DETERMINATION OF BIOACTIVE PEPTIDE PRODUCTION POTENTIALS OF LACTIC ACID BACTERIA ISOLATED FROM SOME TRADITIONAL DAIRY PRODUCTS

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ABSTRACT

Ph.D. Thesis

DETERMINATION OF BIOACTIVE PEPTIDE PRODUCTION POTENTIALS OF LACTIC ACID BACTERIA ISOLATED FROM SOME TRADITIONAL DAIRY PRODUCTS

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The ability of LAB to release bioactive peptides is strain dependent. In the present study, pH, titratable acidity, viable microbial count, WSE protein, peptide profiles, antioxidant, phenolic activity, antibacterial and ACE inhibition activities in fermented milk produced different LAB were examined on the 1st,14th, and 28th day of storage. Sample made with *Lb. bulgaricus* produced the lowest pH. However, sample with *Lc. lactis* NCCB 100539 produced the highest. Samples with *Lb. bulgaricus* 23 and *Lb. helveticus* 11-B2 presented the highest acidity whereas sample with *S. thermophilus* K16 had the least. Sample produced with *S. thermophilus* K16 had the highest viable microbial count while sample with *Lb. bulgaricus* 23 registered the least on day 1. The sample with *Lb. helveticus* 9-B5 presented the highest protein content while the samples with *Lb. paraplantarum* NCCB 100524 had the least. Peptide profiles were identified by RP-HPLC. Increase of peptide number was noticed during storage days and sample with *Lb. brevis* NCCB 100526 produced the highest number of peaks. The results of DPPH showed that the highest antioxidant activity was observed in sample with *S. thermophilus* K16 while sample made with *Lb. brevis* NCCB 100526 produced the least. Sample with *Lb. brevis* NCCB 100526 produced the highest total phenolic content throughout the storage days. Samples with *Lb. paraplantarum* NCCB 100524 had the highest ACE inhibition activities. For antibacterial activity, the highest inhibition was observed against *Escherichia coli* (ATCC 25922) by sample with *Lb. paraplantarum* NCCB 100524. Despite a number of studies about bioactive peptide formations and bioavailability in dairy products especially in cheese, there are limited studies on the functional properties of our own endemic cultures and bioactive peptide formations in fermented milk yet they have functional potentials as illustrated in our findings. According to the results obtained from our study, it is possible to produce traditional functional products using our LAB strains. However, in order to produce naturally traditional products, there is need to determine the molecular and biochemical characteristics of these strains isolated from natural sources to establish their technological properties.

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Key Words: fermented milk, lactic acid bacteria, functional, bioactive peptide, antioxidant, antibacterial, ACE-inhibitor

ÖZET

Doktora Tezi

BAZI GELENEKSEL SÜT ÜRÜNLERİNDEN İZOLE EDİLEN LAKTİK ASİT BAKTERİ SUŞLARININ BİYOAKTİF PEPTİT ÜRETİM POTANSİYELLERİNİN BELİRLENMESİ

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Laktik asit bakterilerinin (LAB) biyoaktif peptitleri meydana getirme yeteneği, bakteri suşuna ve süt işleme koşullarına bağlıdır. Bu çalışmada, çeşitli LAB izolatları ile üretilen fermente sütte pH, asitlik, canlı bakteri sayısı ve suda çözünür peptit profillerinin analizleri yapıldı. Fonksiyonel aktiviteler için antioksidan, fenolik aktivite, antimikrobiyal ve ACE inhibitör özellikleri depolamanın 1., 14. ve 28. günlerinde incelenmiştir. Tüm test ölçümleri ikişer tekrar halinde gerçekleştirildi. Varyans analizi Minitab yazılımının 17. versiyonu (State College, ABD) kullanılarak yapıldı. Sonuçlar ortalama ± standart sapma olarak verildi. Depolama günleri ve farklı LAB starter kültürleri arasındaki önemli farklılıklar %95 güven düzeyinde olan Tukey testi ($P \leq 0.05$) kullanılarak ölçülmüştür.

Lactobacilli suşları 37 °C'de, Lactococci, Streptococci ve Enterococci ise 30 °C'de inkübe edilmiş ve 4.70-4.80 pH aralığı elde edildiğinde inkübasyon sonlandırılmıştır. Fermente süt örneklerinde pH değerleri 3.88-4.81-arasında bulunmuştur. Sonuçlara göre, *Lb. bulgaricus* 23 ve *Lb. helveticus* 11-B2 ile üretilen fermente süt örnekleri depolama süresi sonunda en düşük pH değerini gösterirken, *Lc. lactis* NCCB 100539 örneği depolamanın hem 1. hem de 28. gününde en düşük pH değerlerini göstermiştir. *Lb. helveticus* 11-B2, *Lb. helveticus* 9-B5, *Lb. helveticus* 12-B1 ile üretilen örnekler diğer LAB türlerine göre daha kısa fermantasyon süresine (3-4 saat) sahip bulunmuştur. Tüm fermente süt örneklerinin ortalama titre edilebilir asitliği laktik asit cinsinden %0.16-1.52 laktik asit arasında tespit edilmiştir. *Lb. bulgaricus* 23 ve *Lb. helveticus* 11-B2 ile üretilen örneklerde en yüksek titre edilebilir asitlik değerlerine depolamanın 28. gününde ulaşılmıştır.

Canlı mikrobiyal sayım, yayma plak yöntemiyle gerçekleştirilmiştir. Canlı mikrobiyal sayım sonuçlarına göre, laktobasil örneklerinin canlı bakteri sayısı 8.2-11.1 log KOB/mL arasında tespit edilmiştir. *S. thermophilus* K16 depolamada en yüksek canlı mikrobiyal sayıya sahipken, *Lb. bulgaricus* 23 örneği en az canlılık göstermiştir. Suda Çözünen Azot (SÇA) protein degeri, Qubit™ 4 Florometre yöntemi kullanılarak ölçülmüştür. SÇA'nın pH 4.6'daki ortalama protein miktarı, fermente süt örnekleri arasında önemli ölçüde farklılık göstermiştir ($P \leq 0.05$). Örneklerimizdeki SÇA protein içeriğinin ortalama değeri

