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Characterisation of Antimicrobial Capacity of Fruit Concentrates for the Use in Sugar Reduced Fruit Jams

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by
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Abbreviations

pH	Acidity
a_w	Water activity
FDA	Food and Drug Administration
GRAS	Generally regarded as safe
G/NG	Growth/non-growth ratio
PBS	Phosphate-buffered saline
PCR	Polimerase Chain Reaction
dd	double deionised
bp	base pair

Characterisation of Antimicrobial Capacity of Fruit Concentrates for the Use in Sugar Reduced Fruit Jams

Ertan Ermis

Abstract

The objective of this study was to evaluate the antifungal activity of different fruit extracts to inhibit or delay the growth of xerophilic fungi (*Absidia glauca, Penicillium brevicompactum, Saccharomyces cerevisiae*, and *Zygosaccharomyces bailii*) and by this way, to increase the shelf life of the sugar reduced fruit jam. The follow-up studies focused on the berry extracts and on the isolated moulds (*Penicillium commune* and *Eurotium rubrum*) from jam systems. Sodium benzoate, potassium sorbate, and *p*-hydroxybenzoic acid butyl ester were used at different concentrations to compare the antifungal effect with the berry extracts (cranberry and cowberry extracts). It was found that the cowberry extract was more effective than cranberry extract at a same concentration in sugar reduced jam. The strongest inhibitor was observed as sodium benzoate in jam systems. *A. glauca* was affected by all of the antifungal agents and extracts. It was found that yeast strains were much more resistant to antifungal agents in same medium conditions and at a same concentration than moulds. *Zygosaccharomyces bailii* was able to grow on higher concentration of antifungal agent contained medium than other fungi strains. *A. glauca* was the most sensitive to antifungal agents and extracts and was not able to grow on sugar reduced jam systems.

1. Introduction

`Food with less calories´ is the magic word for a lot of consumers, who care about the functions and metabolism of food and effects of food ingredients on human health, nowadays. Therefore, food industry is trying to make the work needed to prepare less calorific functional food with or without artificial additives or no additives and is developing new concepts and products for this purpose.

Although the preservation technique of preparing jam or marmelade by adding sugar is not that new, the reduced sugar products are relatively new in the market.

One of the most important factors that affect the quality and the safety of sugar reduced jam during its shelf life is fungal contamination and fungal growth. Contamination may occur from environment, people and tools which are used to handle jams after opening the jar by consumer.

Although the concept of `sugar reduced jam´ sounds good, keeping the product for long time after opening the lid of the jar is associated with fungal activities. The main reason for fungal growth is the reduced sugar content. It is known that the addition of more than 60% sugar to food-water systems, most of the biological activities are slowed or even stopped, which gives a longer shelf life to the product. Sugar binds free water in food complexes and therefore a_w (water activity) is decreased. Adding less sugar means not enough sugar molecules to bind free water in the food complex. Therefore less sugar content (lower than critical level), which results at high a_w, leads to fungal growth on the surface of product. The factors that effect the fungal growth are pH, a_w, and natural antifungal compounds which come from fruits in sugar reduced jam.

The main problem in a sugar reduced jam in terms of fungal growth is that it gets spoiled by moulds and yeasts in a certain time interval after opening the lid of the jar. Moulds can easily grow on acidic media and due to this growth colonies can be seen on the surface of the sugar reduced jam. Many moulds may produce toxic substances which effect human health.

Because food is so important to survival, food preservation is one of the oldest technologies used by human beings. As food preservatives are essential for many foods to protect them against spoilage and from technological point of view, usage of these chemical substances should be eliminated in sugar reduced jam. The idea of producing sugar reduced jam aims a natural food without preservatives in the product. Consumers demand food products with fewer artificial food additives but increased safety, quality and shelf-life. These demands have led to renewed interest in the use of natural antifungal agents to preserve foods. However, despite the wide range of potential antifungal agents, relatively few are suitable for use in practice in particular food products. At this point, the idea of using a natural food preservative like berry extracts that are rich in antifungal phenolic compounds could be used to extend the shelf life of sugar reduced jam.

The objective of this study was to evaluate the antifungal activity of different fruit extracts to inhibit or delay the growth of xerophilic fungi and by this way, to increase the shelf life of the sugar reduced jam. The approach to inhibit or delay the growth of xerophilic fungi should be using `hurdle concept´. By changing more than one condition like lowering pH, lowering a_w, and/or adding antifungal agents into product should be taken into consideration. Since the pH and a_w level of the product is not enough to inhibit the fungal growth, a suitable fruit extract which is rich in antifungal compounds should be added to the product. Not only antifungal activities but also other positive effects on health like anti-oxidative and anticarcinogenic effects of these extracts are known.

Due to the antifungal activity of some weak acids, the lowest concentration of sodium salt of benzoic acid, potassium salt of sorbic acid and butyl ester of *p*-hydroxybenzoic acid should be examined as reference in fructose added wort agar medium against selected xerophilic fungi which are mainly responsible for spoilage of and growth on sugar reduced jam.

The isolation of possible fungi strains which may spoil and grow on sugar reduced jam was aimed. As a last step, the antifungal effects of using different fruit extracts should be tried in different sugar reduced jams to evaluate the growth responses of isolated and selected fungi.

2. Literature Review

2.1 Fruit preserves and jam

Jams, jellies, marmelades, and preserves are classified according to their fruit component form which is used in production (Somogyi *et al.*, 1996). In jams, crushed or ground fruit material is used, while jellies are made with strained fruit juice (Somogyi *et al*., 1996). Marmelades are jellies in which pieces or slices of citrus peel are suspended. Preserves are produced with whole fruit or large pieces of fruit (Ahmed, 1981). In fact, regardless of their form, all these forms are basically sugar-acid-pectin gels or low-methoxyl pectin-calcium gels. The complex interaction between function and level of pectin, pH, sugar type and content, setting temperature which effects the pectin gel formation, and calcium content results in their structure, appearance and mouthfeel characteristics (Somogyi *et al*., 1996).

Pectin is the universal choice for jam and jellies due to its presence as a natural fruit ingredient and also because of the characteristic consistency that pectin changes to a gel, although the availability of other alternative gelling agents are possible (Somogyi *et al*., 1996). Pectin is a plant cell wall polysaccharide consisting of a linear chain composed of D-galacturonic acid residues linked via α (1-4)-glycosidic bonds (Toman *et al*., 1976). According to Oakenfull (1987), pectin gels can be described as intermediate between solid and liquid phases. The solvent water, pH, and sugar type and content affect the intermolecular forces which play important role in gel structure. Holdsworth (1983) reports that, the optimum pH is 3.0 to get a better gel structure. This structure prevents the seperation of aqueous phase from gel form (Somogyi *et al*., 1996). May and Stainsby (1986) report that replacement of sucrose with other sugars such as, maltose, glucose or fructose syrups alter the typical rheological properties and setting times of gel models.

Sugar is used in jams and jellies to decrease a_w 0.80 or lower (Holdsworth, 1983). Holdsworth (1983) also reports that the best concentration of sugar is about 67.5% and it should be as little as 60% by adjusting the pectin composition.

The four main and necessary ingredients in manufacturing jams are fruit, pectin, sugar, and acid (Somogyi *et al*., 1996). Spices, buffering agents, preservatives, and antifoaming

agents are optional ingredients (Somogyi *et al*., 1996). In some products it is necessary to add antifungal agents such as sorbic acid to control the growth of fungi (Holdsworth, 1983). The main stages in jam manufacture are as folows (Somogyi *et al*., 1996):

- blending of ingredients to have a uniform structure
- evaporation to get the solid level wanted
- pasteurization of the product by heat treatment

2.2 Spoilage of low a_w foods

Water is not only a very important component of food products, but also a very critical factor effecting quality and safety. Microbiological stability is one of the factors to be considered in quality and safety of food products, which depend on the moisture content and a_w of each component in a complex food system.

Low a_w foods or concentrated foods are defined according to Pitt and Hocking (1999) as both evaporated products and those to which sugars have been added. The list includes jams, confectionary and fruit based products. Such foods are susceptible to spoilage by xerophiles and provide ideal growth conditions for the most xerophilic fungi (Pitt and Hocking, 1999).

Pitt and Hocking (1999) report that, traditional jams are boiled or evaporated down to 0.75 a_w or below and hot filled into jars before closing. The answer to any spoilage problem by xerophilic fungi with a traditional type of jam must rely on that basic condition: to produce jams which will not spoil, a_w of product must be reduced to a safe level. Jams which have much lover levels of added sugars and because of that much higher a_w are produced as sugar reduced and/or diabetic products. Due to low sugar/high a_w level of sugar reduced jams, spoilage by xerophilic fungi may take place and growth conditions may be suitable for growth on the surface of sugar reduced jam. These products can be preserved by food preservatives (Pitt and Hocking, 1999).

According to Defigueiredo and Splittstoesser (1980), the stability of high sugar products depends on the amount of available free water molecules, the concentration and

availability of sugars, the oxigen tension, pH, storage temperature and time, initial cell concentration, contaminating species, and the presence of preservatives.

2.2.1 Xerophilic fungi which spoil low a_w food systems

Fungi are yeasts, which can form ascospores and grow by budding and moulds, which can form conidia, ascospores and sporangiaspores (Yousef and Carlstrom, 2003). They are mostly aerobic and grow slower than other microorganisms. However the colonies of moulds can be seen and distinguished easily after growth, the colonies of yeasts can not be distinguished easily from those of bacteria. They are mostly osmotolerant and acid tolerant (Yousef and Carlstrom, 2003).

Yousef and Carlstrom (2003) report that, fungi are the major cause of big economic losses to the food industry due to spoilage. They are widespread and ubiquitous microorganisms (Yousef and Carlstrom, 2003).

Yeasts are capable to grow between 0-40°C (Stokes, 1971). According to Elliott and Michener (1965), the optimum temperature for growth is between 25 and 35°C. They are mainly aerobic microorganisms depending on their species. Yeasts need more water than moulds for growth. Mossel and Ingram (1955) found a_w=0.88 to be the approximate value below which yeast growth is limited. Although these microorganisms are more acid resistant compared to bacteria, they can not resist acidity as much as moulds can resist. The approximate pH range for growth is between 1.5 and 8.5.

Deak and Beuchat (1996) report that the yeasts which belong to genus *Zygosaccharomyces* are capable of spoiling high sugar/low a_w food systems.

Defigueiredo and Splittstoesser (1980) report that if the storage time is sufficiently long and the conditions are suitable, high concentrations of sugar, organic acids, the exclusion of air, refrigeration, and application of other storage conditions will not safeguard a food from the action of yeasts.

Moulds grow commonly at refrigeration temperatures which are 0-4°C. Some species are thermophiles which can grow at 50°C (Michener and Elliott, 1964). Cochrane (1958) reports that moulds are aerobic organisms which mean they require oxigen under most

conditions. They can grow in a wide range of a_w values down to 0.70 in some conditions and for some species (Defigueiredo and Splittstoesser, 1980). The approximate pH range for growth is between 1 and 11.

Moulds grow slowly and they may make the food unfit to eat before their growth is evident. They are also called filamentous fungi which represant a very important group of food spoilage organisms. They are mostly xerophilic and some strains may be pathogen to human (Defigueiredo and Splittstoesser, 1980).

Some mould species may produce toxins which commonly are referred to as mycotoxins. Mycotoxins include aflatoxins, which are highly carcinogenic; ochratoxin, which leads to kidney toxicity; and T-2 toxin, which causes alimentary toxic aleukia (Yousef and Carlstrom, 2003).

2.2.1.1 Fungi species which may spoil sugar reduced jam

Relativlely a few fungal genera represent the microflora of intermediate and low moisture foods. According to Hocking and Pitt (1981), if jams spoil, *Eurotium* species, *Aspergillus restictus* and related species, xerophilic *Penicillia*, especially *P. corylophilum* are usually responsible. Char *et al*. (2005) studied the growth response of some xerophilic fungi like *P. brevicompactum* in Argentine milk jam. According to this report, potassium sorbate greatly effected the effect of a_w on growth. Char *et al*. (2005) also reports that in the presence of 1,000 ppm potassium sorbate, the mould species which were studied are not able to grow at a_w 0.74.

Hocking and Pitt (1981) have seen the bubbling of lid of the jar after spoilage with *Zygosaccharomyces rouxii*, most probably from contamination after opening. The black yeast-like fungus *Trichosporonoides nigrescens* is also reported from spoiled, fermenting jam by Hocking and Pitt (1981).

Saccharomyces cerevisiae is reported as a source of spoilage in low pH foods by Pitt and Hocking (1999). It grows optimally between 33 and 35°C in 10-30% glucose. The minimum growth temperature is 4°C in 10% glucose and 13°C in 50% glucose (Jermini and Schmidt-Lorenz, 1987). Juven *et al.*(1978) report that *Saccharomyces cerevisiae* can grow at neutral pH down to 0.89 a_w in glucose media. According to Pitt (1974), *S.*

cerevisiae tolerates up to 100 mg/kg benzoic acid at pH 2.5-4.0 and 200 mg/kg sorbic acid at pH 4.0 and can grow down to pH 1.6 in HCl.

