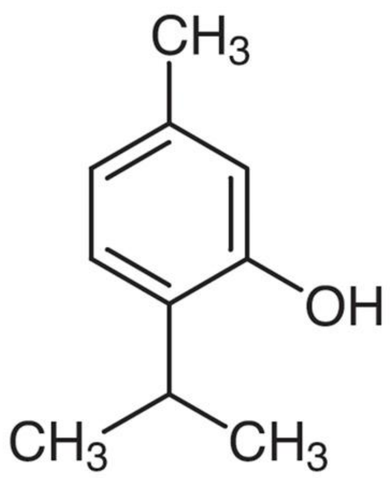

Fig. 12. The formula of thymol [31]

Suwanamornlert et al. discovered that thymol is effective as a yeast and mold inhibitor. 3% and 6% concentrations of thymol were introduced into polylactic acid/polybutylene-succinate-co-adipate mix films. The manufacturing of the biopolymer blend film with thymol contains a heating process. The part of thymol is lost because of film formation by blending components at high temperature (up to 100 °C). The lost quantity was a few percent higher in blend with a higher thymol amount and is equal to approximately one-fifth of primary thymol content. Meaning that direct heat does not impact the volatile compound quality and performance. Only quantity is influenced, but the loss can be minimized by reducing heating time [32].

There is not a lot of research done on this pair synergetic effect, especially against edibles spoilage causing funguses. Yet there is scientifically proven efficacy against the pathogen *Staphylococcus aureus*, which is one of the most common bacteria in animal origin foodstuffs causing infection. This bacteria is transmitted through air or contact way. When bacteria discover a suitable surface, biofilm can be formed, which is a well-organized adherent lifestyle by secreting extracellular polymeric compounds on the surface for protective stay. As it is a frequent issue causing medical devices/implants to fail, a study by Valliammai, A. proved citral and thymol synergetic activity in inhibiting methicillin resistant *Staphylococcus aureus* (MRSA) biofilm formation on titanium [33].

The synergism of these two components was established by a conventional checkerboard pattern, there 1×10^6 of MRSA containing 96 wells were introduced with series doses of citral and thymol (0, 6.25, 12.5, 25, 50, and 100 g/mL). After incubation at 37 °C for 24 hours, each plate was rinsed, air-dried and stained with 0.4% crystal violet. Biofilm cells were stained afterwards with 10% glacial acetic acid and the absorbance was measured at 570 nm for quantification. The FIC index was measured by Eq. 1.

The visualisation of the study culmination is recorded in Fig. 13.

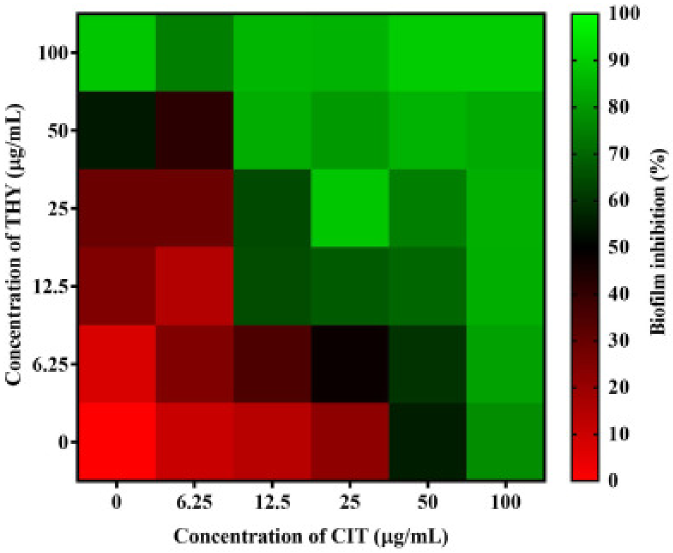

Fig. 13. The combined effect of citral and thymol on MRSA biofilm development [33]

The outcome shows that positive interactions are achieved by these two components' conjunction. 19 of 25 tested diverge concentration mixtures of citral and thymol were able to inhibit MRSA by over 60%. If to liken individual effects, the only highest concentration of 100 µg/ml (one of 5 tested) can achieve the same inhibition reaction. The combined antibiofilm capability of 25 g/mL citral and 25 g/mL thymol against MRSA is the greatest and was calculated to reach a synergetic effect. Biofilm suppression by the synergistic combination is over 90%. Whereas individual concentrations of citral (25 g/mL) and thymol inhibited biofilm by approximately 20-30%, respectively.

1.3.4. Carvacrol and thymol

Carvacrol is a phenolic monoterpenoid found in the essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*) and other plants, among others. This chemical has antibacterial and antifungal properties. Carvacrol has been recommended as a natural food preservative for the food industry due to its taste and antibacterial properties [34]. Its formula can be found in Fig. 14.