0.106 ile 0.390 mg/mL arasında değişmiştir. *Lb. helveticus* 9-B5 en yüksek SÇA protein miktarı içerirken (0.390 mg/mL), *Lb. paraplantarum* NCCB 100524'ün ise en düşük SÇA protein değerine sahip oldugu gözlenmiştir. Peptit profili RP-HPLC kullanılarak belirlenmiş ve sonuçlar proteoliz seviyesinin 28 günlük depolama sırasında giderek arttığını göstermiştir. Farklı bakterilerle üretilen örneklerde depolama günlerinde SÇA'da pik sayıları ve pik yükseklikleri açısından farklılıklar gözlenmistir. En yoğun pikler, *E. faecium* NCCB 100527, *E. faecalis* NCCB 100525, *Lb bulgaricus* 23 ve *Lb. paraplantarum* NCCB 100524 ile üretilen örneklerde belirlenmiştir. Bu durum bahsedilen suşların daha yüksek proteolitik aktivite göstermesi ve dahil edildikleri fermente sütlerde daha fazla biyoaktif peptit üretme potansiyelinde olabileceği şeklinde yorumlanmıştır. Ancak *Lb. helveticus* 9-B5, *Lb. helveticus* 11-B2 ve *Lb. helveticus* 12-B1 ile üretilen fermente süt örnekleri, diğer örneklere göre daha küçük ve daha az pik göstermiştir. Depolama sonunda *L. plantarum* NCCB 100523 ile üretilen örnek en az sayıda ve düşük yükseklikte pikler üretmiş, bunu sırasıyla *E. faecium* NCCB 100527 ve *S. thermophilus* 1-K4 ile üretilen örnekler izlemiştir. *S. thermophilus* 1-K4, *Lb. helveticus* 9-B5, *Lb. helveticus* 11-B2 ve *Lb. helveticus* 12-B1 ile fermente sütün örneklerinin hidrofobik bölgesinde önemli bir pik artışı tespit edilmemiştir.

Farklı LAB suşları ile üretilen fermente süt örneklerinde oluşan peptitlerin biyoaktivitelerinin belirlenmesi kapsamında küçük molekül ağırlıklı peptit fraksiyonları (<3 kDa) ayrılarak analiz edilmiştir. Antioksidan aktiviteyi analiz etmek için DPPH yönteminden yararlanılmıştır. Ölçülen absorbanslar UV/VIS spektrofotometresi ile 517 nm'de belirlenmiş ve sonuçlar μM Trolox eşdeğer antioksidan kapasitesine (μM TEAC) dönüştürülmüştür. Sonuçlara göre tüm örneklerin antioksidan aktivite değeri 152.3-1935.2 μM TEAC arasında değişmektedir. Örnekler arasında ortalama DPPH değerinde önemli farklılıklar olduğu gözlenmiştir ($P \leq 0.05$). *S. thermophilus* K16 içeren fermente süt örnekleri, depolama günleri boyunca en yüksek antioksidan aktivite gösteren örnekler olarak belirlenmiş ve bunu *E. faecium* NCCB 100527 ile üretilen örnek takip etmiştir. En düşük antioksidan aktivite 1. günde *Lb. paraplantarum* NCCB 100524 ile yapılan örneklerde ve depolamanın 14. ve 28. gününde *Lb. brevis* NCCB 100526 ile üretilen orneklerde gözlenmiştir.

Toplam fenolik madde ölçümü Folin-Ciocalteu yöntemi kullanılarak UV/VIS spektrofotometre ile 760 nm'de ölçüm yapılarak belirlenmiştir. Toplam fenolik madde içeriklerin sonuçları, mg gallik asit eşdeğerleri (GAE/g) cinsinden hesaplandı. Fermente süt örneklerinde toplam fenolik madde içeriği 10.68-35.61 arasında değişmektedir. *Lb. brevis* NCCB 100526 ile yapılan örnekler, depolama günleri boyunca en yüksek fenolik içeriği üretirken ve bunu *S. thermophilus* K16 ile yapılan örnekler izlemiştir. Ancak *Lb. paraplantarum* NCCB 100524 ile yapılan fermente süt örnekleri tüm örnekler arasında en az fenolik madde içeriğinde belirlenmiştir.

Fermente süt örneklerinin ACE inhibisyon aktivitesi RP-HPLC kullanılarak belirlenmiş ve sonuçlar yüzde olarak verilmiştir. Genel olarak ACE inhibisyon aktivitesi %40.48-117.81 arasında değişmektedir. Sonuçlara göre, *Lb. paraplantarum* NCCB 100524 ile üretilen örnek, 28. günde en yüksek ACE inhibisyon aktivitesi gösterirken, *S. thermophilus* K16 ile hazırlanan örnek depolamanın 1. gününde tüm numuneler arasında en yüksek ACE inhibisyon aktivitesini göstermiştir.

Antibakteriyel aktivite, farklı patojen bakterilere karşı (*Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella enterocolitica* (ATCC 14028), *Bacillus cereus* (ATCC 14579), *Mycobacterium tuberculosis* (ATCC 7644), *Enterococcus faecalis* (ATCC 29212) Brain Heart Infusion (BHI) agar kullanılarak agar-kuyucuk yöntemi ile belirlenmiştir. Negatif kontrol olarak steril su kullanılmıştır. Antibakteriyel aktivite, inhibisyon bölgesinin alanı (mm) cinsinden ölçülmesi ile belirlenmiştir. *Lb paraplantarum* NCCB 100524 içeren fermente süt örnekleri depolamanın 28. gününde *Escherichia coli*'ye (ATCC 25922) karşı en yüksek inhibisyonu göstermiş, *L. plantarum* NCCB 100523 ile üretilen örnekler ise, depolamanın 1. gününde *Mycobacterium tuberculosis*'e (ATCC 7644) ve depolamanın 28. gününde *Bacillus cereus* 'a (ATCC 14579) karşı en yüksek inhibisyonu göstermiştir.

Çalışmada ülkemize özgü (endemik) suşların süt endüstrisinde ticari kültürlere alternatif olarak kullanım potansiyelleri ve kullanımları durumunda üründe oluşabilecek biyoaktif peptit düzeyleri ile ürünün fonksiyonel özellikleri üzerine sağlayacakları katkılar hakkında ön fikir edinilmiştir. *S. thermophilus* K16, *Lb. helveticus* 11-B2, *E. faecium* NCCB 100527 ve *Lb. brevis* NCCB 100526 suşları biyoaktif peptit üretim potansiyeli bakımından en umut vadeden türler olarak kabul edilirken, *S. thermophilus* 1-K4 ve *Lb. coryniformis* NCCB 100528 ile üretilen örnekler analiz edilen fonksiyonel özellikler bakımından en düşük değerleri göstermiştir. *Lb. bulgaricus* 23, *Lb. helveticus* 11-B2, *Lb. helveticus* 9-B5, *Lb. helveticus* 12-B1, *S. thermophilus* 1-K4 suşları irdelenen teknolojik özelliklerden biri olarak yüksek asitlik üretme özelliğinde bulunmuştur. Sonuç olarak; analiz edilen tüm suşlar arasından hangi LAB'ın üründe daha fazla fonksiyonel etki yaratacağı ve ürünün irdelenen teknolojik özellikleri üzerine etkileri bu çalışma ile belirlenmiştir. Bu sonuçlar çerçevesinde fonksiyonel süt ürünleri endüstrisinde antioksidan aktivite, fenolik içerik, ACE-inhibitör aktivite ve antimikrobiyal aktivite gibi değerlendirilen fonksiyonel etkiler bakımından ürün kalitesini arttıracak aday kültür olarak kullanılabilecek türlerin ve daha yüksek biyoaktif peptit üretme potansiyelinde olabilecek suşların da ön bilgisi edinilmiştir. *S. thermophilus* K16 ve *E. faecium* NCCB 100527 ile üretilen örnekler antioksidan aktivite kapasitesi en yüksek suşlardır. *Lb. brevis* NCCB 100526 *ve S. thermophilus* K16 ile üretilen örnekler toplam fenolik içeriği bakımından en iyi suşlar olarak tespit edilmiştir. *Lb. paraplantarum* NCCB 100524, *L. plantarum* NCCB 100523, *Lb. helveticus* 11-B2 ve *S. thermophilus* K16 ile üretilen örnekler en iyi ACE inhibisyon *aktivitesini* gösteren suştur. Bu 6 suş, aynı zamanda entegre edildikleri fermente sütlerde belirlenen peptit profil kromotagramı hidrofobik bölgelerinde en yoğun piklerin tespit edildiği suşlardır. Bu durum, bu suşların fonksiyonel özelliklerinin yüksek olması ile bağdaştırılabilir.

