İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMPACT OF BEE POLLEN FERMENTATION ON THE PROFILE AND BIOACCESSIBILITY OF PHENOLIC COMPOUNDS

M.Sc. THESIS

Serpil GÖNÜL

Department of Food Engineering

Food Engineering Programme

AUGUST, 2016

İSTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IMPACT OF BEE POLLEN FERMENTATION ON THE PROFILE AND BIOACCESSIBILITY OF PHENOLIC COMPOUNDS

M.Sc. THESIS

Serpil GÖNÜL
(506141529)

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Prof. Dr. Dilek BOYACIOĞLU

AUGUST, 2016

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

POLEN FERMENTASYONUNUN FENOLİK BİLEŞİKLERİN BİYOYARARLILIĞI VE PROFİLLERİ ÜZERİNDEKİ ETKİSİ

YÜKSEK LİSANS TEZİ

Serpil GÖNÜL
(506141529)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Prof. Dr. Dilek BOYACIOĞLU

AĞUSTOS, 2016

Serpil Gönül, a M.Sc. student of İTU Graduate School of Science Engineering and Technology student ID 506141529, successfully defended the thesis/dissertation entitled "IMPACT OF BEE POLLEN FERMENTATION ON THE PROFILE BIOACCESIBILTY OF PHENOLIC COMPOUNDS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Dilek BOYACIOĞLU** ....
İstanbul Technical University

Jury Members : **Assist. Prof. Dr. Dilara ERDİL** ....
İstanbul Technical University

Assist. Prof. Dr. Perihan YOLCİ ÖMEROĞLU ......
Okan University

Date of Submission : 08 September 2016
Date of Defense : 08 August 2016

FOREWORD

I would first like to express my sincere gratitude to Prof. Dr. Dilek Boyacıoğlu for the continuous support of my master study and research, for her patience, motivation, enthusiasm, and immense knowledge. I also would like to express my deepest sense of gratitude to Prof. Dr. Katleen Raes for helping me in countless ways during my Erasmus Programme. Her guidance helped me in all the time of research and writing of this thesis. I sincerely thank to Assoc. Prof. Dr. Esra Çapanoğlu Güven for her valuable guidance as well as supporting me in the scientific and academic field.

I sincerely thank to Nalan Sağlam Demir for her kindly guidance during my experiments. I would like to express my special thanks to SBS Scientific Bio Solutions Industry and Trade Ltd. Co. for supplying bee pollen samples, bee bread and honey. Moreover, it is my pleasure to thank all the master's students and scientific staff in the Laboratory of Food Microbiology and Biotechnology, Department of Industrial Biological Sciences, UGent Campus Kortrijk for their helps.

I also would like to express my gratefulness to my friend Sevil Ercan who was always there for me all these time and helped me.

Finally, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

August 2016

Serpil GÖNÜL
(Food Engineer)

TABLE OF CONTENTS

Page

FOREWORD .... **vii**
TABLE OF CONTENTS .... **ix**
ABBREVIATIONS .... **xi**
LIST OF TABLES .... **xiii**
LIST OF FIGURES .... **xv**
SUMMARY .... **xvii**
ÖZET .... **xix**
1. INTRODUCTION **1**
2. LITERATURE REVIEW **3**
2.1 Bee Pollen 3
2.1.1 Chemical composition of bee pollen 5
2.1.2 Health benefits of bee pollen 9
2.2 Bee Bread 11
2.2.1 Chemical composition of bee bread 12
2.2.2 Health benefits of bee bread 13
2.3 Fermentation 13
2.3.1 Properties of lactic acid bacteria 14
2.3.2 Microbiological properties of bees, bee pollen and bee bread 16
2.4 Antioxidants and Their Benefits 17
2.4.1 Antioxidant properties of bee pollen 19
2.4.2 Antioxidant properties of bee bread 19
2.5 Bioavailability of Phenolic Compounds 19
3. MATERIAL AND METHODS **23**
3.1 Overview of Samples and Experiments 23
3.2 Fermentation 25
3.2.1 Determination of pH 26
3.2.2 Microbiological analysis 26
3.3 Extract Preparation 27
3.4 In Vitro Gastrointestinal Digestion 28
3.5 Chemical Analyses 30
3.5.1 Total phenolic content 30
3.5.2 Total flavonoid content 31
3.5.3 Total antioxidant capacity 32
3.5.3.1 DPPH (Diphenyl-1-pictylhydrazyl) method 32
3.5.3.2 ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method 33
3.5.3.3 CUPRAC (Cupric İon Reducing Antioxidant Capacity) method 34
3.5.3.4 FRAP (The Ferric Reducing Antioxidant Power) method 35
3.5.4 Dry matter content 36
3.5.5 Statistical analysis 37
3.6 Microscopic Analysis 37
4. RESULTS AND DISCUSSION **39**

4.1 Total Phenolics, Total Flavonoids and Total Antioxidant Capacity of Bee Pollen Samples 39
4.2 Total Phenolics, Total Flavonoids and Total Antioxidant Capacity of Bee Bread........ 41
4.3 Fermentation Results........ 43
4.3.1 The pH and acidity results........ 44
4.3.2 Microbiological analysis results........ 46
4.3.3 Dry matter content results 48
4.4 Total Phenolic, Total Flavonoid and Total Antioxidant Capacity of Undigested Bee Pollen, Undigested Fermented Bee Pollen and Undigested Bee Bread Samples 49
4.5 Total Phenolic, Total Flavonoid and Total Antioxidant Capacity of Digested Bee Pollen, Digested Fermented Bee Pollen and Digested Bee Bread Samples.... 58
4.6 Microscopic Analysis 66
CONCLUSION........ 69
REFERENCES 71
APPENDICES 83
APPENDIX A: Calibration curves 83
APPENDIX A 84
CURRICULUM VITAE........ 89

ABBREVIATIONS

ABTS : 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ANOVA : Analyse of Variances
CUPRAC : Cupric İon Reducing Antioxidant Capacity
DPPH : 1,1-diphenyl-2-picrylhydrazil
DM : Dry Matter
FRAP : The Ferric Reducing Antioxidant Power
GAE : Gallic Acid Equivalent
ISO : International Organisation of Standardization
TEAC : Trolox Equivalent

LIST OF TABLES

Page

Table 2.1 : Chemical composition of bee pollen. **6**
Table 2.2 : Amino acid composition of pollen. **7**
Table 2.3 : The basic polyphenolic compounds of bee pollen. **8**
Table 2.4 : List of factors potentially affecting bioavailability of antioxidants in humans. **20**
Table 3.1: Summary of overall analysis **24**
Table 3.2 : Constituents and concentrations of the simulated digestion fluids of *in vitro* digestion. **29**
Table 3.3 : Preparation of enzymes of *in vitro* digestion. **29**
Table 4.1 : Total phenolics, total flavonoids and antioxidant activity in pollen samples (n = 3). **39**
Table 4.2 : Total phenolics, total flavonoids and antioxidant activity in bee bread sample (n=3) **42**
Table 4.3 : The pH value of bee pollen, bee bread and fermented bee pollen samples*. **45**
Table 4.4 : Dry matter content of bee pollen, bee bread and fermented bee pollen samples* **48**
Table 4.5 : Total phenolic content of undigested and digested bee pollen samples, bee bread and fermented pollen samples (mg GAE/100g DM)* **59**
Table 4.6 : Total flavonoid content of undigested and digested bee pollen samples, bee bread and fermented pollen samples (mg catechin/100g DM)* **61**
Table 4.7 : DPPH radical scavenging activities of undigested and digested bee pollen, fermented bee pollen and bee bread samples (mg TEAC/100g DM)* . **64**
Table 4.8 : Total antioxidant capacity of undigested and digested bee pollen, fermented bee pollen and bee bread samples (µmol TEAC /100g DM) based on ABTS method* **64**
Table 4.9 : Total antioxidant capacity of undigested and digested bee pollen, fermented bee pollen and bee bread samples based on CUPRAC method (mg TEAC /100g DM)* **65**
Table 4.10 : Total antioxidant capacity of undigested and digested bee pollen, fermented bee pollen and bee bread samples (µmol Fe+2 /100g DM) based on FRAP method* **65**

LIST OF FIGURES

Page

Figure 2.1 : The bee and its hind legs **3**
Figure 2.2 : Different coloured pollen pellets **3**
Figure 2.3 : Structure of the pollen cell.. .... **4**
Figure 2.4 : Potential therapeutic properties of bee pollen and plausible biological mechanisms by which the pollen compounds act. **9**
Figure 2.5 : Bee bread **11**
Figure 2.6 : Stored bee bread in the comb **11**
Figure 2.7 : Bee pollen **15**
Figure 2.8 : commercial bee bread **15**
Figure 2.9 : Actions of endogenous antioxidants **18**
Figure 2.10 : Definition of bioavailability, bioaccessibility, bioactivity and physiochemical events involved on each stage **20**
Figure 3.1 : Flow diagram of fermented bee pollen production **26**
Figure 3.2 : The structure of diphenyl-1-picrylhydrazyl **33**
Figure 4.1 : Afyon FBP+LB, İzmit FBP+LB and Sivas FBP+LB, respectively…...**44**
Figure 4.2 : Afyon FBP+BB, İzmit FBP+BB and Sivas FBP+BB, respectively...... **44**
Figure 4.3 : Results of microbiological analysis of fermented bee pollen samples.. **46**
Figure 4.4 : Total phenolic content of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples* **50**
Figure 4.5 : Total flavonoid content of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples* **51**
Figure 4.6 : DPPH radical scavenging activities of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples* **53**
Figure 4.7 : Total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples based on ABTS method* **54**
Figure 4. 8 : Total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples based on CUPRAC method* **55**
Figure 4.9 : Total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples according to FRAP method* **56**
Figure 4.10 : Phenolic compounds bioaccessibility in intestinal phase from digested bee pollen, fermented bee pollen and bee bread samples* **59**
Figure 4.11 : Flavonoid compounds bioaccessibility in intestinal phase from digested bee pollen, fermented bee pollen and bee bread samples* **61**
Figure 4.12 : Afyon BF, Afyon AF respectively. **66**
Figure 4.13 : İzmit - BF, İzmit AF respectively.. **66**

Figure 4.14 : Sivas - BF, Sivas AF respectively. **66**

Figure A. 1 : Calibration curve for the total flavonoid content of unfermented pollen samples **84**
Figure A. 2 : Calibration curve for the total flavonoid content of fermented pollen samples **84**
Figure A. 3 : Calibration curve for the total phenolic content of unfermented pollen samples **84**
Figure A. 4 : Calibration curve for the total phenolic content of fermented pollen samples **85**
Figure A. 5 : Calibration curve for the total antioxidant capacity of unfermented pollen samples based on FRAP method **85**
Figure A. 6 : Calibration curve for the total antioxidant capacity of fermented pollen samples based on FRAP method **85**
Figure A. 7 : Calibration curve for the total antioxidant capacity of unfermented pollen samples based on ABTS method **86**
Figure A. 8 : Calibration curve for the total antioxidant capacity of fermented pollen samples based on ABTS method **86**
Figure A. 9 : Calibration curve for the total antioxidant capacity of unfermented pollen samples based on DPPH method **86**
Figure A. 10 : Calibration curve for the total antioxidant capacity of fermented pollen samples based on DPPH method **87**
Figure A. 11: Calibration curve for the total antioxidant capacity of unfermented and fermented pollen samples based on CUPRAC method **87**

IMPACT OF BEE POLLEN FERMENTATION ON THE PROFILE AND BIOACCESSIBILITY OF PHENOLIC COMPOUNDS

SUMMARY

Bee-pollen is well-known by its nutritional and bioactive value in regard to protein, lipids, vitamins or polyphenols. In traditional medicine, alternative diets and supplementary nutrition, bee pollen has been used for many years due to its nutritional properties and health benefits. Reports indicate that pollen is insufficiently digested when entering the human digestive system due to its exine layer. However pollen has a high nutritional value. In this study, fermentation was done with pollen samples collected from local bee producers of three different cities (Afyon, Izmit, and Sivas) in Turkey to increase its digestibility by the degradation of exine layer. As starter culture *Lactococcus lactis* subsp. *Lactis* or commercial bee bread was used for the fermentation experiment. An *in-vitro* gastrointestinal digestion method was done for commercial bee bread, unfermented and fermented bee pollen samples. Total phenolic content, total flavonoid content and total antioxidant capacity analyses (based on the DPPH, ABTS, CUPRAC and FRAP methods) were performed on the unfermented and fermented, either or not digested pollen samples and commercial bee bread.

Successful fermentation result was obtained only in Afyon bee pollen sample because of the high reduction of pH from 4.59 to 3.87-3.82 by the higher activity of LAB. It was seen that the total phenolic and total flavonoid content of unfermented samples decreased due to the fermentation. According to obtained total phenolic content results, decreasing was observed in Afyon fermented bee pollen samples (29%), İzmit fermented bee pollen samples (20%) and Sivas fermented bee pollen samples (50%) on average. In addition to this, according to obtained total flavonoid content results, decreasing was observed in Afyon fermented bee pollen samples (32%), İzmit fermented bee pollen samples (42%) and Sivas fermented bee pollen samples (48%) on average.

In addition to this, fermented pollen samples had a significantly higher antioxidant activity compared to their unfermented sample according to the DPPH and CUPRAC methods. Moreover, according to obtained total antioxidant capacity results based on DPPH method, increasing was observed in Afyon fermented bee pollen samples (52%), İzmit fermented bee pollen samples (44%) and Sivas fermented bee pollen samples (210%) on average. In addition to this, According to obtained total antioxidant capacity results based on CUPRAC method, increasing was observed in Afyon fermented bee pollen samples (36%), İzmit fermented bee pollen samples (58%) and Sivas fermented bee pollen samples (26%) on average. Moreover, the effect of fermentation was not observed on the total antioxidant capacity of the bee pollen based on ABTS and FRAP methods.

A higher total phenolic content, total flavonoid content and total antioxidant capacity were observed compared to unfermented bee pollen samples in all fermented bee pollen samples in all digestion phases according to digestion experiments results. Obtained bioaccessibility results from intestinal phase showed that fermented pollen

samples had a higher phenolic and flavonoid compound bioaccessibility compared to their unfermented samples.

POLEN FERMENTASYONUNUN FENOLİK BİLEŞİKLERİN BİYOYARARLILIĞI VE PROFİLLERİ ÜZERİNDEKİ ETKİSİ

ÖZET

Arı poleni içerdiği protein, yağ, vitamin ve polifenol içeriğine göre sahip olduğu besin ve biyoaktif değerleriyle iyi bilenen bir arı ürünüdür. Sahip olduğu besinsel özellikler ve sağlık etkileri sebebiyle geleneksel tıpta, alternatif diyetlerde ve ilave besinlerde uzun yıllardır kullanılmaktadır. Polen arıların büyüyüp gelişmelerini tamamlayabilmeleri ve salgı bezlerinin gelişmesi için gerekli olan başlıca protein kaynağıdır. Polen yokluğunda koloninin yavru üretip koloninin devamlılığının sağlanması mümkün değildir.

Polen aynı zamanda insan metabolizması için çok değerli besin maddelerini içermektedir. Esas olarak yüksek derecede protein ve karbonhidrat kaynağı olmakla birlikte zengin vitamin ve mineral madde deposudur. Polen insan vücudu için gerekli olan aminoasitlerin de tamamını içermektedir. Polen yüksek besin değerine sahip olmasına rağmen, yapılan çalışmalar sonucu elde edilen veriler göstermiştir ki; polen insanın sindirim sistemine girdiğinde eksin (polenin dış kabuğu) tabakasından dolayı sindirimi yetersiz kalmaktadır. Polenin dış duvarı eksin olarak adlandırılır. Bu tabaka çok nadir olarak bulunan ve çok dayanıklı olan sporopollenin denilen bir yapıdan oluşmaktadır. İç tabaka ise selülozdan yapılmış olup tipik bitki hücre duvarının yapısındadır.

Toplanan arı poleni, bal ve arı tükürüğü arılar tarafından arı ekmeği üretilmek üzere peteklerdeki hücrelerin içerisinde karıştılır ve laktik asit bakterilerinin yardımıyla laktik asit fermentasyonu gerçekleşerek arı poleni arı ekmeğine dönüştürülür. Arı poleni arı ekmeğine dönüştürüldüğünde insan sindirim sisteminde daha kolay sindirilebilir hale gelir bunun sebebi fermentasyonun etkisiyle polenin eksin tabakasında kısmen bir parçalanma olmasıdır. Ayrıca, arı ekmeği arı poleni ile karşılatırıldığında yeni besin madddelerinin polene eklenmesiyle daha zengin hale gelir. Yüksek laktik asit içeriği ve diğer metabolitler arı ekmeğini küflerin diğer mikroorganizmaların sebep olduğu bozunmalara karşı korur.

Arı ekmeğinin içerdiği protein, yağ, mineral, vitamin, flavonoidler ve gerekli aminoasitler ile birlikte arılar ve insanlar için çok önemli bir besin kaynağı olduğu bilinmektedir. Arı ekmeğinin başlıca temel bileşenleri yaklaşık olarak %20 protein, %3 yağlar, %24-53 oranında k.hidratlardır. Bunlara ek olarak, arı ekmeği 25'in üzerinde demir, kalsiyum, fosfor, potasyum, bakır, çinko ve magnesyum gibi farklı makro ve mikro elementleri içerir. Arı ekmeği kansızlık, hepatit diyabet ve mide-bağırsak problemlerinde gibi sağlık sorunları tedavi etmek amacıyla kullanılmaktadır. Bununla birlikte, arı ekmeği antimikrobiyal, antioksidan ve antiradyasyon aktiviteye sahiptir.

Biyoyararlılık, alınan besinin normal fizyolojik fonksiyonlarda kullanılmak ve depolanmak için erişilebilir durumdaki kısmıdır. Flavonoidlerin sindirim kanalından

girişinden sonra emilim işlemi ince bağırsakta gerçekleşmektedir. Emilim derecesi pek çok faktörden etkilenmekte olup flavonoidlerin alt sınıflarında da farklılıklar göstermektedir ve polarite gibi kimyasal özellikler ile ilişkilidir. Ayrıca, alınan dozun ve alım şeklinin, beslenmenin, cinsiyet farklılıklarının, genetik özelliklerin, kolondaki mikrobiyal populasyonun ve tüketilen gıdada mevcut diğer bileşenlerin de emilim ve biyoyararlılığı etkilediği tespit edilmiştir.

Bu çalışmada, eksin tabakasını parçalayarak polenin sindirimini arttırmak için Türkiye'nin 3 farklı ilinden (Afyon, İzmit, ve Sivas) toplanan polen örnekleri fermente edilmiştir. Polen fermentasyonuna başlamadan önce Türkiye'nin 5 faklı ilinden (Antalya, Afyon, Bursa, İzmit and Sivas) toplanan 6 adet farklı polen örneği üzerinde toplam fenolik içeriği, flavonoid içeriği ve toplam antioksidan kapasitesi deneyleri yapılmıştır. Yapılan deneylerin sonuçlarına göre fermentasyon proses için Afyon, İzmit ve Sivas yaz örnekleri seçilmiştir. Fermentasyon prosesi için maya kültürü olarak *Lactococcus lactis* subsp. *Lactis* ya da ticari arı ekmeği kullanılmıştır.

Polenin arı ekmeğinde dönüşmesinde bir çok mikroorganizma görev alır. Bakteriler ve mayalar salgıladıkları enzimleri ile eksinin kısmi parçalanmasını sağlayarak arı ekmeğinin sindirimi kolaylaştırır ve biyoyararlılığını arttırır. Yapılan araştırmalar bal arısının midesinde, arı poleninde ve arı ekmeğinde birbirine benzer mikroorganizmaların etkin olduğunu göstermiştir.

Yapılan çalışmalarda *Lactobacillus, Bifidobacterium* ve *Pasteurelaceae* bakteri türleri izole edilirken, maya olarak *Candida* spp. ve *Torulopsis* spp türleri izole edilmiştir. *Bacilllus* türleri esterases, lipases, proteases, aminopeptidases, phosphatases, ve glycosidases enzimlerini salgılarken, *Candida* Türleri proteases and phospholipases enzimlerini salgılayarak fermentasyona katkı sağlamaktadır.

Ticari arı ekmeği, fermente edilmemiş ve fermente edilmiş arı poleni örnekleri için insan mide-bağırsak sisteminde (ağızda, midede ve ince bağırsakta) sindirim boyunca meydana gelen fizyolojik koşulları taklit etmek amacıyla laboratuvar ortamında mide-bağırsak sindirim modeli uygulanmıştır. Ticari arı ekmeği ile fermente edilmemiş ve fermente edilmiş arı poleni örneklerinin sindirilmemiş ve sindirilmiş örnekleri üzerinde toplam fenolik içeriği, toplam flavonoid içeriği and toplam antioksidan kapasitesi analizleri (DPPH, ABTS, CUPRAC ve FRAP yöntemlerine bağlı olarak) yapılmıştır.

Gıdaların farklı antioksidan içeriklerinden dolayı, hücre içinde de birbirinden farklı tepkimeler oluşmaktadır. Bundan dolayı gıdaların toplam antioksidan kapasitesinin belirlenmesinde tek bir yöntem değil çok sayıda yöntem kullanılmaktadır. Bu yöntemlerle belirlenen kısaca, standart bir antioksidan maddeye göre gıdanın serbest radikali bağlama veya oksidasyonu durdurma gücüdür.

Fermentasyon sonucundan elde edilen sonuçlara göre başarılı fermentasyon sadece Afyon örneğinde gözlenmiştir. Bunun sebebi Laktik asit bakterisinin yüksek aktivesinin etkisiyle pH değerinin 4.59'dan 3.87-3.82 değerlerine düşmesidir. Fermentasyon sonrasında fermente olmuş İzmit örneklerinin pH değerleri 4.09-4.14 ölçülürken, fermente olmuş Sivas örneklerinin pH değerleri 4.05-4.15 olarak ölçülmüştür. Fermente olmuş Afyon örneklerinin hiçbirinde maya üremesi gözlenmemiştir. Bunun sebebi mayaların en iyi 4-6 pH aralığında yüksek üreme kabiliyetine sahip olmalarıdır. Bununla birlikte fermente olmuş İzmit ve Sivas örneklerinde maya üremesi gözlenmiştir.

Fermentasyonun etkisiyle fermente edilmiş Afyon polen örneklerinde Laksit asit bakterilerinin üremesinde 3 logluk artış gözlenirken, fermente edilmiş İzmit ve Sivas örneklerinde sırasıyla 0.5 ve 1 logluk artş gözlenmiştir. Ticari arı ekmeğinde laktik asit bakterisi, maya ve toplam aerobik mezofilik bakteri üremesi gözlenmemiştir.

Fermentasyonun etkisiyle fermente edilmemiş örneklerin toplam fenolik içeriğinde ve toplam flavonoid içeriğinde düşüş gözlenmiştir. Alınan toplam fenolik içeriği sonuçlarına göre, fermente edilmiş Afyon örneklerinde (%29), İzmit örneklerinde (%20) ve Sivas örneklerinde (%50) düşüş gözlendi. Alınan toplam flavonoid içeriği sonuçlarına göre, fermente edilmiş Afyon örneklerinde (%32), İzmit örneklerinde (%42) ve Sivas örneklerinde (%48) düşüş gözlendi.

Buna ek olarak, DPPH ve CUPRAC deneylerinden elde edilen sonuçlara göre fermente edilmiş polen örnekleri fermente edilmemiş polen örneklerine göre önemli derecede yüksek antioksidan aktivitesine sahiptir. DPPH methoduna göre alınan toplam antioksidan kapasitesi sonuçlarına göre, fermente edilmiş Afyon örneklerinde (%52), İzmit örneklerinde (%44) ve Sivas örneklerinde (%210) artış gözlenmiştir. CUPRAC yöntemine göre, fermente edilmiş Afyon örneklerinde (%38), İzmit örneklerinde (%58) ve Sivas örneklerinde (%26) artış gözlendi. Bununla birlikte, ABTS ve FRAP metodlarından alınan sonuçlara göre arı poleninin toplam antioksidan kapasitesi üzerinde fermentasyonun etkisi gözlemlenmemiştir.

Alınan sindirim deneyleri sonuçlarına göre; bütün fermente edilmiş polen örneklerinde fermente edilmemiş polen örneklerine kıyasla bütün sindirim fazlarında daha yüksek toplam fenolik içeriği ve toplam flavonoid içeriği gözlenmiştir. Bütün fermente olmuş polen örneklerinde toplam fenolik içeriği ve toplam flavonoid içeriği değerleri kademeli olarak ağızdan ince bağırsağa doğru artmıştır. İnce bağırsak fazından alınan biyoyararlılık sonuçları fermente edilmiş polen örneklerinin fermente edilmemiş polen örneklerine kıyasla daha yüksek fenolik ve flavonoid bileşen biyoyararlılığına sahip olduğunu göstermiştir.

Toplam antioksidan kapasitesi için alınan sindirim deneyleri sonuçlarına göre; bütün fermente edilmiş polen örneklerinde fermente edilmemiş polen örneklerine kıyasla bütün sindirim fazlarında daha yüksek antioksidan kapasitesi gözlenmiştir. Bütün fermente olmuş polen örneklerinde antioksidan kapasitesi değerlerinin kademeli olarak ağızdan ince bağırsağa doğru arttığı gözlenmiştir.

Fermentasyon öncesinde ve sonrasında yapılan mikroskobik analiz sonuçlarına göre; fermentasyondan sonra her üç polen örneği(Afyon, İzmit ve Sivas) için de eksin tabakasında kısmi bir parçalanma olduğu gözlemlenmiştir. Eksinin kısmi parçalanması ile fermente olmuş polenin sindirimi kolaylaşmış ve insan vücudunda biyoyararlılığı artış göstermiştir. Sivas örneğinin eksin tabakası diğer örneklere kıyasla daha dikenli bir yapıya sahiptir, bu durum eksinin parçalanmasını zorlaştırmaktadır. Fermentasyon öncesinde yapılan toplam antioksidan kapasitesi ve toplam fenolik madde kapasitesi deneylerinde de Sivas yaz örneğinin en düşük değerlere sahip olmasının eksin tabakasının dikenli oluşunun etkisi büyüktür.

Bu yüksek lisans tezinin birinci bölümünde giriş bilgileri; ikinci bölümünde literatür özeti; üçüncü bölümünde kullanılan malzeme ve yöntemler; dördüncü bölümünde çalışmadan elde edilen sonuçlar ve beşinci bölümde ise sonuç ve öneriler konularında bilgi verilecektir.

1. INTRODUCTION

Bee pollen is the male gametophyte of flowers. Bees cover their bodies with pollen dust with the help of their saliva, several combs and hairs when they visit flowers and they then attach the pellets to their hinder legs to transport them to their hives (Campos et al., 2008). Pollen is consumed by the bees and their larvae (LeBlanc et al., 2009). Several traps are designed by beekeepers to collect bee pollen from the hives (Leja et al., 2007).

Bee pollen is an apicultural product containing nutritionally valuable substances, polyphenolic compounds and primarily flavonoids (Kroyer and Hegedus, 2001). It has been used for many years owing to its nutritional properties and health benefits in traditional medicine, alternative diets and supplementary nutrition (Freire et al., 2012). Bee pollen has a protein content ranging from 10 % to 40%, carbohydrates between 13 % and 55 %, and lipids ranging from 1 % to 10 % (Villanueva et al., 2002). Although it has a high nutritional value, it is shown that the availability of nutrients and bioactive compounds of bee pollen is low when the pollen is ingested by humans (Zuluaga et al., 2015). This is owing to the outer layer of bee pollen, known as exine that is very elastic, strong and firm and it is made of sporopollenin which protects the compounds that are within the pollen and ensures chemical and enzymatic resistance to pollen (Atkin et al., 2011; Bogdanov, 2014; Southworth, 1974).