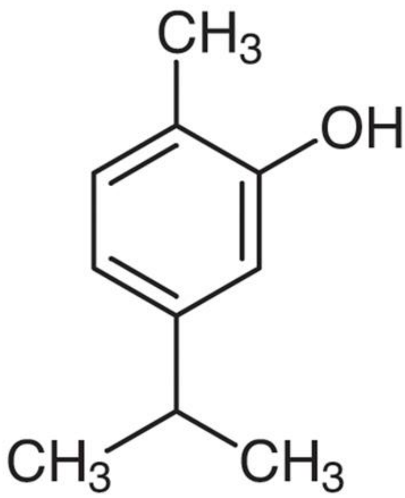

Fig. 14. The formula of carvacrol [35]

A combination of thymol and carvacrol naturally exists in EO of oregano, thyme and et. Carvacrol is known to be a more potent antimicrobial agent than thymol. There is a synergism between the carvacrol and its precursor, which usually can be found together – p-cymene [34]. But along with thymol, carvacrol exhibit even stronger activity of antioxidant, antimicrobial, antihypertensive, immunomodulatory, anticancer and even antiviral, which was tested with the nowadays active virus – COVID-19. These features are also proved by research on many different foodstuffs and the implementation of thymol and carvacrol into their composition or packaging [36].

Another quality these compounds exhibit is their antifungal property. It is not researched as much as other already mentioned traits, but not less functional than others. Research by J. Ju, et al, which was discussed above, proved the antifungal properties and synergism of these components against *Penicillium* genus. An article by C. E. Ochoa-Velasco [37] investigated coatings with carvacrol and thymol for tropical fruits against the most common infection caused by *Colletotrichum gloeosporioides*.

The fungi were secluded from the corrupted epidermis of papaya, placed in a Petri dish with potato-dextrose agar (PDA) and kept in favorable conditions till growth was noticed. Afterwards, *gloeosporioides* spp. was separated and further cultivated. The spore suspension was made by pouring water on the surface of the PDA. The film was made with corn starch, sodium hydroxide and water by stirring and gelatinizing it. Different pH values were set to test the influence of EO components effectiveness. Lastly, glycerol and the mix of thymol and carvacrol of different concentrations were added to the mix, which was dried in the last step. The formed film was infected with 30 µL of spore suspension and kept under ambient conditions. Growth was recorded for 7 days once daily. Afterwards, the MIC concentrations were examined, already mentioned microdilution or checkboard method was used to identify the FIC values. The films were made by the same technique of pH 5.
The outcome is displayed in Table 5.

Table 5. FIC of carvacrol and thymol against *Colletotrichum gloeosporioides* [37]

	Concentration of thymol (mg/l)		
Concentration of carvacrol (mg/l)	375	750	1125
375	-	-	-
750	-	0.85	1.07
1125	0.85	1.07	1.28

The values which are not indicated showed growth of the examined pathogen. None of the tested pairs displayed a synergistic effect. But 2 of them demonstrated additive reaction, which is considered when the FIC value is between 0.5 and 1. It is crucial to note that too high a concentration could lead to no effect. Consequently, each concentration should be selected thoroughly according to the case.

The slightly acidic solution was the best choice for continuing the experiment because the required actives values were the lowest. The appropriate pH should be addressed for the proper operation of edible films containing antimicrobials. This results in increased antibacterial action. Worth to mention that bread, depending on the type, also is slightly acidic.

A synergistic effect could be achieved when one of the components disintegrates the lipid membrane and makes it easier for the other molecule to enter the cytoplasm. [37]

2. Development of solutions / results of experimental research

The experimental part aims to create an antifungal coating which could prolong the shelf life of bread. The scientifically proven mixtures, discussed in the analytical part, will be used to compare their effectiveness and determine which of them has the most potential to prevent mold growth.

The study consists of three stages:

- Bread mold rate study;
- Samples comparison after storage in diverse packaging;
- Antifungal coating efficacy.

The first part of the test aims to choose the type of bread for the final examination. As the main subject of a study is bread, it is vital to test which type of it suits this case the best. The shelf life was considered to be the day when mold spots were detected.

The second fragment of study is needed to evaluate chosen packaging suitability for such a product and test the quality of bread stored in the original package and selected paper packaging in three ways: as it comes, with 1 wall covered with coating and all walls covered with coating.

The third experiment is to analyze the antifungal quality of three picked out EO mixes, incorporated into paper packaging as a coating, on the samples of bread.

The aim of the experiment:

- To determine most microorganism susceptible bread;
- To choose the coating conditions in the last stage of study;
- To test the antifungal qualities of chosen blends containing coating and identify their performance.

2.1. Materials used

2.1.1. Bread

The bread was chosen from the market leader in Lithuania – UAB “Vilniaus duona” – which occupies 21,8% of the market share. Although the sales of baked goods are generally decreasing as it contains flour and sugar, “Vilniaus duona” maintains the constant growth of sales [4]. 3 different brands of bread were selected – “Beatos virtuvė“, „Agotos“ and „Urtės“. All of them are mixed rye bread, which does not have preservatives and contains grains and/or seeds. This type of bread contains a lot of moisture and its price is on the high side because of its ingredients. The compositions of chosen bread types are exhibited in Table 6.