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Anahtar Kelimeler: fermente süt, laktik asit bakterisi, fonksiyonel, biyoaktif peptit, antioksidan, antibakteriyel, ACE-inhibitör

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TABLE OF CONTENTS

THESIS APPROVAL
ETHIC ..... **i**
ABSTRACT ..... **ii**
ÖZET ..... **iii**
FOREWORD ACKNOWLEDGEMENTS ..... **vi**
SYMBOLS AND ABBREVIATIONS ..... **ix**
LIST OF FIGURES ..... **x**
LIST OF TABLES ..... **xi**
1. INTRODUCTION **1**
2. LITERATURE REVIEW **3**
2.1 Sources of Bioactive Peptides **3**
2.2 Production of Bioactive Peptides **4**
2.2.1 Bioactive peptide discovery approaches **4**
2.3 Methods of Production of Bioactive Peptides **7**
2.3.1 Enzymatic hydrolysis of bioactive peptides **7**
2.3.2 Microbial fermentation of bioactive peptides **8**
2.3.3 Combined methods for production of bioactive peptides **9**
2.4 Functional Properties of Milk Derived Bioactive Peptides **10**
2.4.1 Physiological functional properties of bioactive peptides **11**
2.4.1.1 Anti-hypertensive peptides **12**
2.4.1.2 Anti-oxidant peptides **14**
2.4.1.3 Anti-cancer peptides **15**
2.4.1.4 Anti-diabetic peptides **16**
2.4.1.5 Opioid peptides **17**
2.4.1.6 Antimicrobial peptides **18**
2.4.1.7 Immunomodulatory peptides activity **20**
2.4.1.8 Coronavirus (Covid-19) preventive activity of bioactive peptides **21**
2.4.1.9 Mineral-binding peptides activity **22**
2.5 Dairy Products as Carriers of Bioactive Peptides **23**
2.5.1 Peptide occurrence in dairy products **23**
3. MATERIAL AND METHODS **25**
3.1 Material **26**
3.1.1 Raw milk **26**
3.1.2 Chemicals, mediums, standards **26**
3.1.3 Bacterial strains used for fermented milk production **26**
3.2 Methods **27**
3.2.1 Preparation of bacterial strains used **27**
3.2.2 Production of fermented milk **28**
3.3 Analyses of Fermented Milk Samples **29**
3.3.1 pH value **29**
3.3.2 Titratable acidity value **29**

3.3.3 Viable microbial number **30**
3.3.4 Preparation of water soluble extract (WSE) for peptide profile analysis **30**
3.3.5 Water soluble extract (WSE) protein quantity **30**
3.3.6 Determination of peptide profile **31**
3.3.7 Obtaining peptides with molecular size below 3kDa by centrifugation **32**
3.4 Determination of Peptide Bioactivities **32**
3.4.1 Determination of antioxidant activity **33**
3.4.2 Determination of phenolic content **34**
3.4.3 Determination of antibacterial activity **34**
3.4.4 Determination of ACE inhibitory activity **35**
3.5 Statistical Analyses **36**
4. RESULTS AND DISCUSSION **37**
4.1 Properties of Pasteurized Milk Used in Fermented Milk Production **37**
4.2 pH Values of the Fermented Milk Samples **37**
4.3 Titratable Acidity (% Lactic acid) of Fermented Milk Samples **42**
4.4 Viable Microbial Count of the Fermented Milk Samples **47**
4.5 Water Soluble Extract Protein Quantity of the Fermented Milk Samples **52**
4.6 Peptide Profile of Fermented Milk Samples **57**
4.7 Antioxidant Activity (DPPH) of the Fermented Milk Samples **67**
4.8 Phenolic Content in Fermented Milk Samples **73**
4.9 Antibacterial Activity of the Fermented Milk Samples **78**
4.10 Antihypertensive Activity of Fermented Milk Samples **82**
5. CONCLUSION AND FUTURE DIRECTIONS **88**
5.1 Future Directions **91**
REFERENCES **92**
APPENDIX 1 BACTERIA GROWTH CONDITIONS **114**
APPENDIX 2 ANTIOXIDANT ACTIVITY (μM TEAC) WITH DPPH ASSAY **119**
APPENDIX 3 PHENOLIC CONTENT (mg GAE/g) **126**
APPENDIX 4 SAMPLE PH VS TITRATABLE ACIDITY XY CURVE **133**
CURRICULUM VITAE **140**

SYMBOLS AND ABBREVIATIONS

ACE	Angiotensinogen-Converting Enzyme
ACN	Acetonitrile
ATCC	The American Type Culture Collection
BHI	Brain heart infusion
BPs	Bioactive peptides
CFU	Colony forming unit
CVD	Cardiovascular diseases
DM	Dry matter
DPPH	2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6- Sulfonic Acid
g	Gram
GAE	Gallic acid equivalents
GIP	Gastric inhibition polypeptide
GLP-1	Glucagon-like peptide-1
h	Hour
HCl	Hydrochloric acid
HHL	Hippuryl-histidyl-leucine
HPLC	High performance liquid chromatography
LAB	Lactic acid bacteria
mg	Milligram
Mins	Minutes
mL	Milliliter
mm	Millimeter
MRS	De Man Rogosa Sharpe
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCCB	The Netherlands Culture Collection of Bacteria
ND	Not detected
nm	Nanometer
^oC	Degree Celsius
PM	Pasteurized milk
PVDF	Polyvinylidene difluoride
RAS	Rennin-Angiotensin system
RM	Reconstituted milk
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high-performance liquid chromatography
TEAC	Trolox Equivalence Antioxidant Capacity
TFA	Trifluoroacetic acid
UF	Ultrafiltration
UV	Ultra violet
WSN	Water soluble nitrogen
WSPs	Water soluble peptides
α-CN	Alpha casein
κ-CN	Kappa casein