Bee pollen, honey, and bee saliva are mixed by bees into their cells of the honeycomb to produce bee bread that is produced by a lactic acid fermentation (Gilliam, 1979b). Fermentation helps to conserve bee bread from deleterious microorganisms by reducing the pH (Ellis and Hayes, 2009). Bee bread becomes more digestible and enriched with new nutrients by lactic acid fermentation when compared to bee pollen since fermentation causes partly the destruction of the exine layer of pollen (Krell, 1996; Mizrahi and Lensky, 2013). Bee bread is a source of proteins, fats, micro-elements and vitamins for the bees (Marieke et al., 2005).

Bee bread is used for anaemia, hepatitis, diabetes and gastrointestinal problems, in addition, it reduces blood pressure and cholesterol and improves liver functions (Marieke et al., 2005). Moreover, bee bread possesses antimicrobial, antioxidant, hepatoprotective, and antiradiation activity (Ivanišová et al., 2015). Although it is sold at the market because of these health effects, its price is quite expensive.

The aim of this study was to produce fermented bee pollen that is cheaper than bee bread and has the same antioxidant potential and bioavailability of bioactive compounds as commercially available bee bread. To obtain this fermented bee pollen lactic acid bacteria or bee bread as a source of natural yeasts, were used.

Within this context, the objectives of this study were;

(i) to evaluate the fermentation effect on the antioxidant properties, phenolic and flavonoid content of the bee pollen;

(ii) to understand the impact of bee pollen fermentation on the profile and bioaccessibility of phenolic compounds by using an *in vitro* gastrointestinal digestion model.

2. LITERATURE REVIEW

2.1 Bee Pollen

Bee pollen is a fine-powder-like material, originating from flowering plants pollen, and made by worker honey bees by mixing the flowering plants pollen with nectar and bee secretions (Figure 2.1 and Figure 2.2) (Rebiai et al., 2013). Bees ate pollen grains, as this is the most important protein source to survive (Almeida-Muradian et al., 2005). Consumption of pollen is a prerequisite for the development of the brood and for normal colony growth (Ismail et al., 2013).

Figure 2.1 : The bee and its hind legs (anonym, t.y.).

Figure 2.2 : Different coloured pollen pellets (Krell, 1996).

Bees pick up pollen grains from the flowers and store them as pollen pellets on their hind legs with the help of several combs and hairs while collecting during their trips (Almeida-Muradian et al., 2005). Pollen traps of various types are used by beekeepers to obtain the pollen loads from bees when they enter to their hives (Barth et al., 2010). Pollen harvesting, purification and storage are important issues to preserve optimal bee pollen quality. The color of pollen varies from white to black, mostly being yellow, orange or yellowish-brown, but various different colors are possible according to the floral sources (Popov-Raljić et al., 2010). Although spherical shapes predominate, appearance of pollen is in the form of heterogeneous grains with varied shapes and sizes (Popov-Raljić et al., 2010).

Pollen grains can vary quite a lot in size (from about 2.5 to nearly 250 µm) and in diameter. Each pollen grain comprises of vegetative and generative cells surrounded by a double wall of the matrix type which is formed by intine and exine parts (Denisow and Denisow-Pietrzyk, 2016). The inner part, which is called intine, consists primarily of cellulose and pectin, the outer part, which is known as exine, is predominantly formed by a complex carbohydrate sporopollenin (sporoderm) (Denisow and Denisow-Pietrzyk, 2016). The structure of bee pollen is shown in Figure 2.3. Although digestion of sporopollenin and cellulose are very difficult or almost impossible by the honey bee digestive enzymes, Klungness and Peng (1984) found that hemicellulose and pectic acid components could be partially digested by honey bees (Roulston and Cane, 2000).

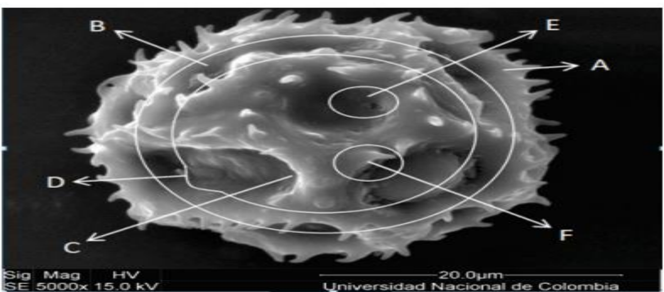

Figure 2.3 : Structure of the pollen cell. Outer part: (A) exine. Inner part ((depicted in imaginary white lines): (B) intine; (C) endoplasmic reticulum; (D) aperture; (E) vegetative nucleus; (F) nucleus of the generative cell (Atkin et al., 2011).

The nutritional requirement to grow colony populations and to maintain their health for honey bee colonies originates from nectar and pollen. Nectar supplies

carbohydrates and the remaining dietary requirements are provided from pollen such as protein, lipids, vitamins, and minerals (Brodschneider and Crailsheim, 2010).

Besides nutritional benefits for bees, bee pollen is a very important nutritional source for human consumption. Fresh bee collected pollen includes nearly 20-30 g water per 100 g (Campos et al., 2010). This high humidity is an ideal culture medium for microorganisms such as bacteria and yeast. Therefore, bee pollen has to be harvested daily and immediately placed in a freezer to prevent spoilage and to preserve its maximum quality or it has to be dried to 7-8 % moisture content and kept in a cool, dark place (Aličić et al., 2014; Campos, et al., 2010). The best drying method is an electric oven, where humidity can continuously run off in order to dry bee pollen (Collin et al., 1995). The maximum temperature for drying is 40°C to prevent the degradation of bee pollen nutrients and the drying time should be as short as possible to avoid losses of volatile compounds (Collin et al., 1995; Krell, 1996).

2.1.1 Chemical composition of bee pollen

The chemical composition of bee pollen can vary owing to their botanical and geographic origin (Almaraz-Abarca et al., 2004). The major components of bee pollen are carbohydrates, crude fibers, proteins and lipids at proportions ranging between 13% and 55%, 0.3% and 20%, 10% and 40%, 1% and 10%, respectively (Villanueva et al., 2002). High ranges are observed in the major composition of bee pollen because they differ in the enviromental conditions, the plant species visited by the bees, collection location, season and year of production (Herbert and Shimanuki, 1978; Serra Bonvehi and Escolà Jordà, 1997; Szczęsna et al., 2002). The overall composition of bee pollen is shown in Table 2.1. (Campos et al.*, 2008; Bogdanov**, 2014), RDI (Required Daily Intake) requirements according to Reports of the Scientific Committee for Food, 2010, average RDI values have been assumed. Amino acid composition of pollen is shown in Table 2.2.

Table 2.1 : Chemical composition of bee pollen(Campos et al.*, 2008; Bogdanov**, 2014).

Main Components	**g* in 100 g**	**% RDI for 15 g Pollen**	**RDI* (g/day)**
Carbohydrates (fructose, glucose, sucrose, fibers)	13-55	1 – 4.6	320
Crude fibers	0.3 – 20	0.3 – 18	30
Protein	10 – 40	5.4 – 22	50
Fat	1 – 13	0.1 – 4	80
Vitamines	**mg* in 100g**	**% RDI for 15 g Pollen**	**RDI (mg/day)**
Ascorbic acid (C)	7 – 56	2 – 15	100
b-Carotene (provitamine A)	1 – 20	30 – 600	0.9
Tocopherol (vitamine E)	4 – 32	8– 66	13
Niacin (B3)	4 – 11	7 – 20	15
Pyridoxin (B6)	0.2 – 0.7	4 – 13	1.4
Thiamin (B1)	0.6 – 1.3	15 – 32	1.1
Riboflavin (B2)	0.6 – 2	12 – 42	1.3
Pantothenic acid	0.5 – 2	2 – 9	6
Folic acid	0.3 – 1	20 – 67	0.4
Biotin (H)	0.05 – 0.07	30 – 42	0.045
Minerals**			
Potassium (K)	400 – 2000	5 – 27	2000
Phosphor (P)	80 – 600		1000
Calcium (Ca)	20 – 300	0.5 – 7	1100
Magnesium (Mg)	20 – 300	2 – 23	350
Zink (Zn)	3 – 25	10 – 79	8.5
Manganese (Mn)	2 – 11	15 – 85	3.5
Iron (Fe)	1.1 – 17	2 – 37	12.5
Copper (Cu)	0.2 – 1.6	4 – 36	1.2

Table 2.2 : Amino acid composition of pollen (Szczêsna, 2006).

Type of Amino Acid	mg/g DM
Aspartic Acid	12.52-28.30
Threonine	5.01-12.49
Serine	6.34-13.26
Glutamic Acid	12.87-29.25
Proline	11.39-32.27
Glycine	5.87-12.76
Alanine	6.80-12.94
Valine	5.74-11.93
Methionine	1.45-4.52
Isoleucine	4.77-10.23
Leucine	8.43-23.10
Tyrosine	2.63-5.87
Phenylalanine	5.03-11.46
Lysine	9.53-21.14
Histidine	3.15-6.16
Arginine	4.68-11.26

The primary amino acids are proline, aspartic acid, phenylalanine and glutamic acid in bee pollen (Roldán et al., 2011). Vitamins and carotenoids, minerals and trace elements and phenolic compounds are other minor components of bee pollen (Bogdanov, 2011). Nevertheless, the composition of bee pollen relies strongly on the plant source as well as on the climatic conditions, soil type, and beekeeper activities (Morais et al., 2011). While there is no significant amount of vitamin C or lipid soluble vitamins in bee pollen, it is rich in B complex vitamins (thiamine, niacin, riboflavin, pyridoxine, pantothenic acid, folic acid and biotin) and carotenoids, which can be provitamin A (de Arruda et al., 2013).

Naringenin, isorhamnetin-3-O-rutinoside, rhamnetin-3-O-neohesperidoside, isorhamnetin, quercetin-3-O-rutinoside, quercetin-3-O-neohesperidoside, kaempferol, quercetin, vanillic acid, protocatechuic acid, gallic acid, p-coumaric acid, hesperidin, rutin, apigenin and luteolin are the main phenolic compounds in bee pollen, which are one of the most critical compounds related to antioxidant activity in pollen and their total amounts varies between 0.3-1.1 % w/w (Bonvehí et al., 2001; Han et al., 2007). The basic phenolic compounds of bee pollen are given in Table 2.3 (Rzepecka-Stojko et al., 2015).

Table 2.3 : The basic polyphenolic compounds of bee pollen (Rzepecka-Stojko et al., 2015).

MAIN POLYPHENOLIC COMPOUNDS OF BEE POLLEN			
Bee Pollen Compound and the Structures of Major Classes		**Free Hydroxyl Groups Positions**	**TEAC [a] (mM)**
1. PHENOLIC ACIDS			
HYDROXYBENZOIC ACIDS:			
Gallic acid		3,4,5	3,0
Protocatechuic acid		3,4	1,2
	Hydroxybenzoic acid		
HYDROXYCINNAMIC ACIDS:			
Caffeic acid		3,4	1,3
Ferulic acid		4	1,9
Chlorogenic acid		3,4	1,3
para-Coumaric acid		4	2,2
ortho-Coumaric acid	Hydroxycinnamic acids	2	1,0
2. FLAVONOIDS			
FLAVONES:			
Luteolin		5,7,3′,4′	2,1
Apigenin		5,7,4′	1,5
Chrysin		5,7	1,4
	Flavones		
FLAVONOLS:			
Quercetin		3,5,7,3′,4′	4,7
Rutin (Q 3-o-rutoside)		5,7,3′,4′	2,4
Kaempherol		3,5,7,4′	1,3
Myricetin		3,5,7,3′,4′,5′	3,1
Galangin		3,5,7	N/D
FLAVANONES:			
Naringenin		5,7,4′	1,5
Pinocembrin		5,7	N/D
	Flavonones		
ISOFLAVONES:			
Genistein		5,7,4′	N/D
	Isoflavones		

[a]Trolox equivalent antioxidant capacity.

The characteristic color, size, morphology, flavor, and composition vary for each pollen pellet specific to the floral species (Di Paola-Naranjo et al., 2004). Composition (minor and major) components of bee pollen are varying a lot due to several reasons: differences in gathering area or time, different processes or storage treatments in

commercial production such as heat-drying, age-related oxidation, ultraviolet (UV) exposure, or irradiation sterilization (Campos et al., 1997; Domínguez- Valhondo et al., 2011).

2.1.2 Health benefits of bee pollen

Nowadays, the products of the honey bee, *Apis Mellifera* L., are of great interest in various fields, e.g. pharmaceutical industries and nutritional applications (Ismail, et al., 2013). Since ancient times bee pollen was used by people for their nutritional benefit despite bee collected pollen began to be used for human nutrition after the second world war (Bogdanov, 2012, chap. 1). Chemical composition of bee pollen is shown in Table 2.1. Bee pollen has an energy content of about 1692 kJ (404.3 kcal) in 100 g and is a good source of energy (Estevinho et al., 2012). Indeed, bee pollen is referred as the ‘‘only perfectly complete food’’ because it includes all the essential amino acids needed for the human body (Pascoal et al., 2014). As well as its nutritional value, bee pollen is considered as a healthy food with a wide range of therapeutic properties, such as antimicrobial, antifungal, antioxidant, anti-radiation, hepatoprotective, chemoprotective/chemopreventive and anti-inflammatory activities (Morais et al., 2011; Pascoal et al., 2014). Potential therapeutic properties of bee pollen and plausible biological mechanisms are shown in Figure 2.4 (Denisow and Denisow-Pietrzyk, 2016).

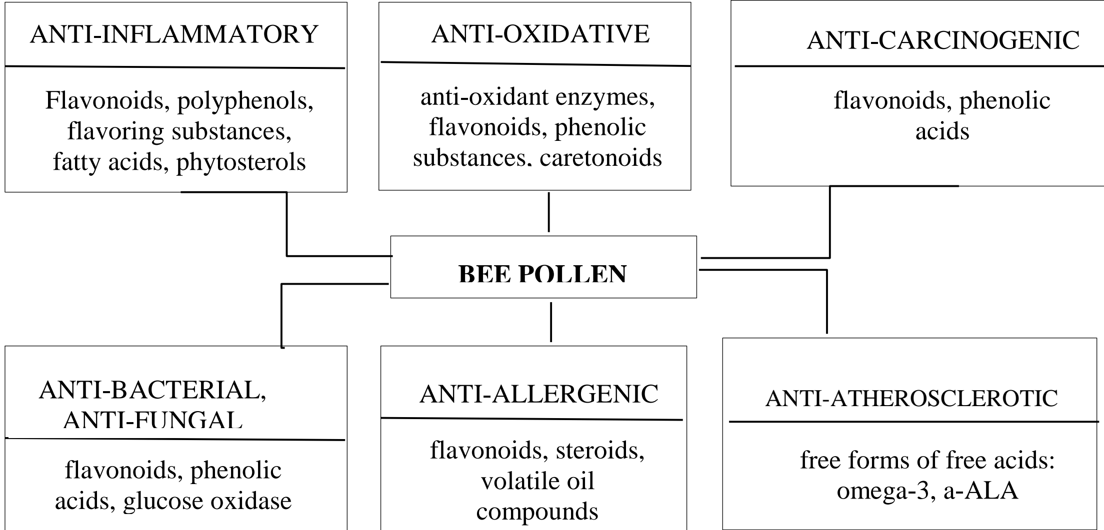

Figure 2.4 : Potential therapeutic properties of bee pollen and plausible biological mechanisms by which the pollen compounds act; abbreviations: ALA – alpha Linolenic acid (Denisow and Denisow- Pietrzyk, 2016).

Bee pollen is used for reducing cravings for sweets and alcohol, as a radiation protectant, blood formation and a cancer inhibitor in Chinese medicine (Ulbricht et al., 2009). Moreover, it has been found that pollen sets off helpful effects in the prevention of prostate problems, arteriosclerosis, gastroenteritis, respiratory diseases, allergy desensitization, improving the cardiovascular and digestive systems, body immunity and delaying aging (Estevinho et al., 2012). Consumption of bee pollen helps to repair of tissues, which results from the acceleration on the mitotic rate (Morais et al., 2011).

Bee pollen has antimicrobial effects (Balch & Balch 1990). Antibacterial activity of Turkish bee pollen was studied against 13 different bacterial species pathogens for plants (*Agrobacterium tumefaciens, A. vitis, Clavibacter michiganensis subsp. michiganensis, Erwinia amylovora, E. carotovora pv. carotovora, Pseudomonas corrugata, P. savastanoi pv. savastanoi, P. syringae pv. phaseolicola, P. syringae pv. syringae, P. syringae pv. tomato, Ralstonia solanacearum, Xanthomonas campestris pv. campestris and X. axonopodis pv. vesicatoria*) (Basim et al., 2006). The results show that the Turkish bee pollen extract have an inhibitory effect against all pathogens and this bee-pollen extract has a potential to become a seed protectant as some of the bacterial pathogens are transmitted through the seeds (Basim et al., 2006). Bee pollen loads collected in 2009 from two locations in Slovakia were tested against pathogenic bacteria, microscopic fungi and yeasts. This research showed that a combination of methanolic and ethanolic extracts of bee pollen samples possessed antibacterial and antifungal effects on bacteria, fungi and yeasts (Kacaniova et. al, 2012). It has been found that bee pollen exhibits antimicrobial properties against pathogenic *Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella enterica and Escherichia coli* (Fatrcová-Šramková et al., 2013).

Pollen has also significant antifungal activity against different pathogens (Koç et al., 2011; Özcan, 2004). The phenolic compounds found in bee pollen are probably responsible for antifungal activity (Cushnie & Lamb, 2005).

According to Kacaniova et al. (2013), it is found that bee pollen supplementation significantly increases the number of *Lactobacillus* spp. and *Enterococcus* spp. in the caecum of broiler chickens. Therefore bee pollen could be used as a potential feed additive with prebiotic activity to the poultry diet. However, the use of bee pollen is low in the industry because of its high price which is around 60 euros per kilogram.

2.2 Bee Bread

Bee bread is composed of pollen, which has been collected by bees and mixed with its digestive enzymes, transported back to the hive, packed into pellets and preserved with a small quantity of honey and bee wax (Nagai et al., 2005). After 2 weeks, this mixture is chemically changed by different enzymes, microorganisms, moisture and temperature (35-36°C) and the fermented pollen is called bee bread (Nagai et al., 2005). A high content of lactic acid and other metabolites protects bee bread from spoilage by molds and by other microorganisms. It is known that bee bread is a source of proteins with essential amino acids, fats, minerals, vitamins, and flavonoids and the most nutritious food for bees (Mutsaers et al., 2005). The chemical composition of bee bread differs slightly from that of pollen; for example, bee bread has a higher acidity as a result of the 6 times higher content of lactic acid compared to pollen. Also it contains larger amounts of vitamin K (Nagai et al., 2005). Bee bread can be stored longer than bee pollen because of the changed composition (Mutsaers et al., 2005). Bee bread and stored bee bread in the comb are given in Figure 2.5 and Figure 2.6.

Figure 2.5 : Bee bread (anonym, t.y.).

Figure 2.6 : Stored bee bread in the comb (anonym, t.y.).

A special instrument that is called a bee bread punch is used by the beekeepers to remove bee bread from the comb (Mutsaers et al., 2005). Fresh bee bread can be stored in the freezer or dried until the moisture content is decreased from 20% to 14% (Mutsaers et al., 2005).

2.2.1 Chemical composition of bee bread

The major components of bee bread are approximately 20% proteins, 3% lipids, 24-35% carbohydrates, 3% minerals and vitamins and it is composed of well-balanced proteins containing all essential amino acids, the full spectrum of vitamins (C, B1, B2, B3, B5, B9, E, H, P) pigments and other biologically active compounds, like enzymes as saccharase, amylase, phosphatase, and flavanoids, carotenoids, hormones (Nagai, et. al, 2005). Additionally, bee bread contains over 25 different micro- and macro-elements such as iron, calcium, phosphorus, potassium, copper, zinc, selenium, magnesium (Nagai, et. al, 2005). Bioactive compounds (flavonoids, phenolic acids and their derivatives) are one of the most critical ingredients related to its bactericidal, antiviral, antifungal and antioxidant effect in bee bread (Čeksterytė et al., 2008).

Some polyunsaturated fatty acids (PUFAs), such as ω-3 and ω-6, are not synthesized in the human body, so they should be consumed with food (Čeksterytė et al., 2008). The ω-3 fatty acids that contain α-linolenic (ALA), docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids are the most important fatty acids in the human diet (Tapiero et al., 2002). Isidorov et al. (2009) studied the fatty acid profile in bee bread samples. Saturated and unsaturated (α-linolenic, linoleic acids) fatty acids were predominant components in the obtained ether extracts. While noticeable amounts (9%) of C16–C18 aliphatic acids and their esters were noted in hexane extracts, small quantities of hexadecanoic, linoleic and α-linolenic acids were identified in methanol extracts of bee bread. In a study of Čeksterytė et al. (2008), twenty-two fatty acids were determined in bee bread. On average, arachidonic and oleic acids were present in 16% and 15%, respectively while the content of arachidic acid was 12%, eicosapentaenoic acid - 8%, α-linolenic acid - 5% and docosahexaenoic - 5%. They identified also capric, lauric, myristic, myristoleic, palmitic, margaric, stearic, oleic, linoleic, γ-linolenic, eicosenoic, eicosatrienoic, behenic, erucic, docosapentaenoic, lignoceric acids.

Baltrušaityte et al. (2007) studied the phenolic fractions of bee bread using a HPLC method. p-coumaric acid, kaempferol, apigenin, and chrysin were identified. Isorhamnetin and trace amounts of ferulic and caffeic acids, as well as naringenin and quercetin as flavonoids were also detected by Isidorov et al. (2009) in ether extracts of five bee bread samples. Kaempferol and apigenin were detected in bee bread samples from Poland in a study of Markiewicz-Żukowska et al. (2013).

2.2.2 Health benefits of bee bread

Bee bread has a positive effect on the immune system of the human body (Markiewicz-Żukowska et al., 2013). Abouda et al. (2011) studied the antibacterial activity of bee bread extracts against some pathogenic bacteria and it has been found that all the samples showed strong antimicrobial activities towards the different bacterial strains tested. Furthermore, the results revealed that the Gram positive bacteria were more sensitive to bee bread than Gram negative bacteria.

Bee bread has high B-vitamin content, this helps to improve the metabolism and the functioning of the nervous system and it also has a positive influence on the production of red blood cells and the haemoglobin count of children and adults (Marieke et al., 2005). It enhances the physical performance of athletes by supplying extra energy (Marieke et al., 2005). According to Kasianenko et al. (2010), when patients take honey in combination with bee bread, it has been shown that a significant hypolipidemic effect was registered in patients (total cholesterol decreased by 15.7%, LDL cholesterol by 20.5%).

Markiewicz-Żukowska et al. (2013) reported that bee bread can also be used as a growth promoter and natural antioxidant in the chicken diets up to 1.5 g BB/kg to enhance growth performance, carcass traits, meat composition, serum constituents and blood hematology, in addition, economical efficiency of Sinai chickens, without negative effects on chickens viability.

2.3 Fermentation

A lot of food preservation methods are used to maintain food at an acceptable level of quality from the time of production to the time of consumption. Fermentation is a process based on the biological activity of microorganisms for the production of a

range of metabolites, which are able to inhibit the growth and survival of undesirable microflora in foods. It is one of the oldest preservation methods (Ross et al., 2002).

2.3.1 Properties of lactic acid bacteria

A starter culture is a microbiological culture that is added to a raw material to produce a fermented food or beverage by accelerating and steering its fermentation process (Leroy and De Vuyst, 2004). Lactic acid bacteria (LAB) have been used to produce fermented foods and beverages for centuries. LAB can decrease the pH by the production of many organic acids such as lactic, acetic and propionic acid, so they help to improve food safety (Salvucci et al., 2016).

They make a contribution to the flavor, microbial safety, improvement of shelf-life, enhancement of texture and sensory profile of the final product (Leroy and De Vuyst, 2004).

Lactococci are homofermentative Gram positive cocci, belonging to the group of LAB, and are generally found on plants and the skins of animals (Casalta and Montel, 2008). *Lactococci* which produce L(+)-lactic acid from glucose grow at 10°C but not at 45°C (Stiles and Holzapfel,1997). *Lactococcus lactis* produces the biggest quantity of lactic acid between 33 °C and 35 °C and at an optimum pH 6.0 (Akerberg et al., 1998).

L. lactis subsp. *lactis* has been extensively used as starter culture for dairy fermentation (i.e. in cheeses, sour cream and butter) (Beresford et al., 2001). Acidification is its main role in dairy fermentation due to L-lactic acid production (Stiles and Holzapfel, 1997). They contribute to the flavor of the food product due to their ability to produce aromatic compounds (alcohols, ketones, aldehydes), citrate, amino acid or fat metabolism, or to the development of texture by producing exopolysaccharides (Smit et al., 2005). Moreover, they are used to preserve food by producing organic acids and bacteriocins and nisin (Delves-Broughton et al., 1996). LAB has an important role by secreting enzymes including esterases, lipases, proteases, aminopeptidases, phosphatases, and glycosidases in the fermentation of bee pollen (Gilliam et al. 1990). These enzymes lead to fermentation and conversion of pollen constituents to form bee bread and they are responsible for softening of the exine wall of pollen before it is ingested (Gilliam et al., 1990). In this study, *L. lactis* subsp. *lactis* (formerly *Lactobacillus xylosus*) (www.bacterio.cict.fr) was focused on

because *L. lactis* subsp. *Lactis* produces esterases, lipases, proteases, aminopeptidases and phosphatases (Chich et al., 1997; Durlu- Ozkaya et al., 2001) and these enzymes could degrade the pollen cell wall during the fermentation. Scanning electron microscope images of bee pollen and commercial bee bread are shown in Figure 2.7 and Figure 2.8 (Bobadilla et al., 2012).

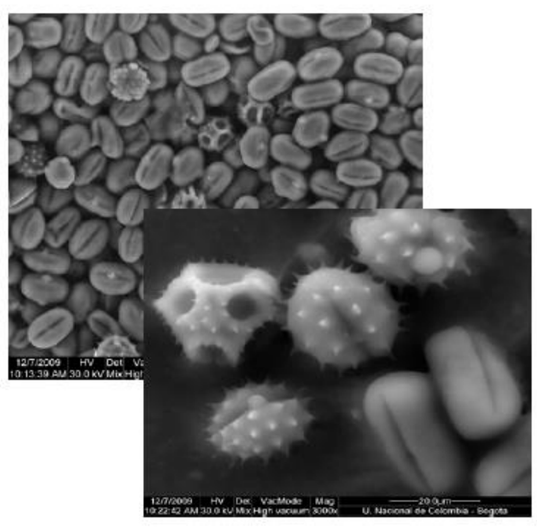

Figure 2.7 : Bee pollen (Bobadilla et al., 2012).

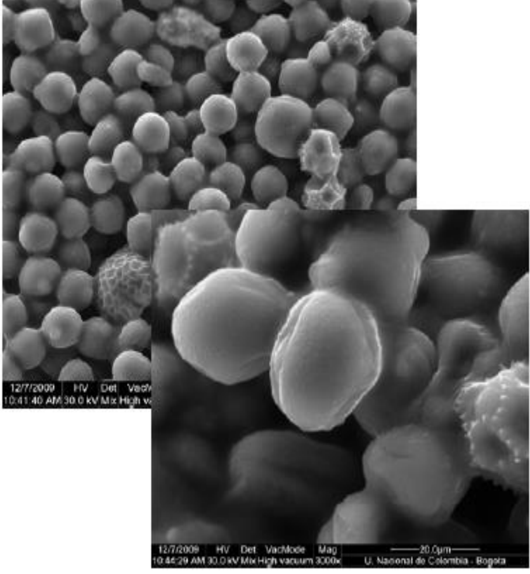

Figure 2.8 : Commercial bee bread (Bobadilla et al., 2012).