One of the most resistant yeast to weak acids is *Zygosaccharomyces bailii* (Pitt and Hocking, 1999). It also tolerates low pH and low a_w as well. Growth was observed by Kalathenos *et al.* (1995) at pH values of 2.8-3.0, 40-45° Brix, and 800 mg/l benzoic acid in packed fruit cordials.

One of the most xerophilic *Penicilia* is *Penicillium brevicompactum* (Pitt and Hocking, 1999). The minimum and maximum temperatures for growth of *P. brevicompactum* are -2 and 30°C, with an optimum near to 23°C (Mislivec and Tuite, 1970). The minimum a_w for germination and growth is 0.78 at 25°C (Hocking and Pitt, 1979).

Another xerophilic fungus which may spoil and grow on sugar reduced jam is *Penicillium corylophilum* (Pitt and Hocking, 1999). Hocking and Pitt (1979) reported germination at 0.80 a_w after 38 days at 25°C. *P. corylohilum* germinates within 7 days at 5°C (Pitt and Hocking, 1999).

According to Pitt and Hocking (1999), some *Absidia* species are also able to grow down to 0.88 a_w. *A. glauca* and *A. corymbifera* are examples to those species. The growth temperatures are minimum 14°C, maximum 50°C, and optimum near 40°C (Pitt and Hocking, 1999).

An example to *Eurotium* species is *Eurotium amstelodami*. The optimal temperature for growth of *E. amstelodami* is 33-35°C (Domsch *et al*., 1980), with a maximum at 43-46°C. It was reported to grow down to 0.70 a_w at 25°C by Armolik and Dickson (1956).

Smith and Hill (1982) reported the temperature range for growth of *Aspergillus restrictus* was minimum 9°C, optimum 30°C and maximum 40°C. Growth of this species has been observed down to 0.75 a_w (Pitt and Hocking, 1999)

Hocking and Pitt (1981) report that, *Trichosporonoides nigrescens* was isolated from jam. This yeast-like fungus is a xerophile, able to grow at 0.75 a_w.

2.3 Methods to inhibit fungal growth in sugar reduced jam

Mould and /or yeast growth in jam depends on formulation, a_w, and environmental factors like storage temperature (Char *et al.*, 2005). The growth could be prevented by an optimum design of the manufacture process using the hurdle technology concept (Gould, 1995; Alzamora *et al*., 1995). Employing the synergy that exists between two or more parameters allow to advance the growth/no growth (G/NG) ratio and to better quantify the hurdle concept (Jay *et al.*, 2005). The hurdles for sugar reduced jam could be assumed as hot filling, a_w, pH, and antifungal organic acids.

From the long list of chemicals inhibitory to xerophilic fungi growth, only a few have been permitted in foods such as sorbates and sodium benzoate (Defigueiredo and Splittstoesser, 1980). The inhibitory effects of these acids depend on the undissociated form (Branen *et al*., 1990). The undissociation and pK_a values of benzic acid and sorbic acid can be seen at Appendix 1. Undissociated form is more permeable to the cell wall. Uptake of these acids and their growth inhibitory effects increases at pH values below their pK_a values (Deak and Beuchat, 1996). The effective pH range of common antifungal agents can be seen at Appendix 1.

Benzoic acid occurs naturally in some berry species, prunes, greengage plums, cinnamon, and ripe cloves (Kimble, 1977). Branen *et al.* (1990) report that benzoic acid and sodium benzoate are suitable for foods that naturally are in a pH range below 4.5 or acid should be added to bring the pH under pK_a value. Somogyi *et al.* (1996) report that benzoic acid is most effective in the pH range of 2.5-4.0 and the salt forms are more soluable than free acid form. Holdsworth (1983) reports that when the concentration of undissociated benzoic acid exceeds 25 mg/ml, yeast development is inhibited.

Sorbic acid and potassium sorbate are very effective for controling fungi (Holdsworth, 1983). The inhibitory microbial activity of sorbates can be effective up to pH 6.5 (Somogyi *et al*., 1996).

Esters of *p*-hydroxybenzoic acids are also in use as antimicrobial agents (Holdsworth, 1983).

Benzoic acid and sodium benzoate are generally regarded as safe (GRAS) by Code of Federal Regulations (1977) up to a maximum permitted level of 0.1% (Jay *et al.*, 2005).

2.3.1 Fruit extracts which contain fungistatic agents to use in sugar reduced jam

The fruits contain different kinds of antimicrobial polyphenolic compounds and high levels of organic acids. Extracts of cranberry and high bush blueberry are effective against various fungi such as *Penicillium* spp. and *Aspergillus niger*. Cippolini and Stiles (1992) report that, the primary sources of antifungal activity were water-soluble, nonalcoloid chemicals such as phenolics and acids. Salunke and Kadam (1995) reported that the antifungal activity of the fall-ripening species such as cranberries is much higher than the summer-ripening species such as high bush blueberries. Content of benzoic acid and *p*-hydroxybenzoic acid of some fruit extracts can be seen in Appendix 2.

Pimiä *et al*. (2004) studied the effects of berries and berry phenolics on selected pathogenic gastrointestional bacteria. They report that phenolic berry extracts inhibited the growth of gram-positive and gram-negative cells. They also report that the most efficient berries are cloudberry and raspberry.

Currently there are some researchs on effects of berries on certain chronic and degenerative diseases. The effects of polyphenolics present in fruits include antioxidant, anti-allergic, anti-diabetic, anti-inflammatory, antiviral, anti-mutagenic, anti-proliferative, antimicrobial, anti-carcinogenic and protection from cardiovascular damage and allergy (Ghosh, D.K., 2005; Hollman P.C.H. and Katan, M.B., 1999; Duthie *et al*., 2000). Ghosh *et al*. (2006) demonstrated the protective effects of boysenberry and blackcurrant extracts against H_2O_2-induced cell toxicity and DNA damage. These results show us that the use of these extracts in sugar reduced jam worth not only in prevention of fungal growth but also in prevention of some health problems.

3. Materials and Methods

3.1 Materials

3.1.1 Strains

Penicillium brevicompactum (DSM 3825), Absidia glauca (DSM 811) Saccharomyces cerevisiae (DSM 1333) and Zygosaccharomyces bailii (DSM 70834) isolates were obtained from DSMZ[1].

3.1.2 Media

Moulds and yeasts were grown on wort agar medium (Malt extract 15 g/L; peptone 0.75 g/L; maltose 12.75 g/L; dextrin 2.75 g/L; glycerol 2.35 g/L; potassium dihydrogen phosphate 0.4 g/L; ammonium chloride 1.0 g/L; agar 20 g/L; pH 5.2).

3.1.3 Chemicals/Preservatives

Fructose (Merck, D(-), 99% pure, granular) was used to lower the a_w of wort medium and HCl (1 N) was used for acidification of the medium. Sodium benzoate (Sigma Chemical), potassium sorbate (Merck), and *p*-hydroxybenzoic acid butyl ester were used as additives in wort agar medium. For this purpose, 100 mg/mL stock solutions of these antifungal agents in destilled water were prepared and were cold sterilized by microfilter.

3.1.4 Sugar reduced jam

Sugar reduced jams which were used in the experiments were obtained from Schwartauer Werke GmbH & Co. KGaA. Strawberry-lime, strawberry-guava, and raspberry-aloevera jams were selected. The jam samples which were used did not contain artificial preservtives.

[1] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)

3.1.5 Friut extracts

Extracts of cranberry and cowberry were obtained from the Plant Foodstuff Institute, University of Hohenheim. The antifungal agent concentrations of extracts were studied by this department and the data were observed from this institute as can be seen in Appendix 2.

3.2 Methods

3.2.1 Culture conditions of yeast and moulds

Tubes with slope shaped wort agar medium were inoculated with yeasts, incubated for 2 to 4 days at 30°C and were kept in refrigeration (0-4°C) conditions for further use. To obtain a uniform and standardized cell number in liquid culture (overnight culture), liquid wort medium was inoculated with yeasts from this culture each time. The cell concentration in overnight culture after 15 hours incubation at 30°C was in the range 1-10*10^6 cell/mL respectively.

To obtain fungal spores, pertri plates filled with approximately 20 mL wort agar medium, were inoculated with moulds and incubated in the dark for 8 to 10 days at 25°C for sporulation.

A counting chamber (Thoma, 3x8 cm) with microsquares (0.0025 mm^2) was used to count the cells in overnight culture and conidia spore suspension. Zeiss brand microscope was used for this purpose. Required dilutions were prepared and 40 µL of the dilution/suspension was taken onto chamber and cells/spores were counted in 20 different microsquares under microscope. The formula which was used to calculate the approximate cell number per mL of liquid culture and of spore suspension:

$N=M*4*10^6$

Where, N is final cell count as cell/mL and M is the mean value of the number of cells that were counted under microscope as cell/square.

3.2.2 Harvesting fungal spores

30 g of sterilized sea sand (0.1-0.3 mm, VWR International) was taken onto wort agar plate which was inoculated with fungal spores and incubated for sporulation. Plate was closed well and shaked to get the spores onto the sand particules. Spores were attached on the surface of sand particules at the end of shaking. Sand with spores was taken into sterile PBS solution (NaCl 8 g/L; KCl 0.2 g/L; Na_2HPO_4 1.44 g/L; KH_2PO_4 0.24 g/L; pH 7) with 0.05% Tween 80. The mixture was shaked well to get spores into PBS solution. Solution with spores was pippetted into another sterile tube. After centrifugation (3750 rpm, 15 min, 4°C), supernatant was removed and the pellet was dissolved in PBS solution. The final conidia concentration was in the range 1-6*10^6 conidia/mL. Spore suspension was kept at refrigeration conditions (0-4°C) for further use.

3.2.3 Determination of antifungal activities of different antifungal agents and fruit extracts against selected fungi

3.2.3.1 Sodium benzoate (pH 4.5)

Wort agar medium (pH 5.2) was dissolved in destilled water and autoclaved at 121°C for 15 minutes. After cooling down to 45°C, drops of sterile sodium benzoate stock solution (100 mg/ml) were added to adjust the sodium benzoate concentration as percentage (w/v) of medium to 0.050, 0.075, 0.100, 0.125, and 0.150 respectively. pH of medium was adjusted to 4.5 with the addition of HCl solution. The pH of the system was measured with a glass electrode (Schott Blueline 21) attached to a pH-meter (WTW pH 537). PH meter was calibrated with buffer solutions before taking the measurements.

After adjusting the pH and sodium benzoate concentrations of the medium, as 20 ml per petri dish was spreaded on each and kept for one day in atmospheric conditions. Three petri dishes were prepared for each sodium benzoate concentration and for each fungi strain. Three petri without sodium benzoate were prepered for each strain for control. Petri dishes were inoculated with the overnight cultures of yeasts as 100 cells per petri dish and with the conidia spore suspensions of moulds as 100 spores per petri dish. Peptone water (0.85% NaCl; 0.1% trypton) was used to dilute the overnight cultures and

spore suspensions. Spread plate method was used and the petries were incubated for 5 days at 30°C for yeasts and 10 days at 25°C for moulds. Growth was observed in the first 3 days of incubation for yeasts and in 5 days for moulds. The experiments were repeated three times with three replicates. Inhibition of growth was determined by no visible mycelium development after incubation period.

3.2.3.2 Sodium benzoate (pH 4.0, 31% fructose)

Wort medium, agar, and fructose were weighed seperately. Wort medium without agar and fructose were dissolved in different jars in destilled water. Agar was dissolved in another jar in distilled water. They were autoclaved seperately. After cooling down to 45°C, fructose and wort medium solutions were mixed and drops of sodium benzoate solution was added to adjust the sodium benzoate content of medium as percentage (w/v) to 0.025, 0.035, 0.060, 0.080, and 0.090 respectively. Petri plate for control was prepared without sodium benzoate. The final pH was adjusted to 4.0 with the addition of HCl. As last step of preperation of medium, autoclaved agar which was kept at 45°C was added to the mixture.

20 g of medium was spreaded on each petri dish and kept at laboratory conditions until using them. Three petri dishes were prepared for each sodium benzoate concentration for each strain to get accurate results. Petri dishes with medium were inoculated with approximately 100 cells/spores per petri dishes. Petries were incubated for 5 days at 30°C for yeasts and 10 days at 25°C for moulds.

3.2.3.3 Potassium sorbate (pH 4.0, 31% fructose)

The same set of experiments as described in 3.2.3.2, potassium sorbate was used instead of sodium benzoate. The final potassium sorbate concentration of medium was adjusted as percentage (w/v) to 0.020, 0.030, 0.045, and 0.065 respectively. Control petries were prepared without potassium sorbate. All petries were inoculated with approximately 100 cells/spores and incubated for 5 days at 30°C for yeasts and 10 days at 25°C for moulds.