LIST OF FIGURES

Figure 2.1 Popular approaches used in the discovery of bioactive peptides from food proteins 5
Figure 2.2 Major bioactive functional compounds derived from milk (Park and Nam 2015)..... 11
Figure 2.3 Physiological functional effects of bioactive peptides 12
Figure 3.1 Preperation of bacteria strains used 28
Figure 3.2 Fermented milk production process 29
Figure 4.1 pH values of the samples during storage days (n=2)..... 41
Figure 4.2 Titratable acidity values of the samples during storage days (n=2) 46
Figure 4.3 Viable microbial counts of the samples during storage days (n=2). 51
Figure 4.4 WSE protein quantity of samples during storage days (n=2)..... 56
Figure 4.5 *Lc. lactis* NCCB 100539 peptide profile during storage days 60
Figure 4.6 *Lb. coryniformis* NCCB 100528 peptide profile during storage days 60
Figure 4.7 *Lb. brevis* NCCB 100526 peptide profile during storage days..... 61
Figure 4.8 *L. plantarum* NCCB 100523 peptide profile during storage days..... 61
Figure 4.9 *E. faecium* NCCB 100527 peptide profile during storage days..... 62
Figure 4.10 *E. faecalis* NCCB 100525 peptide profile during storage days..... 62
Figure 4.11 *Lb. bulgaricus* 23 peptide profile during storage days 63
Figure 4.12 *Lb. paraplantarum* NCCB 100524 peptide profile during storage days 63
Figure 4.13 *S. thermophilus* K16 peptide profile during storage days..... 64
Figure 4.14 *S. thermophilus* 1-K4 peptide profile during storage days 64
Figure 4.15 *Lb. helveticus* 9-B5 peptide profile during storage days 65
Figure 4.16 *Lb. helveticus* 11-B2 peptide profile during storage days 65
Figure 4.17 *Lb. helveticus* 12-B1 peptide profile during storage days 66
Figure 4.18 Pasteurized milk (control) peptide profile during storage day 66
Figure 4.19 DPPH (TEAC μM)) values of the samples during storage days (n=2) 72
Figure 4.20 Phenolic content of the sample during storage days (n=2)..... 77
Figure 4.21 ACE inhibitory activity of the samples during storage days (n=2) 87

LIST OF TABLES

Table 2.1 Some bioactive peptides in dairy products with antimicrobial effects 19
Table 2.2 Biological activities of selected milk hydrolysates and fermented dairy products 24
Table 3.1 Lactic acid bacteria used 27
Table 3.2 RP-HPLC Gradient Program 32
Table 4.1 Properties of pasteurized milk used in production (n=2) 37
Table 4.2 pH values of the samples during the storage days (n=2) 38
Table 4.3 Titratable acidity (% LA) values of the samples during storage days (n=2) 43
Table 4.4 Viable microbial counts (log CFU/mL) values of the samples during storage days (n=2) 48
Table 4.5 Water soluble extract (WSE) protein quantity of the samples during storage days (n=2) 53
Table 4.6 Phenolic content (mg GAE/g) values of the samples during storage days (n=2) 68
Table 4.7 Antibacterial activity of the samples during storage days (n=2) 74
Table 4.8 ACE inhibitory activity (%) of the samples during storage days (n=2) 81
Table 4.9 ACE inhibitory activity (%) of samples during storage days (n=2) 83
Table 5.1 Summary of the functional properties in the samples during the storage 91

1. INTRODUCTION

Research interventions on foods with certain functionalities have gained immense attentiveness in bioactive compounds, particularly peptides with bioactivity in foods due to their influence on health promotion (Munir et. al 2020, Peredo-Lovillo et al. 202). In this perspective, biofunctional properties attributed to bioactive peptides (BP) derived from milk exhibit numerous therapeutic roles: antihypertensive, antioxidant, cholesterol-lowering, anti-inflammation, immunomodulatory, and inhibition against microorganisms (Nguyen et. al. 2020, Dinić et al. 2023) anti-cancer mineral binding properties (Raziq 2015). BP also play a potential preventive role against coronavirus disease (COVID-19) (Savastano et al. 2019, Ni et al. 2020, Pradeep et al. 2021, Ashokbhai et al. 2022). They offer therapeutic aids as a result of their increased biological benefit and are specific to targets, reduced toxicity, structure diversity and low build up in body tissues (Rafiq 2015).

Milk derived BP are found dormant inside the series of the parental protein and may be obtained in parental protein by enzyme digestion, gastrointestinal fermentation and/or in food fermentation by lactic acid bacteria [LAB] cultures with proteolytic activities (Otağ and Hayta 2013, Nongonierma and FitzGerald 2018, Aspri et al. 2018; Kocak et al. 2020). BP can also be produced during food processes for example; during cheese ripening, pressure or heat treatments (Dapkevicius et al. 2021, Peredo-Lovillo et al. 2022) while others can be obtained by a combination of methods (Chen et al. 2007, Chakrabarti et al. 2018, Peredo-Lovillo et al. 2022). The production of certain BP during the fermentation in a controlled approach is quite difficult. This process greatly depends on the ability of the proteolytic bacteria system used to generate the targeted peptide. Therefore, the choice of the bacterium to be used and the control of its proteolytic activity are among the most essential characteristics in the generation of fermented milk products with BP. Dairy products for instance yoghurt, kefir and cheese are among the rich sources of BP with health functions (Amorim et al. 2019, Banihashemi et al. 2020).

Recently, the utilization of LAB which form BP especially in the production of dairy product has become popular as a potential origin of biotherapeutic peptides and peptides that strengthen the immune system. Various sources stated that different proteolytic

enzymes of LAB produce BP with various functional properties from milk proteins (Georgalaki et al. 2002, Broadbent et al. 2011, Ardö et al. 2017, Edebali et al. 2021). Fermented dairy products are rich in bioactive compounds formed during milk fermentation Elkhtab et al. (2017) and the formation and concentration of BP in dairy products vary in regards to the type of products, the production, maturation method and the proteolytic activities of the microbial cultures used.

Some of the LAB used within the scope of the thesis (*Lactococcus lactis, Lactiplantibacillus plantarum, Lb. brevis, Lb. paraplantarum, Lb. coryniformis, Enterococcus faecium, Enterococcus faecalis*) were obtained from Karaman Divle Obruğu Tulum cheese, which was traditionally produced from raw sheep milk without using starter cultures. For all these microbiota, protease and lipase enzyme production activities were also determined qualitatively and quantitatively for each strain (Budak et al. 2016a, 2016b, Ozturkoglu Budak et al. 2016, 2016, Ozturkoglu-Budak et al. 2018). Apart from the microbial strains originating from Divle cheese, we also employed different endemic strains (*Lb. helveticus* 11-B2, *Lb. helveticus* 9-B5, *Lb. helveticus* 12-B1, *S. thermophilus* K16, *S. thermophilus* 1-K4) isolated from products such as traditional cheese and yoghurt from some of our other previous project studies.