Table 6. Ingredients of tested types of bread

Product	Ingredients
Seed bread „Agotos“	Water, **rye** flour, sunflower seeds (15.4%), **wheat** flour, pumpkin seeds (10.3%), watermelon seeds (10.3%), flaxseed (9.0%), sugar, yeast, **wheat gluten**, iodized salt, **wheat** fiber, **rye** malt, caraway seeds, flour treatment agent ascorbic acid
Black sliced bread „Beatos virtuvė“ with seeds	**Rye** flour, water, **wheat** flour, sunflower seeds (13.7%), flaxseed (8.8%), pumpkin seeds (7.6%), sugar, yeast, iodized salt, **wheat gluten**, **rye** malt, **barley** malt extract, **wheat** fiber, flour treatment agent ascorbic acid
7 grain dark sliced whole grain bread „Urtės“	Wholegrain **rye** flour (23.9%), water, **rye** flour, **wheat** flour, sunflower seeds (6.5%), broken **rye** (3.3%), broken **wheat** (3.1%), **barley** malt extract, **wheat gluten**, **barley** groats (2.5%), sugar, flaxseed (1.3%), **soy** grits (1.3%), yeast, iodized salt, peeled **sesame** seeds (0.5%), **wheat** fiber, **rye** malt, caraway seeds, flour treatment agent ascorbic acid

These 3 products are similar in their composition. 11 components are used for all 3 types – water, rye flour, sunflower seeds, wheat flour, flaxseed, sugar, yeast, iodized salt, wheat fiber, rye malt, flour treatment agent ascorbic acid. Consequently, their recipes are alike. „Urtės“ bread stands out as its main ingredient is wholegrain rye flour and this good has more diverse components such as grains and other seeds.

For the first test, all 3 types of bread were purchased on February 28th, 2022, at a local supermarket. Units with the same validity period were selected with a best before the date of March 5th, 2022.

For the second part, 2 packages of “Urtės” bread were purchased on March 23rd, 2022, at a local supermarket. Units with the same validity period were selected with a best before the date of March 28th, 2022.

For the final part, 2 packages of “Urtės” bread were purchased on April 9th, 2022, at a local supermarket. Units with the same validity period were selected with a best before the date of April 14th, 2022.

2.1.2. Essential oils

EO were selected at the „Kvapų namai“ store. The types of plant EO were selected by evaluating sources information and the company‘s provided chromatogram with the exact quantity of each component.

- The selected source of carvacrol is EO of oregano (*Origanum vulgare*) with the main component amount of 57.36% [38];
- The EO of clove (*Syzygium aromaticum)* was used as the source of eugenol with its part in the mixture of 68.18% [39];
- Oil of lemon grass (*Cymbopogon citratus*) provides citral, which takes a 67.54% combined geranial and neral [40];
- Thymol was provided by the EO of thyme (*Thymus zygis*), which percentage in the mixture is 35.06% [41].

2.1.3. Packaging

For the first part of the research, PE bags with ziplock were chosen as a package as they are airtight and ensure equal conditions. 15x24 cm bags were purchased from a local market.

For further research original packaging of the product was saved and used as a control to compare the samples.

The paper packaging, to substitute the plastic, was chosen by the company „Mayr-Melnhof Karton AG“ (MMK). In 2016, this company created an innovative carton board specialized for foodstuff - FOODBOARD™. Its main distinctiveness:

- Is a food-safe carton board with a functional barrier that works;
- Mineral oils, phthalates, and bisphenol A are examples of unwanted ingredients that are protected in packaged meals;
- Cross-contamination is prevented throughout transport, storage, and at the point of sale;
- It is neither an extruded nor laminated compound, but rather a pure carton board solution;
- Is biodegradable, recyclable, and comes with an FSC® or PEFCTM certification;
- is offered on the brown reverse side (GT4) and light reverse side (GT5) recycled carton board (GT1) [42].

This new cartonboard was compared to the same thickness of most popular boards in the packaging industry by I. Bacevičiūtė. MMK carton has poorer mechanical properties in contrast with “Arktika“, „Ensocoat“ and „Korsnäs White“. But its main advantage lies in the way it is made: FOODBOARD™ do not need lamination with plastic. It results in a faster manufacturing process, fewer machines used and most importantly, plastic elimination, leading to 400 times quicker decay in nature. [43]

2.1.4. Base

1 kg of soy wax was purchased from the online store soyacandles.lt. It is made 100% from soybeans, hydrolyzed and comes in the flake form. Batch no. is 0018187963 with the 10th of September, 2021, date of manufacturing. Other important characteristics are indicated in Table 7.