3.2.3.4 *p*-hydroxybenzoic acid butyl ester (pH 4.0, 31% fructose)

The same set of experiments as described in 3.2.3.2, butyl ester of *p*-hydroxybenzoic acid was used instead of potassium sorbate. *p*-hydroxybenzoic acid butyl ester solution in methyl alcohol (100 mg/mL) was added to medium to adjust the final concentrations as percentage (w/v) to 0.005, 0.010, 0.020, and 0.050 respectively. Control petries were prepared without *p*-hydroxybenzoic acid butyl ester. All petries were inoculated as 100 cells/spores from dilutions of overnight culture and spore suspension and incubated for 5 days at 30°C for yeasts and 10 days at 25°C for moulds.

3.2.3.5 Sodium benzoate in strawberry-lime jam (pH 3.0, 31% fructose)

The jam used in this study (Schwartau) contained 31% fructose and had a pH value of 3.0. Calculated amounts of jams were taken into different glass cups and drops of sodium benzoate stock solution were added to adjust the sodium benzoate concentration of jam as percentage (w/w) to 0.020, and 0.040 for each fungus respectively. Control Petries were prepared without sodium benzoate. Jam and sodium benzoate were mixed well.

Two petri dishes were prepared for each antifungal agent concentration and each fungi and 20 g of prepared jam was spreaded on each petri dish. Surfaces of jams in petri dishes were inoculated with the dilutions from overnight cultures of yeasts and from conidia spore suspensions of moulds. 10 µL (1 cell/µL) from dilutions were dropped on the surface of jams in petri dishes at 10 different points to see the growth of colonies and to count them easily. Petri dishes were incubated for 12 days at 30°C for yeasts and 19 days at 25°C for moulds.

3.2.3.6 Cranberry extract and cowberry extract in raspberry-aloevera jam (pH 3.0, 31% fructose)

Calculated amounts of jams were taken into different glass cups and berry extracts were added to the jam to give final concentrations of 0.005, 0.01, 0.03 and 0.04 (%, w/w benzoic acid). Berry extracts had certain amounts of benzoic acid. Concentrations of benzoic acid of extracts were thought as base for calculations in sugar reduced jam systems. The calculated concentrations of berry extracts vs. benzoic acid concentrations can be seen Appendix 3. The same set of experiment was prepared for each extract

seperately. Jam and extracts were mixed well, inoculated and incubated as described in 3.2.3.5. Fungal growth was followed by checking the growth by eye in every 2 days after incubation time started. The experiments were repeated three times with two replicates.

3.2.3.7 Cranberry extract and cowberry extract in strawberry-guava jam (pH 3.0, 31% fructose)

The same set of experiment as described in 3.2.3.6 was repeated with strawberry-guava jam and with the final concentrations of 0.005, 0.01, 0.03 and 0.04 (%, w/w benzoic acid) respectively.

3.2.4 Isolation and determination of moulds/yeasts that are able to grow on sugar reduced jam

Five petri dishes were prepared with 20 g of strawberry-lime jam for this purpose. These 5 petri dishes were distributed to 5 different kitchens of different houses randomly to keep them open for 12 hours. One jar with full of jam was kept open lid in laboratory for 12 hours. They were incubated at 25 °C for 12 days.

3.2.4.1 Amplification of 28s rRNA gene of moulds

One loop of mould spores were taken from wort agar plate and suspended in 500 µL PBS solution. 0.5 g of zirkonia/silica beads (0.5 mm, Biospec products, Inc.) was put into PBS-spore mix. With a beadbeater, the mix was shaked at 5000 rpm for 2 minutes to release DNA structure from fungi spores into the PBS solution. 400 µL from PBS-DNA mix was taken into a new 1.5 ml tube and heated to 96°C and kept at this temperature for 15 minutes. The mixture was centrifugated at 14000 rpm for 1.5 minutes. 300 µL of supernatant (DNA solution) was taken with a micropipette into a new tube to use for getting PCR product.

50 µL of DNA solution was taken into a thin-wall thermal cycling tube with a micropipette. All reagents were added in the order as follows: 5 µL PCR buffer (incl. $MgCl_2$), 0.25 µL PCR primer P1 (5'-ATC AAT AAG CGG AGG AAA AG-3') (50 pmol/µL), 0.25 µL PCR primer P2 (5'-CTC TGG CTT CAC CCT ATT C-3') (50

pmol/µL), 1 µL dNTPb (dGTP, dATP, dTTP, dCTP), 0.5 µL DNA polymerase, 42 µL sterile dd H_2O. The thermal cycler (MWG, Primus 96 plus) was programmed as follows: 94°C for 4 min; 35 cycles of 94°C for 30 sec, 62°C for 60 sec, 72°C for 60 sec; and 72°C for 3 min. PCR pruduct was kept at 15°C for further use.

5 µL of PCR product was taken into a tube and mixed with 2 µL SAP (Shrimp Alkaline Phosphatase) and 0.5 µL Exonuclease I. The mixture was incubated for 15 minutes at 37°C and heated to 80°C for 15 minutes.

33 nanogram (50 fmol) templates from ExoSAP-mix were taken into a tube and other reagents were added as follows: 2 µL Quick Start Mix DTSC (Beckman), 1 µL primer P1 (5 pmol/µL), and filled with sterile dd H_2O to 10 µL. The thermocycler was programmed as follows: 30 cycles of 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min. Final pruduct (cycle sequencing preperate) was kept at 4°C for further use.

10 µL cycle sequencing preperate was taken and mixed with reagents as follows: 10 µL sterile dd H_2O, 2 µL EDTA solution (100mM, pH 8.0), 2 µL Sodium acetate (3 M, pH 5.2), 1 µL glycogen (Beckman) and 60 µL 100% ethanol. Mixture was centrifugated at 14000 rpm for 15 minutes at 20°C and supernatant was removed. Pellet was rinsed with 200 µL ethanol (70%) and cenrtifugated. Rinsing and centrifugation was applied two times. After removing supernatant, pellet was dried at room temperature for 15 minutes. Pellet was dissolved in 30 µL sample load solution (deionised formamide) and sequenced with a sequencer (Beckman CEQ 8000). LFR1 method (85 minutes, 15 second injection time, 45°C capillar temperture) was used. Raw data was processed and reported by a software program (CEQ 8000 V 9.0).

3.2.4.2 Identification of species

For determining the closest relatives of 28S rRNA sequences, a search of the GenBank DNA database was conducted by the BLAST algorithm (NCBI, 2006). A similarity of >98% to 28S rRNA sequences of type strains (Appendix 5 and 6) was used as the criterion for identification.

3.2.5 Determination of antifungal activities of different fruit extracts against isolated and selected fungi

3.2.5.1 Cranberry extract and cowberry extract in strawberry-lime jam (pH 3.0, 31% fructose)

The same set of experiment as described in 3.2.3.6 was repeated with strawberry-lime jam and with the final concentrations of 0.005, 0.01, 0.02 and 0.04 (%, w/w benzoic acid) respectively.

4. Results

4.1 Determination of antifungal activities of different antifungal agents and fruit extracts against selected fungi

4.1.1 Sodium benzoate (pH 4.5)

The effect of sodium benzoate on growth of *Z. bailii, S. cerevisiae*, *P. brevicompactum*, and *A. glauca* in wort gar medium at pH 4.5 is presented in Table 1. Mean value of the numbers of colony forming units (CFU) on three plates of the same concentration levels were calculated and Table 1 was illustrated as mean value of CFU vs. different concentrations of sodium benzoate. As was expected, the higher the concentration, the greater the inhibitory effect of sodium benzoate on selected fungi. Data indicated that *Z. bailii* was more resistant than were *S. cerevisiae*, *P. brevicompactum*, and *A. glauca* at equivalent concentration of benzoate. The critical inhibitory concentration of sodium benzoate on fungi strains seemed to occur between 0 and 0.05% benzoate on A. glauca; 0.075 and 0.1% benzoate on both *S. cerevisiae* and *P. brevicompactum*; 0.125 and 0.150% benzoate on *Z. bailii.*

Table 1: Antifungal effect of sodium benzoate (in wort medium, pH 4.5).

	Number of cfu / sodium benzoate (%, w/v)					
Name of fungi	0.000	0.050	0.075	0.100	0.125	0.150
Absidia glauca	34	0	0	0	0	0
Penicillium brevicompactum	39	25	9	0	0	0
Saccharomyces cerevisiae	79	52	19	0	0	0
Zygosaccharomyces bailii	86	84	56	26	12	0

A comparison of effects of sodium benzote on growth of fungi strains at pH 4.5 are presented in Figure 1. Sodium benzote was effective in inhibition of growth of all strains at different concentrations in defined incubation time. At the 0.1% concentration, fungistatic effects were observed on all strains except *Z. bailii* which was not affected in growth but affected in the size of colonies. At zero benzoate concentration, all strains grew well. At 0.15% benzoate, all cultures were inhibited.

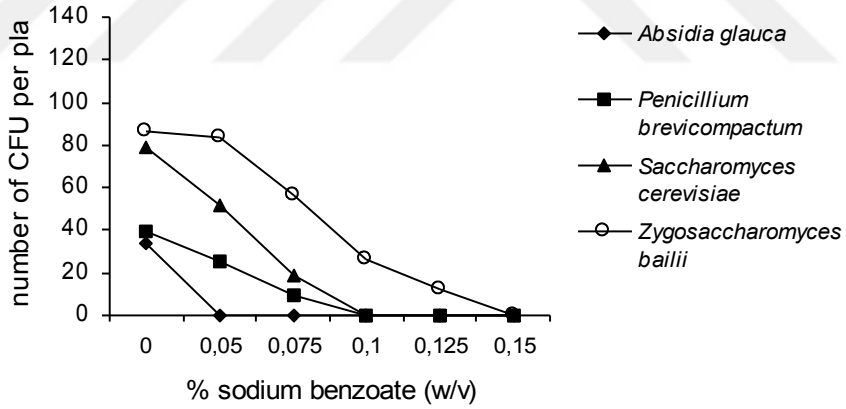

Figure 1: Growth inhibition of different fungi by sodium benzoate at pH 4.5.

4.1.2 Sodium benzoate (pH 4.0, 31% fructose)

The effect of sodium benzoate on growth of *Z. bailii, S. cerevisiae*, *P. brevicompactum*, and *A. glauca* at pH 4.0 and 31% fructose is presented in Table 2. Data has shown that , inhibition of growth of fungi by sodium benzoate was concentration, pH, and a_w dependent. The inhibitory effect of 0.09% was noticable for all strains while 0.035 and

0.06% sodium benzoate were effective in inhibition of the growth of *S. cerevisiae*, *P. brevicompactum*, and *A. glauca.*

Table 2: Antifungal effect of sodium benzoate (in wort medium, pH 4.0, 31% fructose).

	Number of cfu / sodium benzoate (%, w/v)					
Name of fungi	0.000	0.025	0.035	0.060	0.080	0.090
Absidia glauca	29	0	0	0	0	0
Penicillium brevicompactum	74	14	0	0	0	0
Saccharomyces cerevisiae	98	45	2	0	0	0
Zygosaccharomyces bailii	121	89	73	48	6	0

A comparison of growth inhibitions of selected fungi by different concentrations of sodium benzoate at pH 4.0 and 31% fructose were presented in Figure 2. Growth capabilities appeared to be the same at 0% sodium benzoate, whereas that in the 0.025% sodium benzoate-treated medium was slightly lower for *S. cerevisiae*, *P. brevicompactum*, and *A. glauca*. In the 0.06% sodium benzoate-treated medium was lower for *Z. bailii.*

While the growth inhibition rate of *Z. bailii* is 86% at 0.125% sodium benzote-treated medium at pH 4.5, in 31% fructose added medium at pH 4.0, it was observed as 95% at 0.08% sodium benzoate. The same values were calculated for other strains as follows: for *S. cerevisiae,* %75 at 0.075% sodium benzoate-treated medium at pH 4.5, while 97% in 0.035% sodium benzoate-treated and 31% fructose added medium at pH 4.0; for *P. brevicompactum,* 76% in 0.075% sodium benzoate-treated medium at pH 4.5, while 81% in 0.025% sodium benzoate-treated and 31% fructose added medium at pH 4.0; *A. glauca* was not able to grow at any concentration of sodium benzoate.

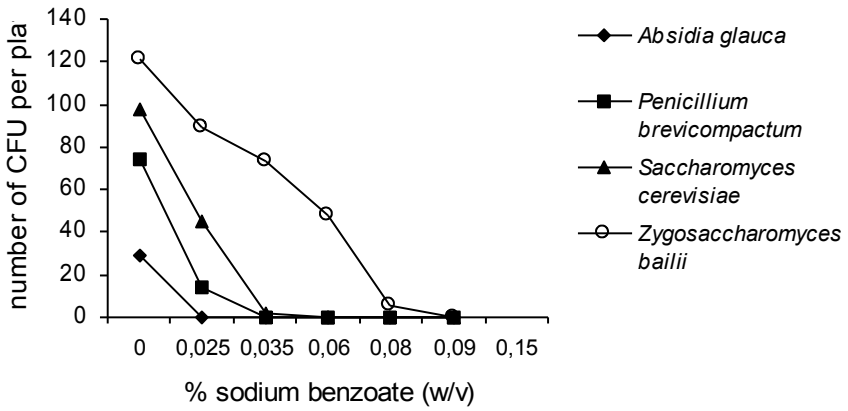

Figure 2: Growth inhibition of different fungi by sodium benzoate at pH 4.0 and 31% fructose.