Although there are studies in the literature about bioactive peptide formations and bioavailability of these peptides in dairy products, especially cheese produced around the world, there are limited studies on the functional properties of our own endemic cultures and bioactive peptide formations. The LAB isolates obtained from traditional fermented dairy products used in our study are isolates that have not been studied by researchers before in terms of their bioactive peptide production potential. As a result, the purpose of this thesis is to determine some functional and technological characteristics of a variety of native LAB strains in fermented milk samples that they integrated and find out the most suitable ones in terms of the bioactive peptide production capacity. This data will be useful for the detection of the best candidate that could be used as culture in the dairy industry for the production of functional dairy products.

2. LITERATURE REVIEW

2.1 Sources of Bioactive Peptides

Bioactive peptides located in food protein matrices are considered to be encrypted widely in dormant state in the parental protein matrix and therefore necessitates to be hydrolyzed in order to exploit their biological benefits (Li et al. 2015, Hati et al. 2018, Parmar et al. 2018, Mora et al. 2019, Kinariwala et al. 2020). BP have short sequences spanning from 2-20 amino acids (Amigo and Hernández-Ledesma 2020, Banihashemi et al. 2020, Turan and Durak 2020, Folliero et al. 2022, Okoye et al. 2022). They also have low molecular weight compounds (<10 kDa) (Dingess et al. 2019).

Regardless of their sources, BP are mainly categorized as internal or external. The internal peptides are obtained by enzymatic method with the help of microorganisms whereas external peptides are released from outside sources for example diet, additives and supplements (Chakrabarti et al. 2018, Okoye et al. 2022). BP in food are on a great extent from either animal-derived or plant-derived BP sources (Chaudhary et al. 2021). Animal based protein is an essential source of valuable BP and could be derived from bovine milk and among other dairy products (Wali et al. 2020, Ali Redha et al. 2022), eggs, fish, bovine blood and gelatin (Yu et al. 2021, Okoye et al. 2022). Bp can be obtained from plants such as soy, maize, sorghum, amaranth and mushrooms respectively (Chauhan and Kanwar 2020).

Milk traditionally obtained from cow, goat, sheep, buffalo, camel and yak has widely been used as a fundamental BP source with cow milk as the most predominate. Dairy products for instance; cheese and bovine milk have by far been considered as rich origins of protein with bioactivity from food. However, bioactive peptides may also be delivered from animal products such as cow blood, fish, eggs and meat whereas peptides of plant origin plant can include maize, soy, rice, sorghum, pumpkin and mushrooms (Chauhan and Kanwar 2020).

Among the food sources, dairy products like cheese and other milk byproducts are known as the greatest sources of BP (Lemes et al. 2016, Mohanty et al. 2016, Chauhan and Kanwar 2020). BP can be produced through gastrointestinal tract digestion or food processes. Low and medium molecular weight BP for instance phosphopeptides performance and opioid have been reported in milk from human beings by pressure liquid chromatography-mass spectrometry (HPLC-MS) techniques and tandem mass spectrometry methods. Lactoferrin a serin protease which is rich in BP in particular and can be found in milk from mammals with iron-binding glycoprotein, antimicrobial and has immunomodulating effects. (Moller et al. 2008). Bovine colostrum is regarded as an essential source of bioactive peptide after gastrointestinal digestion (Jorgensen et al. 2010, Chauhan and Kanwar 2020).

2.2 Production of Bioactive Peptides

Production of BP coming from food protein can be achievable through hydrolysis by digestive enzymes during gastrointestinal digestion or by fermentative starter cultures. However, a number of studies highlight the use of a combined methods to generate BP. Furthermore, techniques like heating or chemical digestion by acids or alkalines are among the other various ways of BP production (Hafeez et al. 2013, Ahtseh 2018, Jakubczyk et al. 2020).

2.2.1 Bioactive peptide discovery approaches

The popular methods in peptide discovery and formation are categorically shown in Fig. 2. 1. The empirical method (*in vitro* method) (Saavedra et al. 2013) includes various approaches such as; selecting necessary food protein source, isolation of protein fractions, peptide generation from fragments by proteolysis with the aid of the internal enzymes, external enzymes or through food fermentation, pre- bioactivity selection, purifying and fractionating, extra evaluation of the bio-activity of the screened peptides, identification of peptides by mass spectrometry method, and *in vitro* or *in vivo* approval of bioactivity. Even though the empirical methodology is the broadly utilized way, it is time- consuming because it demands for intensive sample preparation in form of suitable sample

pretreatment, fractionating, and purification. This approach does not allow distinctive identification of single BPs. However, to overcome setback of the empirical methodology, bioinformatics methods have lately been initiated (Capriotti et al. 2016).