2.3.2 Microbiological properties of bees, bee pollen and bee bread

Pollen undergoes the lactic acid fermentation by bacteria and yeasts during the conversion of pollen to bee bread (Foote, 1957; Haydak, 1958). The same species of bacteria and yeasts were found in pollen, beebread and the guts of workers (Gilliam, 1979a).

According to Olofsson and Vásquez (2008), *Lactobacillus* and *Bifidobacterium* phylotypes are dominated in the honey stomach. It has been found that the LAB flora in the honey stomach consists of 10 different phylotypes, five of ten were most closely related to the *L. kunkeei*, *Bifidobacterium asteroids*, and *Bifidobacterium coryneforme* and the other 5 phylotypes were most closely related to the *Lactobacillus* genus.

In the study of Vásquez and Olofsson (2009), it has been found that six *Lactobacillus* phylotypes and two *Bifidobacterium* phylotypes were detected in bee pollen. Moreover, two different phylotypes belonging to the *Pasteurelaceae* family were identified in the bee pollen. All of them were most closely related to either the LAB or the bacteria belonging to the *Pasteurelaceae* family that were detected within the honey stomach of the honeybee *Apis mellifera* by Olofsson and Vásquez (2008) and Vásquez et al. (2009).

Vásquez and Olofsson (2009) studied the detection of the honey stomach LAB flora in two weeks old Swedish bee bread and two month old American bee bread.

It has been found that, six *Lactobacillus* phylotypes and three *Bifidobacterium* phylotypes were identified from the two weeks old Swedish bee bread. Moreover, one phylotype belonging to the *Pasteurelaceae* family was identified. All of them were most closely related to either the LAB or the bacteria belonging to the *Pasteurelaceae* family that were detected within the honey stomach of the honeybee *Apis mellifera* by Olofsson and Vásquez (2008) and Vásquez et al. (2009). Neither LAB nor bacteria belonging to the *Pasteurelaceae* family were detected from the two month old American bee bread (Vásquez and Olofsson, 2009).

It has been shown that *Candida* spp. and *Torulopsis* spp. were dominant yeast flora in honey bees (Gilliam et al., 1974). In the study of Gilliam (1979a), *Candida guilliermondii* var. *guilliermondii*, *Torulopsis magnolia*, *Metschnikowia pulcherrima, Rhodotorula glutinis* var. *glutinis* and *Rhodotorula pallida* were isolated as yeast flora

from bee pollen. Moreover, *Torulopsis magnolia*, *Cryptococcus flavus*, *Cryptococcus laurentii* var. *magnus* and *Rhodotorula glutinis* var. *glutinis* from one week old bee bread, *Torulopsis magnolia*, *Cryptococcus albidus* var. *albidus* from three weeks old bee bread and *Torulopsis magnolia, Cryptococcus albidus* var. *diffluens* and *Cryptococcus albidus* var. *albidus* from six weeks old bee bread were identified as yeast flora.

Yeasts (Gilliam, 1979a) and bacteria belonging to the genus *Bacillus* (Gilliam, 1979b) have an important role in the modification of pollen to bee bread. In the study of Gilliam et al. (1990), it was shown that *Bacillus* species help to convert from bee pollen to bee bread by producing a variety of enzymes including esterases, lipases, proteases, aminopeptidases, phosphatases, and glycosidases. In addition to this, *Bacillus* species could also produce chemicals such as antibiotics and fatty acids to prevent growing of microorganisms that could lead to spoilage of stored food (Gilliam et al., 1990). Moreover, proteases and phospholipases are produced by *Candida* spp. (de Souza Ramos et al., 2015). These enzymes produced by LAB are responsible for softening of the exine wall of pollen (Gilliam et al., 1990) so they may help to degrade the exine layer of pollen. In our study, *L. lactis* subsp. *lactis* was used to imitate the 'lab standardised' beebread production since *L. lactis* subsp. *Lactis* produces esterases, lipases, proteases, aminopeptidases and phosphatases (Chich et al., 1997; Durlu-Ozkaya et al., 2001) and they may aid the degradation of the exine layer of bee pollen.

Although the literature that is available is quite old, especially for the yeast, they are cited in new articles because of the lack of studies about yeast.

2.4 Antioxidants and Their Benefits

Antioxidants are compounds that are used in foods to prevent or slow deterioration, rancidity, or discoloration caused by oxidation (Nagai et al., 2005). Oxidation that is a chemical reaction in which a substance loses an electron so it can produce free radicals, which start chain reactions, resulting in a damage of the cells (Campos et al., 2010). Increasing the concentration of reactive oxygen species (ROS) lead to oxidative stress in cells while exogenous (environmental) and endogenous factors (i.e., the superoxide anion, a natural by-product of the metabolism) result in ROS (Denisow and Denisow-Pietrzyk, 2016). Oxidative stress causes the development of chronic and degenerative diseases e.g. rheumatoid arthritis, cancer, neurodegenerative diseases, aging, cataract,

cardiovascular and autoimmune disorders (Pham-Huy et al., 2008). There are several enzyme systems that catalyze reactions to neutralize reactive oxygen species and free radicals (MatÉs et al., 1999). Endogenous antioxidants: enzymes – catalase (CAT), superoxide dismutase (SOD), and peroxidases are defense systems against ROS in human cells (Derochette et al., 2013). Actions of endogenous antioxidants are shown in Figure 2.9 (Marino, 1998).

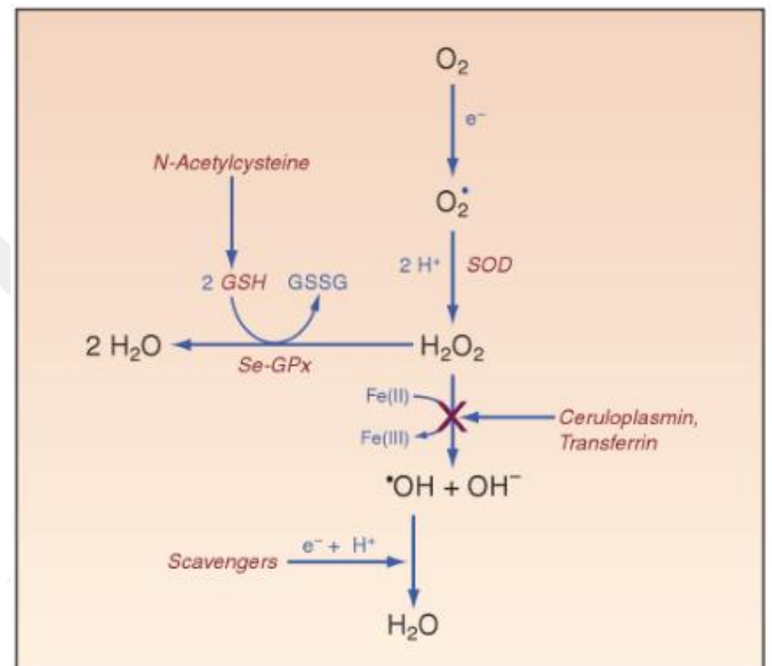

Figure 2.9 : Actions of endogenous antioxidants (highlighted in red). SOD= superoxidase dismutase, Se-GPx= selenium-glutathione peroxidase complex, GSH= reduced glutathione, GSSG= oxidized glutathione (a dipeptide connected by a disulfide bridge) (Marino, 1998).

Antioxidants act as radical scavengers and participate in this cycle in which oxidation reactions can produce free radicals in a way to help in the elimination of the dangerous free radicals and their intermediates so they can inhibit other oxidation reactions by being oxidized themselves (Campos et al., 2010). The natural antioxidants found in foods are phenolic compounds, ascorbic acid, carotenoids, some protein-based compounds, Maillard reaction products, phospholipids and sterols (Choe and Min, 2009).

2.4.1 Antioxidant properties of bee pollen

Several researchers have been reported a close relationship between pollen antioxidant bioactivity and phenolic compounds (Almaraz-Abarca et al., 2004; Campos et al., 2003; LeBlanc et al., 2009). According to these studies, it was found that antioxidant activity of bee pollen depends on pollen species and independent of its geographical origin. Antioxidative effects of bee pollen is associated to the activity of antioxidant enzymes, the content of secondary plant metabolites such as phenolic substances, carotenoids, vitamin C, vitamin E, and glutathione (Carpes et al., 2007). Quercetin, caffeic acid, caffeic acid phenethyl ester (CAPE), rutin, pinocembrin, apigenin, chrysin, galangin, kaempferol, and isorhamnetin are found in bee pollen (Llnskens and Jorde, 1997; Tomás-Lorente et al., 1992). According to Pascoal et al. (2014), flavonoids present in bee pollen provide inactivation of electrophiles, scavenge free radicals, reactive oxygen species (ROS) so they can prevent them from becoming mutagens. Flavonoids may remove toxic metals from the body by binding metal ions (Llnskens and Jorde, 1997).

2.4.2 Antioxidant properties of bee bread

According to Nagai et al. (2004) and Baltrušaitytė, et al. (2007), bee bread possess high antioxidant and free radical scavenging abilities against these radicals such as superoxide anion radical and hydroxyl radical. Both phenolic compounds and flavonoids contribute to the antioxidant potential of natural food products (Larson, 1988). Bee bread has remarkable amounts of proteins, vitamins, flavonoids and phenolic compounds as natural antioxidants (Zuluaga et al., 2015).

2.5 Bioavailability of Phenolic Compounds

Bioavailability has been defined as the fraction of an ingested nutrient or compound which reaches the systemic circulation and the specific sites where it can exert its biological action (Porrini and Riso, 2008). This definition includes three main steps: release from the carrier matrix, intestinal absorption and tissue uptake (Porrini and Riso, 2008). There are a lot factors that influence bioavailability of antioxidants in humans (Porrini and Riso, 2008). They are listed in Table 2.4.

Table 2.4 : List of factors potentially affecting bioavailability of antioxidants in humans (Porrini and Riso, 2008).

Related to the antioxidant	Chemical structure; species/form; molecular linkage; concentration in foods; amount introduced; interaction with other compounds
Related to the food/preparation	Matrix characteristics; technological processing; presence of positive effectors of absorption: fat, protein, lecithin; presence of negative effectors of absorption: fiber, chelating agents; duration of storage
Related to the host	Disorders and/or pathologies; enzyme activity; gender and age; genetics; hormonal status; intestinal transit time; microflora; nutritional and antioxidant status; physiological condition; secretion of HCl
External	Exposure to different environments; food availability

Bioaccessibility has been described as the fraction that is released from food matrix and is available for intestinal absorption (typically based on *in vitro* procedures) (Parada and Aguilera, 2007).

Bioactivity involves tissue uptake and the consequent physiological response (such as antioxidant, anti-inflammatory) is the specific impact upon exposure to a substance (Carbonell- Capella et al., 2014).

Physiochemical events occurred on each stage and definition of bioaccessibility, bioavailability and bioactivity are given in Figure 2.10.

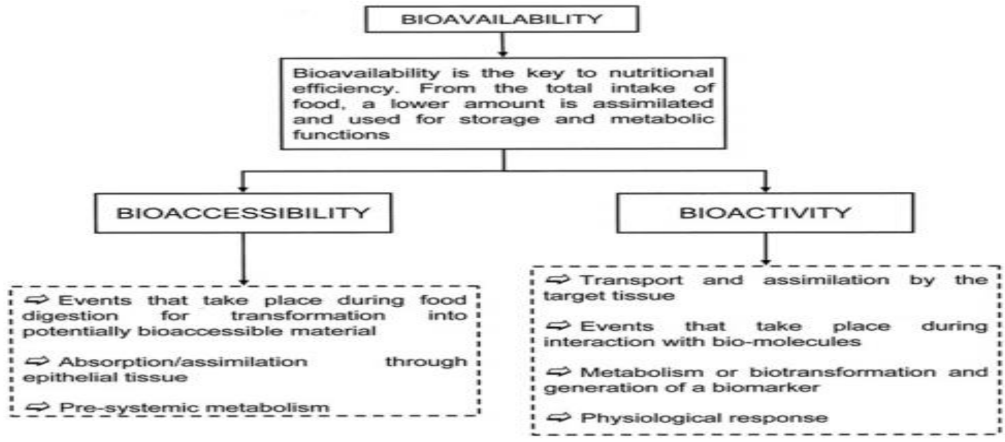

Figure 2.10 : Definition of bioavailability, bioaccessibility, bioactivity and physiochemical events involved on each stage (Fernández-García et al., 2009).

Phenolic compounds that include an aromatic ring, one or more hydroxyl substituents are the main classes of secondary metabolites in plants (Bravo, 1998). Phenolic compounds are classified as simple phenols or polyphenols depending on their number of phenol units in the molecule (Khoddami et al., 2013). When phenolic compounds involve two or more phenolic units, they are called as polypehols. Their biological properties such as bioavailability, antioxidant activity, specific interactions with cell receptors and enzymes are affected by the chemical structure of polyphenols (Scalbert and Williamson, 2000). It is required to know the nature of the main polyphenols ingested, their dietary origin, the amounts consumed in different diets, their bioavailability and the factors controlling their bioavailability to understand their influence upon human health (Scalbert and Williamson, 2000).

The flavonoids are known as secondary plant compounds that have different important physiological and pharmacological activities (Bogdanov, 2014). They have various biological properties such as antioxidant, antiaging, anticarcinogen, antiinflammatory, antiatherosclerosis, cardioprotective and enhance the endothelial function (Bogdanov, 2014). According to Han et al. (2007), they aid indirect protection by activating endogenous defensive systems and by modulating different physiological processes. Variations in the heterocyclic ring C bring about flavonols, flavones, catechins, flavonenes, anthocyanidins and isoflavonoids (Hollman and Katan, 1997). Different types of flavonoids have different rates of absorption and bioavailability.

For example, while isoflavones are the best absorbed, flavanols, flavanones and flavonol glycosides are intermediate and proanthocyanidins, flavanol gallates and anthocyanins are the worst absorbed dietary flavonoids (Viskupicova et al., 2008). Moreover, genetic properties, dosage, diet, sex differences, and the microbial population of the colon influence the absorption (Heim et al., 2002; Kim et al., 2007).

Flavonoids are present primarily as glycosides and the nature of the saccharide in food and status of substitution are significant factors for intestinal absorption (Depeint et al., 2002). Glycosides are exposed to deglycosylation before absorption (Day et al., 1998). Intracellular cytoplasmic β-glucosidase carries out the hydrolysis of the saccharide of flavonoids (Depeint et al., 2002). There are three different types of β-glucosidase in humans: a broad-specificity cytosolic β-glucosidase, lactase phloridzin hydrolase and glucocerebrosidase (William et al., 1996). Big differences in β-

glucosidase activity may play an important role in the bioactivity of flavonoids (Németh, et al., 2003). Passive diffusion of the resulting flavonoid aglycone is the next step after deglycosylation through epithelial cells, which is supported by increased hydrophobicity (Day et al., 1998).

The bioavailability of phenolic compounds are affected by variations in cell wall structure, differences in concentration within plant tissues, their structure and conjugation (Balasundram and et al., 2006; Scalbert and Williamson, 2000).

Several studies has been conducted to evaluate the antioxidant activity of phenolic compounds *in vitro* studies. Although it has shown that phenolic compounds are powerful antioxidants *in vitro* studies, there is still a dispute whether *in vitro* similar effects can be obtained *in vivo* because of the lack of knowledge about regarding whether phenolic compounds can stay with effective chemical forms at enough time in human body (Karakaya, 2004). Therefore, it is of critical importance to evaluate the flavonoid and the phenolic compound absorption and bioavailability in the human gastrointestinal system besides the investigation of the total phenolic and total flavonoid contents of food materials.

Different analytical techniques have been applied to measure bioaccessibility of nutrients and bioactive compounds both *in vivo* and *in vitro* studies. *In-vivo* studies carried out in animals or human are complex, time consuming and expensive (Yesiltas et al., 2014). However, studies carried out in-vitro systems permit the analysis of multiple samples and may supply data about relative potential bioavailability of different polyphenolic components (McDougall et al., 2005). *In vitro* studies have been developed to mimic the physiologic conditions that occur in the human gastrointestinal system (mouth, stomach, and intestine) during digestion (Fernández-García et al., 2009). The chemical and enzymatic composition of saliva, gastric juice, duodenal juice, and bile juice, temperature and shaking or agitation are the basic characteristics of the *in vitro* gastrointestinal methods (Wittsiepe et al., 2001).

3. MATERIAL AND METHODS

3.1 Overview of Samples and Experiments

Pollen samples were collected from local bee producers of five different cities (Antalya, Afyon, Bursa, Izmit, and Sivas) in Turkey by SBS Scientific Bio Solutions Industry and Trade Ltd. Co.. Also one bee bread sample from India was used in this study. There were two types of Sivas pollen, summer and winter. Total phenolic content, DPPH, ABTS and FRAP analyses were done on these 6 different samples (Antalya, Afyon, Bursa, Izmit, Sivas winter and Sivas summer). Based on the results obtained on the 6 different pollen samples, 3 (Afyon, Izmit and Sivas summer) were choosen to work further with in the fermentation studies. All samples (unfermented and fermented) were ground using a laboratory scale grinder, and stored at -20 °C until analysis.

For the fermentation experiment as starter culture *Lactococcus lactis* subsp. *Lactis (*LMG6890) (from Belgian Co-ordinated Collections of Micro-organisms, Laboratory for microbiology, Gent, Belgium) or bee bread was used. Honey sample from a local bee producer from Antalya in Turkey was also purchased. An *in-vitro* gastrointestinal digestion method was performed for unfermented and fermented bee pollen samples.

Various analyses were done on the unfermented and fermented, either or not digested pollen samples (Afyon, Izmit and Sivas). A summary of the analyses performed is shown in Table 3.1.

Table 3.1: Summary of overall analysis.

Samples		Fermentation	Microbiology (MRS, PCA and RBA)	pH	Dry Matter Content	*In vitro* digestion	Extraction	Total phenolic content	Total flavonoid content	DPPH	FRAP	ABTS	CUPRAC
Bee pollen samples	ABP	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	IBP	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	SBP	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	BB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Fermented bee pollen samples	AFBP+BB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	IFBP+BB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	SFBP+BB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	AFBP+LB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	IFBP+LB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	SFBP+LB	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

*ABP: Afyon bee pollen, IBP: Izmit bee pollen, SBP: Sivas bee pollen, BB: Bee bread, AFBP+BB: Bee bread added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen

3.2 Fermentation

The fermentation process was done according to the method proposed by Dany (1988). Three different bee pollen samples (Afyon, Izmit and Sivas) were used for the fermentation process. Two types of fermented bee pollen samples were prepared. In the first method, bee bread as a natural starter of yeasts, was added to the mixture of bee pollen, next to honey and sterilized water. In the second method, starter culture (*Lactococcus lactis* subsp. *Lactis*) was added to the mixture of bee pollen, honey and sterilized water. Lactococcus culture was prepared 24 hours before inoculation was performed in MRS-medium at 30°C.

Firstly, 10 grams of pollen sample were mixed with 5 ml sterilized water and the mix was soaked for 90 minutes in the fermentation flasks. Then, 2.5 ml sterilized water was heated, stirred in 1.5 grams honey and the mixture was boiled for at least 5 minutes. The mixture was cooled, and, when the temperature was approximately 30-32^0C, 1 ml 10^7 cfu/ml starter culture (*Lactococcus lactis* subsp. *Lactis*) or 0,5 grams bee bread was added to the fermentation flasks. The flasks were closed and inoculated at 30^0C. After 3 days, the flasks were removed and placed at 22ºC and the fermentation process was completed 8 days later. All fermented pollen samples were stored at -20°C until further analyses, except for microbiological analysis and pH measurement which was done immediately after sampling. All fermentations were done in triplicate. A flow diagram of fermented bee pollen production is presented in Figure 3.1.

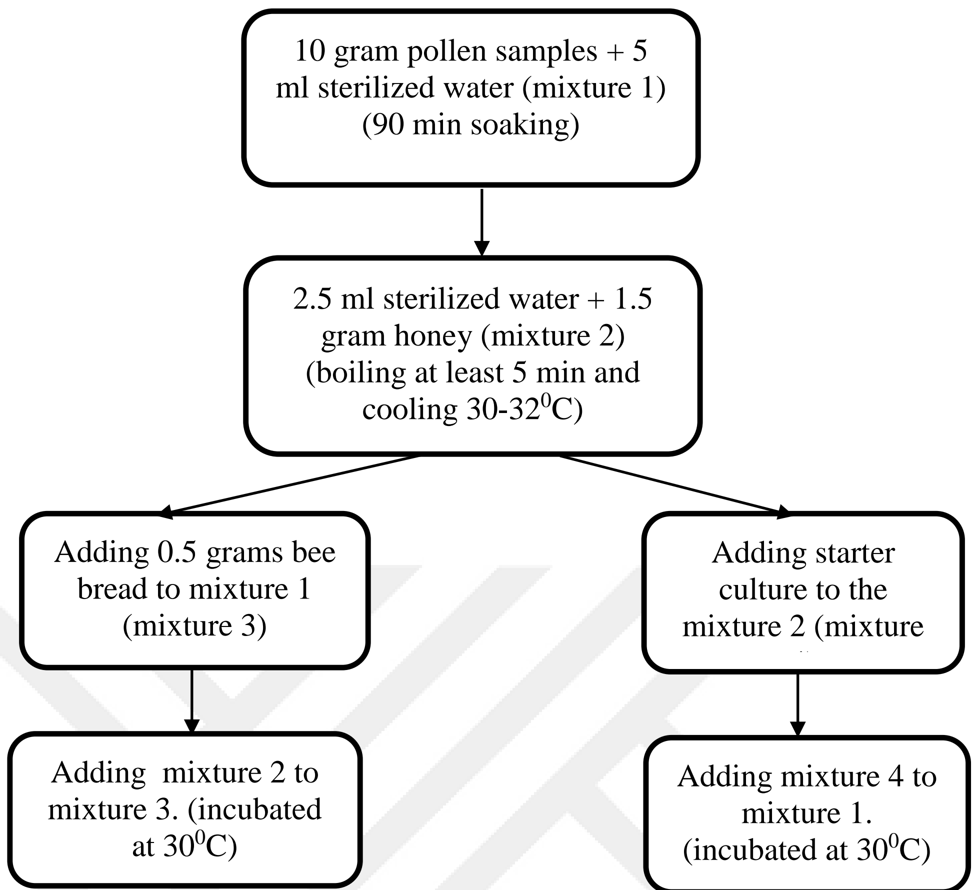

Figure 3.1 : Flow diagram of fermented bee pollen production.

3.2.1 Determination of pH

The measurement of the pH of bee pollen and bee bread samples was performed according to Degrandi-Hoffman, et al. (2013). Therefore, 0,3 g sample of pollen or bee bread were weighed and dissolved in 300 μl of distilled water. The pH value of the bee pollen, bee bread and fermented pollen samples were measured with a pH meter.

3.2.2 Microbiological analysis

Chemicals:

de Man, Rogosa and Sharpe (MRS) agar, plate count agar (PCA), Rose-Bengal chloramphenicol agar (RBA) and bacteriological agar were purchased from Oxoid Limited (Basingstoke, United Kingdom).

Instruments:

Stomacher

Incubator

MRS (de Man, Rogosa and Sharpe agar) medium

MRS medium was prepared for counting lactic acid bacteria. 5.2 g MRS and 7.5 g agar were added to 500 ml distilled water in 500 ml schott flask, and then autoclaved for 20 min at 121°C. 15 ml MRS agar was dispensed in each sterile petri plate. All the plates were stored at 4°C.

PCA (plate count agar) medium

PCA medium was prepared for counting total aerobic mesophilic bacteria. 8.75 g plate count agar was added to 500 ml distilled water in 500 ml schott flask, and then autoclaved for 20 min at 121°C. 15 ml PCA was dispensed in each sterile petri plate. All the plates were stored at 4°C.

RBA (Rose-Bengal chloramphenicol agar) medium

RBA medium was prepared for counting yeast. 16 g Rose-Bengal chloramphenicol agar was added to 500 ml distilled water in 500 ml schott flask, and then autoclaved for 20 min at 121°C. 15 ml RBA was dispensed in each sterile petri plate. All the plates were stored at 4°C.

Procedure:

A 1 g fermented bee pollen sample (AFBP+BB, IFBP+BB, SFBP+BB, AFBP+LB, IFBP+LB, SFBP+LB) or bee bread sample were blended in 9 ml physiological water for 1 min in a stomacher, and then a 1:10 dilution was made. Sequential ten-fold dilutions of the homogenate were made afterwards. Then, 0.1 ml of the appropriate decimal dilutions were plated onto the MRS agar, PCA and RBC agar and incubated at 30^{0}C, 22^{0}C and 22^{0}C for 48-72 h for counting lactic acid bacteria, total aerobic mesophilic bacteria and yeast respectively. The colonies appeared on the plates (30-300 colonies per plate) were counted and expressed as colony forming units (CFU) per 1 g of the sample.

3.3 Extract Preparation

Chemicals:

Pure methanol was purchased from VWR International LLC (Fontenay-sous-Bois, France).

Instruments:

Ultra-turrax

Sonication

Procedure:

All pollen samples and bee bread sample, as well as fermented bee pollen samples underwent an extraction to obtain a concentrated extract of phenolic compounds. According to the method proposed by Mărghitaş et al. (2009), 15 ml methanol was added to the tubes containing 2 g of sample (bee pollen, bee bread and fermented bee pollen). The mixture was homogenized using an ultraturrax for 30s at 7000 rpm. The homogenized mixture was left at room temperature for 1h. After sonication (15min), the mixture was filtered. This procedure was repeated three times and the liquid phase of the extract was collected. Collected liquid phase was evaporated to dryness under vacuum. The resulting dried extracts were dissolved in 10 ml methanol and stored until analysis (-20°C).

3.4 *In Vitro* Gastrointestinal Digestion

Chemicals:

Potassium chloride, sodium chloride, magnesium chloride hexahydrate, sodium hydroxide from VWR International LLC (Leuven, Belgium); α-amylase, pepsin, pancreatin and bile from Sigma-Aldrich Chemie GmBH (Steinheim, Germany); hydrochloric acid from Chem-Lab NV (Zedelgem, Belgium); calcium chloride dihydrate from Chem-Lab NV (Zedelgem, Belgium); monopotassium phosphate from VWR International LLC (Fontenay sous Bois, France); ammonium chloride from VWR International LLC (Haasrode, Belgium) and sodium bicarbonate from Janssen Chimica Company (Geel, Belgium) were purchased.

Instruments:

Shaking water bath

Procedure:

An i*n-vitro* gastrointestinal digestion method was performed according to the procedure described by Minekus et al. (2014). Salivary, gastric, intestinal fluids and enzymes were prepared according to Table 3.2 and Table 3.3. All digestion fluids were completed to 400 ml with distilled water. The pH of the fluids were adjusted to

appropriate values which are indicated in Table 6. Total phenolic and total flavonoid content, as well as total antioxidant capacity were determined on all samples, after each digestion phase, i.e. oral phase, gastric phase and intestinal phase.

Table 3.2 : Constituents and concentrations of the simulated digestion fluids of *in vitro* digestion.