Table 7. Parameters of soybean wax NatureWax C-3 [44]

Criterion	Value
Storage temperature	15 - 25°C
Melting point	45°C
Maximum heating temperature	71.1 – 93.3°C

2.2. Methodology

2.2.1. Preparation of samples for bread type selection test

The crust was cut off to achieve the same conditions for all the samples. Each piece was cut into 5x5 cm square and put into designated packaging.

Specimens were stored the same – at the room's temperature (about 20-23°C) at the place without direct sun contact. Their visual changes were captured each day till the mold is spotted on any sample. This would be considered the expiration date of the bread.

2.2.2. Cardboard surface comparison

This investigation was performed using BYK Gardner digital microscope DPM 300, pictured in Fig. 15.

Fig. 15. Digital microscope DPM 300 by BYK Gardner

By using it, coating coverage was evaluated and set side by side with the original carton board. All taken photos were made with a scale of 1:230.

2.2.3. Calculation of water loss

To test the air permeability of the paper package and select the most optimal type of coating, water loss was calculated. The weights of the 1^{st} day and 5^{th} day were compared.

Same samples were used for the texture quality test to check both of these properties and select the best type of coating for the last study part.

The weighing was performed and measured using AND GR-202 electronic scales, which are demonstrated in Fig. 16.

Fig. 16. AND GR-202 electronic scales [45]

Scales accuracy is 0.1 mg and their weighing platform is in a closed glass rectangular chamber to ensure more precise answers.

2.2.4. Comparison of texture properties

Texture profile analysis (TPA) was performed with a TA.XTPlus texture analyzer. The picture of it is exhibited in Fig. 17.

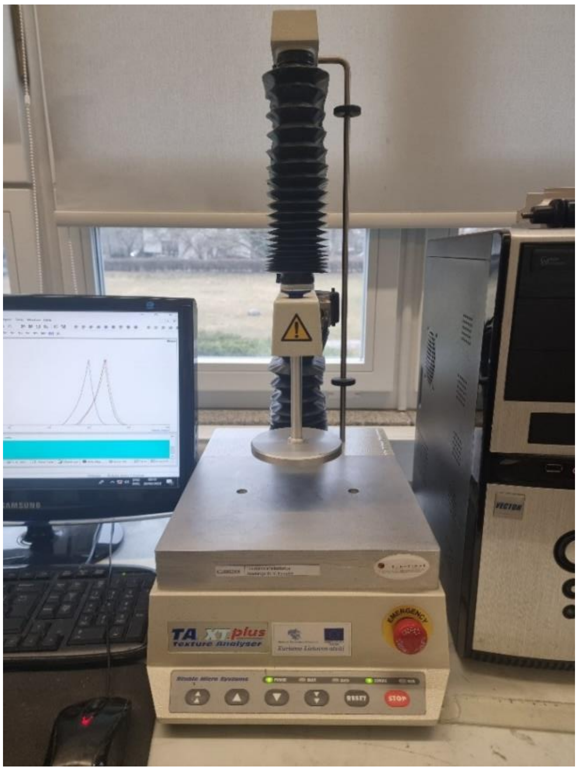

Fig. 17. TA.XTPlus texture analyzer

Samples were cut into the same size of 7 cm in length, 4 cm in width and 0,5 cm in height to prepare samples of the same size for the accuracy of the results. The test was performed twice: the day bread was purchased, with fresh bread, and after 5 days, the last day the batch is valid for consumption, after keeping the samples in three different conditions:

- In the original packaging;
- In the MMK carton box without coating;
- In the MMK carton box with all walls covered with coating.

9 exact samples were prepared and 3 of each were kept under the same conditions to estimate possible errors and achieve a more accurate outcome.

Texture analyzer parameters: the samples were compressed to 40% of the full height at a rate of 1 mm per second. The following tests were performed to determine and compare the textural properties of the bread: hardness, springiness, cohesiveness, resilience, gumminess, and chewiness. A short description of each quality is submitted in Table 8.

Table 8. Information about properties used for texture profile analysis [46]

Characteristic	**Definition**
Hardness	The maximum force of the 1st compression
Springiness	The distance of the detected height during the second compression divided by the original compression distance
Cohesiveness	The area of work during the second compression is divided by the area of work during the first compression
Resilience	The division of the upstroke energy of the first compression by the downstroke energy of the first compression
Gumminess	Hardness x Cohesiveness
Chewiness	Gumminess x Springiness

2.2.5. Calculation of essential oils containing coating

The coating was prepared with the base of soy wax and the main element – the required number of drops of two essential oils.

First of all, 3 drop samples of each EO, used for the study, were measured using the same AND GR-202 electronic scales as in the previous method. The drop weights are given in Table 9.

Table 9. The average weight of essential oils drops

Oil	**Average drop weight, g**
Oregano	0.0293
Clove	0.0286
Lemon grass	0.0315
Thyme	0.0234
Average	**0.0282**

The average weight of drop is 0.0282g and it was used as a standard. Evaluating info about the loss of oil weight during heating from P. Suwanamornlert et. al article [23], 1/5 of mass evaporate during the fabrication process and it will be eliminated:

$$x = 0.0282 \times \frac{4}{5} = 0.02256\ g = 0.023\ g \quad (2)$$

where: *x* is the weight of 1 drop of EO including evaporation after heating, g.