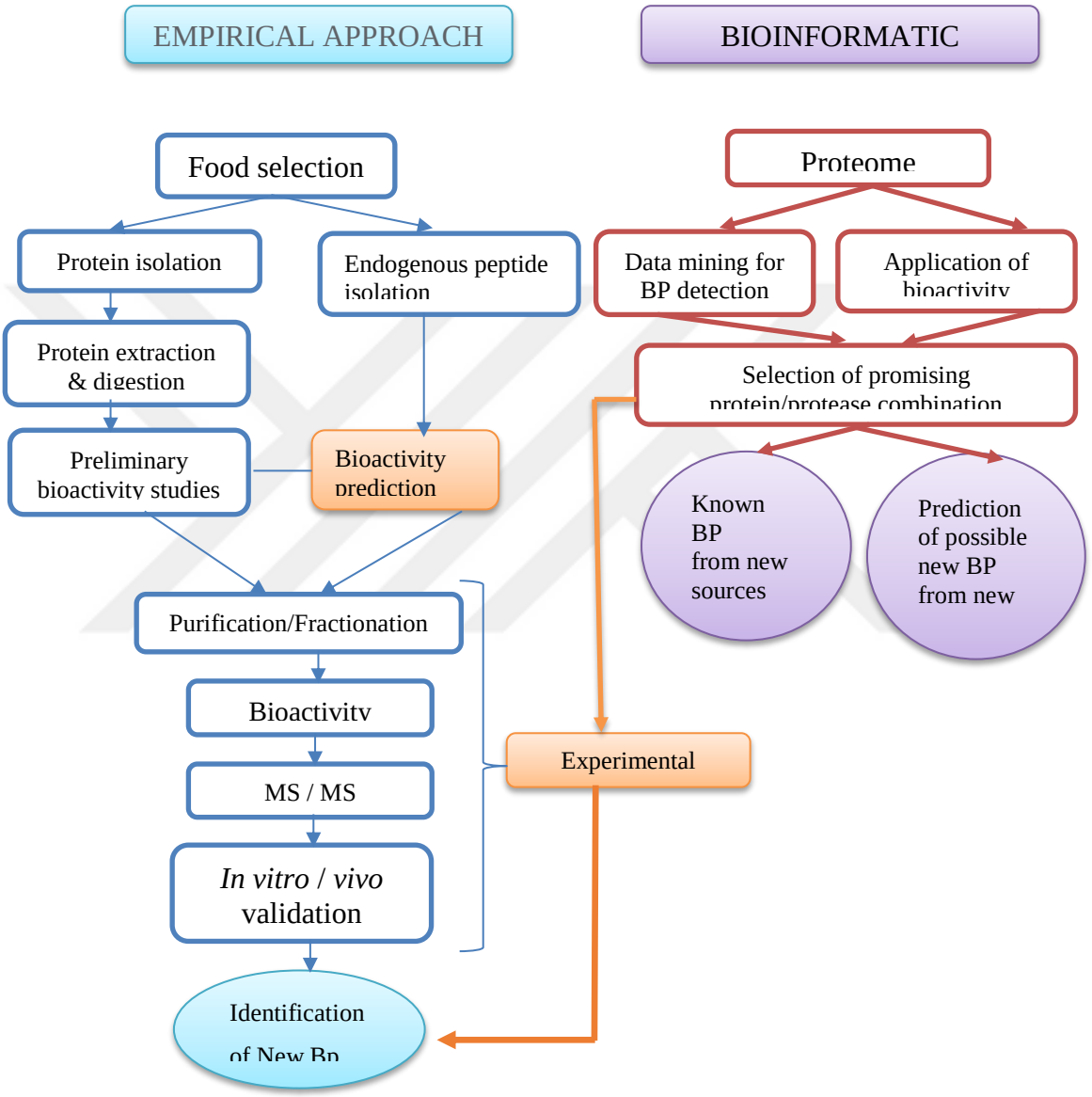


Figure 2.1 Popular approaches used in the discovery of bioactive peptides from food proteins

The bioinformatic approach allows the establishment of potential description of biological activity of protein particles, quantitative descriptors measurements to estimate target parent proteins of BP, and the estimation of bonds vulnerable to breakdown by protein endopeptidases. Following sample dissolution, it is probable to clarify the difficulty of the primary sample since it is quite easy to evaluate the biologically active peptide instantly (Sauer et al. 2015). The bioinformatics approach offers a particular list of candidates to be subjected to further individual approval that demands the production of these given peptides and test their individua bioactivity. Bioinformatics methodology generally takes advantage of the data given by multiple databanks like BIOPEP Minkiewicz et al. (2008) PepBank, PeptideDB, peptide databases of antimicrobial (APD2) and the Collection of AntiMicrobial Peptide (CAMP) Sànchez-Rivera et al. (2014) to allocate a bio- activity to the recognized peptides. The peptide property classifications obtained in the PeptideDB and the BIOPEP databank contains peptide hormones, peptides with antimicrobial activity, cytokines and growth boosters, and toxic peptides, on the contrary, CAMP and APD2 are only restricted to antibacterial ones, peptides with antiparasitic activity, antivirus, and anti-fungal peptides. These databanks encompass AAs together with peptides whose AAs range from 2–14 and above. BIOPEP is the most applied databank in food experiments. However, MilkAMP is the specified and most recent database accepted to be used for milk and dairy products by Théolier and colleagues, which covers 371 listings of which, 9 are hydrolysates, 299 for antimicrobial peptides, 23 peptides estimated with antimicrobial activities, and 40 considered as inactive peptides (Théolier et al. 2014).

Bioactive peptides obtained in cheeses in pasteurized and raw bovine milk utilizes enzyme-prediction (http://bioware.ucd.ie/ ~enzpred/Enzpred.php) and the BIOPEP databank to flourishingly estimate target BP. In that experiment with 187 established sequences 37 were considered to have ACE inhibitory activity and immunomodulating and indicated differences not only in the particular order but also in their comparative quantities between the two studied cheese groups. The BIOPEP databank was applied to confirm specified enzymes responsible for the secretion of a number of peptides with antimicrobial activity; the bioinformatic experiments permits one to hypothetically assume the likely proteolytic bonds to hydrolyzed in casein (CN) and single out

thermolysin-like enzymes as distinctly possible contender leading to caseicin A from α_{s1}-CN antimicrobial peptide (Guinane et al. 2015).

Bioinformatic approach is more advantageous than the classical method for BP discovery but peptides are accepted as biologically active if the particular serie is acknowledged /accepted by the databank information. In that way, reliableness of bioinformatic information are factually based on the employed studying databank. Qualification of the ascribed BP series are paramount. However, study work of structural bioactivity linkage of peptides larger than 4 AAs is quite obstructed by the increased expenses in terms of the chemicals required (Capriotti et al. 2016).

2.3 Methods of Production of Bioactive Peptides

2.3.1 Enzymatic hydrolysis of bioactive peptides

Enzymatic hydrolysis confined into two kinds of proteolytic enzymes pertaining to their hydrolyzing mechanism: endopeptidases which breaks peptide bonds present in the protein molecules and the exoprotease which hydrolyze N or C terminal bonds of the peptide. Subsequent to enzyme cleavage, further treatment of the hydrosates by ultrafiltration, heat application or the treatment of activated carbon necessary to regulate the molecule size and get rid of bitterness in the obtained hydrolysate (Mukhopadhya and Sweeney, 2016, Peredo-Lovillo et al. 2022).

It is suggested to do enzymatic breakdown of protein at suitable pH and temperature (Peredo-Lovillo et al. 2022). Similar to reduce side reactive effect and to keep the amino acid composition indistinguishable from the initial matter, protein hydrolysis should be carried out in pH range of 6-8 and at temperature 40-60 4 °C (Mukhopadhya and Sweeney 2016). In respect to bioactive peptides production, enzymatic hydrolysis is more preferable compared to microbial fermentation simply because of its instant reaction time, uncomplicated scalability and prediction (Daliri et al. 2017, Karami and Akbari-adergani 2019). Various proteolytic enzymes can be taken advantage of in the protein hydrolysis

to generate hydrolysates of short peptide sequences at suitable enzyme, pH and temperature (Peredo-Lovillo et al. 2022). In a study by Chakrabarti et al. (2018), ACE-inhibition peptides were obtained from fermented milk with *Lb. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* each for 72 hours individually. Another research by Abd El-Fattah et al. (2017) indicated that peptides with ACE-inhibitory properties were obtained during milk fermentation by protease (*Aspergillus oryzae*) for only 1 hour.

The fact that food grade enzymes are generally recognized as safe (GRAS), intake of some enzymes might lead to allergic or toxic responses; hence, it is therefore essential to control enzymes usage for hydrolysis process. Enzymes for instance trypsin, chymotrypsin and pepsin, are widely applied in milk proteins hydrolysis (Mukhopadhya and Sweeney 2016, Chauhan and Kanwar 2020, Akkurt and Tomasula 2022). Peptide successions and peptide biological properties vary and can on a great extent depend on the kind of the enzyme used, enzyme-substrate ratio, the time for hydrolysis and the temperature. These factors might influence both on the extent fermentation and the kind of peptides which is released at the end of production (Choi et al. 2012). For instance, BP production during kefir fermentation by using a three- stage model activates with pepsin, α-amylase and intestinal step generated peptides of ACE-inhibitor β-casein and its mass increased 10,000-times following digestion (Daliri et al. 2017, Peredo-Lovillo et al. 2022). Despite the advantages of enzymatic BP production method, there are some shortcomings as it is sensitive to heat in water, which prevents the creation of peptides with macro-bonds. In this way, application of protease enzyme in BP production under favorable environment needed to ease peptide bond generation without considering peripheral hydrolysis (Chauhan and Kanwar 2020).