	Simulated Digestion Fluids		
Stock solutions:	**Simulated Salivary Fluid (SSF) (pH:7)**	**Simulated Gastric Fluid (SGF) (pH:3)**	**Simulated Intestinal Fluid (SIF) (pH:7)**
KCl 37.5 g/L	15.1 ml	6.9 ml	6.8 ml
KH_2PO_4 68 g/L	3.7 ml	0.9 ml	0.8 ml
$NaHCO_3$ 84 g/L	6.8 ml	12.5 ml	42.5 ml
NaCl 117 g/L	-	11.8 ml	9.6 ml
$MgCl_2(H2O)_6$ 30.5 g/L	0.5 ml	0.4 ml	1.1 ml
$(NH_4)_2Cl$ 48 g/L	0.06 ml	0.5 ml	-
HCl 6 mol/L	0.09 ml	1.3 ml	0.7 ml

Table 3.3 : Preparation of enzymes of *in vitro* digestion.

Enzymes		
Enzyme	**Desired Activity**	**Preparation**
α-amylase	1500 U/mL	15 mg α-amylase + 10 mL SSF
Pepsin	25000 U/mL	100 mg pepsin + 10 mL SGF
Pancreatin	800 U/mL	80 mg pancreatin + 10 mL SIF
Bile	160 mM	250 mg bile + 10 mL SIF

Briefly, 0.5 g minced bee pollen/bee bread or fermented bee pollen samples were weighted in a 50 ml schott flask for the oral phase. Then, 3.5 ml salivary fluid, 0.5 ml α-amylase solution, 25 µl $CaCl_2$ and 975 µl distilled water were added to the flasks respectively and the mixture was agitated in the shaking water bath for 2 min at 37 °C. Then, 2.5 ml sample was taken. To simulate gastric digestion, 6 ml of gastric fluid, 1.28 ml pepsin solution, 4 µl $CaCl_2$, 0.16 ml 1 M HCl and 0.556 ml distilled water

were added to the flask respectively and the mixture was agitated in the shaking water bath for 2 hours at 37 °C. Then, 2.5 ml sample was taken. For the intestinal phase, 7.7 ml intestinal fluid, 3.5 ml pancreatin solution, 1.75 ml bile solution, 28 µl $CaCl_2$, 0.105 ml 1 M NaOH, 0.917 ml distilled water were added to the flask respectively and the mixture was agitated in the shaking water bath for 2 hours at 37 °C. Then, 2.5 ml sample was taken. All samples were stored at -20°C until further analysis.

3.5 Chemical Analyses

3.5.1 Total phenolic content

The measurement of the total phenolic content (TPC) was performed according to Singleton, et al. (1999).

Chemicals:

Folin-Ciocalteau from Chem-Lab NV (Zedelgem, Belgium); sodium carbonate from VWR International LLC (Leuven, Belgium); gallic acid from Sigma-Aldrich Chemie GmBH (Steinheim, Germany); methanol from VWR International LLC (Fontenay-sous-Bois, France) were purchased.

0.2 N Folin-Ciocalteau reactive

10 ml Folin-Ciocalteau was dissolved in 100 ml double distilled water.

20% Na_2CO_3

40 g Na_2CO_3 was dissolved in 200 ml distilled water.

Instruments:

Spectrophotometer

Principle:

The Folin-Ciocalteau reagent is a solution of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids. It oxidizes phenolates, reducing the heteropoly acids to a blue Mo-W complex. The phenolates are only present in alkaline solution but the reagent and products are alkali unstable. Hence a moderate alkalinity and a high reagent concentration are used in the procedure.

Procedure:

Gallic acid was used as a standard to determine the total phenolic content. To 1 ml of different concentrations of gallic acid (ranging between 0 and 50 mg/L) or to 1 ml of sample (undigested and digested bee pollen, bee bread and fermented bee pollen), 1

ml of deionized water was added. Then, 0.5 ml of 0.2 N Folin-Ciocalteau reagent was added, and the contents were vortexed. After 6 min incubation, 1.5 ml of Na_2CO_3 (20%) solution and 1 ml of deionized water were added, and, after vortexing, the mixture was incubated for 2 h at room temperature in the dark. The absorbance was measured at 760 nm after 2 hours. The results were expressed in mg gallic acid equivalents (GAE)/ 100 g of dry weight samples based on the obtained standard curve.

3.5.2 Total flavonoid content

Total flavonoid content was performed based on Dewanto, et al. (2002).

Chemicals:

Sodium nitrite from UCB Company (Brussels, Belgium); aluminium chloride and sodium hydroxide from VWR International LLC (Leuven, Belgium); catechin from Sigma-Aldrich Chemie GmBH (Steinheim, Germany); methanol from VWR International LLC (Fontenay-sous-Bois, France) were purchased.

5% $NaN0_2$

5 g $NaNO_2$ was dissolved in 100% methanol.

10%$AlCl_3$

10 g $AlCl_3$ was dissolved in distilled water.

1 M NaOH

4 g NaOH was dissolved in distilled water.

Instruments:

Spectrophotometer

Principle:

The principle is based on the attachment of Al into the ring structure, and causing a color change by the effect of NaOH. The role of $NaNO_2$ is providing the nitration of any aromatic ring bearing a catechol group with its three or four positions unsubstituted or not sterically blocked (Pekal and Pyrzynska, 2014).

Procedure:

Catechin was used as standard to determine the total flavonoid content. To 1 ml of different concentrations of catechin (ranging between 10 and 100 mg/L) or to 1 ml of sample (undigested and digested bee pollen, bee bread and fermented bee pollen), 1

ml of deionized water was added. Then, 75 µl $NaN0_2$ (5%) was added, and the contents were vortexed.

After 6 min incubation, 150 µl $AlCl_3$ (10%) was added, and, after 5 min incubation, 0.5 ml NaOH was added. The mixture was incubated for 15 minutes at room temperature in the dark. The absorbance was measured at 510 nm after 15 minutes. The concentration of total flavonoid compound content was calculated as mg of catechin equivalents (catechin)/ 100 g of dry weight samples based on the obtained standard curve.

3.5.3 Total antioxidant capacity

The total antioxidant capacity was estimated by four different assays. The 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, 1,1-diphenyl-2-picrylhydrazil, ferric reducing antioxidant power and cupric ion reducing antioxidant capacity assays were performed according to Re et al. (1999), Kumaran and Karunakaran (2006), Benzie and Strain (1996), and Apak et al. (2004), respectively.

3.5.3.1 DPPH (Diphenyl-1-pictylhydrazyl) method

Chemicals:

DPPH (Diphenyl-1-pictylhydrazyl) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma-Aldrich Chemie GmBH (Steinheim, Germany) and methanol from VWR International LLC (Fontenay-sous-Bois, France) were purchased.

0.1 mM DPPH solution

0.003943 g DPPH was dissolved in 100 ml 100% MeOH.

Instruments:

Spectrophotometer

Principle:

The principle of the reduction of DPPH free-radical assay is that antioxidants react with the stable DPPH radical and convert it into 1,1-diphenyl-2-picrylhydrazine. The ability to scavenge the stable DPPH radical is measured by a decrease in the

absorbance. The absorbance is measured at 517 nm after 30 minutes. Figure 3.2 shows the structure of diphenyl-1-picrylhydrazyl (DPPH).

Figure 3.2 : The structure of diphenyl-1-picrylhydrazyl (Kumarun & Karunakaran, 2007).

Procedure:

To 100 µL of standard Trolox (ranging between 0 and 100 mg/L) or to 100 µL diluted sample (undigested and digested bee pollen, bee bread and fermented bee pollen), 2 ml DPPH solution were added, and mixed for 5 seconds by vortex. A 100% MeOH solvent was used as blank. Absorbance at 517 nm was determined after storing the samples for 30 min in dark at room temperature. The results were expressed as mM Trolox equivalent (TEAC)/100g of dry weight, based on the obtained standard curve. Scavenging activity was calculated as follows (Kumaran & Karunakaran, 2007):

$$(Abs_{blank} - Abs_{sample}) / Abs_{blank} * 100$$

3.5.3.2 ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) method

Chemicals:

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulfate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) from Sigma-Aldrich Chemie GmBH (Steinheim, Germany) were purchased.

7 mM ABTS radical cation stock solution

0.0384 g ABTS was dissolved in 10 mL bidistilled water (Solution A).

2.45 mM $K_2S_2O_8$ (potassium persulfate)

0.0066 g potassium persulfate was dissolved in 10 mL bidistilled water (Solution B).

Instruments:

Spectrophotometer

Principle:

Antioxidants react with ABTS radical resulting in the decolourization of the ABTS radical in the aqueous phase. The loss of colour is measured at 734 nm after 5 minutes.

Procedure:

To evaluate the ABTS radical scavenging activity, equal amounts of solutions A and B were mixed and allowed to stand in the dark at room temperature for 12-16 hours before use (solution C). Work solution (fresh ABTS-solution D) was prepared by diluting the ABTS radical cation (solution C) with 100% methanol to an absorbance of 0.70(± 0.02) at 734 nm. Trolox was used as standard. To 20 µL of standard Trolox (ranging between 0-1.44 mM) or to 20 µl sample (undigested and digested bee pollen, bee bread and fermented bee pollen), 2 ml of ABTS radical cation work solution (solution D) were added and mixed, shaken for 5 seconds by vortex. 100% MeOH was used as blank. Absorbance at 734 nm was determined after 5 minutes. The results were expressed as mM Trolox equivalent (TEAC)/100g of dry weight, based on the obtained standard curve.

3.5.3.3 CUPRAC (Cupric İon Reducing Antioxidant Capacity) method

Chemicals:

Ammonium acetate from VWR International LLC (Leuven, Belgium), neocuproine, copper (II) chloride and trolox Sigma-Aldrich Chemie GmBH (Steinheim, Germany) were purchased.

Copper(II) chloride solution (10^{-2} mM)

0.4262 g $CuCl_2.2H_2O$ was dissolved in 250 ml distilled water.

Ammonium acetate buffer (pH 7.0)

19.27 g of ammonium acetate was dissolved in 250 ml distilled water.

Neocuproine (Nc) solution ($7.5x10^{-3}$ M)

0.039 g of Nc was dissolved in 25 ml 96% ethanol.

Instruments:

Spectrophotometer

Principle:

With this method, the copper (II) (or cupric) ion reducing ability of polyphenols is measured. The method comprises mixing of the antioxidant solution (directly or after acid hydrolysis) with a copper (II) chloride solution, a neocuproine alcoholic solution, and an ammonium acetate aqueous buffer at pH 7 and subsequent measurement of the developed color at an absorbance at 450 nm after 30 minutes.

Procedure:

To 100 µL of standard Trolox (ranging between 0.01 and 0.10 mg/ml) or sample (undigested and digested bee pollen, bee bread and fermented bee pollen), 1 ml of copper(II) chloride solution, 1 ml of Nc solution, 1 ml of ammonium acetate buffer and 1 ml of H_2O were put respectively, and, shaken for 5 seconds by vortex. The absorbance is measured at 450 nm after 30 minutes. The results were expressed as mM Trolox equivalent (TEAC)/100g of dry weight, based on the obtained standard curve.

3.5.3.4 FRAP (The Ferric Reducing Antioxidant Power) method

Chemicals:

Ferrous sulfate heptahydrate and sodium acetate trihydrate from VWR International LLC (Leuven, Belgium); acetic acid, hydrochloric acid and ferric chloride hexahydrate from Chem-Lab NV (Zedelgem, Belgium); 2,4,6-tripyridyl-s-triazine (TPTZ) from Sigma-Aldrich Chemie GmBH (Steinheim, Germany) were purchased.

Acetate buffer (pH 3.6)

0.775 g sodiumacetate trihydrate and 4 ml acetic acid were dissolved in 250 ml distilled water.

TPTZ-solution

0.31233 g TPTZ and 250 µl 12 N HCl were dissolved in 100 ml distilled water.

Ferric chloride hexahydrate-solution

0.5404 g ferric chloride hexahydrate was dissolved in 100 ml distilled water.

Instruments:

Spectrophotometer

Principle:

The antioxidant capacity of the sample solution is determined by its ability to reduce ferric to ferrous ion. When iron is complexed with 2,4,6-tripyridyl-s-triazine (TPTZ) in sodium acetate solution at an acidic pH, its reduction results in a color change of the solution, from pale rust to blue. The absorbance of the solution at 593 nm reflects the extent of reduction.

Procedure:

Fe^{II} solution was used as standard. Acetate buffer (pH 3.6), TPTZ-solution and ferric chloride hexahydrate-solution were prepared and they were mixed in proportion to 10:1:1, respectively to prepare FRAP solution. Briefly, 100 µl standard (ranging between 0-1000 µmol/l) or sample (undigested and digested bee pollen, bee bread and fermented bee pollen) was mixed with 300 µl HPLC water and 3 ml FRAP was added, and the contents were vortexed for 5 seconds. The mixture was incubated for 20 min at room temperature in the dark. The absorbance was measured at 593 nm at the end of the incubation period. The results were expressed as µmol Fe^{+2}/100g of dry weight, based on the obtained standard curve.

3.5.4 Dry matter content

Chemical:
Ethanol from Chem-Lab NV (Zedelgem, Belgium) were purchased.
Instruments:

Oven at 105 °C

Desiccator

Procedure:

The dry matter content of samples was determined by ISO 1442-1973 method. Briefly, 15 grams of sea sand was weighed to aluminum foil recipients and recipients were placed in a preheated oven at 105 °C during one hour, after which they were cooled down in a desiccator for at least 30 minutes and weighed (=M_0). Then, 1 gram of bee pollen, bee bread and fermented bee pollen sample was added to the aluminum recipients and weighed again (=M1). Samples were then mixed with 5 ml of 95% ethanol and placed in the oven for 3.5 hours after which they were cooled down in a desiccator for about 45 minutes to one hour and weighed (=M_2). Dry matter content was calculated as seen in equation 3.1.

$$\%DM= [(M_2-M_0)x100]/(M_1-M_0) \quad 3.1$$

Where:

% DM = gram dry matter per 100 g sample

M_0= mass of the preheated sea sand (g)

M_1= mass of the sea sand and sample before drying (g)

M_2= mass of the sea sand and sample after drying (g)
All samples were performed in triplicate.

3.5.5 Statistical analysis

All analyses were performed as three replicates. The standard deviation of the technical replicates was always lower than 5% of the mean value. Data were subjected to statistical analysis using SPSS for the analysis of variance (ANOVA). Tukey's test was used to analyze differences between samples ($p<0.05$).

3.6 Microscopic Analysis

Chemicals:

Basic fuchsine from Sigma-Aldrich Chemie GmBH (Steinheim, Germany); glycerin from Resources of Nature (South Plainfield, USA); gelatin from VWR International LLC (Fontenay-sous-Bois, France); ethanol and phenol from Chem-Lab NV (Zedelgem, Belgium) were purchased.

Instruments:

Microscope
Balance
Heater

Procedure:

40 grams gelatin were weighted, 210 ml distilled water was added to gelatin. The mixture was mixed for 2 hours by using heater (<40oC). After then, 250 ml glycerin and 1 gram phenol were added to the mixture. The mixture was heated for 15 minutes. Basic fuchsine ethanol (% 0,1(w/v)) was added to the mixture and pollen pellets were dyed for microscopic analyses.

4. RESULTS AND DISCUSSION

4.1 Total Phenolics, Total Flavonoids and Total Antioxidant Capacity of Bee Pollen Samples

Five different spectrophotometric analyses were performed on methanolic extracts of the pollen samples collected from various cities in Turkey to obtain insight in the phenolic compounds and their antioxidative capacity. The data obtained for total phenolic, total flavonoid and total antioxidant contents of pollen samples are shown in Table 4.1. Total phenolic and total flavonoid contents in pollen samples ranged from 756 to 1261 mg GAE/100g of DM and from 559 to 778 mg catechin/100g of DM, respectively. Antioxidant activity values of pollen samples were determined in the range of 4517-8239 µmol TEAC/100g of DM, 275-922 mg TEAC/100g of DM, 6320-13151 µmol Fe^{+2}/100g of DM according to the methods ABTS, DPPH and FRAP, respectively. For all measured parameters, the different pollen samples were significantly different ($p < 0.05$), except for the flavonoid content and the total antioxidant activity obtained based on ABTS method. In the present study, total flavonoid content values were found to be lower than total phenolic content values because flavonoids are subgroups of phenolics.

Table 4.1 : Total phenolics, total flavonoids and antioxidant activity in pollen samples (n = 3).

Sample	Total Phenolics (mg GAE/100g DM)	Total Flavonoids (mg catechin/100g DM)	Total Antioxdant Activity		
			ABTS (µmol TEAC/100g DM)	DPPH (mg TEAC/100 g DM)	FRAP (µmol Fe^{+2}/100g DM)
Antalya	1188±114^{ab}	677±124^{a}	7294±1331^{a}	762±104^{a}	12845±1141^{a}
Afyon	1261±143^{a}	648±68.2^{a}	8239±1250^{a}	922±93.2^{a}	13151±1251^{a}
Bursa	971±256^{abc}	662±102^{a}	4517±1084^{a}	615±158^{ab}	9889±2309^{ba}
İzmit	940±57,6^{abc}	778±32.5^{a}	4955±1940^{a}	841±252^{a}	8498±366^{bc}
Sivas_winter	756±77.2^{c}	559±36.8^{a}	6269±1114^{a}	613±69.1^{ab}	8044±873^{bc}
Sivas_summer	852±118^{bc}	754±130^{a}	6925±1582^{a}	275±44.3^{b}	6320±678^{c}

^{a,b,c,d} Values with a different superscript in a column are significantly different ($p < 0.05$)

Afyon and Antalya pollen sample had a significantly higher phenolic compounds (1261±143 and 1188±114 mg GAE/100g of DM respectively) compared to the Sivas samples. This difference or ranking in pollen based on the phenolic compounds was not visible in the flavonoid content. İzmit pollen sample had the highest value of total flavonoids (778 mg catechin/100g of DM) and Sivas winter the lowest (559 mg catechin/100g of DM).

Afyon and Antalya pollen, showing the highest total phenolic content, also had the highest antioxidative capacity among all tested pollen samples, as measured by the ABTS, DPPH and FRAP method. The ranking of the different pollen samples in terms of antioxidative capacity is not the same for all used measurements. Indeed, the different methods of the antioxidative capacity are based on different principles, and different phenolic compounds react different with the reagents used (Yesiltas et al., 2014).

The phenolic compounds have a high ability to neutralize the active oxygen species because of their structure such as the conjugated double bonds and the number of hydroxyl groups in the aromatic ring (Leja et al., 2007). There are several reports investigating the total phenolic and total flavonoid contents of various bee pollen samples. Almaraz-Abarca et al.(2004) studied the variability of antioxidant activity of pollen samples extracted in ethanol-water solution (50% v/v) and it is found that a wide range of total phenolic and total flavonoid values are observed, based on the type of pollen, geography and several climate conditions. In the study of Mărghitaş et al. (2009), the total phenolic content of pollen samples was found between 4.4-16.4 mg GAE/g of DM. LeBlanc et al. (2009) and Morais et al. (2011) obtained total phenolic content values changing between 10.5-34.85 mg GAE/g of FW for methanolic extracts of pollen samples collected form US and Portuguese. Carpes et al. (2007) analysed pollen samples collected from Mexico and a total phenolic content was reported to be in the range of 3.6-10.9 mg GAE/g of DM. On the other hand, the flavonoid contents in different pollen species were reported by Pascoal et al. (2014) in the range of 3.71-10.14 mg catechin/g of FW. Our total phenolic content values coincide with the values of Mărghitaş et al. (2009), although obtained values by other researchers are higher than our values. This might be as a result of geography. Moreover, flavonoid content values are strongly supported by the good agreement between our values and those from the literature.

There have been several different techniques applied for the evaluation of total antioxidant capacity of bee pollen samples. The antioxidant properties of bee pollen cannot be assessed by just one method because of their complex matrix. A close relationship observed between bee pollen and total antioxidant capacity has been found in several studies (Campos et al., 2003; Leja et al., 2007; Le Blanca et al., 2009). It was also reported that the bee pollen antioxidant activity is species-specific (AlmarazAbarca et al., 2004; Leja et al., 2007; Le Blanca et al., 2009; Mărghitaş et al., 2009). In the study of Mărghitaş et al. (2009), the total antioxidant capacity of pollen samples was found between 0.135-2.814 mmol Trolox/g of DM, 0.546-6.838 mmol Trolox/g of DM, 0.255-5.355 mmol Fe^{+2}/g of DM according to the methods DPPH, ABTS and FRAP, respectively. The total antioxidant capacity was found between 5.7-15.2 mg TEAC/g of FW based on DPPH method (Yesiltas et al., 2014).

Our total antioxidant capacity values except Sivas summer were found within the values obtained by Yesiltas et al. (2014). In the study of Zhang et al. (2015), the total antioxidant capacity was found 0.01-1.06 mmol TEAC/g of FW according to ABTS method. Our values obtained based on ABTS method are within the values found by Zhang et al. (2015). Moreover, our values are totally different from the total antioxidant results found by Mărghitaş et al. (2009). The various results were obtained based on the methods of DPPH, FRAP and ABTS in comparison with the antioxidant content of bee pollen samples analysed. This might be as a result of a specific polyphenolic compounds that have different actions as antioxidant against various free radicals or antioxidants respond differently in different measurement methods which involve mechanisms of action and specific reaction conditions (Mărghitaş et al., 2009).

4.2 Total Phenolics, Total Flavonoids and Total Antioxidant Capacity of Bee Bread

Five different spectrophotometric analyses were performed on methanolic extract of the bee bread collected from India to obtain insight in the phenolic compounds and its antioxidative capacity. The total phenolic, total flavonoid and total antioxidant contents of bee bread are shown in Table 4.2. All data are given on 100 g of the dry matter basis.

Table 4.2 : Total phenolics, total flavonoids and antioxidant activity in bee bread sample (n=3).

Sample	Total Phenolics (mg GAE[1]/100g DM)	Total Flavonoids (mg catechin/100g DM)	Total Antioxdant Activity		
			ABTS (µmol TEAC[2]/100g DM)	DPPH (mg TEAC/100g DM)	FRAP (µmol Fe^{+2}/100g DM)
Bee Bread	1004±52.5	599±41.6	7513±1425	911±87.7	11578±850

Zuluaga et al. (2015), obtained values for the total phenolic content ranging between 2.5 - 13.7 mg GAE/g of DM for ethanolic extracts of bee bread sample collected from Colombia. The content of polyphenolic compounds in methanolic extracts were found 15.3 mg GAE/g of DM in bee bread by Cocan et al. (2009). Moreover, total phenolic compounds was determined 13.9 mg GAE/g of DM in the ethanolic extract of bee bread in the study of Stanciu et al. (2008). The results obtained in this study are within the range of reported values in literature. Possible differences in content might be due to the type of solvent used and different extraction methods.

According to study of Stanciu et al. (2009), different solvents have a significant influence on the total phenolic content of bee bread samples. Total flavonoid content in methanolic extract of bee bread sample was reported 5.13 mg QE/g of DM by Cocan et al. (2009). Total flavonoid content for ethanolic extract of bee bread sample collected from Ukraine ranged from 13.6 to 18.2 µg QE/g of DM in the study of Ivanišová et al. (2015). Whereas our values for total flavonoid content coincide with the values of Cocan et al. (2009), they are higher than other data obtained by Ivanišová et al. (2015). The variations observed between the total flavonoid contents of bee bread samples can be related to their botanical origin (Cocan et al., 2009).

In the study of Zuluaga et al. (2015), which evaluated Colombian bee bread, the total antioxidant capacity of bee bread was found between 46.1 - 76.3 µmol TEAC/ g of DM and 35.0 - 70.1 µmol TEAC/ g of DM for ethanolic extracts of bee bread sample according to the methods ABTS and FRAP, respectively. High antioxidant activity

[1] GAE: Gallic acid equivalent
[2] TEAC: Trolox equivalent antioxidant capacity

was also reported by (Ivanišová et al., 2015) in bee bread samples collected from Ukraine, values from their study ranged from 2.5 to 15.8 mg TEAC/g of DM for ethanolic extracts of bee bread based on DPHH method. In the study of Stanciu et al.(2008), the antioxidant capacity of ethanolic extract of bee bread was found 0.521±0.04 mmol Fe^{+2}/L sample solution based on FRAP method and 0.21± 0.05 mmol TEAC/L sample solution according to ABTS method. In addition to this, it was also reported that the antioxidant capacity of methanolic extracts of bee bread was determined 0.404 ± 0.02 mmol Fe^{+2}/L sample solution based on FRAP method and 0.43 ± 0.02 mmol TEAC/L sample solution based on ABTS method (Stanciu et al., 2009). In this study, obtained results for total antioxidant activity based on DPPH and ABTS methods are within those values as obtained by Zuluaga et al. (2015) and (Ivanišová et al. (2015). On the other hand, our values obtained from FRAP method are different from literature values. This value could not be compared to literature data because the results were expressed in different units. There have been several techniques applied to measure antioxidant capacities of bee bread. These methods are different from each other with regard to reaction mechanisms, oxidant species, reaction conditions and the way the final results were expressed (Moniruzzaman et al., 2012). The differences observed between the antioxidant activities of bee bread samples are based on the presence of natural antioxidants, mainly phenolic compounds that differed depending on the region and type (Ivanišová et al. 2015; Sati et al., 2013; Tlili et al., 2014). It was demonstrated that bee bread is a very good source of bioactive compounds with antioxidant effect in the study of Ivanišová et al. (2015), Stanciu et al. (2008) and Zuluaga et al. (2015).

4.3 Fermentation Results

Two types of fermented bee pollen samples were prepared from bee pollen samples originating from Afyon, İzmit and Sivas. While one of them was prepared by using bee bread as a natural starter of yeasts, LAB (*Lactococcus lactis* subsp. *lactis*) was used for producing the other fermented pollen sample. Each bee pollen sample was mixed with honey, water and bee bread or *Lactococcus lactis* subsp. *lactis*. Pictures taken after the fermentation process are shown in Figure 4.1 and Figure 4.2.

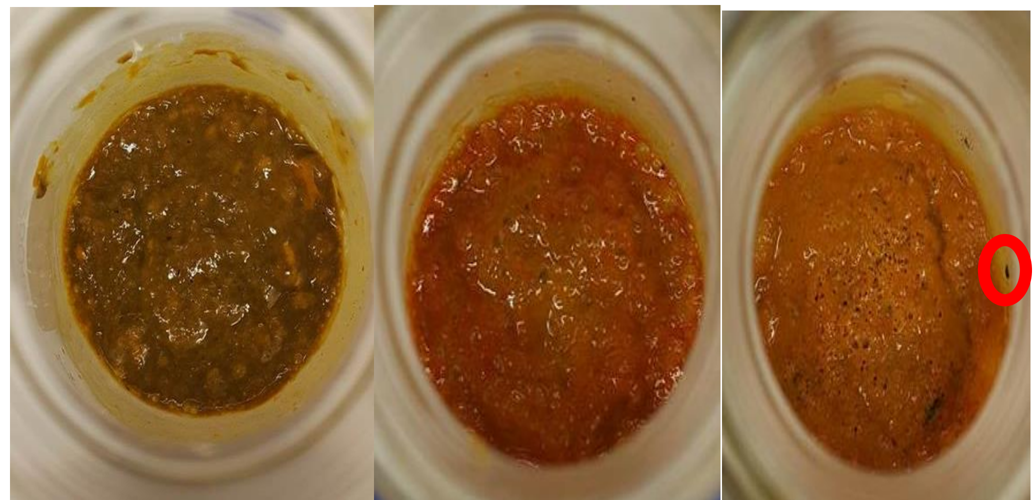

Figure 4.1 : Afyon FBP+LB, İzmit FBP+LB and Sivas FBP+LB, respectively.