After having a final weight of the drops, components concentration in each oil were calculated by using the chromatograms data from „Kvapų namai“ [30,31,32,33]. Each component amount in 1 drop of the mixture was calculated by proportion:

$$a = \frac{x \times z_a}{100} \quad (3)$$

$$b = \frac{x \times z_b}{100} \quad (4)$$

where: a is the first component a weight in 1 drop of EO (g); b is the second component b weight in 1 drop of EO (g); x is the weight of 1 drop of EO (g); z_a is the component a percentage in selected type of OE from chromatogram data (%); z_b is the component b percentage in selected type of OE from chromatogram data (%).

Components weights in 1 drop of EO will be used to calculate that element percentage in the whole mixture, which consists of 100g of soybean wax and minimum of 2 drops of essential oil (1 pcs of each). It is calculated by proportion:

$$p_a = \frac{a \times n_a \times 100}{100 + (0.023 \times (n_a + n_b)} \quad (5)$$

$$p_b = \frac{b \times n_b \times 100}{100 + (0.023 \times (n_a + n_b)} \quad (6)$$

where: p_a is the percentage of component a in the mixture (%); p_b is the percentage of component b in the mixture (%); a is the first component a weight in 1 drop of EO (g); b is the second component b weight in 1 drop of EO (g); n_a is the component a number of drops in the mixture (pcs); n_b is the component b number of drops in the mixture (pcs).

p value should surpass the minimum concentration of required component % in the coating mixture, r, which is taken from the study of J. Ju, et al. [17]. To convert mg/ml to percent, the amount was divided by 10 times:

$$r_a = 1 \frac{mg}{ml} = 0,1\ \% \quad (7)$$

$$r_a = 1 \frac{mg}{ml} = 0,1\ \% \quad (8)$$

where: r_a is the concentration of component a in the coating mixture according to study (%); r_b is the concentration of component b in the coating mixture according to study (%).

If p value is not enough to exceed concentration r, the additional drop is added of this component, p value is calculated again after adding an additional drop of EO to increase the components concentration and again contrasted with r value until p value is the same or higher, meaning that the required concentration is reached.

The summary of the calculations is given in Table 10.

Table 10. Calculations for essential oils drops required for coating

	Citral (a) + Eugenol (b)	**Citral (a) + Thymol (b)**	**Carvacrol (a) + Thymol (b)**
z_a, %	67.54	67.54	57.36
a, g	0.016	0.016	0.013
n_a, pcs	1	1	1
p_a, %	0.016	0.016	0.013

	Citral (a) + Eugenol (b)	Citral (a) + Thymol (b)	Carvacrol (a) + Thymol (b)
r_a, %	0.017	0.017	0.006
p_a exceeds r_a?	No	No	**Yes**
n_a, pcs	2	2	-
p_a, %	0.031	0.031	-
p_a exceeds r_a?	**Yes**	**Yes**	-
z_b, g	68.18	35.06	35.06
b, g	0.016	0.008	0.008
n_b, pcs	1	1	
p_b, %	0.016	0.008	0.008
r_b, %	0.006	0.017	0.017
p_b exceeds r_b?	**Yes**	No	No
n_b, pcs	**-**	2	2
p_b, %	-	0.016	0.016
p_b exceeds r_b?	-	No	No
n_b, pcs	-	3	3
p_b, %	-	0.024	0.024
p_b exceeds r_b?	-	**Yes**	**Yes**

The calculations show that:

- The first mixture will need 1 drop of clove and 2 drops of lemon grass EO;
- The second mix will be composed of 2 drops of lemon grass and 3 drops of thyme EO;
- The third duo is made of 1 drop of oregano and 3 drops of thyme EO.

2.2.6. Preparation of final package

12 boxes altogether were used in the last stage of this study:

- 3 control boxes – inner surface covered with 2 layers of soy wax;
- 3 citral and eugenol incorporated boxes – inner surface covered with 2 layers of the mixture consisting of 100g soy wax and 2 drops of lemon grass and 1 drop of clove EO;
- 3 citral and thymol incorporated boxes – inner surface covered with 2 layers of the mixture consisting of 100g soy wax, 2 drops of lemon grass and 3 drops of thymol EO;
- 3 carvacrol and thymol incorporated boxes – inner surface covered with 2 layers of the mixture consisting of 100g soy wax, 1 drop of oregano and 3 drops of thyme EO.

Preparation of coating

100g of wax flakes were melted in the pot on the stove. When all wax flakes reached a liquid state, the required quantity of respective EO were dripped into the pot with wax. The blend was mixed to ensure the even distribution of ingredients. Once mixed, the paint roller, which width is 9cm, was immersed into the mixture to absorb the coating. After pulling out it was shaken to eliminate excess and ready for a start.