2.3.2 Microbial fermentation of bioactive peptides

Microbial enzymatic bioactive peptides formation constitutes fermentation with starter culture or non-starter cultures, or by aging processes (Udenigwe 2014, Peredo-Lovillo et al. 2022, Galdino et al. 2021, Dapkevicius et al. 2021). Fermented dairy products are basically considered as product's produced by fermentation using controlled microorganisms that can be viably active and adequately available during product shelf-

life (Yu et al. 2021, Galli et al. 2022). BP can be produced by a joint mixture of internal and external peptidase enzymes, for example during milk fermentation (Mora et al. 2019). Safety precautions have to be put into consideration with concern to pathogenicity and toxicity regarding microorganisms applied in the fermentation processes (Mukhopadhya and Sweeney 2016).

Large variety of BP produced by LAB is dependent on the specificity of proteolysis (Rana et al. 2018). It is recommended that bacteria to be used has to be in the aggressive growth stage prior to their harvest, clean-washed, and then put into glucose with sterilized distilled water, which works as an initial inoculant for the protein substrate (Chakrabarti et al. 2018).

Microbial fermentation includes the use of *Lactobacilli* spp., *Lactococci* spp. and *Streptococci* spp. in the generation of hydrolysates from milk. These proteolytic (LAB) constitutes cell endopeptidases, amino-peptidases, cell-envelope associated proteinases, dipeptidases and tripeptidases linked with the production of hydrolysates (Choi et al. 2012). The extent of hydrolysis is generally influenced by bacterial species, conformation of protein and fermentation period. For instance, *Lb. brevis* provides a higher ACE inhibition activity than other strains such as *Lb. lactis*, *Lb. acidophilus*, *Lb. bifermentan*, *Lb. casei*, *Lb. helveticus*, and *L. plantarum*. This therefore indicates that functions of protein hydrolysates functions may vary among the different starter cultures due to the differences in their proteolytic systems (Daliri et al. 2017, Akkurt and Tomasula 2022). Over 40% of peptide bondings in α-$_{S1}$ and β-caseins are generated by LAB proteinases, producing oligopeptides within a range of (4 to 40) amino acid residues. The fermentation factors such as enzyme/substrate ratio, pH, heating, temperature use, and composition of medium and carbon-nitrogen ratio can significantly affect the production of metabolites from dairy proteins (Mukhopadhya and Sweeney 2016).

2.3.3 Combined methods for production of bioactive peptides

A mixture of traditional approaches have been employed to promote hydrolysis with the focus on facilitation of BP generation and to increase their bio-activities. According to a

study by Chen et al. (2008), BP release during milk fermentation using *St. thermophilus* and *Lb. bulgaricus* showed almost 5.6-fold increase after 5 hours digestion by flavourzyme enzyme. Enzymes, or microbial fermentation, subsequently followed by *in vitro* hydrolysis is among the popularly used combined approaches. This combined method promotes both BP production and it is utilized to measure their firmness upon BP consumption. For instance, peptides with ACE-inhibition activity produced from soy-milk fermentation with *L. plantarum* C2 was found to be stable after *in vitro* hydrolysis due to its amino acid structure. In addition, an increase in antioxidant activity was also observed. This increase in antioxidant in that study was thought to be due to proteolysis by fermenting enzymes such as pancreatin pepsin and trypsin (Singh and Vij 2018). In another study conducted on Parmigiano-Reggiano cheese which was subjected to the influence of aging period and simulated hydrolysis on peptide profile, the results showed that BP may vary following simulated digestion and can be categorized basing to the bioactivity and evolution tendency according to the storage period. ACE-inhibition activity from peptides exhibited an increased attendance following hydrolysis in accordance to the ripening time and also antimicrobial activity from peptides illustrated an increasing after digestion, however its antimicrobial activity remained stable after 18 months of ripening period. On the contrary, peptides with opioid activity showed a decreasing shift after digestion with ripening time (Martini et al. 2020). These outcomes are in support with those investigated by Liu and Pischetsrieder (2017), who reported an increasing ACE-inhibition activity for peptides (ca. 10,000-fold) after fermentation of kefir in the laboratory (Peredo-Lovillo et al. 2022).

2.4 Functional Properties of Milk Derived Bioactive Peptides

Food derived proteins provides a numerous of multiple nutrition, bioactivity, and functional potential as a result of the availability of certain amino acid sequences which render particular anatomical benefits (Li et al. 2015). Most BP obtained from dairy products, have a multiple biological activities (Ozturkoglu Budak et al. 2016). However, the functional properties depend on the series of the present peptides (Mora et al. 2019). BP are correlated to numerous biologically functional activities such as; antihypertensive, antioxidant, antimicrobial and have the capacity to reduce cholesterol (Banihashemi et al.

2020, Peredo-Lovillo et al. 2022) type-2 diabetes, obesity, brain ailments, and inflammatory discomfort (Lee et al. 2021, Udenigwe et al. 2021), antithrombotic, opioid management, provide satiety (Mora et al. 2019, and anti-fatigue (Guo et al. 2021). BPs are reported to have preventive activity on cardiovascular related illnesses such as heart muscles hardening (Ali Redha et al. 2022, Georgalaki et al. 2017). BPs are also reported to have anti-aging property (Sah et al. 2016, de lima et al. 2018, Abdel-Hamid et al. 2020). Some selected major bioactive components present in milk are shown in Figure. 2.2 (Park and Nam 2015)