*FBP: fermented bee pollen

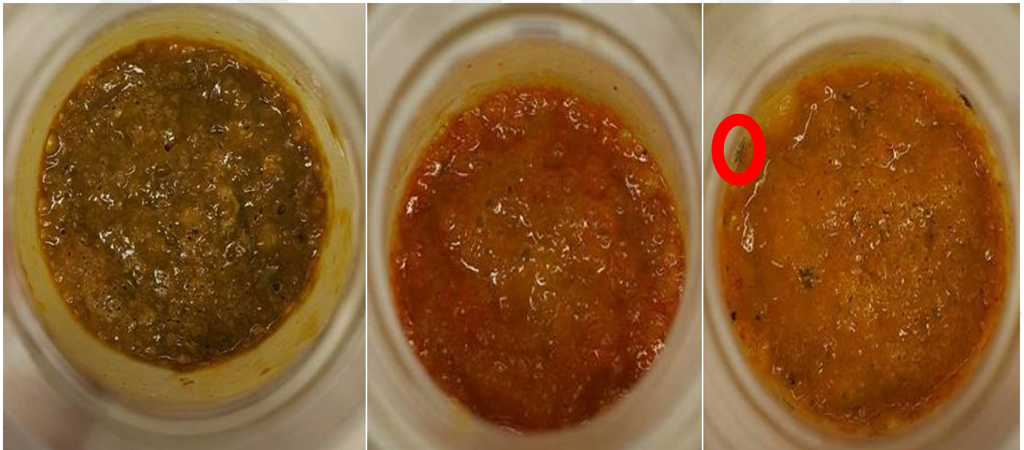

Figure 4.2 : Afyon FBP+BB, İzmit FBP+BB and Sivas FBP+BB, respectively.

*BB: bee bread, FBP: fermented bee pollen

After the fermentation process, white and black molds were seen in all Sivas fermented bee pollen samples (They are circumscribed in red). This might be as a result of contamination of Sivas bee pollen during collecting from the bee hives.

4.3.1 The pH and acidity results

The pH value of bee pollen, bee bread and fermented bee pollen samples are shown in Table 4.3. While the pH value of bee pollen samples ranged between 4.03 and 4.59, the pH value of fermented bee pollen samples were decreased to a pH of 3.82-4.15. The pH value of bee bread was found 3.85±0.005.

Table 4.3 : The pH value of bee pollen, bee bread and fermented bee pollen samples*.

Sample Name	Mean Value ± Standard Deviation
Afyon bee pollen	4.59±0.01
İzmit bee pollen	4.03±0.01
Sivas bee pollen	4.22±0.005
Bee bread	3.85±0.005
AFBP+BB	3.87± 0.04
AFBP+LAB	3.82± 0.09
IFBP+BB	4.09± 0,01
IFBP+LAB	4.14± 0.01
SFBP+BB	4.15± 0.04
SFBP+LAB	4.05± 0.05

*Data ar given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LAB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, , IFBP+LAB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LAB: LAB added Sivas fermented bee pollen

Bees mix pollen with honey and their saliva secretions to produce bee bread with the help of LAB and the pH reduces during fermentation to conserve bee bread from deleterious microorganisms (Ellis and Hayes Jr, 2009). Obtained pH results are similar to that of bee bread (3.8 – 4.3), which is lower than that of pollen (4.1 – 5.9) (Herbert and Shimanuki, 1978). The pH value of bee pollen samples collected from Portugal were found in the range of 4.3 and 5.2 by Feás et al. (2012). The pH results obtained in this study for unfermented bee pollen samples (4.03-4.59) are within the range of reported values (4.1 – 5.9) in literature. In addition to this, the pH results obtained for fermented bee pollen samples (3.82- 4.15) are within the range of reported values (3.8 – 4.3) in literature. According pH results, unfermented Afyon and Sivas bee pollen samples have higher pH values compare to their fermented pollen samples.

However, it was seen that only a successful fermentation was observed with the Afyon samples as after fermentation a clear reduction in pH was observed, comparable to the values of commercial bee bread. For Sivas a very low reduction of 0.1 pH units is observed of which the relevance is doubtful and no reduction at all was obtained by Izmit fermented samples. This might be as a result of activity of LAB. Obtained pH results are in parallel with microbiological data, which will be discussed more in detail in 4.3.2. Regarding to microbiological results, the highest LAB activity was observed in Afyon samples. As a result of this, a clear reduction in pH was observed in Afyon samples. Moreover, the exine layer of İzmit bee pollen may be harder than other

samples so the destruction of exine layer is more difficult by LAB and this may prevent to decrease the pH of İzmit fermented pollen samples.

4.3.2 Microbiological analysis results

Bee bread and fermented bee pollen samples were analyzed for lactic acid bacteria, total aerobic mesophilic bacteria and yeasts. Microbiological analysis results of bee bread and fermented bee pollen samples are given in Figure 4.3. All data are expressed as colony forming units (CFU) per g of the sample.

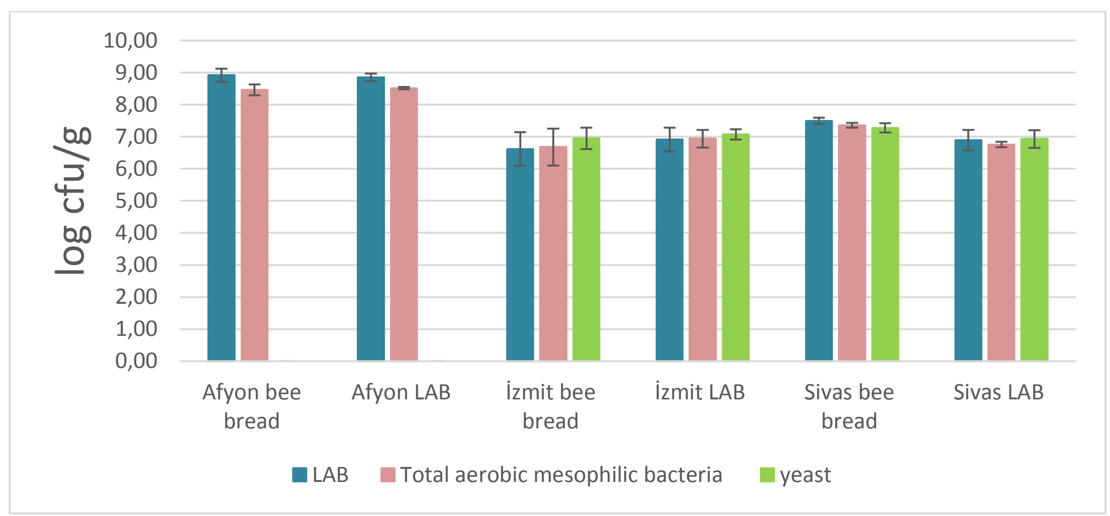

Figure 4.3 : Results of microbiological analysis of fermented bee pollen samples (n = 3).

Afyon fermented bee pollen samples had the highest LAB and total aerobic mesophilic bacteria loads among all fermented pollen samples. However, yeasts were not detected meaning that they were present in an amount lower than $<1,0x10^6$ in Afyon samples.

These microbiological data are in agreement with the pH values of the fermented Afyon bee pollen samples (3.85-3.87), which were the lowest value among all fermented pollen samples. The optimum pH range can vary from pH 4 to 6 for yeast growth and yeast growth is based on temperature, the presence of oxygen, and the strain of yeast (Narendranath and Power, 2005). It appeared that the unfavorable conditions resulting from low pH in Afyon samples contributed to the decline of the yeast growth as fermentation progressed. In addition to this, yeasts were detected in İzmit and Sivas fermented samples since they have favorable pH values to the growth of yeast. İzmit and Sivas fermented pollen samples have similar pH values and LAB, total aerobic mesophilic bacteria and yeast loads. Inoculation was 10^6 cfu/g for LAB in

the beginning. According to this, approximately a 3 log increased of LAB was observed in Afyon LALB fermented samples, while approximately 0.5 and 1 log increase was measured in İzmit and Sivas LAB fermented samples respectively. LAB results are in parallel with the pH results. Regarding to this results, Afyon fermented samples was more stable because high LAB growth was observed. As a result of this, a high reduction of pH was obtained. Moreover, very low reduction of pH in Sivas fermented samples and no reduction in İzmit fermented samples were observed due to low LAB growth.

During bee pollen fermentation, the exine layer of pollen are destructed by LAB so bee bread becomes more digestible (Krell, 1996). Esterases, lipases, proteases, aminopeptidases, phosphatases, and glycosidases are secreted by LAB in the fermentation of bee pollen and this enzymes cause fermentation and conversion of pollen constituents to form bee bread and they are responsible for softening of the exine wall of pollen before it is ingested (Gilliam et al., 1990). LAB growth is lower in İzmit and Sivas pollen samples than Afyon samples. This may be as a result of the chemical structure of their exine layer. The exine layer of pollen contains the macromolecule sporopollenin that is highly resistant to chemical, physical and biological degradation procedures (Wallace et al., 2011). This layer can be different for each pollen samples based on the sporopollenin structure (Wallace et al., 2011). Indeed, LAB could not break down the exine layer of İzmit and Sivas samples as good as Afyon samples to reach sugar inside pollen samples to growth.

All microorganisms analysed here in bee bread were lower than detection limit (detection limit was <1 cfu/g). Vásquez and Olofsson (2009) studied the detection of the honey stomach LAB flora in two weeks old Swedish bee bread and two month old American bee bread. According to these study results, while *Lactobacillus* phylotypes, *Bifidobacterium* phylotypes and *Pasteurelaceae* family were identified in two weeks old Swedish bee bread, neither LAB nor bacteria belonging to the *Pasteurelaceae* family were detected from the two month old American bee bread.

This could be explained by the fermentation process of the bee bread which result in the production of large amounts of lactic acid and antimicrobial substances leading to create a very acidic environment and hostile which generally prevents all bacterial growth (Vásquez and Olofsson, 2009). Any LAB or yeast was not found in commercial bee bread. It was seen that commercial bee bread could not contribute to the fermentation because of this. Inoculum could be coming from LAB in pollen. In the

study of Vásquez and Olofsson (2009), *Lactobacillus* phylotypes, *Bifidobacterium* phylotypes and phylotypes belonging to the *Pasteurelaceae* family were found in bee pollen samples. It should be verified by analyzing bee pollen samples for lactic acid bacteria.

4.3.3 Dry matter content results

The dry matter contents of the bee pollen, bee bread and fermented bee pollen samples are shown in Table 4.4. The dry matter content of all fermented pollen samples ranged between 40.0% and 49.6%. Each bee bread added fermented sample had higher dry matter content than LAB added fermented sample that was fermented by pollen of the same origin. Whereas the dry matter contents in different pollen species were found in the range of 85.1-89.4%, it was determined 84.4 ± 0.62% for bee bread. Bee bread added Afyon fermented bee pollen sample had higher dry matter than other bee bread added fermented pollen samples. Moreover, LAB added Afyon fermented bee pollen sample had a higher dry matter content than other LAB added fermented pollen samples.

Table 4.4 : Dry matter content of bee pollen, bee bread and fermented bee pollen samples*.

Sample Name	Mean Value ± Standard Deviation
Afyon bee pollen	89.4 ± 1.42
İzmit bee pollen	87.4 ± 0.76
Sivas bee pollen	85.1 ± 0.94
Bee bread	84.4 ± 0.62
AFBP+BB	49.6 ± 1.32
AFBP+LAB	44.9 ± 1.20
IFBP+BB	42.9 ± 0.61
IFBP+LAB	40.0 ± 0.81
SFBP+BB	45.3 ± 1.09
SFBP+LAB	40.8 ± 0.29

*Data represent average values ± standard deviation of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen

4.4 Total Phenolic, Total Flavonoid and Total Antioxidant Capacity of Undigested Bee Pollen, Undigested Fermented Bee Pollen and Undigested Bee Bread Samples

The total phenolic content of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples are shown in Figure 4.4. All data were given in terms of mg GAE/100 g on the dry matter basis. The 2-way interaction term (origin x treatment) is significantly different ($p<0.05$) for the total phenolic content, as well as for both main factors origin and treatment ($p<0.05$). Bee bread added Afyon fermented bee pollen sample had the highest value of total phenolic content (1257±207 mg GAE/100g of DM) and LAB added Sivas fermented bee pollen the lowest (394±109 mg GAE/100g of DM) among all tested fermented pollen samples. Reduction on phenolic content was observed 51% for Sivas fermented bee pollen samples on average.

In general, LAB added fermented pollen samples, independent of the origin of the pollen, had a significantly lower amount of phenolic compounds compared to the other samples of the same origin that were treated differently i.e. unfermented or bee bread added fermented samples ($p<0.05$). No difference was observed between the unfermented pollen samples and bee bread added fermented pollen samples based on the phenolic compounds except Sivas samples. Unfermented Afyon bee pollen sample had the highest antioxidative capacity (1261±143 mg GAE/100g of DM) among all tested unfermented pollen samples. Commercial bee bread sample (1004±52.5 mg GAE/100g of DM) had a significantly higher phenolic compound content compared to bee bread added Sivas fermented bee pollen samples and LAB added Sivas fermented bee pollen samples (423±71.5 and 395±109 mg GAE/100g of DM, respectively) ($p<0.05$). On the other hand, significant difference is not observed between commercial bee bread and fermented Afyon and fermented İzmit samples (both bee bread and LAB) ($p > 0.05$).

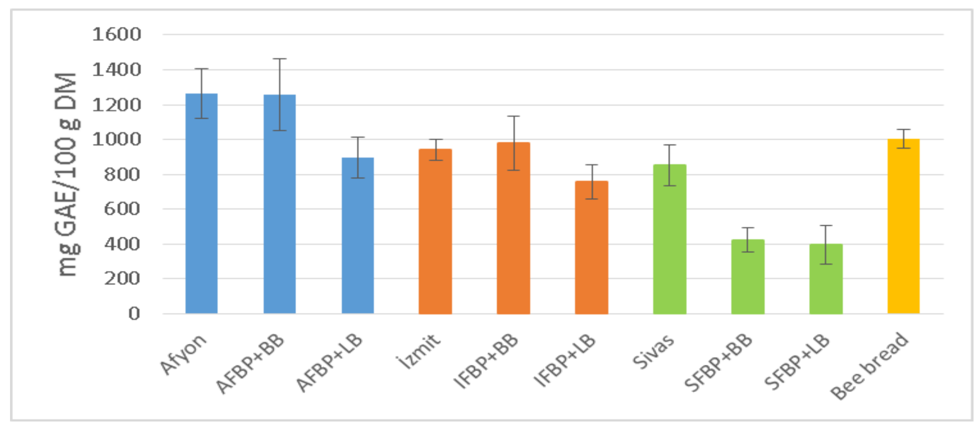

Figure 4.4 : Total phenolic content of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. GAE: Gallic acid equivalent

The Folin-Ciocalteau method is strongly based on the reduction of the Folin-Ciocalteau reagent. Folin-reagents has some interferences with sugars and reducing compounds in the Folin-Ciocalteau assay (Singleton et al., 1999) and this can lead to an increase in total phenolic content. Sugars in pollen (and added honey) samples are used by LAB during the fermentation process. According to obtained pH and microbiological results, successful fermentation was not observed in Sivas bee pollen sample because of the very low reduction of pH from 4.22 to 4.15-4.05 by lower activity of LAB compare to Afyon sample. Because of that, it was not expected to see a decrease in total phenolic compounds for fermented Sivas pollen samples. Therefore, the decrease in phenolic content measured was not expected, as it can be assumed that the sugar was not used, as the LAB activity was very low. However, a clear decreases in the total phenolic compounds was observed in Sivas fermented bee pollen samples compare to unfermented sample. On the other hand, it was expected to see a decrease in total phenolic content for bee bread added and LAB added Afyon fermented bee pollen sample because of use of the sugar by LAB according to the obtained pH and microbiological results. Indeed the pH clearly reduced from 4.59 to 3.87-3.82. However, total phenolic content remained the same. It may be result of decomposition of exine layer by higher activity of LAB compare to İzmit and Sivas samples. In addition to this, honey that contains high amount of sugar was added to fermented pollen samples and this can lead to interference with Folin-reagents and influence the

total phenolic content results. On the other hand, increasing total phenolic content is also possible in fermented bee pollen samples. If the fermentation opens the exine layer, then sugars and phenolic compounds can be released, thus giving an increased value, so this was also expected from the fermentation.

The total flavonoid content of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples are given in Figure 4.5. All data were given in terms of mg catechin/100 g on the dry matter basis. The 2-way interaction term (origin x treatment) is not significantly different ($p>0.05$) for the total flavonoid content. There is no difference in flavonoid content in the unfermented pollens samples depending on the origin of the pollen ($p>0.05$). However, the effect of treatment had a significant effect on the total flavonoid content ($p<0.05$). Unfermented pollen samples had a significantly higher amount of flavonoid compounds compared to the other samples that were fermented ($p<0.05$). No difference was observed between the bee bread added fermented pollen samples and LAB added fermented pollen samples based on the phenolic compounds ($p>0.05$). Reduction on flavonoid content was observed 32,5%, 42,7% and 49% for Afyon, İzmit and Sivas fermented bee pollen samples respectively on average.

Commercial bee bread sample had a significantly higher flavonoid compounds (599±41.6 mg catechin/100g of DM) compared to all fermented bee pollen samples (422±29.7 mg catechin/100g of DM on average) ($p<0.05$).

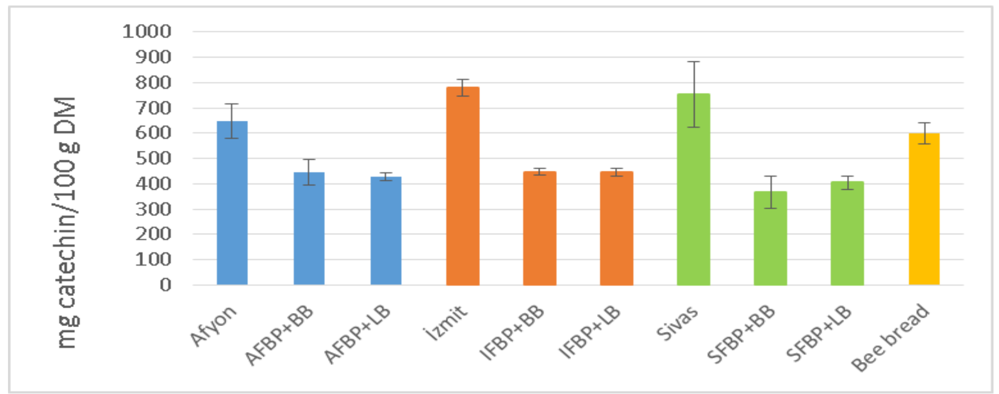

Figure 4.5 : Total flavonoid content of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen

According to the obtained pH and microbiological results, it was expected to see an increase of the total flavonoid content of bee bread added Afyon fermented bee pollen sample because of the higher LAB activity and reduction of pH value. However, no significant difference was observed among fermented bee pollen samples. It was understood that the fermentation could not open the exine layer and as a result of this, flavonoid compounds could not be released from the exine layer.

The results of DPPH radical scavenging activity in mg TEAC/100 g DM are shown in Figure 4.6. No significant effect of the 2-way interaction terms (origin x treatment) was observed ($p>0.05$). However, both main effects, type of treatment and origin, were highly significant on the antioxidant capacity as measured by the DPPH radical scavenging activity ($p<0.05$). Each unfermented pollen sample had a significantly lower antioxidant activity (based on the DPPH-method) compared to its fermented samples ($p<0.05$), no difference was observed between the bee bread added fermented pollen samples and LAB added fermented pollen samples based on antioxidant capacity ($p>0.05$). Each origin is significantly different from the others ($p<0.05$). Afyon samples (bee pollen, bee bread added fermented bee pollen and LAB added fermented bee pollen) had the highest value of total antioxidant capacity (922±93.2, 1433±73.1 and 1373±123 mg TEAC/100g of DM, respectively) among all origin samples. Moreover, LAB added Sivas fermented bee pollen had the lowest (851±55.4 mg TEAC/100g of DM) among all tested fermented pollen samples. Increase on antioxidant content was observed 52.1%, 42% and 213% for Afyon, İzmit and Sivas fermented bee pollen samples respectively on average.

Commercial bee bread sample had a significantly lower antioxidant capacity (911±87.7 mg TEAC/100g of DM) compared to bee bread added Afyon fermented bee pollen, LAB added Afyon fermented bee pollen samples and bee bread added İzmit fermented bee pollen (1433±73.1, 1373±123 and 1247±227 mg TEAC/100g of DM, respectively) ($p<0.05$). On the other hand, no significant difference is observed between the commercial bee bread and other fermented bee pollen samples ($p>0.05$).

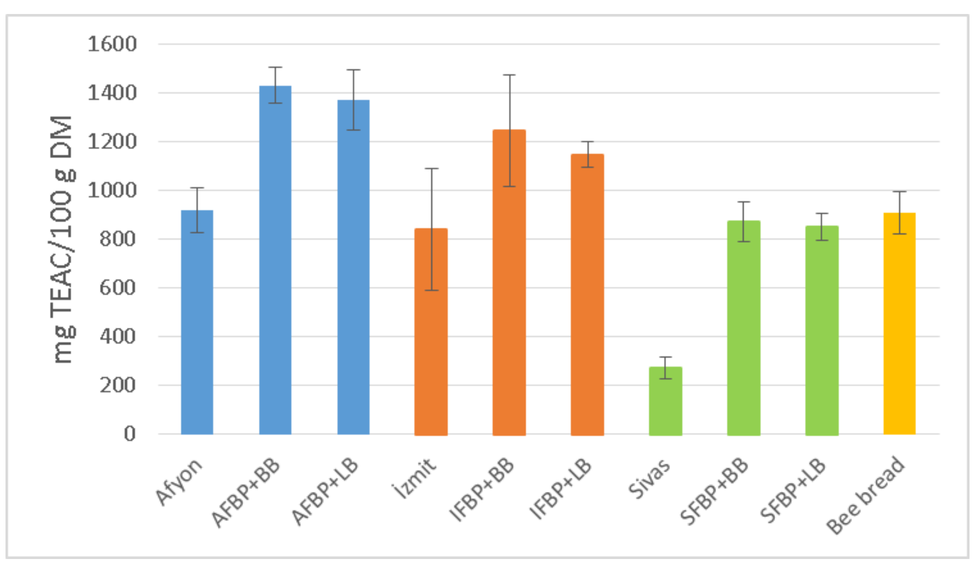

Figure 4.6 : DPPH radical scavenging activities of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

The total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples are given in Figure 4.7 according to the ABTS method. All data were given in terms of µmol TEAC/100 g on dry matter basis. The 2-way interaction term (origin x treatment) is not significantly different ($p>0.05$) for the total antioxidant capacity, as well as both main factors origin and treatment ($p>0.05$). LAB added fermented bee pollen samples had the highest value of total antioxidant capacity for İzmit and Sivas (8677±4055 and 8620±2425 µmol TEAC/100g of DM, respectively) compared to the other samples that were treated. Moreover, no significant difference is observed between commercial bee bread and all tested fermented bee pollen samples on the total antioxidant capacity based on ABTS method ($p>0.05$).

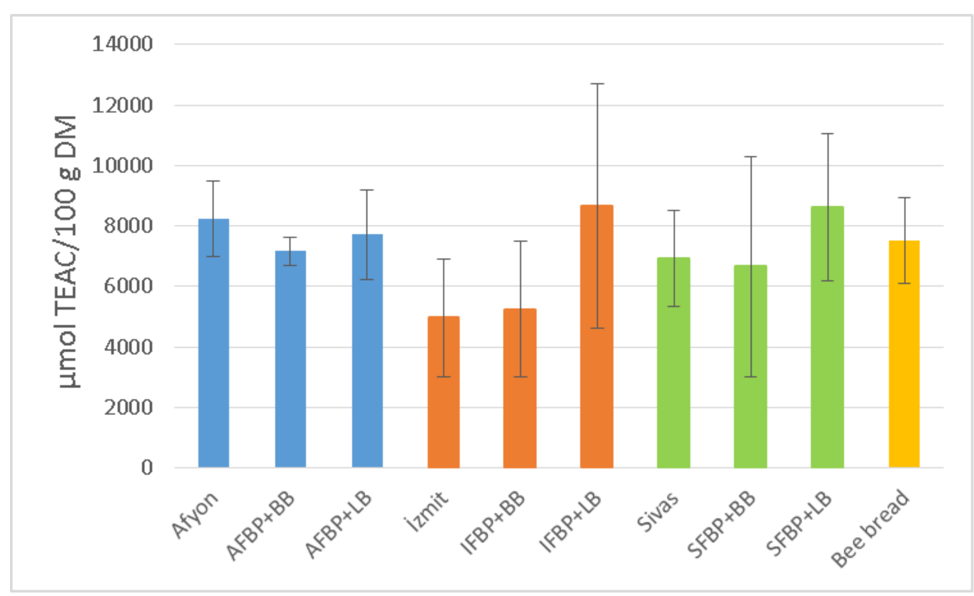

Figure 4.7 : Total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples based on ABTS method*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen,SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

The total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples are given in Figure 4.8 according to CUPRAC method. No significant effect of the 2-way interaction terms (origin x treatment) was observed (p>0.05). However, both main effects, type of treatment and origin, were highly significantly different in terms of the antioxidant capacity (p<0.05). Each origin is significantly different from others (p<0.05). Afyon samples (bee pollen, bee bread added fermented bee pollen and LAB added fermented bee pollen) had the highest value of total antioxidant capacity (2386±277, 3202±201 and 3308±229 mg TEAC/100g of DM, respectively) among all origin samples.

Unfermented pollen samples had a significantly lower amount of antioxidant capacity as measured by the CUPRAC method, compared to the other samples that were treated differently (i.e. fermented) (p<0.05). In addition to this, no difference was observed between the bee bread added fermented pollen samples and LAB added fermented pollen samples based on the total antioxidant capacity (p>0.05). Increase on antioxidant content was observed 36%, 61% and 26% for Afyon, İzmit and Sivas fermented bee pollen samples respectively on average.

Bee bread sample had a significantly higher antioxidant capacity (2312±447 mg TEAC/100g of DM) compared to bee bread added Sivas fermented bee pollen (1485±149), moreover, it had a significantly lower antioxidant capacity compared to bee bread added Afyon fermented bee pollen samples and LAB added Afyon fermented bee pollen (3202±201 and 3308±229 mg TEAC/100g of DM respectively) ($p<0.05$).

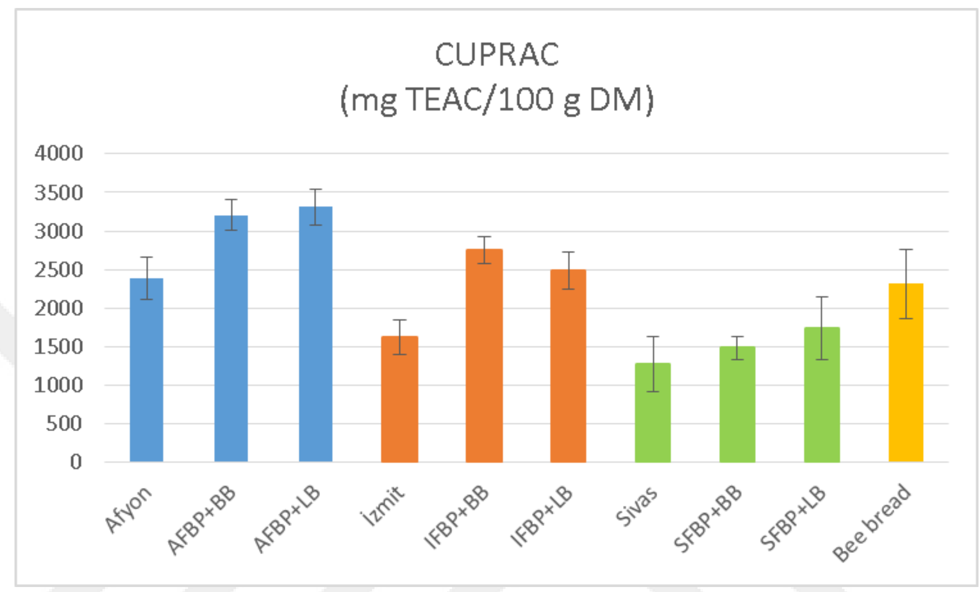

Figure 4. 8 : Total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples based on CUPRAC method*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen,SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

The total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples are given in Figure 4.9 based on FRAP method. All data were given in terms of µmol Fe^{+2}/100 g on the dry matter basis. No significant effect of the 2-way interaction terms (origin x treatment) is observed ($p>0.05$) for total antioxidant capacity, as well as no influence of the treatment ($p > 0.05$). This means that fermentation had no real impact on the measurements based on the FRAP-method compared to unfermented samples. However, the type of origin is highly significantly different in their antioxidant capacity based on the FRAP-method ($p<0.05$).