One layer of coating was applied on the whole inner surface in 3 strokes, starting from one side and finishing on another, as it is demonstrated in Fig. 18.

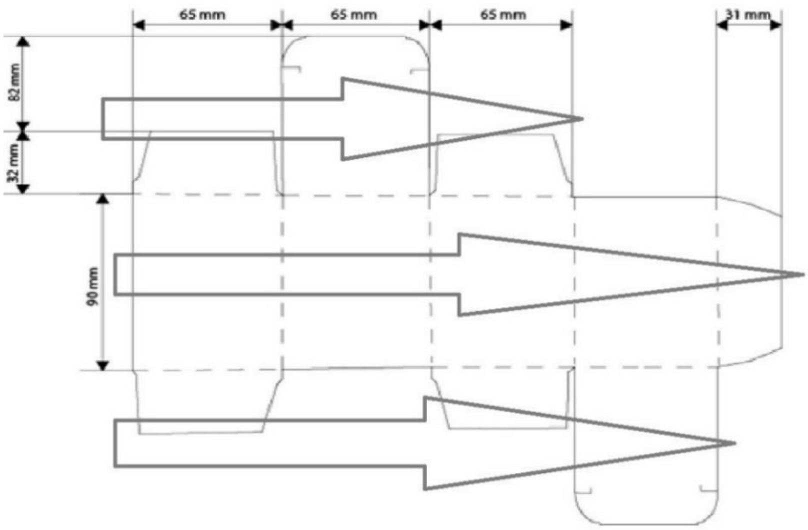

Fig. 18. Application of coating with roller

While slightly pressing the roller on the surface, the liquid coating is squeezed off the roller cover and evenly distributed on the surface of the box. Any surplus is pushed down out of the box surface on the auxiliary paper base. After applying the first layer with 3 strokes, a few minutes waited for it to become solid and the second layer was applied in the same method.

Once both layers are applied and fully dried, the box was folded through bending lines. Wax crumbs resulting from folding were wiped off the surface. Once boxes were prepared for samples, each piece of bread was captured on both sides for comparison after 8 days. Pictured slices were inserted into the designated box. The gaps of the box were sealed with insulating tape to ensure equal conditions.

Once all is set, boxes are placed in the same room with a temperature of about 22°C and without any direct sunlight. On the 9th day, boxes were opened to check the visual changes and see the results, which are presented in the next section.

2.3. Results and their analysis

2.3.1.1.

The pictures of bread on the 1st, 3rd, 6th and 9th day are presented in Table 11.

Table 11. Results of bread shelf-life comparison

	„Agotos“	**„Beatos virtuvė“**	**„Urtės“**
1st day			
3rd day			
6th day			
9th day			

„Agotos“ and „Urtės“ bread had visual inhibition spotted on the 9th day of supervision. „Agotos“ have 2 small areas with white spots. „Urtės“ specimen had one big field. However „Beatos virtuvė“ sample was intact. As “Urtės” specimen had the biggest affected space by the unwanted fungus activity, this leads to the conclusion that this type of bread is most susceptible to decay. It was chosen to continue further tests.

2.3.2. Comparison of bread kept in original and paper packaging

Cardboard surface comparison

The captures of a microscope to compare carton and wax surfaces are in Fig. 19 and Fig. 20.