4.1.3 Potassium sorbate (pH 4.0, 31% fructose)

Table 3 depict the effects of the different concentrations of potassium sorbate in 31% fructose added wort medium at pH 4.0, respectively. Initial populations of *Z. bailii* (94 CFU per plate), *S. cerevisiae* (81 CFU per plate), *P. brevicompactum* (35 CFU per plate), and *A. glauca* (29 CFU per plate) were each reduced into 0 in 0.02% potassium sorbate-treated medium for *S. cerevisiae*, *P. brevicompactum*, and *A. glauca*; and into 2 CFU per plate in 0.045% potassium sorbate-treated medium for *Z. bailii*.

Table 3: Antifungal effect of potassium sorbate (in wort medium, pH 4.0, 31% fructose).

	Number of cfu / potassium sorbate (%, w/v)				
Name of fungi	0.000	0.020	0.030	0.045	0.065
Absidia glauca	29	0	0	0	0
Penicillium brevicompactum	35	0	0	0	0
Saccharomyces cerevisiae	81	0	0	0	0
Zygosaccharomyces bailii	94	92	78	2	0

Figure 3 present a comparison of the growth inhibition by different concentrations of potassium sorbate at pH 4.0 and 31% fructose. Treatment of 0.02% sodium benzoate led to zero growth for *S. cerevisiae*, *P. brevicompactum*, and *A. glauca*. However in the range 0.045-0.065% potassium sorbate was required to inhibit the growth of *Z. bailii.*

While the growth inhibition rate of *Z. bailii* is 95% at 0.08% sodium benzoate in 31% fructose added medium at pH 4.0, it was observed as 97% at 0.045% potassium sorbate in the same conditions of the medium, respectively. *S. cerevisiae, P. brevicompactum,* and *A. glauca* were not able to grow in 0.02% potassium sorbate treated-medium.

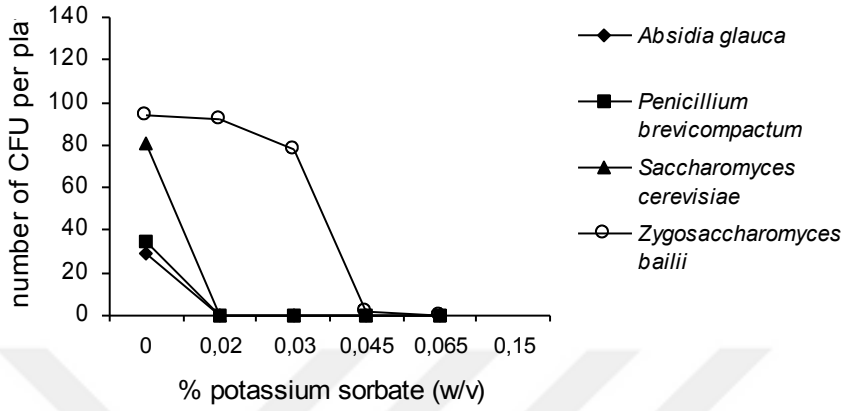

Figure 3: Growth inhibition of different fungi by potassium sorbate at pH 4.0 and 31% fructose.

4.1.4 *p*-hydroxybenzoic acid butyl ester (pH 4.0, 31% fructose)

Table 4 illustrates the results obtained. The initial populations of the fungi strains decreased by different concentrations of *p*-hydroxybenzoic acid butyl ester in 31% fructose added medium at pH 4.0. Table 4 also depicts the effect of *p*-hydroxybenzoic acid butyl ester on the growth of mould and yeast strains. A concentration of 0.01% was required to reduce to zero the number of detectable CFU of mould strains while concentration of 0.02% was required for *S. cerevisiae* and 0.05% for *Z. bailii.*

Table 4: Antifungal effect of *p*-hydroxybenzoic acid butyl ester (in wort medium, pH 4.0, 31% fructose).

	Number of cfu / *p*-hydroxybenzoic acid butyl ester **(%, w/v)**				
Name of fungi	0.000	0.005	0.010	0.020	0.050
Absidia glauca	29	25	0	0	0
Penicillium brevicompactum	27	11	0	0	0
Saccharomyces cerevisiae	89	56	39	0	0
Zygosaccharomyces bailii	62	58	46	37	0

The growth of selected fungi strains in wort medium supplemented with fructose (31% w/v) and *p*-hydroxybenzoic acid butyl ester with different concentrations at pH 4.0, show that, yeast strains resisted a concentration level up to 0.05%, while it was 0.01% for mould strains (Figure 4).

While the growth inhibition rate of *Z. bailii* is 97% at 0.045% potassium sorbate in 31% fructose added medium at pH 4.0, it was observed as 25% at 0.02% *p*-hydroxybenzoic acid butyl ester and 100% at 0.05% *p*-hydroxybenzoic acid butyl ester. The same values were calculated for other strains as follows: for *S. cerevisiae* %100 at 0.02% potassium sorbate-treated medium, while 50% at 0.035% *p*-hydroxybenzoic acid butyl ester and %100 at 0.02%; for *P. brevicompactum* 100% in 0.02% potassium sorbate-treated medium, while 59% at 0.005% *p*-hydroxybenzoic acid butyl ester and 100% at 0.01%; and for *A. glauca* 100% in 0.02% potassium sorbate-treated medium, while 13% at 0.005% *p*-hydroxybenzoic acid butyl ester and 100% at 0.01%, respectively.

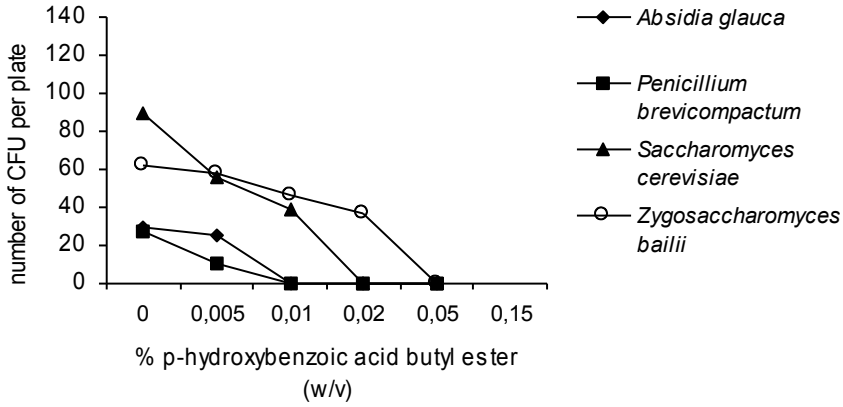

Figure 4: Growth inhibition of different fungi by *p*-hydroxybenzoic acid butyl ester at pH 4.0 and 31% fructose.

4.1.5 Sodium benzoate in strawberry-lime jam (pH 3.0, 31% fructose)

Results of the study concerning the combined effects of sodium benzoate, pH, fructose, and antifungal compounds which exist naturally in sugar reduced jam are shown in Table 5. With the exception of *A. glauca*, sugar reduced jam did not inhibit the growth of selected fungi, however a slight decrease in number of CFU were observed.

Table 5: Antifungal effect of sodium benzoate (in strawberry-lime jam, pH 3.0, 31% fructose).

	Number of cfu / sodium benzoate (%, w/w)			
Name of fungi	Control [2]	0.000	0.020	0.040
Absidia glauca	16	0	0	0
Penicillium brevicompactum	23	3	0	0
Saccharomyces cerevisiae	65	12	0	0
Zygosaccharomyces bailii	72	49	3	0

Data from experiments designed to evaluate the combined effects of sodium benzoate, pH, jam itself, and fructose on growth inhibition of test fungi are presented in Figure 5. The growth of all of the fungi strains affected by sodium benzoate in sugar reduced jam

[2] Wort agar medium (pH 4.0)

system. *Z. bailii* was observed as the most resistant fungi to sodium benzoate in jam system.

While the growth inhibition rate of *Z. bailii* is 95% at 0.08% sodium benzoate in 31% fructose added medium at pH 4.0, it was observed as 95% at 0.02% sodium benzoate in jam system. The same values were calculated for other strains as follows: for *S. cerevisiae* 97% in 0.035% sodium benzoate-treated and 31% fructose added wort medium at pH 4.0, while 100% in 0.02% sodium benzoate-treated jam; for *P. brevicompactum* 81% in 0.025% sodium benzoate-treated and 31% fructose added medium at pH 4.0, while 86% in jam without sodium benzoate; and *A. glauca* was not able to grow on jam.

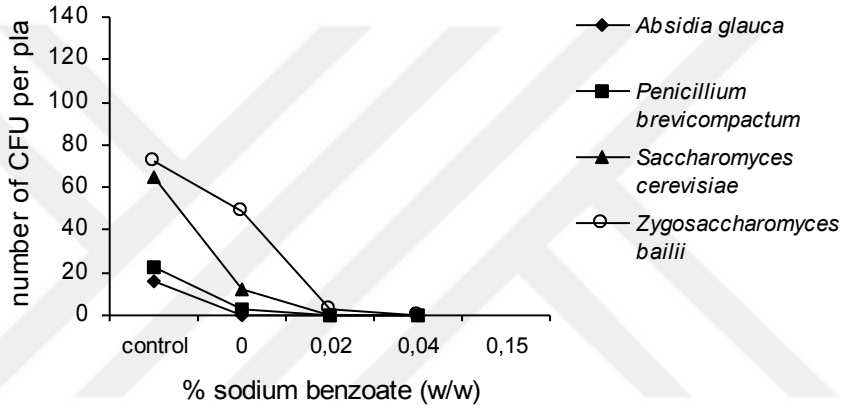

Figure 5: Growth inhibition of different fungi by sodium benzoate in in strawberry-lime jam at pH 3.0 and 31% fructose.

4.1.6 Cranberry extract and cowberry extract in raspberry-aloevera jam (pH 3.0, 31% fructose)

Table 6 and Table 7 display the growth of fungi strains as number of CFU vs. different concentrations of berry extracts in raspberry-aloevera sugar reduced jam systems. The growth of *A glauca* was inhibited by jam without extract. The growth of *P. brevicompactum* was strongly inhibited by 0.005% benzoic acid (3% cranberry extract) in raspberry-aloevera jam.

Table 6: Antifungal effect of cranberry extract in raspberry-aloevera jam at pH 3.0 and 31% fructose.

	Number of cfu / benzoic acid from cranberry (% in jam, w/w)					
Name of fungi	control	0.000	0.005	0.01	0.03	0.04
Absidia glauca	18	0	0	0	0	0
Penicillium brevicompactum	25	4	0	0	0	0
Saccharomyces cerevisiae	71	15	12	6	0	0
Zygosaccharomyces bailii	70	57	56	54	55	40

The growth inhibition rate for *S. cerevisiae* is 78% in jam, and 83% in 0.005% benzoic acid (3% cranberry extract)-treated jam. The rate is lower for *Z. bailii* at the same levels of concentration. 18% in jam, and nearly the same in 0.005% benzoic acid (3% cranberry extract)-treated jam. In 0.04% benzoic acid (24% cranberry extract), the inhibition rate was observed as 42%.

While the growth inhibition rate of *Z. bailii* is 95% in 0.02% sodium benzoate-treated strawberry-lime jam, it was observed as 42% in 0.04% benzoic acid (24% cranberry extract) in raspberry-aloevera jam. The same values were calculated for other strains as follows: for *S. cerevisiae* 100% in 0.02% sodium benzoate-treated strawberry-lime jam, while 91% in 0.01% benzoic acid (6% cranberry extract)-treated raspberry-aloevera jam; for *P. brevicompactum* 86% in strawberry-lime jam without sodium benzoate, while 84% in raspberry-aloevera jam without extract; and *A. glauca* was not able to grow on jam.

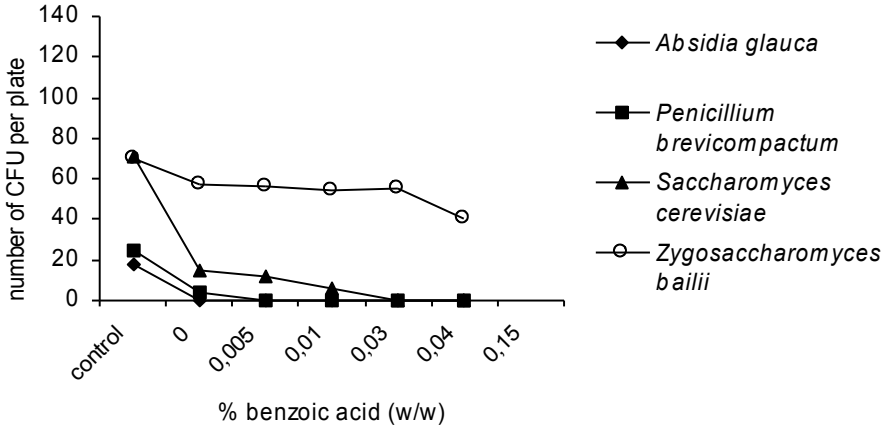

Figure 6: Growth inhibition of different fungi by cranberry extract in raspberry-aloevera jam at pH 3.0 and 31% fructose.