Each origin is significantly different from the others ($p<0.05$). Afyon samples (bee pollen, bee bread added fermented bee pollen and LAB added fermented bee pollen) had the highest value of total antioxidant capacity (13151±1251, 12756±512 and 12139±439 µmol Fe^{+2}/100g of DM, respectively) among all origin samples. Moreover, LAB added Sivas fermented bee pollen had the lowest of total antioxidant capacity (4212±75,4 µmol Fe^{+2}/100g of DM) among all tested fermented pollen samples. Reduction on antioxidant content was observed 5%, 6% and 30% for Afyon, İzmit and Sivas fermented bee pollen samples respectively on average.

Commercial bee bread had a significantly higher antioxidant capacity (11578±850 µmol Fe^{+2}/100g of DM) compared to LAB added İzmit fermented bee pollen, bee bread added Sivas fermented bee pollen and LAB added Sivas fermented bee pollen samples (8064±1008, 4530±1740 and 4212±75.4 µmol Fe^{+2}/100g of DM respectively) ($p<0.05$). On the other hand, no significant difference is observed between the commercial bee bread and the other fermented bee pollen samples ($p> 0.05$).

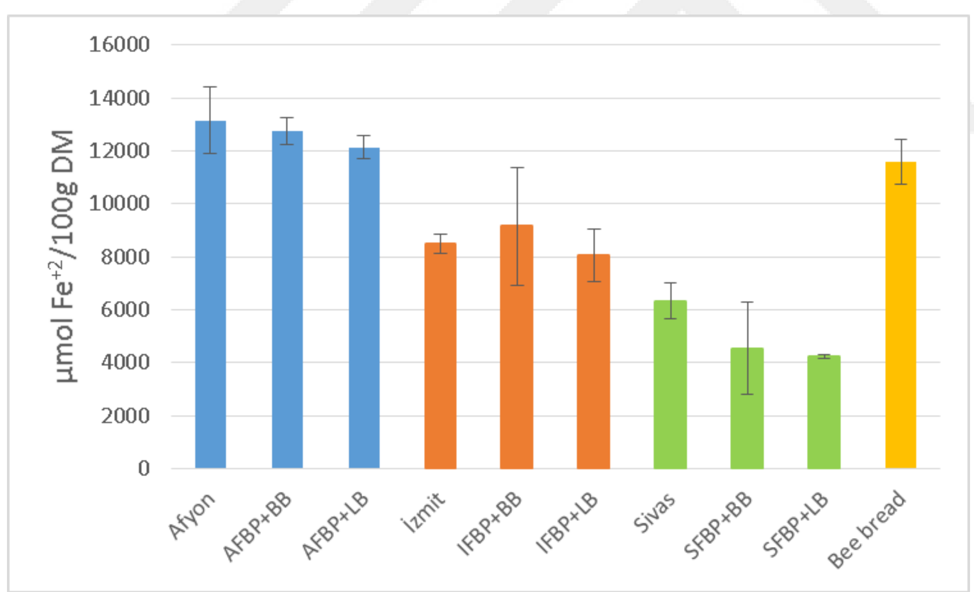

Figure 4.9 : Total antioxidant capacity of undigested bee pollen, undigested fermented bee pollen and undigested bee bread samples according to FRAP method*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen.

According to the obtained total antioxidant capacity results from the four different assays, no significant effect of the 2-way interaction terms (origin x treatment) is observed ($p>0.05$). On the other hand, type of origin is highly significant on the antioxidant capacity ($p<0.05$) except for the results obtained with the ABTS method. Afyon fermented bee pollen samples had the highest value of total antioxidant capacity except for the ABTS method among all tested fermented bee pollen samples based on origin.

While there is a significant difference among treatments based on DPPH and CUPRAC methods ($p<0.05$), no significant effect was observed according to ABTS and FRAP methods ($p>0.05$) based on treatment. According to obtained pH and microbiological results, the best fermentation was observed in Afyon bee pollen sample because of the reduction pH from 4.59 to 3.87-3.82 by higher activity of LAB. Increasing total antioxidant capacity was observed in fermented bee pollen samples compare to their unfermented pollen samples based on DPPH and CUPRAC methods. It might be as a result of activity of LAB because LAB is responsible for secreting enzymes including esterases, lipases, proteases, aminopeptidases, phosphatases, and glycosidases in the fermentation of bee pollen and these enzymes have an important role by softening of the exine wall of pollen (Gilliam et al., 1990). It seems that microbiological treatment achieved the opening grain of the exine layer, releasing antioxidative compounds linked to this and this leads to increasing the total antioxidant capacity of fermented bee pollen samples for DPPH and CUPRAC assays. On the other hand, the total antioxidant capacity almost remained the same except for LAB added İzmit and Sivas fermented bee pollen samples in ABTS method. Moreover, the total antioxidant capacity almost remained the same except for unfermented Sivas samples in which unfermented pollen sample had highest amount of antioxidant capacity in the FRAP method. This might be as a result of the methods that have a different principle to measure the total antioxidant capacity. The ranking of the different pollen samples and fermented pollen samples in terms of antioxidative capacity is not the same for all used measurements. Several techniques have been used for the assessment of total antioxidant capacity of honeybee products. Each method has a different principle including the radical or the necessary detection time, moreover, each method possesses own advantages and disadvantages (Capanoglu, et al., 2008). Therefore, using at least

two methods is desirable for assessing and comparing the antioxidant capacity of a sample (Sakanaka & Ishihara, 2008).

4.5 Total Phenolic, Total Flavonoid and Total Antioxidant Capacity of Digested Bee Pollen, Digested Fermented Bee Pollen and Digested Bee Bread Samples

Total phenolic, total flavonoid, and total antioxidant capacity were determined on all samples (bee pollen samples, bee bread and LAB fermented pollen samples), after each digestion phase, i.e. oral phase, gastric phase and intestinal phase. The total phenolic content of all samples after the application of the *in-vitro* digestion method are shown in Table 4.5. All data are given in terms of mg GAE/100 g on dry matter basis. Phenolic compounds absorption values obtained in intestinal phase relative to the total value obtained in the unfermented undigested pollen are given in Figure 4.10.

According to digestion experiments results, total phenolic content of bee pollen samples was 39.7 mg GAE/100g DM, 139 mg GAE/100g DM and 307 mg GAE/100g DM on average from oral phase, gastric phase and intestinal phase, respectively. Moreover, average total phenolic content of fermented bee pollen samples was 68.6 mg GAE/100g DM, 219 mg GAE/100g DM and 530 mg GAE/100g DM from oral phase, gastric phase and intestinal phase, respectively. No difference was observed among tested bee pollen samples, as well as among fermented bee pollen samples.

Digested commercial bee bread sample had similar total phenolic content values to pollen samples. Moreover, commercial bee bread sample had lower values (40.4±0.19, 152±0.98 and 333±1.35 mg GAE/100g of DM) compare to average total phenolic content of fermented bee pollen samples (68.8, 219 and 530 mg GAE/100g DM) in oral, gastric and intestinal phase, respectively.

The bioaccessibility results showed that the lowest bioaccessibility was observed in Afyon fermented bee pollen samples (40%) compare to İzmit fermented bee pollen samples (61%) and Sivas fermented bee pollen samples (61%) on average based on total phenolic content. However, no difference was observed between İzmit and Sivas fermented samples. Moreover, bioaccessibility of each unfermented sample was lower than its fermented samples. Each LAB added fermented sample had higher bioaccessibility than bee bread added fermented sample that was fermented by pollen of the same origin. Commercial bee bread sample had lower bioaccessibility according to fermented bee pollen samples. These results should be verified by HPLC. In this

way it could be understood whether the measured values and thus bioaccessibility were due to interference of sugars with the Folin reagents or not.

Table 4.5 : Total phenolic content of undigested and digested bee pollen samples, bee bread and fermented pollen samples (mg GAE/100g DM)*.

Sample	Before digestion	After digestion		
		Simulated Salivary Fluid SSF	Simulated Gastric Fluid SGF	Simulated Intestinal Fluid SIF
Afyon	1261±143	37.7±0.64	137±0.76	290±1.37
AFBP+BB	1257±208	66.5±0.24	211±4.21	490±6.3
AFBP+LAB	897±116	70.9±3.17	213±1.16	516±2.91
İzmit	940±57.6	37.9±0.57	130±1.31	307±12.09
İFBP+BB	980±155	66,8±0,27	219±0.84	547±1.96
İFBP+LAB	755±99	71.7±2.23	235±1.17	598±10.99
Sivas	852±118	43.6±0.37	149±2.72	325±1.87
SFBP+BB	423±71.5	65.0±0.72	205±1.70	490±3.80
SFBP+LAB	395±110	70.9±1.13	233±3.98	542±9.76
Bee Bread	1004±52.5	40.4±0.19	152±0.98	333±1.35

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LAB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LAB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LAB: LAB added Sivas fermented bee pollen. GAE: Gallic acid equivalent

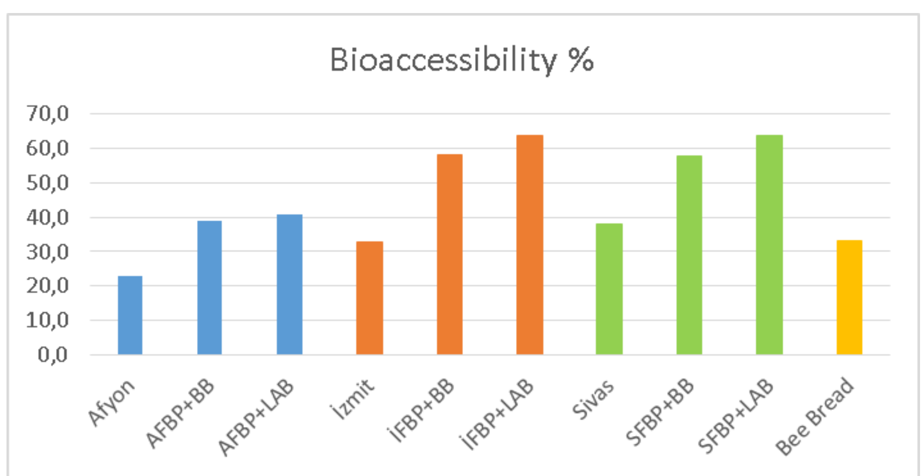

Figure 4.10 : Phenolic compounds bioaccessibility in intestinal phase from digested bee pollen, fermented bee pollen and bee bread samples*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen

The total flavonoid content of all samples after the application of *in-vitro* digestion method are shown in Table 4.6. All data are given in terms of mg catechin/100 g on

dry matter basis. Flavonoid compounds absorption values obtained in intestinal phase relative to the total value obtained in the unfermented undigested pollen are given in Figure 4.11. Average total flavonoid content of bee pollen samples was 31.8 mg catechin/100g DM, 48.3 mg catechin/100g DM and 84.4 mg catechin/100g DM from oral phase, gastric phase and intestinal phase, respectively according to digestion experiments results. Sivas bee pollen sample had the highest value of total flavonoid content (45.6±8.30, 67.9±10.86 and 101±2.5 mg catechin/100g of DM) from oral phase, gastric phase and intestinal phase, respectively and İzmit bee pollen the lowest (16.0±2.73, 22.0±3.47 and 61.2±9.76 mg catechin/100g of DM) among all tested pollen samples. Moreover, total flavonoid content of fermented bee pollen samples was 36.1 mg catechin/100g DM, 77.1 mg catechin/100g DM and 170 mg catechin/100g DM on average from oral phase, gastric phase and intestinal phase, respectively. İzmit fermented bee pollen samples had the lowest value of total flavonoid content except gastric phase and LAB added fermented Afyon bee pollen the highest (56.8±6.56, 85.9±9.54and 224±4.71mg catechin/100g of DM) among all tested fermented bee pollen samples. Each LAB added fermented bee pollen sample had higher value of total flavonoid content than bee bread added fermented bee pollen sample that was fermented from pollen with the same origin except fermented İzmit sample in oral phase.

Commercial bee bread had a similar total flavonoid content (33.8±4.33 mg catechin/100g of DM) compared to the average total flavonoid content of fermented bee pollen samples (36.1 mg catechin/100g of DM) in oral phase, although it had lower values (35.1±3.09 and 112±21.87 mg catechin/100g of DM) compared to the average total flavonoid content of fermented bee pollen samples (77.1 and 170 mg catechin/100g DM) in gastric and intestinal phase, respectively.

According to obtained bioaccessibility results from intestinal phase, the highest flavonoid compound bioaccessibility was observed in Afyon fermented bee pollen samples (32%) compare to İzmit fermented bee pollen samples (16%) and Sivas fermented bee pollen samples (24%) on average. In addition to this, bioaccessibility of each unfermented sample was lower than its fermented samples. LAB added each fermented sample had higher bioaccessibility rate than bee bread added fermented sample that was fermented of pollen from the same origin. Commercial bee bread

samples had higher bioaccessibility relative to fermented İzmit bee pollen samples as well as lower compare to Afyon and Sivas fermented samples.

Table 4.6 : Total flavonoid content of undigested and digested bee pollen samples, bee bread and fermented pollen samples (mg catechin/100g DM)*.

Sample	Before digestion	After digestion		
		Simulated Salivary Fluid SSF	Simulated Gastric Fluid SGF	Simulated Intestinal Fluid SIF
Afyon	648±68.2	33.8±3.85	54.9±2.35	91.0±3.93
AFBP+BB	446±49.2	37.7±1.20	77.7±2.64	192±8.85
AFBP+LAB	428±17.1	56.8±6.56	85.9±9.54	224±4.71
İzmit	778±32.5	16.0±2.73	22.0±3.47	61.2±9.76
İFBP+BB	446±13.4	26.2±2.82	75.0±9.34	115±2.80
İFBP+LAB	446±16.9	25.0±5.09	78.5±6.07	128±3.10
Sivas	754±130	45.6±8.30	67.9±10.9	101±2.5
SFBP+BB	365±63.3	32.5±6.89	65.3±8.26	161±35.4
SFBP+LAB	404±26.6	38.7±2.96	80.2±16.2	202±19.0
Bee Bread	598±41.6	33.8±4.33	35.1±3.09	112±21.9

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

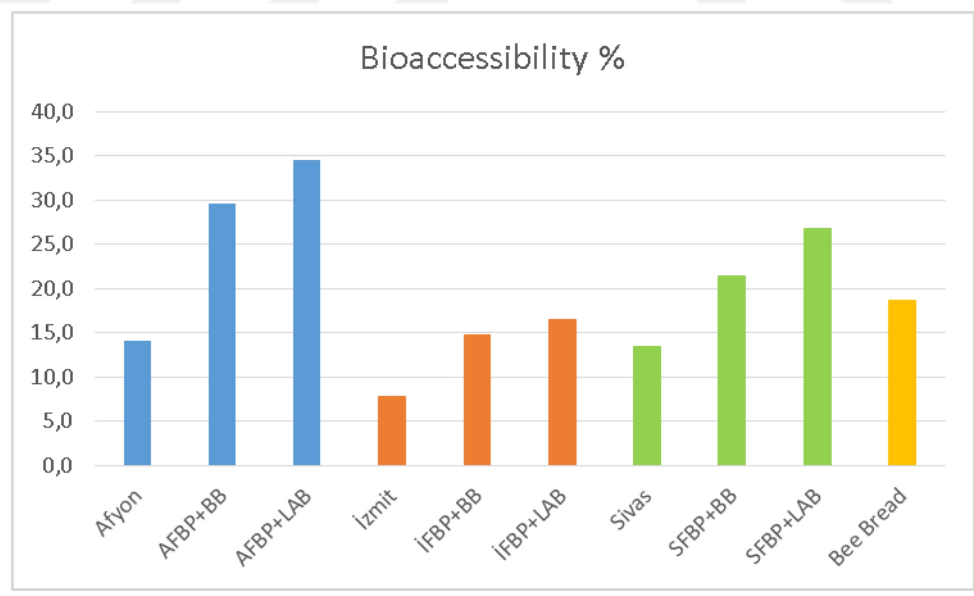

Figure 4.11 : Flavonoid compounds bioaccessibility in intestinal phase from digested bee pollen, fermented bee pollen and bee bread samples*.

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen

The total antioxidant capacity of all samples after the application of *in-vitro* digestion method are shown in Table 4.7, Table 4.8, Table 4.9 and Table 4.10 based on DPPH, ABTS, CUPRAC and FRAP methods, respectively. Sivas bee pollen sample had the highest value of total antioxidant capacity as measured by DPPH, ABTS and CUPRAC methods from oral phase, İzmit bee pollen the lowest among all tested pollen samples according to all methods. Moreover, according to obtained values from gastric phase, Afyon bee pollen sample had the highest value of total antioxidant capacity as measured by DPPH, CUPRAC and FRAP methods, İzmit bee pollen the lowest among all tested pollen samples according to all methods. In addition to this, Sivas bee pollen sample had the highest value of total antioxidant capacity as measured by DPPH and ABTS methods from intestinal phase, Afyon bee pollen the lowest among all tested pollen samples according to DPPH and ABTS methods.

According to the obtained values from oral phase, bee bread added Afyon fermented bee pollen sample had the highest value of total antioxidant capacity as measured by ABTS and CUPRAC methods, bee bread added Sivas fermented bee pollen sample the lowest among all tested fermented bee pollen samples. Moreover, LAB added Afyon fermented bee pollen sample had the highest value of total antioxidant capacity according to DPPH and FRAP methods in oral phase. In addition to this, LAB added Afyon fermented bee pollen sample had the highest value of total antioxidant capacity based on ABTS and CUPRAC methods in gastric phase, bee bread added Sivas fermented bee pollen sample the lowest based on DPPH, ABTS and FRAP methods. According to obtained values from intestinal phase, LAB added Afyon fermented bee pollen sample the highest according to CUPRAC and FRAP methods, Sivas fermented bee pollen sample had the lowest value of total antioxidant capacity as measured by based on all methods.

Commercial bee bread sample had the lowest antioxidant value (87.0±18.0 mg TEAC/100g DM, 714±8.45 µmol TEAC /100g DM and 617±57.8 µmol Fe^{+2} /100g DM in oral phase and 184±8.52 mg TEAC/100g DM, 994±37.6 µmol TEAC /100g DM and 1238±21.3 µmol Fe^{+2} /100g DM in gastric phase) compared to the average values of fermented bee pollen samples based on DPPH (152 and 235 mg TEAC/100g DM), ABTS(1238 and 1295 µmol TEAC /100g DM) and FRAP (775 and 1652 µmol Fe^{+2} /100g DM) methods, respectively in oral phase and gastric phase, respectively. Moreover, it had the lowest antioxidant value (10804±602 µmol TEAC/100g DM,

749±84.5 mg TEAC /100g DM and 2218±186 µmol Fe^{+2} /100g DM) in intestinal phase compare to average values of fermented bee pollen samples based on ABTS (18434 µmol TEAC/100g DM), CUPRAC (876 mg TEAC /100g DM) and FRAP (3097 µmol Fe^{+2} /100g DM) methods, respectively.

In general, a higher total phenolic content, total flavonoid content and total antioxidant capacity were observed compared to unfermented bee pollen samples in all fermented bee pollen samples in all digestion phases. In addition to this, the total phenolic content, total flavonoid content and total antioxidant capacity substantially increased form oral phase to intestinal phase in all digested samples. Although total phenolic content and total flavonoid content of fermented bee pollen samples before digestion were lower than unfermented bee pollen samples except bee bread added İzmit fermented bee pollen sample, fermented bee pollen samples had higher bioaccessibility compare to unfermented bee pollen samples. This observation can be explained by the activation of enzymes that were added in each digestion phase and agitating time during phases. Samples were agitated for 2 minutes by using α-amylase solution, for 2 hours by using pepsin solution and then, again 2 hours by using pancreatin and bile solutions in oral, gastric and intestinal phases, respectively. Observing lower amount of antioxidant capacity in pollen, bee bread and fermented bee pollen samples during the oral phase of digestion might be as a result of short interaction time of α-amylase enzyme with samples. Moreover, digestive enzymes may help in the degradation of the outer layer of the pollen grain making the phenolic, flavonoid and antioxidative compounds more available**.** In general, higher amount of total antioxidant capacity was observed in Afyon fermented bee pollen samples and lower amount in bee bread added Sivas fermented bee pollen sample. It might be as a result of the degradation of exine layer by the help of digestion enzymes. Franchi (1987) studied pollen digestibility by the *in vitro* digestion method. Pepsin, papain and diastase, pancreatin and pancreatic lipase were used as digestive enzymes and from that study it was concluded that digestion is time dependent. Moreover, it was shown that substances placed on the external surface of the grains and in the poral area, are easily reached by the enzymes and are digested (Knox and Heslop-Harrison, 1970).

Table 4.7 : DPPH radical scavenging activities of undigested and digested bee pollen, fermented bee pollen and bee bread samples (mg TEAC/100g DM)*.

Sample	Before digestion	After digestion		
		Simulated Salivary Fluid SSF	Simulated Gastric Fluid SGF	Simulated Intestinal Fluid SIF
Afyon	922±93.1	96.0±16.5	200±2.93	246±30.2
AFBP+BB	1433±73.1	154±12.6	234±8.36	271±75.3
AFBP+LAB	1373±123	197±64.6	244±6.45	243±39.2
İzmit	841±252	57.1±4.09	153±8.33	345±76.9
İFBP+BB	1247±227	123±8.48	219±25.4	280±53.5
İFBP+LAB	1148±53.7	139±5.38	251±2.36	326±72.2
Sivas	275±44.3	99.6±13.8	183±3.62	394±13.6
SFBP+BB	873±81.9	130±12.9	214±3.65	217±52.1
SFBP+LAB	851±55.4	171±20.4	253±12.8	298±53.7
Bee Bread	911±87.7	87.0±18.0	184±8.52	461±68.1

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

Table 4.8 : Total antioxidant capacity of undigested and digested bee pollen, fermented bee pollen and bee bread samples (μmol TEAC /100g DM) based on ABTS method*.

Sample	Before digestion	After digestion		
		Simulated Salivary Fluid SSF	Simulated Gastric Fluid SGF	Simulated Intestinal Fluid SIF
Afyon	8239±1250	796±24.9	1101±20.6	10817±431
AFBP+BB	7166±4648	1354±62.2	1412±120	16838±348
AFBP+LAB	7717±1489	1341±146	1591±198	17925±1743
İzmit	4955±1940	627±62.8	844±107	10979±279
İFBP+BB	5233±2246	1097±61.3	1219±142	19628±826
İFBP+LAB	8667±4055	1321±117	1162±92.1	21296±885
Sivas	6925±1582	807±57.9	1179±159	11029±1253
SFBP+BB	6661±3640	989±70.9	1134±290	16519±507
SFBP+LAB	8620±2425	1327±40.5	1253±52.0	18401±598
Bee Bread	7513±1425	714±8.45	994±37.6	10804±602

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

Table 4.9 : Total antioxidant capacity of undigested and digested bee pollen, fermented bee pollen and bee bread samples based on CUPRAC method (mg TEAC /100g DM)*.

Sample	Before digestion	After digestion		
		Simulated Salivary Fluid SSF	Simulated Gastric Fluid SGF	Simulated Intestinal Fluid SIF
Afyon	2386±277	240±17.1	474±10.6	696±83.7
AFBP+BB	3202±201	263±9.8	505±36.5	860±83.2
AFBP+LAB	3308±229	254±25.3	547±5.7	951±53.6
İzmit	1619±223	160±23.3	356±46.9	709±78.5
İFBP+BB	2750±169	196±32.2	489±50.9	873±104
İFBP+LAB	2484±238	198±25.5	424±51.3	867±40.0
Sivas	1271±354	258±7.8	425±49.8	569±72.7
SFBP+BB	1485±149	185±32.8	443±32.9	767±88.9
SFBP+LAB	1736±408	221±25.8	491±14.1	943±82.8
Bee Bread	2311±447	344±24.3	526±57.1	749±84.5

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen. TEAC: Trolox equivalent antioxidant capacity

Table 4.10 : Total antioxidant capacity of undigested and digested bee pollen, fermented bee pollen and bee bread samples (µmol Fe^{+2} /100g DM) based on FRAP method*.

Sample	Before digestion	After digestion		
		Simulated Salivary Fluid SSF	Simulated Gastric Fluid SGF	Simulated Intestinal Fluid SIF
Afyon	13151±1250	849±67.0	1960±117	2398±108
AFBP+BB	12756±512	911±31.2	2096±82.3	3481±300
AFBP+LAB	12139±439	980±78.2	2063±10.6	3508±371
İzmit	8497±366	455±42.3	982±55.5	1744±91.5
İFBP+BB	9151±2219	521±29.4	1377±20.1	2690±289
İFBP+LAB	8064±1008	620±24.2	1463±36.4	3125±210
Sivas	6320±678	822±49.7	1375±41.9	1952±172
SFBP+BB	4530±1740	660±28.5	1365±133	2649±294
SFBP+LAB	4212±75.4	958±129	1549±156	3132±147
Bee Bread	11578±850	617±57.8	1238±21.3	2218±186

*Data were given as the mean values±standard deviations of triplicates. AFBP+BB: Bee bread added Afyon fermented bee pollen, AFBP+LB: LAB added Afyon fermented bee pollen, IFBP+BB: Bee bread added Izmit fermented bee pollen, IFBP+LB: LAB added Izmit fermented bee pollen, SFBP+BB: Bee bread added Sivas fermented bee pollen, SFBP+LB: LAB added Sivas fermented bee pollen

4.6 Microscopic Analysis

Microscopic analyses results were shown in Figure 4.12, 4.13 and 4.14 for unfermented and fermented Afyon, İzmit and Sivas bee pollen samples, respectively.

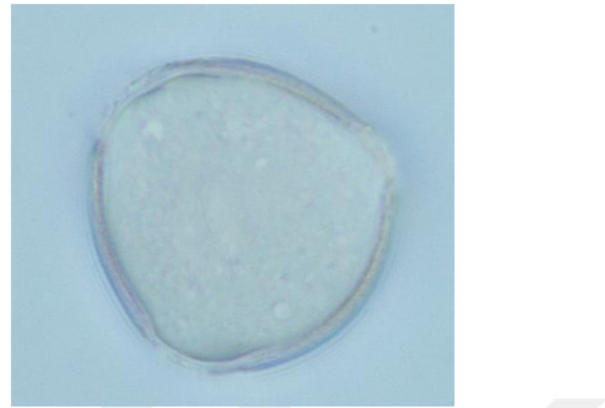
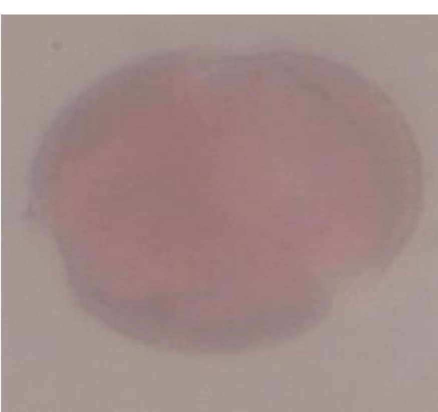

Figure 4.12: Afyon BF, Afyon AF respectively.