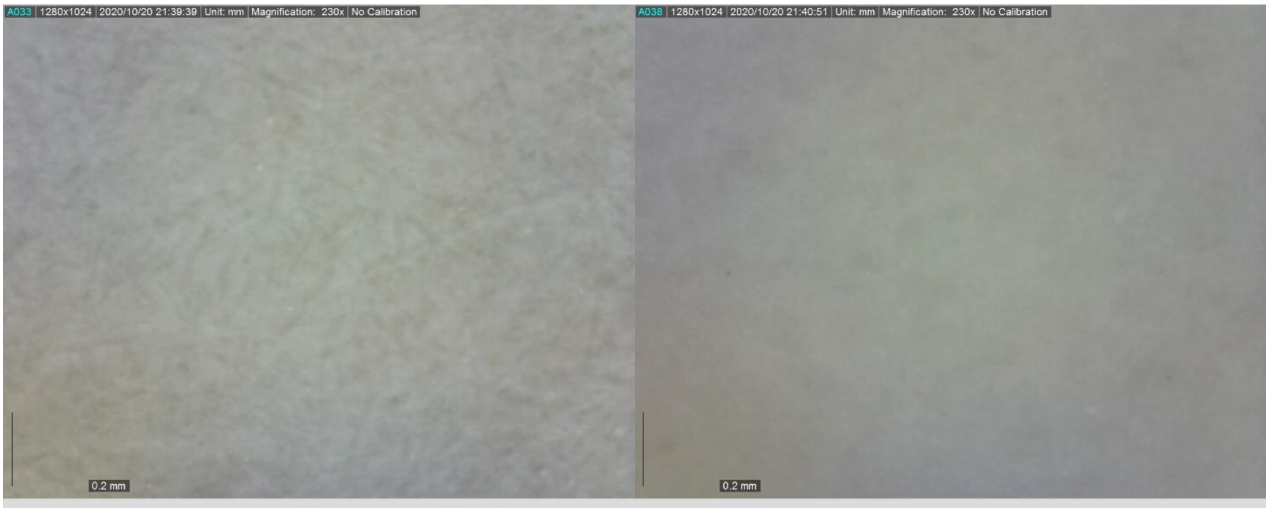

Fig. 19. Original MKK carton (on the right) with 1 layer of wax coating (on the left)

Fig. 20. Original MKK carton (above) and with 1 layer of wax coating (bellow)

The carton surface has a visible texture. There are lines and it can be presumed, that board thickness may differ depending on the fibre’s arrangement. Uneven grooves can accumulate water, which is more easily absorbed into the material and evaporates. However, the wax coating creates a smooth surface with difficult to see or completely invisible texture. That leads to the presumption that wax coating can create a waterproof and impermeable surface. On the other hand, this kind of coverage can be easily damaged by sharp objects or during folding as can be seen in Fig. 21.

Fig. 21. Wax coating after folding on the paper box surface

Not removed crumbs appear on the surface of the bread, can be mixed with mold spots and cause an unattractive appearance. That is why it can be suitable only on rigid surfaces, which retain their shape.

Water loss observation

The results are presented in Table 12.

Table 12. Moisture loss of bread kept in different packaging

	Original packaging	**All walls covered with the coating**	**1 wall covered with the coating**
Sample weight on 1st day no. 1, g	20.0482	18.4315	18.9092
Sample weight on 1st day no. 2, g	18.8562	19.1831	20.9992
Sample weight on 1st day no. 3, g	20.3427	16.2191	16.7256
Average samples weight on 1st day, g	*19.7490*	*17.9446*	*18.8780*
Sample weight on 5th day no. 1, g	17.1895	14.3795	11.5036

	Original packaging	All walls covered with the coating	1 wall covered with the coating
Sample weight on 5th day no. 2, g	16.9760	15.3485	12.8100
Sample weight on 5th day no. 3, g	17.7338	12.2381	10.3708
Average samples weight on 5th day, g	*17.2998*	*13.9887*	*11.5615*
Average weight loss, g	**2.4490**	**3.9559**	**7.3165**
Average weight loss, %	**12.40**	**22.04**	**38.76**

As can be seen from the results, the bread in the original packaging lost the least of water – 12.40%. When the coating was applied to the inner paper package surface, water loss was 10% bigger than kept in the original packaging – 22.04%. Although only 1 wall was coated the results were the worst, bread lost even more – 38.76% - which is more than 2 times more compared with a fully covered paper box.

It can be seen, that the barrier of MMK without plastic is permeable to water vapour. Though that only one wall coating may be enough for active packaging was denied. To achieve better resistance closer to the original plastic bag, it was decided to apply 2 layers of coating on all walls of the inner surface.

Comparison of texture properties

TAX of bread kept under divergent conditions is given in Table 13.

Table 13. Texture profile analysis of bread on 1st and 5th day after storing

	Day of purchase	**After 5 days in:**	
		Original package	**Paper package with inner wall coating**
Hardness, N			
1st	235.109	350,162	350,672
2nd	189.531	341,212	350,670
3rd	229.435	343,845	350,671
Average	218.025	345,073	350,671
Springiness			
1st	0.782	0,807	0,998
2nd	0.820	0,799	0,999
3rd	0.748	0,737	0,998
Average	0.890	0,781	0,998
Cohesiveness			
1st	0.566	0,526	1,000
2nd	0.561	0,547	1,000
3rd	0.564	0,581	1,000
Average	0.564	0,526	1,000
Resilience			
1st	0.309	0,288	0,104

	Day of purchase	After 5 days in:	
		Original package	Paper package with inner wall coating
2nd	0.280	0,306	0,072
3rd	0.301	0,331	0,088
Average	0.297	0.308	0,088
Gumminess			
1st	133.156	184.253	350.674
2nd	106.364	186.685	350.670
3rd	129.302	199.646	350.672
Average	122.940	190.195	350.672
Chewiness			
1st	104.122	148.713	349.872
2nd	87.183	149.160	350.233
3rd	96.724	147.098	350.120
Average	96.010	148.324	350.075

Bread become harder by about 130N after 5 days of storing. The minimal difference is observed after storing in a paper box with and without coating. Resilience decreased of bread in carton board by more than 2 times white original package maintained pretty much similar value and being a better choice for sustaining it. Gumminess and chewiness increased for all the samples after storing, but for the samples kept in paper box with coating – more than by 3 times.