Both cranberry extract and cowberry extract reduced the tolerance of fungi to sugar reduced jam. However, the effect of cowberry extract was observed more effective than

that of cranberry extract at a same concentration level due to different benzoic acid and other possible antifungal compound concentrations in extracts (Figure 6 and Figure 7).

Table 7: Antifungal effect of cowberry extract in raspberry-aloevera jam at pH 3.0 and 31% fructose.

	Number of cfu / benzoic acid from cowberry (% in jam, w/w)					
Name of fungi	Control	0.000	0.005	0.01	0.03	0.04
Absidia glauca	18	0	0	0	0	0
Penicillium brevicompactum	25	4	0	0	0	0
Saccharomyces cerevisiae	71	15	5	0	0	0
Zygosaccharomyces bailii	70	57	55	49	15	8

While the growth inhibition rate of *Z. bailii* is 42% in 0.04% benzoic acid (24% cranberry extract)-treated raspberry-aloevera jam, it was observed as 88% in 0.04% benzoic acid (22% cowberry extract)-treated raspberry-aloevera jam. The same values were calculated for other strains as follows: for *S. cerevisiae* 91% in 0.01% benzoic acid (6% cranberry extract)-treated raspberry-aloevera jam, while 100% in 0.01% benzoic acid (5.6% cowberry extract)-treated raspberry-aloevera jam; for *P. brevicompactum* 86% in raspberry-aloevera jam without sodium benzoate; and *A. glauca* was not able to grow on jam.

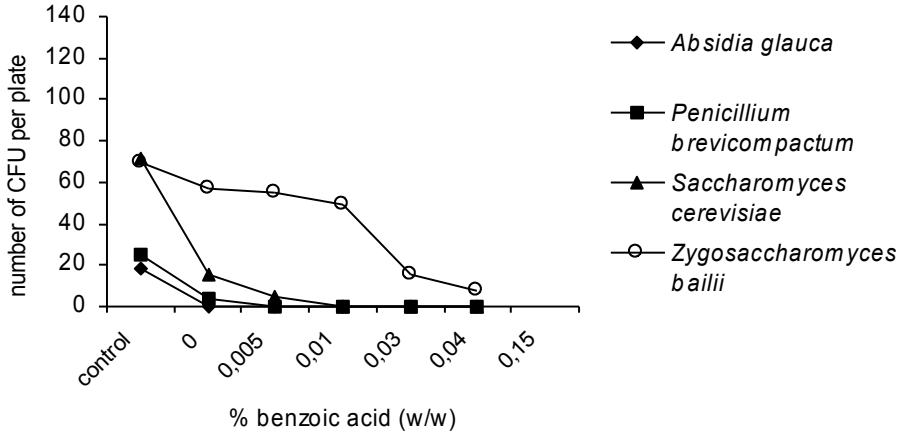

Figure 7: Growth inhibition of different fungi by cowberry extract in raspberry-aloevera jam at pH 3.0 and 31% fructose.

4.1.7 Cranberry extract and cowberry extract in strawberry-guava jam (pH 3.0, 31% fructose)

The repetition of the experiments with the same extracts in different jam resulted not big differences in results (Table 8 and Table 9).

Table 8: Antifungal effect of cranberry extract in strawberry-guava jam at pH 3.0 and 31% fructose.

	Number of cfu / benzoic acid from cranberry (% in jam, w/w)					
Name of fungi	Control	0.000	0.005	0.01	0.03	0.04
Absidia glauca	23	0	0	0	0	0
Penicillium brevicompactum	27	2	0	0	0	0
Saccharomyces cerevisiae	72	22	11	5	0	0
Zygosaccharomyces bailii	80	67	59	54	57	46

While the growth inhibition rate of *Z. bailii* is 42% in 0.04% benzoic acid (24% cranberry extract)-treated raspberry-aloevera jam, it was observed as nearly the same in 0.04% benzoic acid (24% cranberry extract)-treated strawberry-guava jam. The same values were calculated for other strains as follows: for *S. cerevisiae* 91% in 0.01% benzoic acid (6% cranberry extract)-treated raspberry-aloevera jam, while 93% in 0.01% benzoic acid (6% cranberry extract)-treated strawberry-guava jam; for *P. brevicompactum* 84% in raspberry-aloevera jam and 92% in strawberry-guava jam systems without benzoic acid; and *A. glauca* was not able to grow on jam.

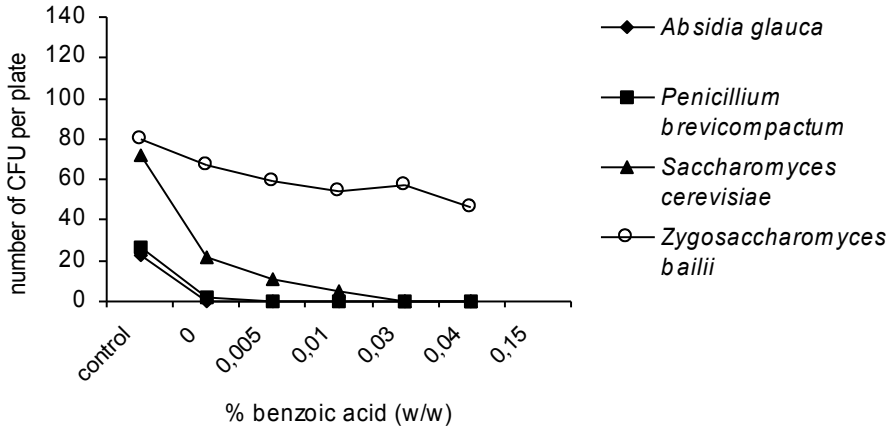

Figure 8: Growth inhibition of different fungi by cranberry extract in strawberry-guava jam at pH 3.0 and 31% fructose.

Comparison of the inhibition effects of different concentrations of berry extracts on different fungi strains reveal that at any treated concentration level of extracts, Z. bailii resisted the highest concentration levels (Figure 8 and Figure 9). At 0.005% benzoic acid (2.8% cowberry extract), the inhibition rate of *S. cerevisiae* is nearly 100%. However, for *Z. bailii*, at 0.04% benzoic acid (22.4% cowberry extract) the rate is nearly 90%.

Table 9: Antifungal effect of cowberry extract in strawberry-guava jam at pH 3.0 and 31% fructose.

	Number of cfu / benzoic acid from cowberry (% in jam, w/w)					
Name of fungi	Control	0.000	0.005	0.01	0.03	0.04
Absidia glauca	23	0	0	0	0	0
Penicillium brevicompactum	27	2	0	0	0	0
Saccharomyces cerevisiae	72	22	5	0	0	0
Zygosaccharomyces bailii	80	67	62	58	20	9

While the growth inhibition rate of *Z. bailii* is 42% in 0.04% benzoic acid (24% cranberry extract)-treated strawberry-guava jam, it was observed as 88% in 0.04% benzoic acid (22% cowberry extract)-treated strawberry-guava jam. The same values were calculated for other strains as follows: for *S. cerevisiae* 93% in 0.01% benzoic acid (6% cranberry extract)-treated strawberry-guava jam, while 93% in 0.005% benzoic acid (2.8%

cowberry extract)-treated strawberry-guava jam; for *P. brevicompactum* 92% in strawberry-guava jam without sodium benzoate; and *A. glauca* was not able to grow on jam.

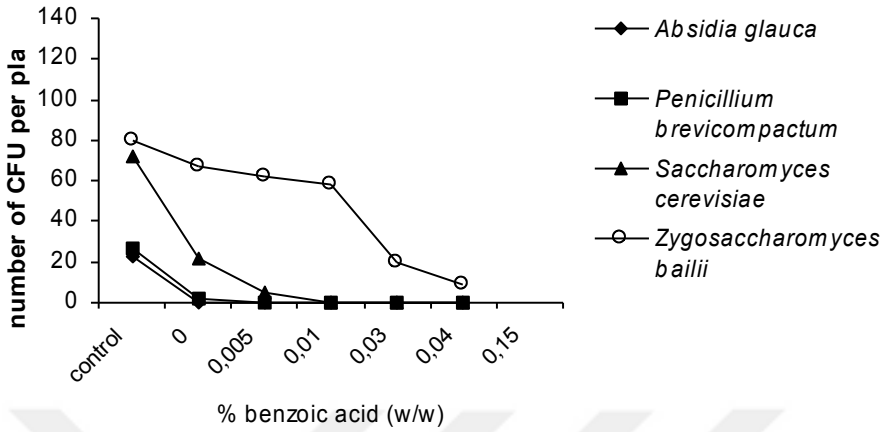

Figure 9: Growth inhibition of different fungi by cowberry extract in strawberry-guava jam at pH 3.0 and 31% fructose.

A significant change in color of the jam occured when the extract was added to the jam systems at different concentrations (Figure 10). With the addition of 3% cranberry extract into strawberry-guava jam, a distinguishable change in color was observed. The change was stronger with the addition of higher concentrations of extracts.

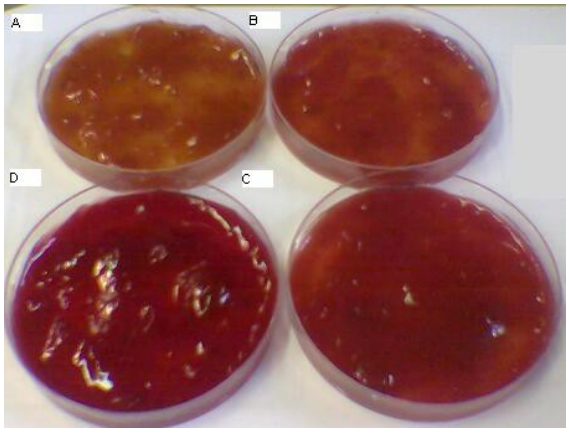

Figure 10: Color difference of strawberry-guava jam with different concentrations (w/w) of cranberry extract. (A- no extract, B- 3% , C- 12%, D- 24%).

4.2 Isolation and determination of moulds/yeasts that are able to grow on sugar reduced jam

Two different mould species were identified according to GenBank database. One species was isolated from one of the petri dish which were put in the kitchens and kept open for 12 hours. After searching for similarities of sequences, it was found as *Eurotium rubrum* with the similarity of 100%. The colonies of *E. rubrum* showed difference in colony structure on wort agar medium at pH 4.5 and on jam at pH 3.0 (Figure 11).

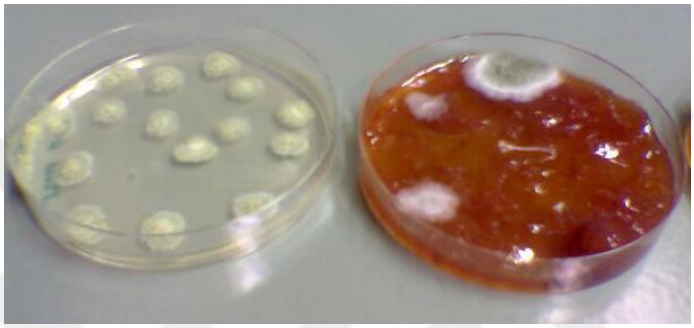

Figure 11: Colonies of *E. rubrum* on wort medium (pH 4.5) and Strawberry-Lime jam (pH 3.0, 31%fructose (w/w), 5.62% cranberry extract (w/w),).

Another mould species was able to grow on jam in jar which was put in the kitchens and kept open for 12 hours. After searching for similarities of sequences, it was found as *Penicillum commune* with the similarity of 99%. Each mould is distinct from other and produces a characteristic colony when grown in wort agar medium at pH 4.5 and on jam systems (Figure 11 and Figure 12).

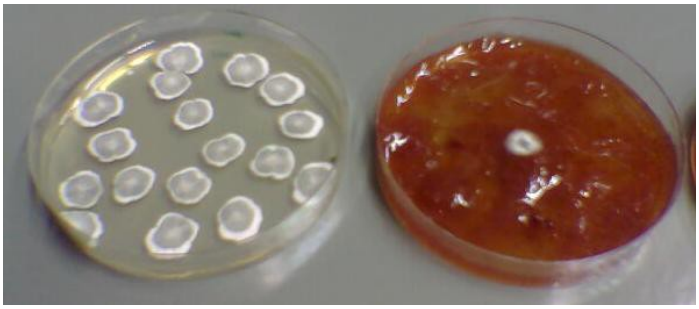

Figure 12: Colonies of *P. commune* on wort medium (pH 4.5) and Strawberry-Lime jam (pH 3.0, 31%fructose (w/w), 5.62% cranberry extract (w/w)).

4.3 Determination of antifungal activities of different fruit extracts against isolated and selected fungi

4.3.1 Cranberry extract and cowberry extract in strawberry-lime jam (pH 3.0, 31% fructose)

The effect of berry extracts on growth of *A. glauca, P. brevicompactum, P. commune,* and *E. rubrum* are presented in Table 10 and Table 11. The higher the concentration, the greater the inhibitory effect of extracts on mould strains. However, *P. commune,* and *E. rubrum* were more resistant to extracts than *A. glauca,* and *P. brevicompactum* (Figure 13 and Figure 14).