*BP: Before Fermentation, AF: After Fermentation

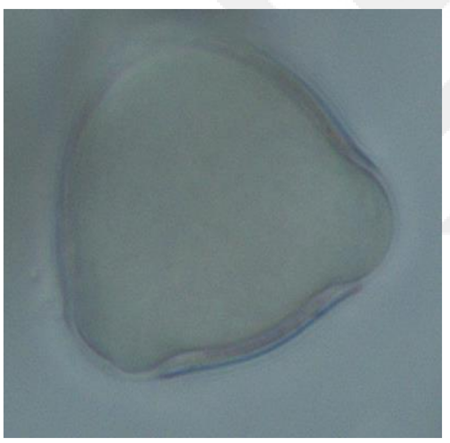
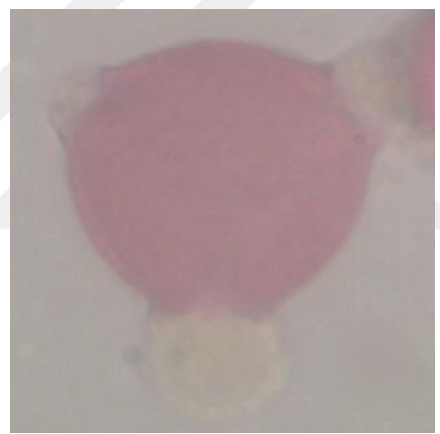

Figure 4.13: İzmit - BF, İzmit AF respectively.

*BP: Before Fermentation, AF: After Fermentation

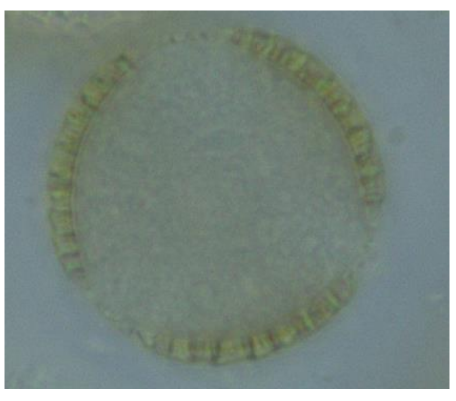
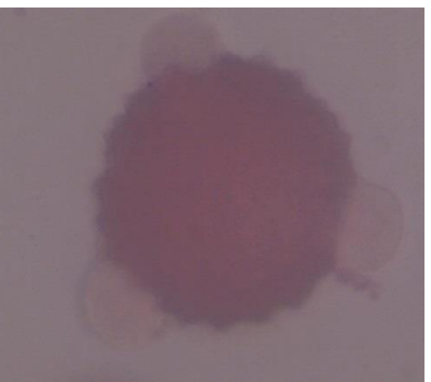

Figure 4.14: Sivas - BF, Sivas AF respectively.

*BP: Before Fermentation, AF: After Fermentation

According to microscopic analyses, it was seen that fermentation process causes partly the destruction of the exine layer of bee pollen samples. Sivas bee pollen sample has spines type exine layer. Because of that, it is more difficult to break down exine layer of Sivas pollen sample. Ayfon sample has soft exine layer compare to other samples. This helps to release antioxdative compounds linked to this.

CONCLUSION

In conclusion, successful fermentation was observed only in Afyon bee pollen sample as a very high reduction of pH by the higher activity of LAB was observed.

Decreasing on the phenolic and flavonoid content of the bee pollen was observed by the effect of fermentation. According to obtained total phenolic content results, decreasing was observed in Afyon fermented bee pollen samples (29%), İzmit fermented bee pollen samples (20%) and Sivas fermented bee pollen samples (50%) on average. In addition to this, according to obtained total flavonoid content results, decreasing was observed in Afyon fermented bee pollen samples (32%), İzmit fermented bee pollen samples (42%) and Sivas fermented bee pollen samples (48%) on average. Moreover, it was observed that unfermented pollen sample had a significantly lower antioxidant activity (based on the DPPH and CUPRAC methods) compared to its fermented samples. According to obtained total antioxidant capacity results based on DPPH method, increasing was observed in Afyon fermented bee pollen samples (52%), İzmit fermented bee pollen samples (44%) and Sivas fermented bee pollen samples (210%) on average. In addition to this, According to obtained total antioxidant capacity results based on CUPRAC method, increasing was observed in Afyon fermented bee pollen samples (36%), İzmit fermented bee pollen samples (58%) and Sivas fermented bee pollen samples (26%) on average. On the other hand, fermentation had no real impact on the measurements compared to unfermented samples regarding to the ABTS and FRAP methods.

Digestion experiments results showed that a higher total phenolic content, total flavonoid content and total antioxidant capacity were obtained in all fermented bee pollen samples in all digestion phases compared to unfermented bee pollen samples. In addition to this, the total phenolic content, total flavonoid content and total antioxidant capacity substantially increased form oral phase to intestinal phase in all digested samples.

In general, it can be said that fermentation of bee pollen has different effects on the antioxidant properties, phenolic and flavonoid content. Future studies, e.g. the investigation of the phenolic compounds by HPLC, are necessary to examine the exact effect of fermentation. In this study, bioaccessibility of the bioactive compounds were analyzed and evaluated by *in vitro* gastrointestinal digestion model. However, it would be interesting to focus on other bioaccessibility/bioavailability techniques by *in vitro* and *in vivo* studies. *Lactococcus lactis* subsp. *Lactis* demonstrated its adaptability to bee pollen and showed to be effective in terms of performance in the production of acidity and decrease in pH for Afyon pollen sample. Future experiments should be focused on different types of LAB as well as yeasts. Moreover, the effect of microorganisms associated with pollen should be investigated to understand the effect of bee pollen on the fermentation process.

REFERENCES

Abouda, Z., Zerdani, I., Kalalou, I., Faid, M., and Ahami, M. T. (2011). The antibacterial activity of Moroccan bee bread and bee-pollen (fresh and dried) against pathogenic bacteria. *Research Journal of Microbiology*, *6*(4), 376.

Akerberg, C., Hofvendahl, K., Zacchi, G., and Hahn-Hägerdal, B. (1998). Modelling the influence of pH, temperature, glucose and lactic acid concentrations on the kinetics of lactic acid production by Lactococcus lactis ssp. lactis ATCC 19435 in whole-wheat flour. *Applied microbiology and biotechnology*, *49*(6), 682-690.

Aličić, D., Šubarić, D., Jašić, M., Pašalić, H., and Ačkar, Đ. (2014). Antioxidant properties of pollen. *HRANA U ZDRAVLJU I BOLESTI*, *3*(1), 6-12.

Almaraz-Abarca, N., da Graça Campos, M., Ávila-Reyes, J. A., Naranjo-Jiménez, N., Herrera-Corral, J., and González-Valdez, L. S. (2004). Variability of antioxidant activity among honeybee-collected pollen of different botanical origin. *INTERCIENCIA-CARACAS-, 29*, 574-578.

Almeida-Muradian, L. B., Pamplona, L. C., Coimbra, S., and Barth, O. M. (2005). Chemical composition and botanical evaluation of dried bee pollen pellets. *Journal of food composition and analysis*, *18*(1), 105-111.

Apak, R., Güçlü, K., Özyürek, M., and Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *Journal of Agricultural and Food Chemistry*, *52*(26), 7970-7981.

Atkin, S. L., Barrier, S., Cui, Z., Fletcher, P. D., Mackenzie, G., Panel, V., Sol, V., and Zhang, X. (2011). UV and visible light screening by individual sporopollenin exines derived from Lycopodium clavatum (club moss) and Ambrosia trifida (giant ragweed). *Journal of Photochemistry and Photobiology B: Biology*,*102*(3), 209-217.

Balasundram, N., Sundram, K., and Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food chemistry*, *99*(1), 191-203.

Balch, J. F., and Balch, P. A. (1990). Prescription for nutritional healing. NewYork: Avery Publishing Group Inc. (pp. 12–39).

Baltrušaitytė, V., Venskutonis, P. R., and Čeksterytė, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. Food Chemistry, *101*(2), 502-514.

Barth, O. M., Freitas, A. S., Oliveira, É. S., Silva, R. A., Maester, F. M., Andrella, R. R., and Cardozo, G. M. (2010). Evaluation of the botanical origin

of commercial dry bee pollen load batches using pollen analysis: a proposal for technical standardization. *Anais da Academia Brasileira de Ciências*, *82*(4), 893-902.

Basim, E., Basim, H., and Özcan, M. (2006). Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. *Journal of Food Engineering*, *77*(4), 992-996.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., and Cogan, T. M. (2001). Recent advances in cheese microbiology. *International Dairy Journal*, *11*(4), 259-274.

Bee Bread (n.d). photograph, Retrieved May 15,2016, from < http://rubezhnoe.all.biz/en/bee-bread-g1669044#.VzjOxTWLTIU>.

Benzie, I. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry*, *239*(1), 70-76.

Bobadilla, C. A. F., Díaz-Moreno, A. C., Zuluaga-Domínguez C. M., and Quicazán, M. C. (2012). *Solid State Fermentation of Bee-Collected Pollen Induced by Lactic Acid Starter Cultures with Probiotic Bacteria.* II International Symposium on Bee Products –Annual meeting of the International Honey Commission, Bragança, Portugal, September 9-12.

Bogdanov, S. (2011). Pollen: Nutrition, Functional Properties, Health: A Review. Bee Product Science, Available online: http://www.bee-hexagon.net/files/file/fileE/Health/PollenBook2Review.pdf, (accessed on 11 May 2012).

Bogdanov, S. (2012). Pollen: Collection, harvest, composition, quality. The bee pollen book (pp. 1-13) http://www.bee-hexagon.net.

Bogdanov, S. (2014). Pollen: production, nutrition and health: a review. *Bee Product Science.*

Bonvehí, J.S., Torrentó, M.S., and Lorente, E.C. (2001). Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain, *J. Agr. Food Chem. 49*, 1848-1853.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition reviews*, *56*(11), 317-333.

Brodschneider, R., and Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, *41*(3), 278-294.

Campos, M., Markham, K. R., Mitchell, K. A., and da Cunha, A. P. (1997). An approach to the characterization of bee pollens via their flavonoid/phenolic profiles. *Phytochemical Analysis*, *8*(4), 181-185.

Campos, M. G., Webby, R. F., Markham, K. R., Mitchell, K. A., and Da Cunha, A. P. (2003). Age-induced diminution of free radical scavenging capacity in bee pollens and the contribution of constituent flavonoids. *Journal of agricultural and food chemistry*, *51*(3), 742-745.

Campos, M. G., Bogdanov, S., de Almeida-Muradian, L. B., Szczesna, T., Mancebo, Y., Frigerio, C., and Ferreira, F. (2008). Pollen

composition and standardisation of analytical methods. *Journal of Apicultural Research*, *47*(2), 154-161.

Campos, M., Frigerio, C., Lopes, J., and Bogdanov, S. (2010). What is the future of Bee-Pollen. *Journal of ApiProduct and ApiMedical Science*, *2*(4), 131-144.

Capanoglu, E., Beekwilder, J., Boyacioglu, D., Hall, R., and De Vos, R. (2008). Changes in antioxidant and metabolite profiles during production of tomato paste. *Journal of Agricultural and Food Chemistry*, *56*(3), 964-973.

Carbonell-Capella, J. M., Buniowska, M., Barba, F. J., Esteve, M. J., and Frígola, A. (2014). Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Comprehensive Reviews in Food Science and Food Safety*, *13*(2), 155-171.

Carpes, S. T., Begnini, R., Alencar, S. M. D., and Masson, M. L. (2007). Study of preparations of bee pollen extracts, antioxidant and antibacterial activity.*Ciência e Agrotecnologia*, *31*(6), 1818-1825.

Casalta, E., and Montel, M. C. (2008). Safety assessment of dairy microorganisms: the Lactococcus genus. *International journal of food microbiology*, *126*(3), 271-273.

Čeksterytė, V., Račys, J., Kaškonienė, V., and Venskutonis, P. R. (2008). Fatty acid composition in beebread. *Biologija*, *54*(4), 253-257.

Chich, J. F., Marchesseau, K., and Gripon, J. C. (1997). Intracellular esterase from Lactococcus lactis subsp. lactis NCDO 763: purification and characterization. *International Dairy Journal*, *7*(2), 169-174.

Choe, E., and Min, D. B. (2009). Mechanisms of antioxidants in the oxidation of foods. *Comprehensive Reviews in Food Science and Food Safety*, *8*(4), 345-358.

Cocan, O., Marghitas, L. A., & Dezmirean, D. (2009). Total polyphenols, flavonoids and radical scavenging activity of beepollen and beebread collected from Transylvania area. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 62.*

Collin, S., Vanhavre, T., Bodart, E., & Bouseta, A. (1995). Heat treatment of pollens: impact on their volatile flavor constituents. *Journal of Agricultural and Food Chemistry*, *43*(2), 444-448.

Cushnie, T. T., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International journal of antimicrobial agents*, *26*(5), 343-356.

Dany, B. (1988). Selbstgemachtes aus Bienenprodukten. Ehrenwirth Verlag, Mu~nchen, 174 pp. Krell, R. (1996). Retrieved from Value-added products from beekeeping (No. 124). Food & Agriculture Org..

Day, A. J., DuPont, M. S., Ridley, S., Rhodes, M., Rhodes, M. J., Morgan, M. R., and Williamson, G. (1998). Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS letters*, *436*(1), 71-75.

de Arruda, V.A.S., Pereira, A.A.S., Freitas, A.S., Barth, O.M., and Almeida-Muradian, L.B. (2013). Dried bee pollen: B complex vitamins, physicochemical and botanical composition, *Journal of Food Composition and Analysis 29*, 100-105.

Degrandi-Hoffman, G., Eckholm, B. J., and Huang, M. H. (2013). A comparison of bee bread made by Africanized and European honey bees (Apis mellifera) and its effects on hemolymph protein titers. *Apidologie*, *44*(1), 52-63.

de Souza Ramos, L., Barbedo, L. S., Braga-Silva, L. A., dos Santos, A. L. S., Pinto, M. R., and da Graça Sgarbi, D. B. (2015). Protease and phospholipase activities of Candida spp. isolated from cutaneous candidiasis. *Revista Iberoamericana de Micología*, *32*(2), 122-125.

Delves-Broughton, J., Blackburn, P., Evans, R. J., and Hugenholtz, J. (1996). Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek*, *69*(2), 193-202.

Denisow, B., and Denisow-Pietrzyk, M. (2016). Biological and therapeutic properties of bee pollen. A review. *Journal of the Science of Food and Agriculture.*

Depeint, F., Gee, J. M., Williamson, G., and Johnson, I. T. (2002). Evidence for consistent patterns between flavonoid structures and cellular activities. *Proceedings of the Nutrition Society, 61*(01), 97-103.

Derochette, S., Franck, T., Mouithys-Mickalad, A., Deby-Dupont, G., Neven, P., and Serteyn, D. (2013). Intra-and extracellular antioxidant capacities of the new water-soluble form of curcumin (NDS27) on stimulated neutrophils and HL-60 cells. *Chemico-biological interactions*, *201*(1), 49-57.

Dewanto, V., Wu, X., Adom, K. K., and Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of agricultural and food chemistry*, *50*(10), 3010-3014.

Di Paola-Naranjo, R. D., Sánchez-Sánchez, J., González-Paramás, A. M., and Rivas-Gonzalo, J. C. (2004). Liquid chromatographic–mass spectrometric analysis of anthocyanin composition of dark blue bee pollen from Echium plantagineum. *Journal of Chromatography A*, *1054*(1), 205-210.

Domínguez-Valhondo, D., Bohoyo Gil, D., Hernández, M. T., and González-Gómez, D. (2011). Influence of the commercial processing and floral origin on bioactive and nutritional properties of honeybee-collected pollen. *International Journal of Food Science & Technology*, *46*(10), 2204-2211.

Durlu-Ozkaya, F., Xanthopoulos, V., Tunail, N., and Litopoulou-Tzanetaki, E. (2001). Technologically important properties of lactic acid bacteria isolates from Beyaz cheese made from raw ewes' milk. *Journal of Applied Microbiology*, *91*(5), 861-870.

Ellis, A. M., and Hayes Jr, G. W. (2009). An evaluation of fresh versus fermented diets for honey bees (Apis mellifera). *Journal of apicultural research*, *48*(3), 215-216.

Estevinho, L.M., Rodrigues, S., Pereira, A.P., and Feás, X. (2012). Portuguese bee pollen: palynological study nutritional and microbiological evaluation. *International Journal of Food Science and Technology, 47*, 429–435.

Fatrcová-Šramková, K., Nôžková, J., Kačániová, M., Máriássyová, M., Rovná, K., and Stričík, M. (2013). Antioxidant and antimicrobial properties of monofloral bee pollen. *Journal of Environmental Science and Health, Part B*, *48*(2), 133-138.

Feás, X., Vázquez-Tato, M. P., Estevinho, L., Seijas, J. A., and Iglesias, A. (2012). Organic bee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules*, *17*(7), 8359-8377.

Fernández-García, E., Carvajal-Lérida, I., and Pérez-Gálvez, A. (2009). In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency. *Nutrition Research*, *29*(11), 751-760.

Foote, H. L. (1957). Possible use of microorganisms in synthetic bee bread production. Amer. Bee J, 97, 476-478. Gilliam, M. (1979). Microbiology of pollen and bee bread: the yeasts. *Apidologie, 10*(1), 43-53.

Franchi, G. G. (1987). Researches on pollen digestibility. *Plant Embryology. The Tuscan Contribution*, 43-52.

Freire, K. R., Lins, A., Dórea, M. C., Santos, F. A., Camara, C. A., and Silva, T. (2012). Palynological origin, phenolic content, and antioxidant properties of honeybee-collected pollen from Bahia, Brazil. *Molecules, 17*(2), 1652-1664.

Gilliam, M., Wickerham, L. J., Morton, H. L., and Martin, R. D. (1974). Yeasts isolated from honey bees, Apis mellifera, fed 2, 4-D and antibiotics. *Journal of invertebrate pathology, 24*(3), 349-356.

Gilliam, M. (1979a). Microbiology of pollen and bee bread: the yeasts. *Apidologie, 10(*1), 43-53.

Gilliam, M. (1979b). Microbiology of pollen and bee bread: the genus Bacillus. *Apidologie, 10*(3), 269-274.

Gilliam, M., Roubik, D. W., and Lorenz, B. J. (1990). Microorganisms associated with pollen, honey, and brood provisions in the nest of a stingless bee, Melipona fasciata [Bacillus. megaterium, Bacillus. circulans, Bacillus. alvei]. *Apidologie (France).*

Han, X., Shen, T., and Lou, H. (2007). Dietary Polyphenols and Their Biological Significance. *Int.J.Mol.Sci.* 8: 950-988.

Haydak, M. H. (1958). Pollen-pollen substitutes-beebread. *Amer. Bee J, 98*(4), 145-146.

Heim, K. E., Tagliaferro, A. R., and Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of nutritional biochemistry, 13*(10), 572-584.

Herbert, E. W., and Shimanuki, H. (1978). Chemical composition and nutritive value of bee-collected and bee-stored pollen. *Apidologie, 9*(1), 33-40.

Hollman, P. C. H., and Katan, M. B. (1997). Absorption, metabolism and health effects of dietary flavonoids in man. *Biomedicine & Pharmacotherapy, 51*(8), 305-310.

Isidorov, V. A., Isidorova, A. G., Sczczepaniak, L., and Czyżewska, U. (2009). Gas chromatographic–mass spectrometric investigation of the chemical composition of beebread. *Food Chemistry, 115*(3), 1056-1063.

Ismail, A. H. M., Owayss, A. A., Mohanny, K. M., & Salem, R. A. (2013). Evaluation of pollen collected by honey bee, Apis mellifera L. colonies at Fayoum Governorate, Egypt. Part 1: Botanical origin. *Journal of the Saudi Society of Agricultural Sciences, 12*(2), 129-135.

Ivanišová, E., Kačániová, M., Frančáková, H., Petrová, J., Hutková, J., Brovarskyi, V., Velychko, S., Adamchuk, L., Schubertová, Z., and Musilová, J. (2015). Bee bread-perspective source of bioactive compounds for future. *Potravinarstvo, 9*(1), 592-598.

Kacaniova, M., Vukovic, N., Chlebo, R., Hascik, P., Rovna, K., Cubon, J., Dzugan, M. and Pasternakiewicz, A. (2012). The antimicrobial activity of honey, bee pollen loads and beeswax from Slovakia. *Archives of biological sciences, 64*(3), 927-934.

Kacaniova, M; Rovna, K; Arpasova, H; Hleba, L; Petrova, J; Hascik, P; Cubon, J; Pavelko, A; Chlebo, R; Bobkova, A and Stiricik, M (2013). The effects of bee pollen extracts on the broiler chicken's gastrointestinal microflora. *Research in Veterinary Science 95* (1): 34-37.

Karakaya, S. (2004). Bioavailability of phenolic compounds. *Critical reviews in food science and nutrition, 44*(6), 453-464.

Kasianenko, V. I., Komisarenko, I. A., and Dubtsova, E. A. (2010). Correction of atherogenic dyslipidemia with honey, pollen and bee bread in patients with different body mass. *Terapevticheskii arkhiv, 83*(8), 58-62.

Khoddami, A., Wilkes, M. A., and Roberts, T. H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules, 18*(2), 2328-2375.

Kim, Y. S., Lee, H. J., Cho, K. H., Bae, H. S., Park, E. K., and Kim, D. H. (2007). The role of intestinal microflora on daidzein excretion in urine in humans treated with chungpesagantang. *Archives of pharmacal research, 30*(4), 481-485.

Klungness, L. M., and Peng, Y. S. (1984). A histochemical study of pollen digestion in the alimentary canal of honeybees (Apis mellifera L.). *Journal of Insect Physiology, 30(*7), 511-521**.**

Knox, R. B., and Heslop-Harrison, J. (1970). Pollen-wall proteins: localization and enzymic activity. *Journal of Cell Science, 6*(1), 1-27.

Koç, A. N., Silici, S., Kasap, F., Hörmet-Öz, H. T., Mavus-Buldu, H., and Ercal, B. D. (2011). Antifungal activity of the honeybee products against Candida spp. and Trichosporon spp. *Journal of Medicinal Food, 14*(1-2), 128-134.

Krell, R. (1996). *Value-added products from beekeeping* (No. 124). Food & Agriculture Org..

Kroyer, G., and Hegedus, N. (2001). Evaluation of bioactive properties of pollen extracts as functional dietary food supplement. *Innovative Food Science & Emerging Technologies, 2*(3), 171-174.

Kumaran, A., and Karunakaran, R. (2006). Antioxidant and free radical scavenging activity of an aqueous extract of Coleus aromaticus. *Food Chemistry, 97*(1), 109-114.

Kumaran, A., and Karunakaran, R. J. (2007). In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. *LWT-Food Science and Technology, 40*(2), 344-352.

Larson, R. A. (1988). The antioxidants of higher plants. *Phytochemistry, 27*(4), 969-978.

LeBlanc, B. W., Davis, O. K., Boue, S., DeLucca, A., and Deeby, T. (2009). Antioxidant activity of Sonoran Desert bee pollen. *Food Chemistry, 115*(4), 1299-1305.

Leja, M., Mareczek, A., Wyżgolik, G., Klepacz-Baniak, J., and Czekońska, K. (2007). Antioxidative properties of bee pollen in selected plant species. *Food Chemistry, 100*(1), 237-240.

Leroy, F., and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology, 15*(2), 67-78.

Llnskens, H. F., and Jorde, W. (1997). Pollen as food and medicine—a review.*Economic Botany, 51*(1), 78-86.

Mărghitaş, L. A., Stanciu, O. G., Dezmirean, D. S., Bobiş, O., Popescu, O., Bogdanov, S., and Campos, M. G. (2009). In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania. *Food Chemistry, 115*(3), 878-883.

Marieke, M., Blitterswijk, H., Leven, L., Kerkvliet, J., and Waerd, J. (2005). Bee Products: properties, processing and marketing. *Wageningon University, Netherland. 45p*.

Markiewicz-Żukowska, R., Naliwajko, S. K., Bartosiuk, E., Moskwa, J., Isidorov, V., Soroczyńska, J., and Borawska, M. H. (2013). Chemical composition and antioxidant activity of beebread, and its influence on the glioblastoma cell line (U87MG). *Journal of Apicultural Science, 57*(2), 147-157.

Marino, P. L. (1998). *The ICU book*. Lippincott Williams & Wilkins.

MatÉs, J. M., Pérez-Gómez, C., and De Castro, I. N. (1999). Antioxidant enzymes and human diseases. *Clinical biochemistry, 32*(8), 595-603.

McDougall, G. J., Dobson, P., Smith, P., Blake, A., and Stewart, D. (2005). Assessing potential bioavailability of raspberry anthocyanins using an in vitro digestion system. *Journal of Agricultural and Food Chemistry, 53*(15), 5896-5904.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C. N., Singh, R.P., Vegarud, G.E., Wickham, M. S. J., Weitschies, W. and Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food & function, 5*(6), 1113-1124.

Mizrahi, A., and Lensky, Y. (Eds.). (2013). *Bee products: properties, applications, and apitherapy.* Springer Science & Business Media.

Moniruzzaman, M., Khalil, M. I., Sulaiman, S. A., and Gan, S. H. (2012). Advances in the analytical methods for determining the antioxidant properties of honey: A review. *African Journal of Traditional, Complementary and Alternative Medicines, 9*(1), 36-42.

Morais, M., Moreira, L., Feas, X., and Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology, 49*(5), 1096-1101.

Mutsaers M., van Blitterswijk H., van 't Leven L., Kerkvliet J., van de Waerdt J. (2005). Bee bread. In: Mutsaers M. (Ed.), Bee products properties, processing and marketing. Agromisa Foundation. Wageningen. pp. 34-35.

Nagai, T., Nagashima, T., Myoda, T., and Inoue, R. (2004). Preparation and functional properties of extracts from bee bread. *Food/Nahrung, 48*(3), 226-229.

Nagai, T., Nagashima, T., Suzuki, N., and Inoue, R. (2005). Antioxidant activity and angiotensin I-converting enzyme inhibition by enzymatic hydrolysates from bee bread. *Zeitschrift für Naturforschung C, 60*(1-2), 133-138.

Narendranath, N. V., and Power, R. (2005). Relationship between pH and medium dissolved solids in terms of growth and metabolism of Lactobacilli and Saccharomyces cerevisiae during ethanol production. *Applied and environmental microbiology, 71*(5), 2239-2243.

Németh, K., Plumb, G. W., Berrin, J. G., Juge, N., Jacob, R., Naim, H. Y., Williamson, G., Swallow, D. M., and Kroon, P. A. (2003). Deglycosylation by small intestinal epithelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *European journal of nutrition, 42*(1), 29-42.

Olofsson, T. C., and Vásquez, A. (2008). Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee Apis mellifera. *Current microbiology, 57*(4), 356-363.

Özcan, M. (2004). Inhibition of Aspergillus parasiticus NRRL 2999 by pollen and propolis extracts. *Journal of medicinal food, 7*(1), 114-116.

Pascoal, A., Rodrigues, S., Teixeira, A., Feas, X., and Estevinho, L. M. (2014). Biological activities of commercial bee pollens: antimicrobial, antimutagenic, antioxidant and anti-inflammatory. *Food and Chemical Toxicology, 63(*0), 233-239.