These qualities demonstrate the correlation between moisture loss: hardness, gumminess and chewiness increased after storage. However, resilience even increases slightly after storing samples in a plastic bag, giving it a great advantage over the paper box. Springiness and cohesiveness results are not taken into account as the values are the same for all 3 samples in the paper package.

2.4. Antifungal coating efficacy

Samples pictures, captured in the boxes with an only wax coating, before and after 8 days are displayed in Table 14.

Table 14. Control samples on the 1st and 9th day

		1st day	9th day
1st sample	1st side		

		1st day	**9th day**
	2nd side		
2nd sample	1st side		
	2nd side		
	Other		
3rd sample	1st side		

		1st day	9th day
	2nd side		

2 of 3 control samples had visual changes. 1 spot of fungi was spotted on 1st sample. 2nd sample did not had any differences on crumb, but were was one big fuzzy mold spot on the crust. The last sample hadn't any noticeable changes after this timeline. All in all, the affected area was not as large as in the first study.

The samples in boxes containing citral and eugenol EO mix coating at two designated times are exhibited in Table 15.

Table 15. Samples, kept in boxes with citral and eugenol coating, on 1st and 9th day

		1st day	9th day
1st sample	1st side		
	2nd side		
2nd sample	1st side		

		1st day	9th day
	2nd side		
3rd sample	1st side		
	2nd side		

The bread slice in the 1^{st} box does not have any noticeable dissimilarities. However other 2 had observable changes on the crumb: 2^{nd} sample had a big fuzzy mold grown on the non-contact side and 3^{rd} had noticeable spots on both halves demonstrating a growth of the same quantity as in control samples.

The photos before and after storage with a coating including citral and thymol are present in Table 16.

Table 16. Samples, kept in boxes with citral and thymol coating, on 1st and 9th day

		1st day	9th day
1st sample	1st side		

		1st day	**9th day**
	2nd side		
2nd sample	1st side		
	2nd side		
3rd sample	1st side		
	2nd side		

No noticeable changes were recorded on samples after storage on all 3 samples and their both sides as can be observed in the photos above.

Samples of bread before and after keeping in a paper box containing carvacrol and thymol EO with wax are presented in Table 17.

Table 17. Samples, kept in boxes with carvacrol and thymol coating, on 1st and 9th day

		1st day	**9th day**
1st sample	1st side		
	2nd side		
2nd sample	1st side		
	2nd side		
3rd sample	1st side		

		1st day	9th day
	2nd side		

Any noticeable alterations were observed on these samples either.

Conclusions and recommendations

In the performed experiment, wax coating with synergetic EO mix capacity was evaluated. 2 layers of the wax coating were enough to reduce the paper's air permeability, keep the bread moist and susceptible to mold.

2 of 3 tested pairs, citral with thymol and carvacrol with thymol, showed no growth after 8 days of storing in an active coating covered box. Both blends contain thymol, meaning it could be the main constituent determining the effectiveness. Control and eugenol-thymol coating samples demonstrated similar results, which means the mixture was not effective.

On the other hand, it has not been established if all of the samples had sufficient spores on the surface. To ensure that, spore suspension should be used to infect the sample with the same amount and prove the outcome afterwards.

To be able to use this coating method in the industry, a few points should be considered. The first of them is the application of coating. It should be applied with avoiding the edges which are folded later on. A bit shorter length roller should be used than the box length. Netherneless, this would increase the chance of uneven surface coating as the excess is trapped between the foldable lines. Another technique could be removing the surplus after the procedure with pressured air. Or simply this coating could be applied as an additional packaging part providing its antifungal benefits.

Second, precise concentrations, not approximate ones, are very important to create the synergistic effect. Only specific values of both components can establish synergism. A bigger amount of coating should be made or more precise equipment should be used in order to put the exact values of the materials.

Lastly, a sensory analysis should be performed. Even though small amounts of EO were used, they are highly concentrated and have a great smell. Usual bread samples should be compared with those in contact with the coating to ensure if there is a sensory difference. If there is, it may be acceptable or not for consumers depending on the odor and could become an advantage or a flaw.

Conclusions

1. “Urtės“ bread had the largest mold growth noticed on the 9th day compared with „Agotos and „Beatos virtuvė“ and it was used for coating evaluation. Moisture loss comparison and TPA results showed, that 1 wall or 1 layer of inner walls coating was not sufficient to reach the same values as the samples had kept in the original packaging.
2. A methodology for the impregnation of paper packages with an essential oil antifungal coating was developed. A soy wax base and the required amount of EO drops were used to reach the required concentration of its main ingredient. It was applied by 2 layers on the inner paper box surface and provided reduced air permeability, which is needed to sustain bread’s moisture.
3. Combinations of essential oils were analyzed against common bread molds. Studies have shown the effectiveness of citral-eugenol, citral-thymol and carvacrol-thymol duos. Both thymol containing coatings were effective as no growth was captured on samples after 8 days of storage compared with control and citral-eugenol samples.

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