The critical inhibitory concentration of cranberry extract on fungi strains seemed to occur between 0% and 0.04% benzoic acid (0% and 24% cranberry extract), using 100% inhibition level for extracts on strains.

Table 10: Antifungal effect of cranberry extract in strawberry-lime jam at pH 3.0 and 31% fructose.

	Number of cfu / benzoic acid from cranberry (% in jam, w/w)					
Name of fungi	Control	0.000	0.005	0.01	0.02	0.04
Absidia glauca	18	0	0	0	0	0
Penicillium brevicompactum	28	2	0	0	0	0
Penicillium commune	32	8	3	1	1	0
Eurotium rubrum	49	15	3	2	1	0

The inhibition rate of *E. rubrum* is 98% at 0.02% benzoic acid (%12 cranberry extract); of *P. commune* 97% at 0.02% benzoic acid (%12 cranberry extract); and that of *P. brevicompactum* is 92% in jam without extract. *A. glauca* was not able to grow on jam.

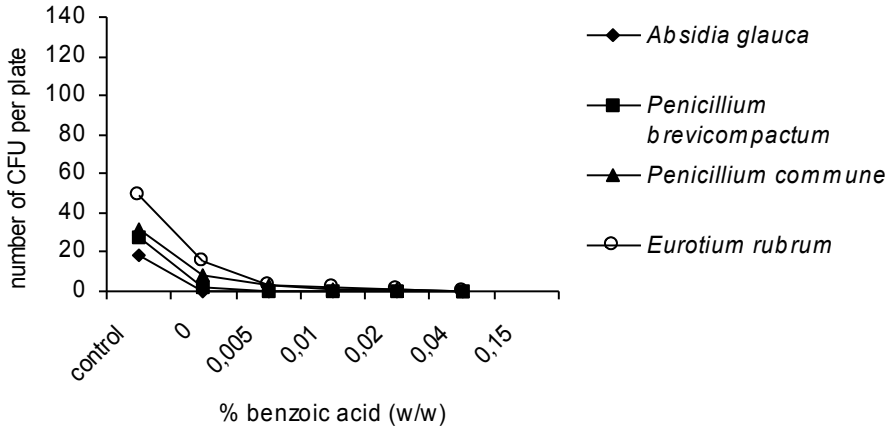

Figure 13: Growth inhibition of different fungi by cranberry extract in strawberry-lime jam at pH 3.0 and 31% fructose.

The critical inhibitory concentration of cowberry extract on fungi strains seemed to occur between 0% and 0.02% benzoic acid (0% and 11% cowberry extract), using 100% inhibition level for extracts on strains.

Table 11: Antifungal effect of cowberry extract in strawberry-lime jam at pH 3.0 and 31% fructose.

	Number of cfu / benzoic acid from cowberry (% in jam, w/w)					
Name of fungi	Control	0.000	0.005	0.01	0.02	0.04
Absidia glauca	18	0	0	0	0	0
Penicillium brevicompactum	28	2	0	0	0	0
Penicillium commune	32	8	3	2	0	0
Eurotium rubrum	49	15	3	1	0	0

While the growth inhibition rate of *E. rubrum* is 98% in 0.02% benzoic acid (12% cranberry extract)-treated strawberry-lime jam, it was observed as 100% in 0.02% benzoic acid (11% cowberry extract)-treated strawberry-lime jam. The same values were calculated for other strains as follows: the growth inhibition rate of *P. commune* 97% in 0.02% benzoic acid (12% cranberry extract)-treated strawberry-lime jam, while 100% in 0.02% benzoic acid (11% cowberry extract)-treated strawberry-lime jam; and that of *P. brevicompactum* is 93% in strawberry-lime jam without extract. *A. glauca* was not able to grow on jam.

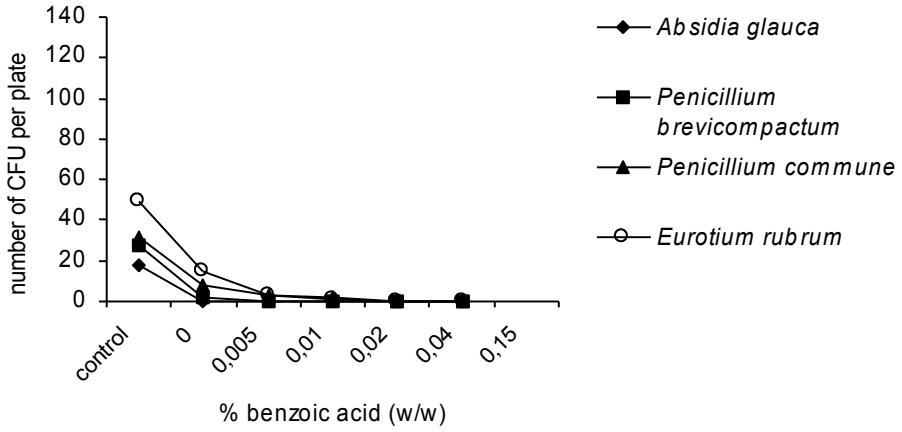

Figure 14: Growth inhibition of different fungi by cowberry extract in strawberry-lime jam at pH 3.0 and 31% fructose.

4.4 Comparison of the effects of different fungistatic agents on different fungi strains

Antimicrobial activities of different antifungal agents and different berry extracts on selected fungi strains was studied on wort agar medium and on sugar reduced jam. In the first step of the study, all the antifungal agents were tested against selected fungi strains (*A. glauca, P. brevicompactum, S. cerevisiae*, and *Z. bailii*). The follow-up studies focused on the berry extracts and on the isolated moulds from jam systems.

The most efficient extract on growth inhibition of *A. glauca* was cowberry extract (Figure 15). When antifungal agents and berry extracts at different concentrations were used, *A. glauca* was clearly affected by all of the antifungal agents and extracts, extracts stronger than agents except sodium benzoate in jam systems. While other agents (sodium benzoate, potassium sorbate, and *p*-hydroxybenzoic acid butyl ester in wort agar medium) and extracts (cranberry and cowberry extracts) inhibit the growth of *A. glauca*, the strongest inhibitor was observed as sodium benzoate in jam systems. The order of the growth inhibition effect of antifungal agents and extracts on *A. glauca* is as follows: sodium benzoate in wort agar medium at pH 4.5< sodium benzoate in 31% fructose added wort agar medium at pH 4.0< potassium sorbate in 31% fructose added wort agar medium at pH 4.0< *p*-hydroxybenzoic acid butyl ester in wort agar medium at pH 4.0 and 31% fructose< sugar reduced jam and extract added jam systems.

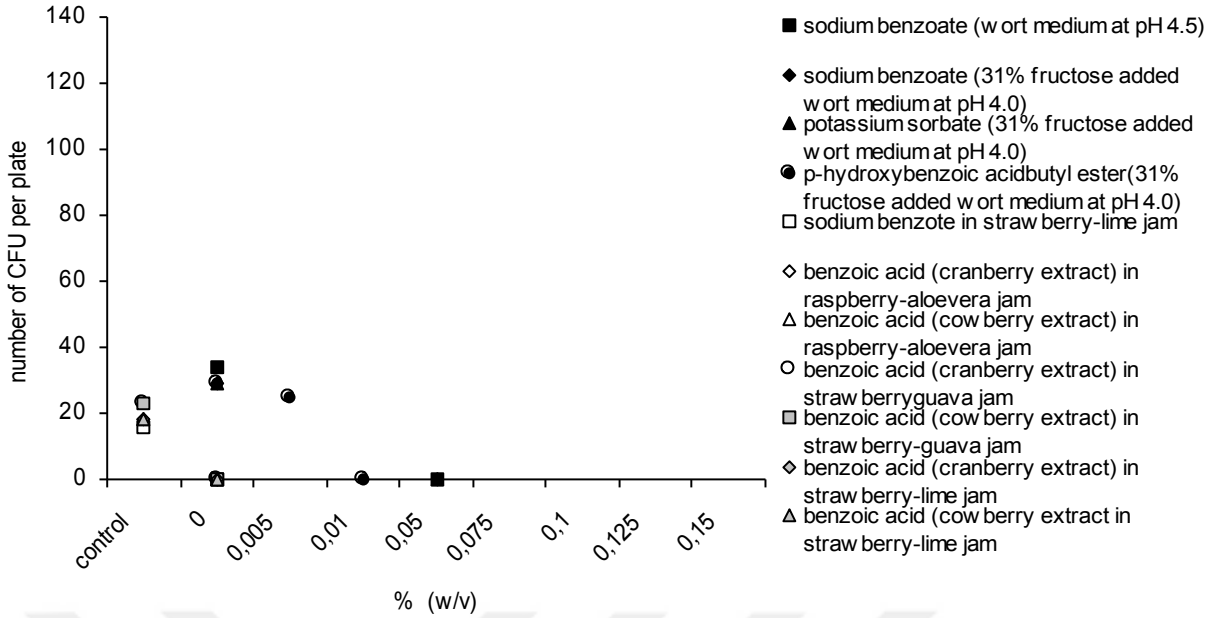

Figure 15: Comparison of antifungal effects of different antifungal agents and berry extracts on *A. glauca*.

Inhibitory effects of antifungal agents on fungi strains clearly increased when the conditions were changed (lowering pH by adding HCl and lowering a_w by adding fructose, and using higher concentration of antifungal agents and extracts).

P. brevicompactum behaved more resistant than *A. glauca* to antifungal agents and extracts (Figure 16). The inhibitory effect of antifungal agents and extracts on the growth of *P. brevicompactum* behaved much the same way as on the growth of *A. glauca*, however *P. brevicompactum* showed a stronger resistance. The order of the growth inhibition effect of antifungal agents and extracts on *P. brevicompactum* is as follows: sodium benzoate in wort agar medium at pH 4.5< sodium benzoate in 31% fructose added wort agar medium at pH 4.0<cranberry extract in raspberry-aloevera jam< *p*-hydroxybenzoic acid butyl ester in 31% fructose added wort agar medium at pH 4.0 < cowberry extract in strawberry-guava jam< cowberry extract in strawberry-lime jam.

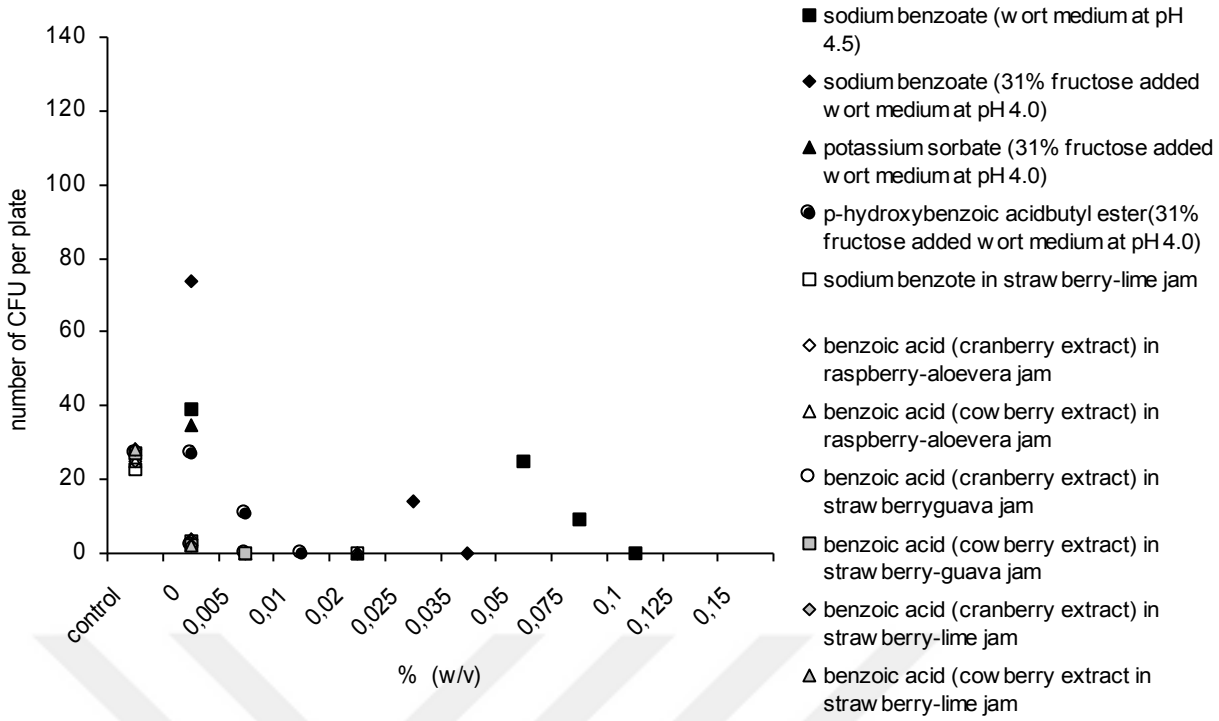

Figure 16: Comparison of antifungal effects of different antifungal agents and berry extracts on *P. brevicompactum*.