Parada, J., and Aguilera, J. M. (2007). Food microstructure affects the bioavailability of several nutrients. *Journal of food science, 72*(2), R21-R32.

Pekal, A., and Pyrzynska, K. (2014). Evaluation of aluminium complexation reaction for flavonoid content assay. *Food Analytical Methods, 7(*9), 1776-1782.

Pham-Huy, L. A., He, H., and Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *Int J Biomed Sci, 4*(2), 89-96.

Popov-Raljić, J. V., Laličić-Petronijević, J. G., Georgijev, A. S., Popov, V. S., and Mladenović, M. A. (2010). Sensory evaluation of pralines containing different honey products. *Sensors, 10*(9), 7913-7933.

Porrini, M., and Riso, P. (2008). Factors influencing the bioavailability of antioxidants in foods: A critical appraisal. *Nutrition, Metabolism and Cardiovascular Diseases, 18*(10), 647-650.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine, 26*(9), 1231-1237.

Rebiai, A., Lanez, T., and Belfar, M. L. (2013). Determination of caffeic acid and gallic acid in Algerian bee pollen by HPLC method. 4 Phytochem & BioSub Conference. *At Bechar, Algeria*.

Roldán, A., van Muiswinkel, G.C.J., Lasanta, C., Palacios, V., and Caro, I. (2011). Influence of pollen addition on mead elaboration: Pysicochemical and sensory characteristics, *Food Chemistry 126*, 574-582.

Roulston, T. A. H., and Cane, J. H. (2000). Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution, 222*(1-4), 187-209.

Ross, R. P., Morgan, S., and Hill, C. (2002). Preservation and fermentation: past, present and future. *International journal of food microbiology, 79*(1), 3-16.

Rzepecka-Stojko, A., Stojko, J., Kurek-Górecka, A., Górecki, M., Kabała-Dzik, A., Kubina, R., Moździerz, A., and Buszman, E. (2015). Polyphenols from Bee Pollen: Structure, Absorption, Metabolism and Biological Activity. *Molecules, 20*(12), 21732-21749.

Sakanaka, S., and Ishihara, Y. (2008). Comparison of antioxidant properties of persimmon vinegar and some other commercial vinegars in radical-scavenging assays and on lipid oxidation in tuna homogenates. *Food Chemistry, 107*(2), 739-744.

Salvucci, E., LeBlanc, J. G., and Pérez, G. (2016). Technological properties of Lactic acid bacteria isolated from raw cereal material. *LWT-Food Science and Technology, 70*, 185-191.

Sati, P., Pandey, A., Rawat, S., and Rani, A. (2013). Phytochemicals and antioxidants in leaf extracts of Ginkgo biloba with reference to location, seasonal variation and solvent system. *Journal of Pharmacy research, 7*(9), 804-809.

Serra Bonvehi, J., and Escolà Jordà, R. (1997). Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. *Journal of Agricultural and Food Chemistry, 45*(3), 725-732.

Scalbert, A., and Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of nutrition, 130*(8), 2073S-2085S.

Smit, G., Smit, B. A., and Engels, W. J. (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews, 29*(3), 591-610.

Southworth, D. (1974). Solubility of pollen exines. *American Journal of Botany*, 36-44.

Stanciu, O., Marghıtas, L. A., and Dezmirean, D. (2008). A comparison of methods used to define the antioxidant capacity of bee pollen and beebread from Romania. *In Proceedings. 43rd Croatian and 3rd International Symposium on Agriculture. Opatija. Croatia* (Vol. 751, p. 754).

Stanciu, O., Marghitas, L. A., and Dezmirean, D. (2009). Examination of antioxidant capacity of beebread extracts by different complementary assays. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 64*(1-2).

Stiles, M. E., and Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International journal of food microbiology, 36*(1), 1-29.

Stored Bee Bread in the Comb (n.d). photograph, Retrieved May 15,2016, from <http://nordicfoodlab.org/blog/2015/9/4/bee-bread>.

Szczęsna, T., Rybak-Chmielewska, H., and Chmielewski, W. (2002). Sugar composition of pollen loads harvested at different periods of the beekeeping season. *Journal of Apicultural Science, 46*(2), 107-115.

Szczêsna, T. (2006). Protein content and amino acid composition of bee-collected pollen from selected botanical origins. *Journal of Apicultural Science, 50*(2), 81-90.

The Bee and its hind legs (n.d). photograph, Retrieved May 15,2016, from <https://urbanremedy.com.au/what-is-bee-pollen/ >.

Tlili, N., Mejri, H., Yahia, Y., Saadaoui, E., Rejeb, S., Khaldi, A., and Nasri, N. (2014). Phytochemicals and antioxidant activities of Rhus tripartitum (Ucria) fruits depending on locality and different stages of maturity. *Food chemistry,160*, 98-103.

Tomás-Lorente, F., Garcia-Grau, M. M., Nieto, J. L., and Tomás-Barberán, F. A. (1992). Flavonoids from Cistus ladanifer bee pollen. *Phytochemistry, 31*(6), 2027-2029.

Ulbricht, C., Conquer, J., Giese, N., Khalsa, K. P. S., Sklar, J., Weissner, W., and Woods, J. (2009). An evidence-based systematic review of bee pollen by the Natural Standard Research Collaboration. *Journal of dietary supplements, 6*(3), 290-312.

Vásquez, A., and Olofsson, T. C. (2009). The lactic acid bacteria involved in the production of bee pollen and bee bread. *Journal of apicultural research, 48(*3), 189-195.

Vásquez, A., Olofsson, T. C., and Sammataro, D. (2009). A scientific note on the lactic acid bacterial flora in honeybees in the USA—a comparison with bees from Sweden. *Apidologie, 40*(1), 26-28.

Villanueva, M. O., Marquina, A. D., Serrano, R. B., and Abellán, G. B. (2002). The importance of bee-collected pollen in the diet: a study of its composition. *International journal of food sciences and nutrition, 53*(3), 217-224.

Viskupicova, J., Ondrejovic, M., and Sturdik, E. (2008). Bioavailability and metabolism of flavonoids. *Journal of Food and Nutrition Research, 47*(4), 151-162.

Wallace, S., Fleming, A., Wellman, C. H., and Beerling, D. J. (2011). Evolutionary development of the plant spore and pollen wall. *AoB Plants, 2011*, plr027.

William, S. H., Jenıson, S. A., Yamada, T., and Pastuszyn, A. (1996). Primary structure of the cytosolic β-glucosidase of guinea pig liver. *Biochemical Journal, 319*(3), 829-837.

Wittsiepe, J., Schrey, P., Hack, A., Selenka, F., and Wilhelm, M. (2001). Comparison of different digestive tract models for estimating bioaccessibility of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) from red slag 'Kieselrot'. *International journal of hygiene and environmental health, 203*(3), 263-273.

Yesiltas, B., Capanoglu, E., Firatligil-Durmus, E., Sunay, A. E., Samanci, T., and Boyacioglu, D. (2014). Investigating the in-vitro bioaccessibility of propolis and pollen using a simulated gastrointestinal digestion System. *Journal of Apicultural Research, 53*(1), 101-108.

Zhang, H., Wang, X., Wang, K., and Li, C. (2015). Antioxidant and tyrosinase inhibitory properties of aqueous ethanol extracts from monofloral bee pollen. *Journal of Apicultural Science, 59*(1), 119-128.

Zuluaga C. M., Serrato J. C., and Quicazan M. C. (2015). Bee-Pollen Structure Modification by Physical and Biotechnological Processing: Influence on the Availability of Nutrients and Bioactive Compounds. *Chemical Engineering Transactions, 43*, 79-84.

Zuluaga, C. M., Serrato, J. M., Quicazan, M. C. (2015). Chemical, Nutritional and Bioactive Characterization of Colombian Bee-Bread. *Chemical Engineering Transactions*, vol. 43, p. 175-180.

APPENDICES

APPENDIX A: **Calibration curves**

APPENDIX A

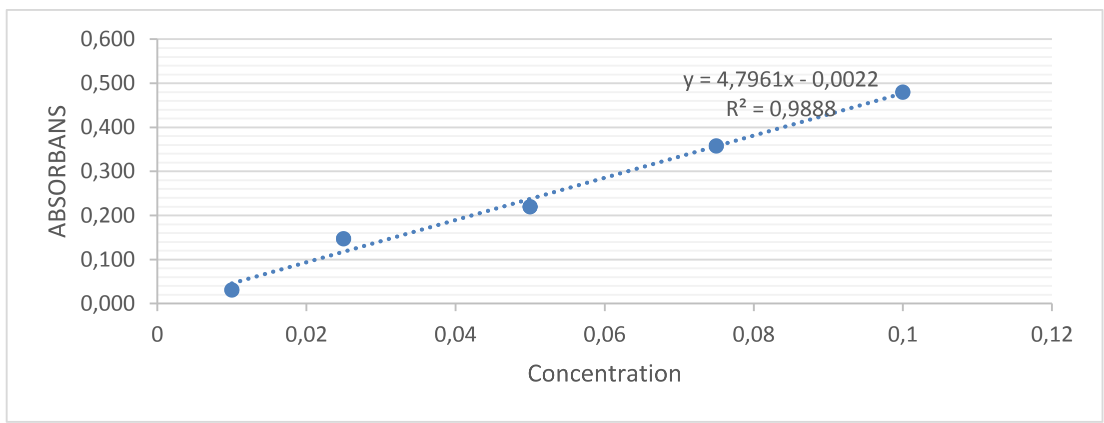

Figure A. 1 : Calibration curve for the total flavonoid content of unfermented pollen samples

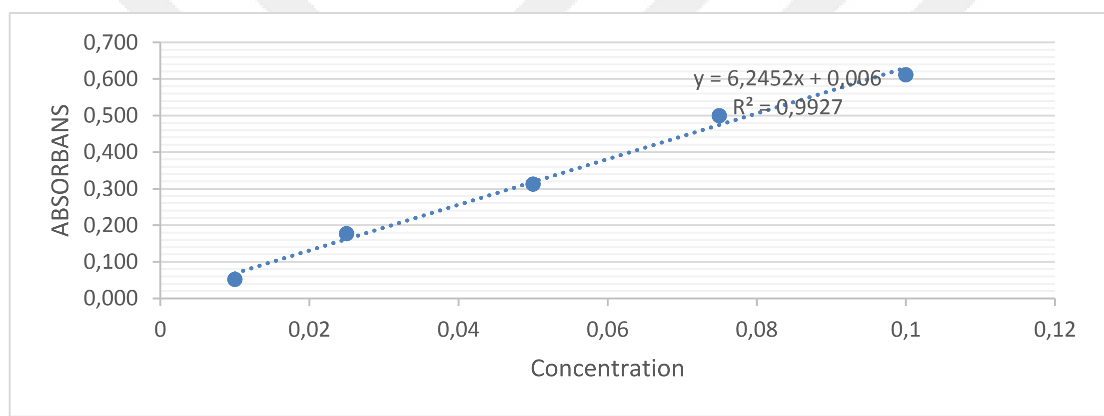

Figure A. 2 : Calibration curve for the total flavonoid content of fermented pollen samples

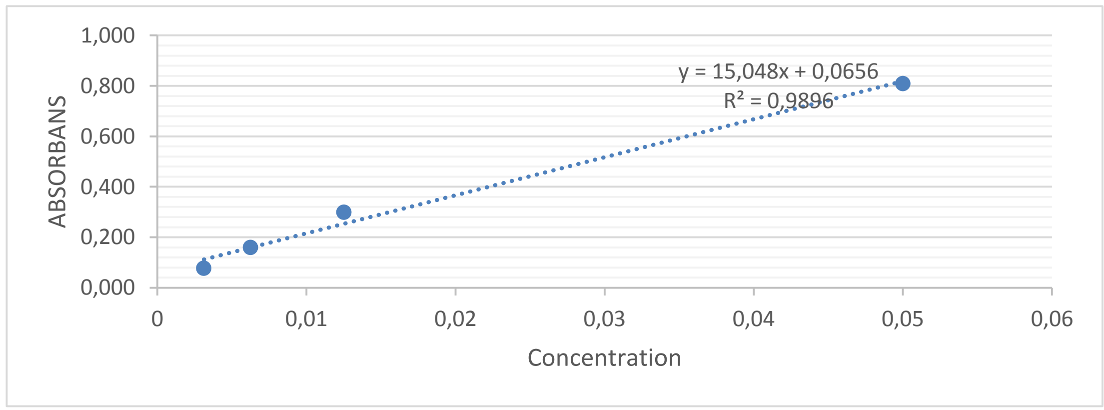

Figure A. 3 : Calibration curve for the total phenolic content of unfermented pollen samples

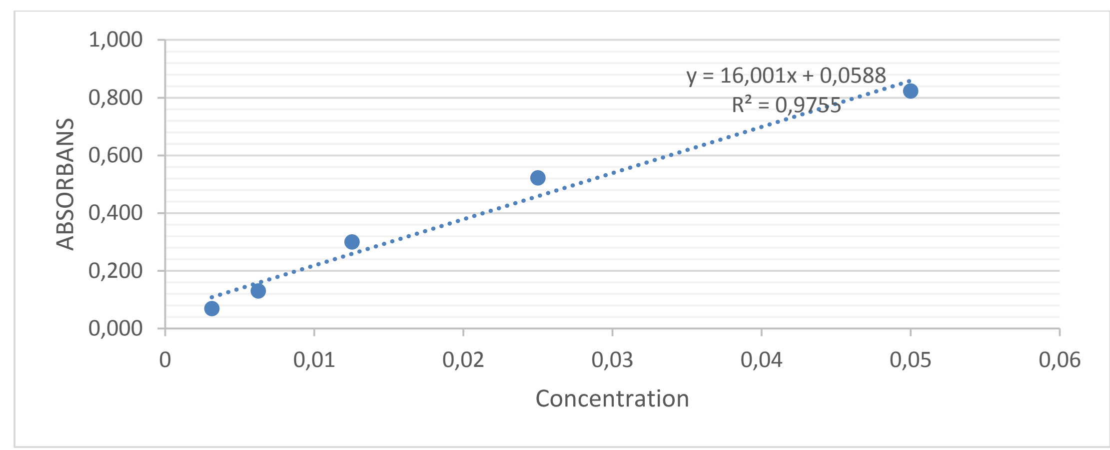

Figure A. 4 : Calibration curve for the total phenolic content of fermented pollen samples

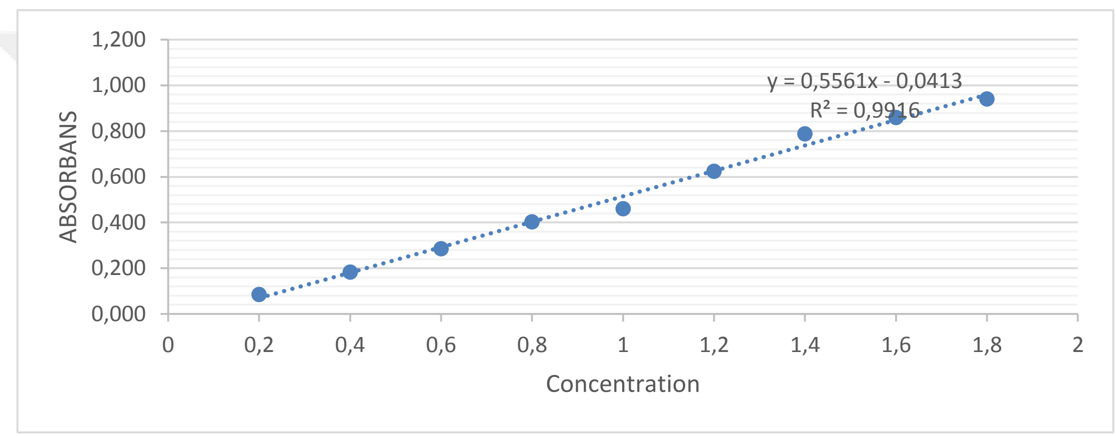

Figure A. 5 : Calibration curve for the total antioxidant capacity of unfermented pollen samples based on FRAP method

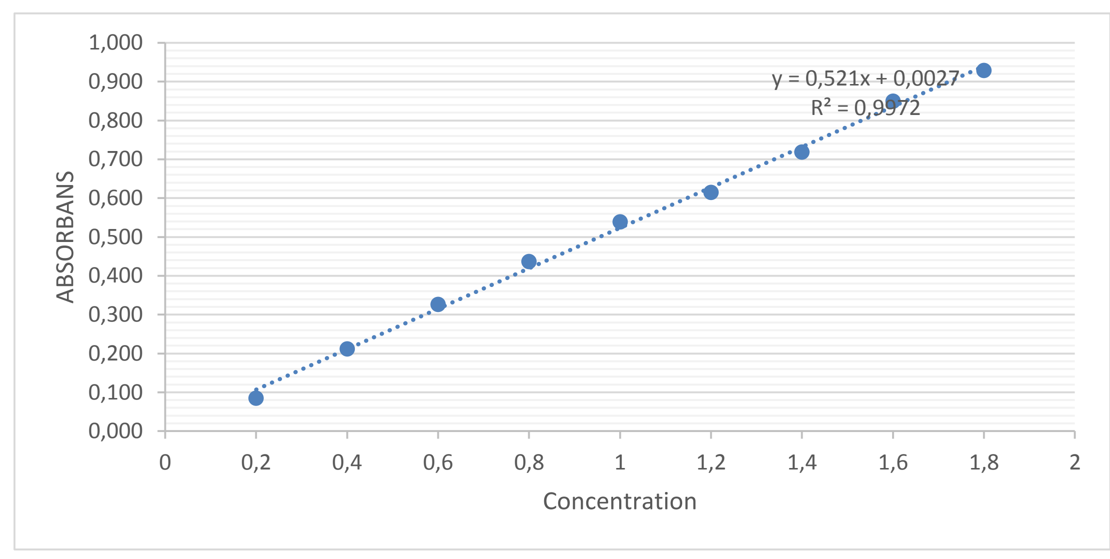

Figure A. 6 : Calibration curve for the total antioxidant capacity of fermented pollen samples based on FRAP method

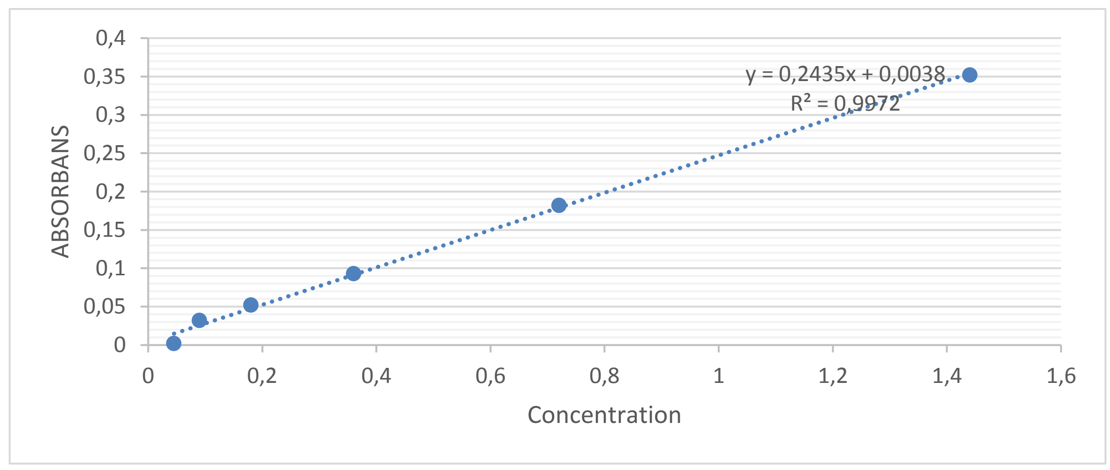

Figure A. 7 : Calibration curve for the total antioxidant capacity of unfermented pollen samples based on ABTS method

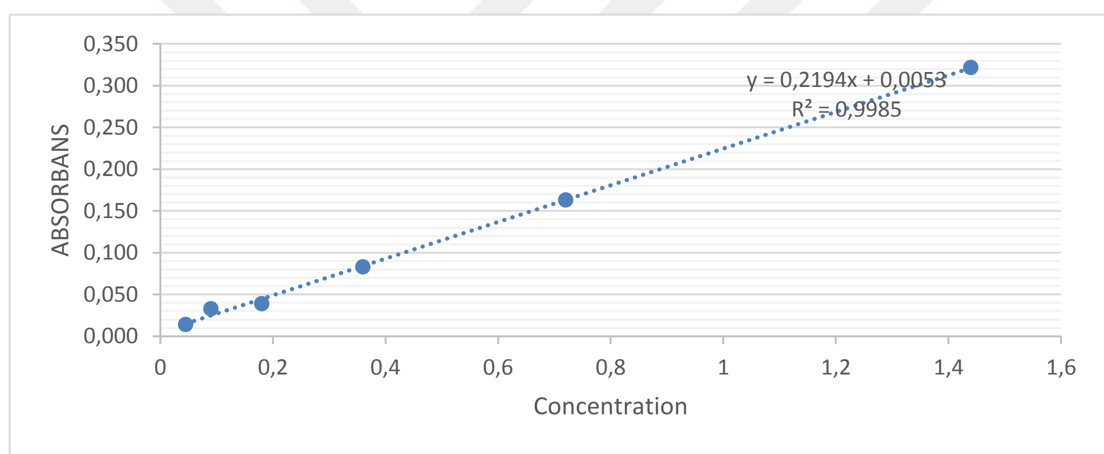

Figure A. 8 : Calibration curve for the total antioxidant capacity of fermented pollen samples based on ABTS method

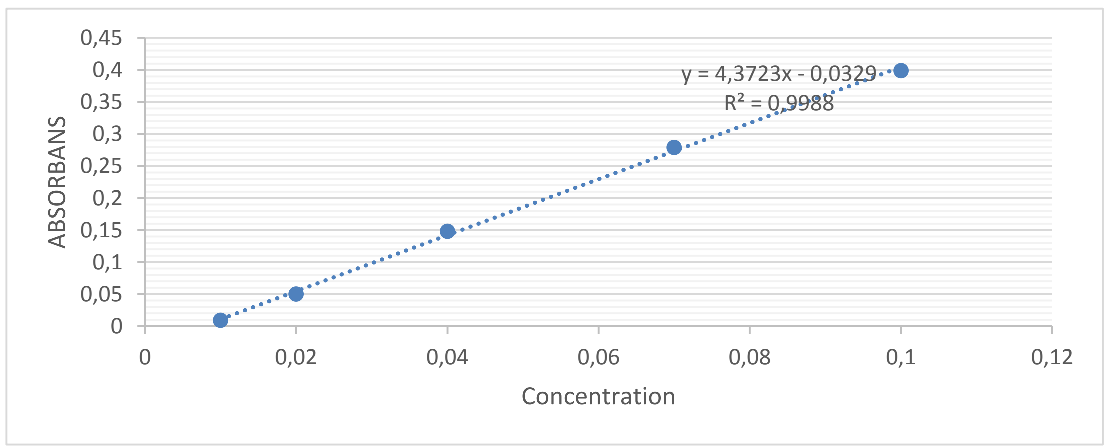

Figure A. 9 : Calibration curve for the total antioxidant capacity of unfermented pollen samples based on DPPH method

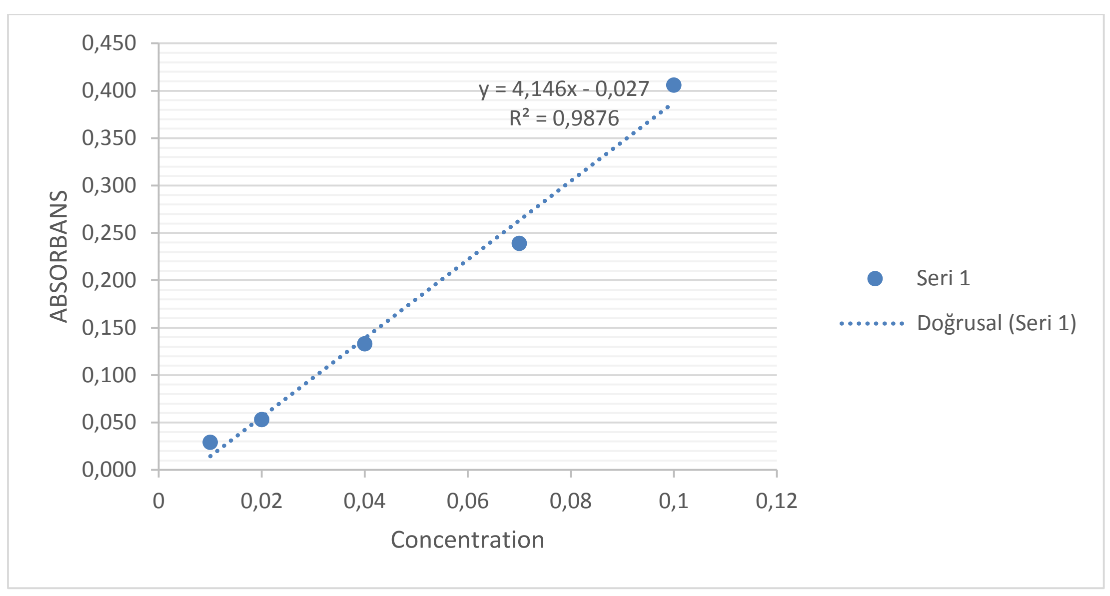

Figure A. 10 : Calibration curve for the total antioxidant capacity of fermented pollen samples based on DPPH method

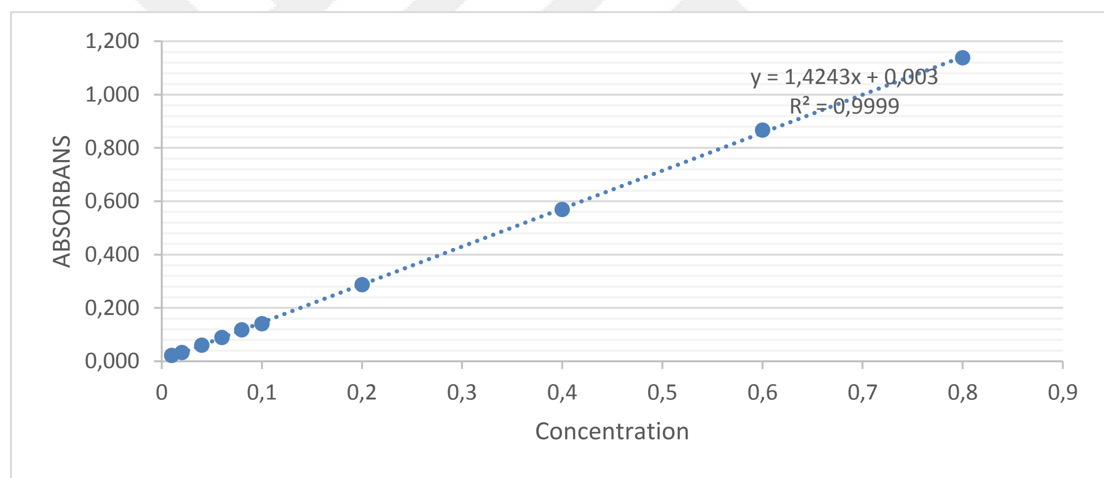

Figure A. 11: Calibration curve for the total antioxidant capacity of unfermented and fermented pollen samples based on CUPRAC method

CURRICULUM VITAE

Name Surname : Serpil Gönül

Place and Date of Birth : Silivri (İstanbul) / 20.05.1991

E-Mail : gonul@itu.edu.tr

EDUCATION :

- **B.Sc.** : 2014, Istanbul Technical University, Chemical-Metallurgical Faculty, Food Engineering Department