The growth of fungi strains were inhibited by both of berry extracts, at different concentrations. The growth of *S. cerevisiae* was strongly inhibited, and the number of cultivable cells decreased with the increase of concentration levels of antifungal agents and extracts (Figure 17). *S. cerevisiae* showed more resistance than mould strains to antifungal agents and extracts. The order of the growth inhibition effect of antifungal agents and extracts on *S. cerevisiae* is as follows: sodium benzoate in wort agar medium at pH 4.5< sodium benzoate in 31% fructose added wort agar medium at pH 4.0 <cranberry extract in strawberry-guava jam< sodium benzoate in strawberry-lime jam< cowberry extract in strawberry-guava jam.

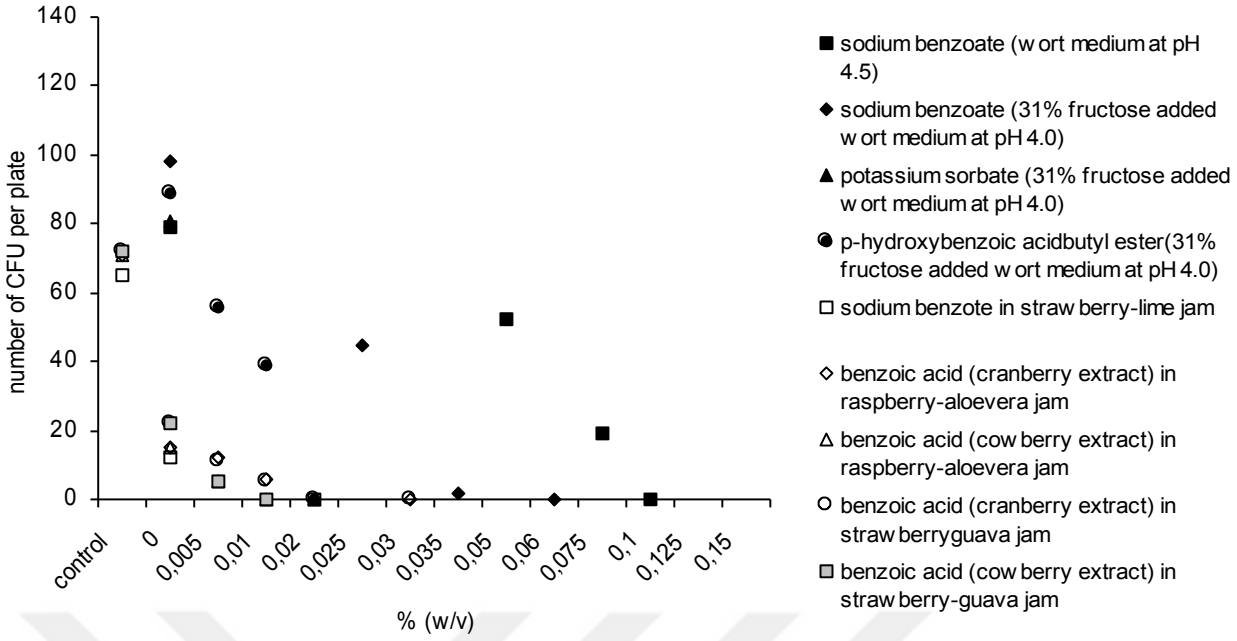

Figure 17: Comparison of antifungal effects of different antifungal agents and berry extracts on *S. cerevisiae*.

Z. bailii was the only exception, showing more resistance, and at the end of cultivation growth was observed even at high berry extract concentrations (24% cranberry and 22% cowberry extract) in jam systems. However, growth of this yeast strain was inhibited by antifungal agents at different concentrations (Figure 18). The order of the growth inhibition effect of antifungal agents and extracts on *Z. bailii* is as follows: sodium benzoate in wort agar medium at pH 4.5< sodium benzoate in 31% fructose added wort agar medium at pH 4.0 <potassium sorbate in 31% fructose added wort agar medium at pH 4.0 < *p*-hydroxybenzoic acid butyl ester in 31% fructose added wort agar medium at pH 4.0 <benzoic acid (cowberry extract) in jam systems< sodium benzoate in strawberry-lime jam

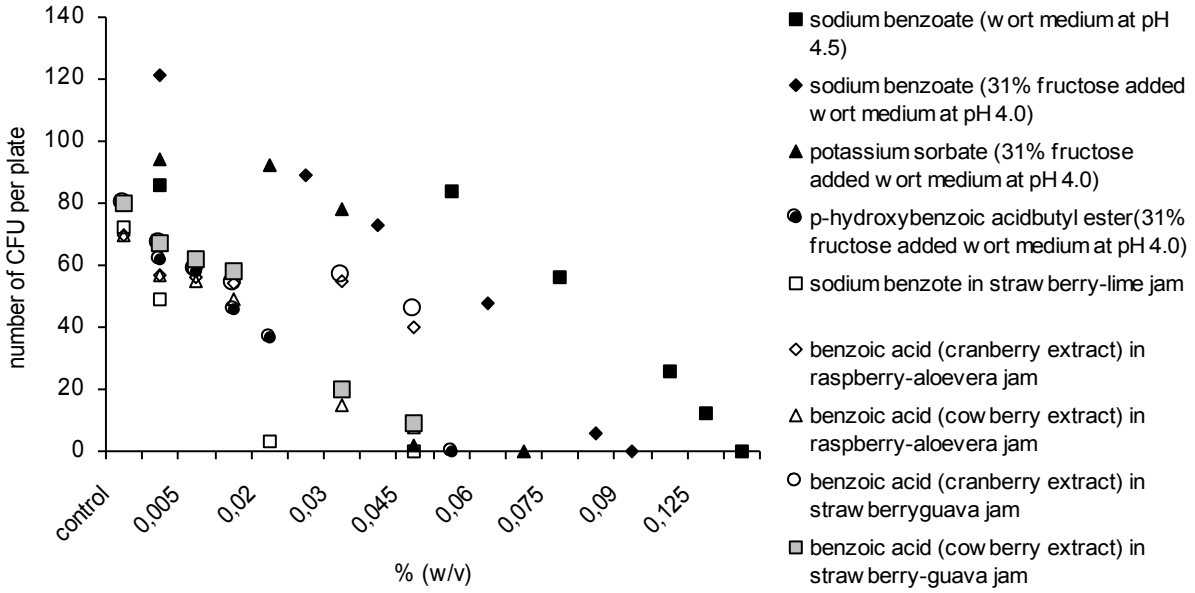

Figure 18: Comparison of antifungal effects of different antifungal agents and berry extracts on *Z. bailii*.

The other studied mould strains (*P. commune* and *E. rubrum*) as well as *A. glauca* and *P. brevicompactum* were sensitive to berry extracts. However, *P. commune* and *E. rubrum* showed higher resistance to berry extracts (Figure 19 and Figure 20). At concentration values of 12% cranberry extract, and 6% cowberry extract, berry extracts were strong inhibitors of *P. commune*. It is the same for *E. rubrum* at the same concentration of extracts.

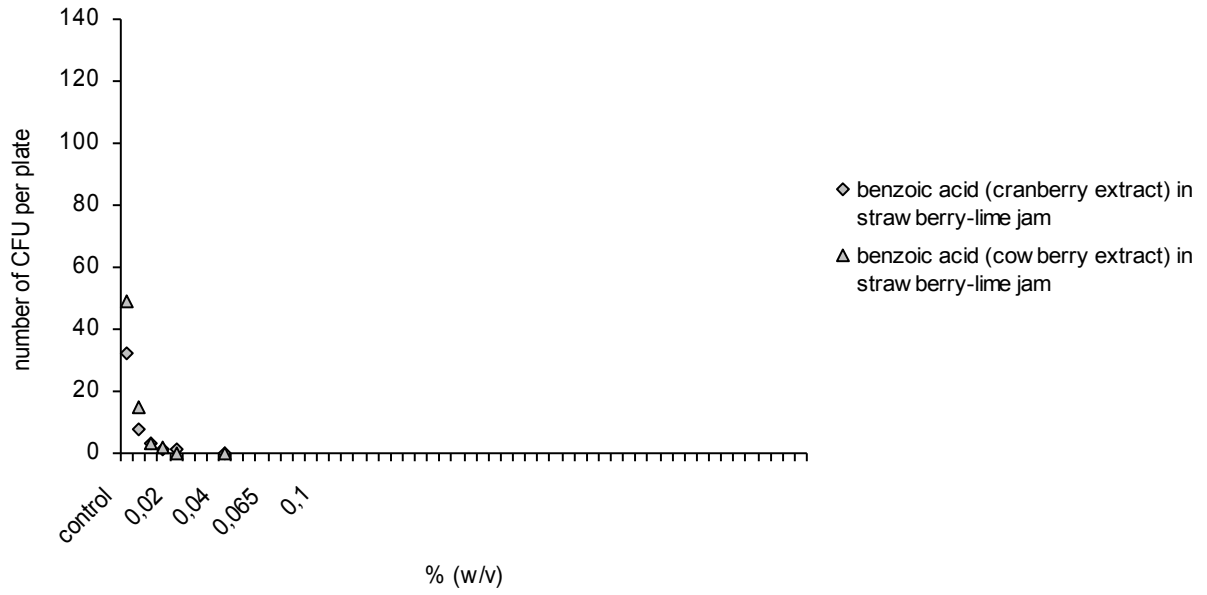

Figure 19: Comparison of antifungal effects of different berry extracts on *P. commune*.

Cowberry extract showed stronger growth inhibition effect than cranberry extract on both *P. commune* and *E. rubrum*. While the growth inhibition rate of *P. commune* is %97 at %0.02 benzoic acid (%12 cranberry extract), it is %100 at %0.02 benzoic acid (%11 cowberry extract). *E. rubrum* behaved the same as *P. commune* against extracts. While the growth inhibition rate of *E. rubrum* is %98 at %0.02 benzoic acid (%12 cranberry extract), it is %100 at %0.02 benzoic acid (%11 cowberry extract).

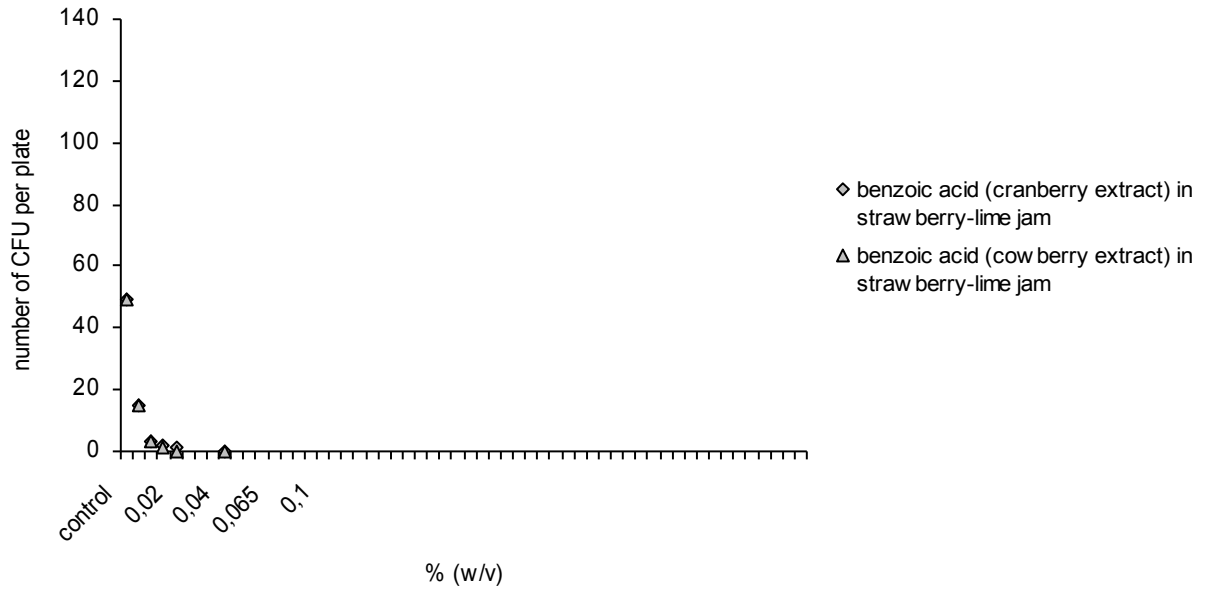

Figure 20: Comparison of antifungal effects of different berry extracts on *E. rubrum*.

The growth of all fungi strains were affected by all antifungal agents and extracts. However the effect of antifungal agents on the growth of *Z. bailii* was the weakest and on the growth of *A. glauca* was the strongest when compared to other strains.

5. Discussion

Information of the antimicrobial properties of the berry extracts and phenolics is scarce in general. In the first part of this study the antimicrobial activities of benzoic acid, sorbic acid, and *p*-hydroxybenzoic acid butyl ester in different conditions in wort agar medium was studied against selected fungi (*A. glauca*, *P. brevicompactum*, *S. cerevisiae*, and *Z. bailii*). In general, these antifungal agents were found to inhibit the growth of selected fungi in different concentrations. Sorbic acid potassium salt and butyl ester of *p*-hydroxybenzoic acid were strong inhibitors for the selected fungi strains.

Gock *et al.* (2003) report that no growth during 100 days for *E. rubrum* in laboratory media (pH 5.5, 25 C) at a_w 0.74. a_w of the systems which was studied in this study were much higher than that value. Because of high a_w, *E. rubrum* could grow well on the sugar reduced jam without/with low concentrations of berry extracts at pH 3.0. Marin *et al.* (2003) observed no growth of *E.chevalieri* in analoge samples with a_w 0.75, pH 6.0 and 0 to 2000 ppm potassium sorbate incubated at 25 C while higher growth rates were reached at a_w from 0.80 to 0.90. Hocking and Pitt (1979) reports that, the minimum a_w for germination and growth is 0.78 at 25°C for *P. brevicomctum*. These authors observed no clear improvement of antifungal activity by a decreasing pH from 4.5 to 3.0 and addition fructose/lowering a_w.

According to Pitt (1974), *S. cerevisiae* tolerates up to 100 mg/kg (0.01%) benzoic acid at pH 2.5-4.0 and 200 mg/kg (0.02%) sorbic acid at pH 4.0 and can grow down to pH 1.6 in HCl. In this study it was observed that *S. cerevisiae* was able to tolerate up to 1000 mg/l (0.1%) sodium benzoate at pH 4.5 and up to 350 mg/l (0.035%) in 31% fructose added wort medium with at pH 4.0. Kalathenos *et al.* (1995) observed growth at pH values of 2.8-3.0, 40-45° Brix, and 800 mg/l benzoic acid in packed fruit cordials. Holdsworth (1983) reports that, yeast development is inhibited when the concentration of undissociated benzoic acid exceeds 25 mg/ml (2.5%). Holdsworth (1983) do not specify differences in growth responses of different yeast species and medium conditions. In this study, *Z. bailii* was able to grow up to 1.5 mg/ml (0.15%) sodium benzoate in wort agar

medium at pH 4.5. It could not grow over this concentration. For *S. cerevisiae,* the concentration was lower than that for *Z. bailii.*

Char *et al.* (2005) observed approximately 2 cm in diameter colony growth of *P. brevicompactum* at 0.83 a_w in milk jam system (pH 6.0) with 1000 ppm (0.1%) potassium sorbate in 20 days at 35 C. The growth was inhibited when the pH decreased to 5.5. Also at 0.79 a_w, *P. brevicompactum* was not able to grow. In this study, *P. brevicompactum* could not grow at 200 ppm (0.02%) potassium sorbate at pH 4.0 in 31% fructose added wort agar medium at pH 4.0. Gock *et al.* (2003) also observed no growth of *E. rubrum* at 0.74 a_w in media

In the second part the antimicrobial activities of sodium benzoate, cranberry and cowberry extracts were studied in different jams against selected and isolated moulds (*P. commune*, *E. rubrum*, *P. brevicompactum*, *A. glauca*). Cowberry extract was the best inhibitor, and *A. glauca* and *P. brevicompactum* were the most sensitive fungi against antifungal agents. Antifungal agents, especially potassium sorbate and *p*-hydroxybenzoic acid butyl ester, showed to be strong antifungal compounds against selected moulds in wort agar medium. Growth inhibition of selected and isolated fungi partly caused by antifungal agents and most of the inhibition seemed to originate from other compounds such as organic acids and other conditions like pH and low a_w in sugar reduced jam systems with berry extracts. Antifungal compounds in berry extracts affected the growth of different fungi species in different mechanisms, not well understood yet.

Mould and yeast strains were clearly inhibited by all antifungal agents and berry extracts indicating that the inhibition was neither strain nor species dependent. However, the concentrations for inhibition of yeasts were much higher than that of moulds. In the recent studies and present experiments, especially the phenolic compounds showed to be responsible for the strong antifungal effects of the berry extracts.

A. glauca and *P. brevicompoactum* were more sensitive to berry extracts, which showed different mode of action, in sugar reduced jam system than antifungal agents in wort agar medium. Berry extracts inhibited the growth of selected and isolated fungi more clearly at the beginning of incubation period of time, and weakening of inhibitory effects were detected during the incubation period. This kind of effect was weaker with extracts in jam

systems than with antifungal agents in wort agar medium. The results suggest that there may be several types of antimicrobial compounds against jam spoilage fungi present in the berry extracts, and these compounds may show different mechanism of action in growth inhibition of the fungi.

Berry extracts inhibited the growth of yeast cells in a different concentration of action compared to mould cells, because inhibition did not weaken during the incubation time, and in many cases no viable cells were detected in first 2 days of incubation. The difference in growth inhibition between yeasts and moulds by the berry extracts was very well illustrted in different shapes of the curves of CFU numbers vs. concentrations.

Comparison of the antifungal effects of antifungal agents and berry extracts clearly showed that phenolic compounds and especially benzoic acid of cranberry and cowberry extracts were responsible for the strong antimicrobial effects against the yeasts and moulds. There was also a strong effect of pH in inhibition of growth as well as that of a_w. However, benzoic acid was only partially responsible for the growth inhibition of fungi and most of the antimicrobial effects probably originated from other compounds, such as other phenolic compounds and conditions, such as low pH and a_w. Benzoic acid has been used in food preservation for a long time. In their undissociated form (in pH below the pK_a value of the acid) the acids may function as permeabilizers of outer membrane of yeast cells and mould spores. It may act as potentiator of the effects of other antimicrobial substances (Brul and Coote, 1999; Alakomi *et al.* 2000). The pK_a of most of organic acids lies between pH 3 and 5, which is the pH area of experiments of this study. Pimiä *et al*. (2004) suggests that one mechanism of action against Gram-negative cells, such as Salmonella is weakening of the outer membrane by the phenolic compounds and organic acids present in the berries. However this has to be further studied and confirmed for fungi.

In the experiments of this study, all strains were affected by antifungal agents and berry extracts. Both of the extracts inhibited the growth of fungi, with the exception of *Z. bailii*. This yeast was also affected by antifungal activity of extracts but the concentration of extract to inhibit the growth was not reached due to requirement of big amount of extract in sugar reduced jam system. More than 25% (w/w) extract was needed, which is not feasible to use in jam. According to Pimiä *et al*. (2004), cranberry contains some specific

soluable compounds, which inhibited the growth of *Listeria* strains. Pimiä *et al*. (2004) also report that, only cranberry extract inhibited the growth from a list of other berries. In this study, both cranberry and cowberry extracts inhibited the growth of fungi in different concentrations. The antifungal activity of other berry extracts should also be evaluated.

The medium pH may have in general great impact on the antimicrobial activity of different phenolic compounds and antifungal agents, and there may be complex interactions between pH of the growth media and antimicrobial effects of the berry phenolics varying in different fungal species and in different berry phenolics.

6. Conclusion

It was observed that lowering pH of the wort medium resulted in lower concentration of sodium benzoate needed to inhibit the growth of fungi. That means, the lower the pH, the lower the amounts of sodium benzoate that is required to inhibit the growth of indicator moulds and yeasts in wort medium.

It was found that, using of granulated fructose to decrease the a_w of the wort medium affected the growth of yeasts and moulds and resulted in lower concentrations of sodium benzoate needed to inhibit the growth in defined incubation period of time.

It was observed that potassium sorbate was more effective than sodium benzoate and *p*-hydroxy benzoic acid butyl ester was more effective than potassium sorbate in same medium conditions and at a same concentration of antifungal agents on fungi strains.

It was found that yeast strains were much more resistant to antifungal agents in same medium conditions and at a same concentration than moulds. *Zygosaccharomyces bailii* was able to grow on higher concentration of antifungal agent contained medium than other fungi strains. *A. glauca* was the most sensitive to antifungal agents and extracts and was not able to grow on sugar reduced jam systems.

Two different species (*P. commune* and *E. rubrum*) of moulds were isolated from the surface of sugar reduced jam system. These species were xerophilic moulds and could grow at high concentrations of berry extracts in sugar reduced jam when compared to other studied moulds in this study.

In total, two kinds of berry extracts (cranberry and cowberry) were studied to understand the effects on inhibition of the growth of moulds on sugar reduced jam. It was observed that the kind of extract used and a well mixed homogen structure of jam and extract itself is very important on the results obtained.

It was observed that different berry extracts have different concentrations of benzoic acid and may have other phenolic compounds and showed diferent antifungal effects at a same concentration in jam systems. Cowberry extract as more effective than cranberry extract at a same concentration in sugar reduced jam.

7. Further Work

More tests should be done to find more precise and accurate concentrations of extracts which inhibit the growth of isolated moulds on sugar reduced jam and the incubation time might be longer to understand the adaptation of moulds to the berry extracts and antifungal agents in it.

The growth inhibition mechanism of berry extracts on fungi should be further studied and possible other antifungal compounds should be studied against fungi strains.

The sensory characteristics of the product after adding berry extracts might be evaluated by a sensory test panel to rank the difference of taste and color of jam and the effects on final quality of the product.

Acknowledgements

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Appendix 1: Undissociation, **pK_a, and pH** values of some antifungal organic acids.

Antifungal agent	Dissociation Constant(K_a)	pK_a[3]	% Undissociated at pH 4.5	pH Range
Benzoic acid	6.46×10^{-5}	4.18	13	2.5-4.0
p-Hydroxybenzoic acid	3.31×10^{-5}	4.48	na[4]	na
Sorbic acid	1.73×10^{-5}	4.76	65	3.0-6.5

Source: Ohio-State, 2006

Appendix 2: Benzoic aicd and *p*-hydroxybenzoic acid content of some fruit extracts.

Name of fruit extract	Genus	*p*-hydroxybenzoic acid (mg/kg extract)	Benzoic acid (g/kg extract)
Blackberry	genus *Rubus*	15.06	na
Strawberry	genus *Fragaria*	40.54	na
Raspberry	genus *Rubus*	27.82	na
Sour Cherry	-	26.37	na
Red Currant	*Ribes sativum* and *R. rubrum*	38.93	na
Cranberry	*Vaccinium oxycoccos* and *V. Macrocarpon*	na	1.65
Cowberry	*Vaccinium vitis-idaea*	na	1.78
Checkerberry	*Gaultheria procumbens*	na	0.87

Source: Plant Foodstuff Institut, University of Hohenheim, 2006

[3] pK_a=-log(Dissociation constant)

[4] not available

Appendix 3: Calculated concentration values of fruit extracts in order to their benzoic acid concentrations in sugar reduced jam systems.

Sodium benzoate (% in jam as w/w)	**Cranberry concentrate** (% in jam as w/w**)**	**Cowberry concentrate** (% in jam as w/w**)**
0.001	0.61	0.56
0.005	3.03	2.81
0.01	6.06	5.62
0.02	12.12	11.24
0.03	18.18	16.85
0.04	24.24	22.47

Appendix 5: Nucleotide sequence of 559 bp fragment of *P. Commune* 28S rRNA gene.

AAGCGGCAAGAGCTCAAATTTGAAAGCTGGCTCCTTCGGGGTCCGCATTGTAATTTGCAGAGGATGC
TTCGGGAGCGGTCCCCATCTAAGTGCCCTGGAACGGGACGTCATAGAGGGTGAGAATCCCGTATGG
GATGGGGTGTCCGCGCCCGTGTGAAGCTCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAA
ATGGGTGGTAAATTTCATCTAAAGCTAAATATTGGCCGGAGACCGATAGCGCACAAGTAGAGTGATC
GAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAGGGAAGCGCTTG
CGACCAGACTCGCTCGCGGGGTTCAGCCGGCATTCGTGCCGGTGTATTTCCCCGCGGGCGGGCCA
GCGTCGGTTTGGGCGGTCGGTCAAAGGCCCTCGGAAGGTAACGCCCCTAGGGGCGTCTTATAGCCG
AGGGTGCAATGCGACCTGCCTAGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCATAATGGTCGT
AAGCGAACCCGTTCTTGAAACACGGACCA

Appendix 6: Nucleotide sequence of 573 bp fragment of *E. Rubrum* 28S rRNA gene.

TCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAATTTGAAATCTGGCCCCTTCGGGGTCCGAGTTGT
AATTTGTAGAGGATGCTTCGGGTGCGGCCCCCGTCTAAGTGCTCTGGAACGGGCCATCGGAGAGGG
TGAGAATCCCGTCTGGGACGGGGTGTCCGCGTCCATGTGAAGCTCCTTCGACGAGTCGAGTTGTTT
GGGAATGCAGCTCTAAATGGGTGGTAAATTTCATCTAAAGCTAAATACTGGCCGGAGACCGATAGCG
CACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAACAGCACGTGAAATTGTTG
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CCGGGGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAAAGGCCCCTGGAATGTAACGCCCCTCGG
GGCGCCTTATAGCCAGGGGTGTCATGCGGCCAGCCTGGACCGAGGAACGCGCTTCGGCACGGACG
CTGGCATAATGGTCGTAAACGACCCGTCTTGAAACACGGACCA

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