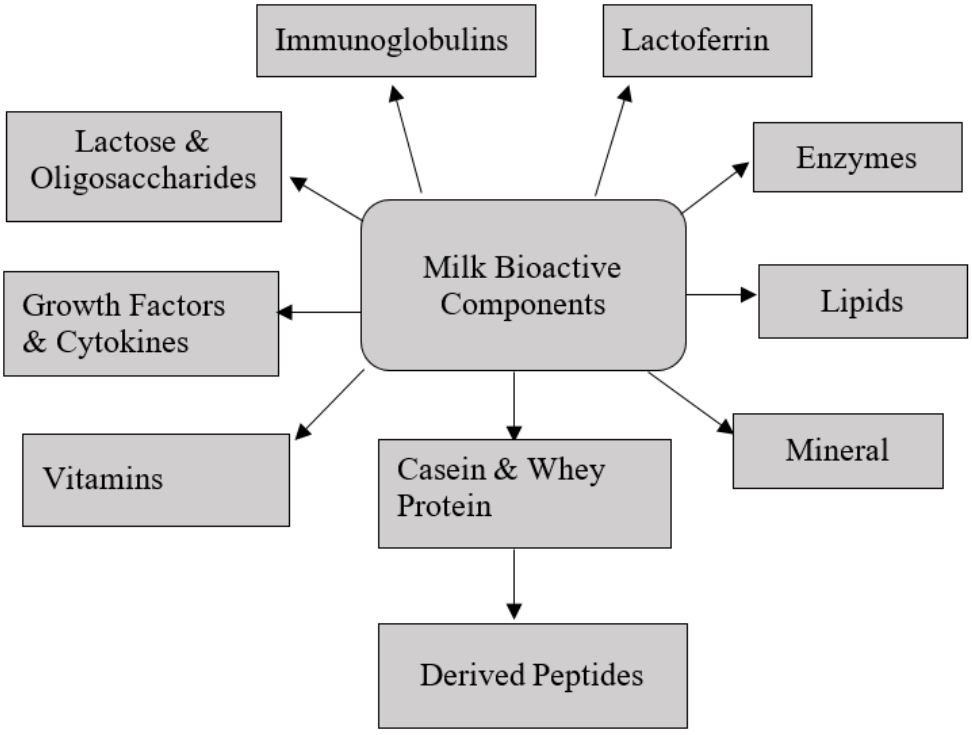


Figure 2.2 Some major bioactive functional compounds derived from milk

2.4.1 Physiological functional properties of bioactive peptides

Components in milk such as milk-caseins, whey-proteins and other micro-elements exert essential biochemical and physiological importance in human body reactions and well-being (Gobbetti et al. 2007, Park and Nam 2015). The effects of peptides from milk are given in Figure 2.3 (generated from data from Manfredini et al. 2021).

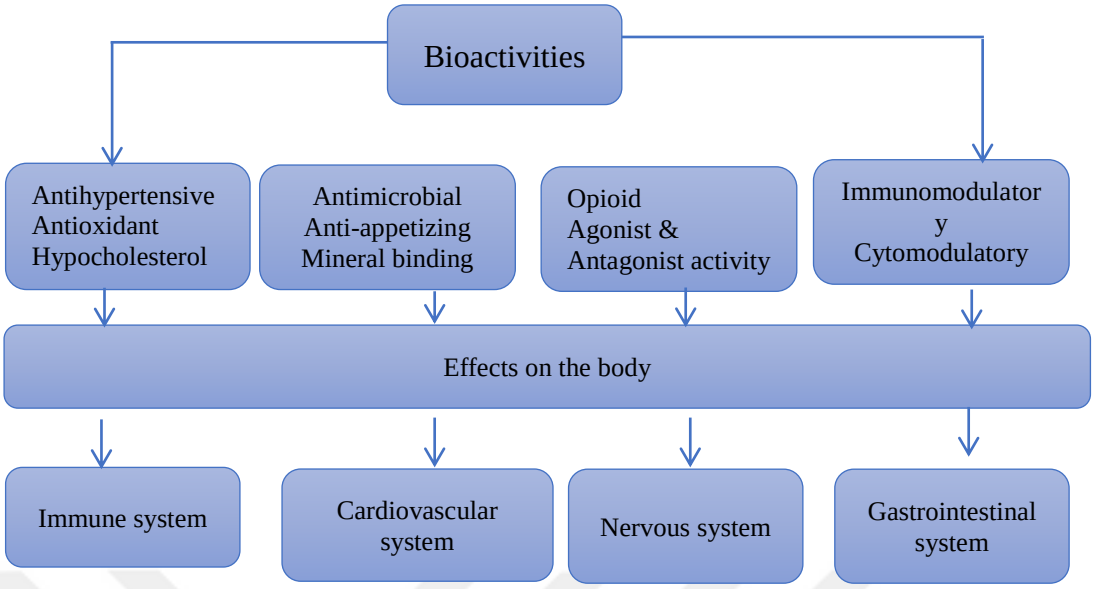


Figure 2.3 Physiological functional effects of bioactive peptides (generated from data from Manfredini et al. 2021)

2.4.1.1 Anti-hypertensive peptides

Hypertension is referred to as a chronic medical disease (Shivanna and Nataraj 2020) characterized by a systolic blood pressure (≥ 130 mmHg) and/or diastolic blood pressure (≥ 80 mmHg) (Okamoto et al. 2020). It is a silent killer disease with over 9.4 million death worldwide (Xiao et al. 2020, Okamoto et al. 2020). The hypertension etiology is complex with multifaceted underlying risks linked with obesity, sedentary lifestyle, smoking, genetics, environment, stress, high salt and alcohol intake (Mudgil et al. 2019). It is recommended that a good lifestyle, diet and oral intake of fermented milk fortified with bioactive peptides has significant effect in the control of hypertension. ACE (peptidyl-dipeptide hydrolase; EC 3.4.15.1) is a multipurpose ectoenzyme which plays a crucial role in the kallikrein-kinin, renin-angiotensin, and body immune system boost (FitzGerald et al. 2004, de la Sierra 2019, Chauhan and Kanwar 2020). These ACE-inhibition peptides are mainly derived from animal proteins particularly dairy food products (Sulejmani et al. 2020).

The ACE mechanism for the regulation of the arterial vaso-constriction and cell surface fluid volumes contributing to the inactivation of the vasodilator bradykinin or by

activating the alteration of angiotensin I to the vaso-constrictor angiotensin II. In this way, the prevention of ACE leads to blood pressure decreases hence controlling hypertension (Rai et al. 2017, Chauhan and Kanwar 2020). Inactivation of ACE enzyme is very significant for the management of hypertension. It has been reported that ACE inhibitor peptides can released from the benign protein by protease enzymes during fermentation and subsequently taken up in the basolateral membrane of the human intestines (Muro Urista et al. 2011, Aspri et al 2018).

ACE-inhibitory are peptides produced during the intensive proteolysis which takes place during food fermentation (Vermeirssen et al. 2003, Anne et al. 2010, Rai et al. 2017). The released peptides confer antihypertensive effect in living organism especially if they find their way to the cardiovascular system in a stimulated phase (Vermeirssen et al. 2004). ACE inhibition potentials are attained when milk protein is hydrolyzed especially casein into peptides by proteinase. Several studies have shown that ACE inhibitors can be released by proteolysis of milk during fermentation by some strains of LAB such as *Lc. lactis ssp. diacetylactis*, *Lb. delbrueckii ssp. bulgaricus*, *Lb. helveticus*, and *St. salivarius ssp. thermophilus* among others. A good number of the produced peptides have demonstrated to lower hypertension both in experiments conducted in rats with hypertensive state and in some human using milk fermented products (Mizushima et al. 2004, Tuomilehto et al. 2004, Pihlanto et al. 2010). It was thought that the decrease of blood pressure by peptides could be due to the ionic calcium release from fermented milk (Al haj et al. 2017, Solanki and Hati 2018).

Several clinical studies have shown that peptides such as casokinins and lactokinins which are obtained from milk protein have significant and remarkable ACE inhibitory activity potential for reducing hypertension (Bahadori et al. 2019, Okamoto et al. 2020). Moreover, enterococci of dairy origin have been considered to have the capacity hydrolyzing casein into peptides with ACE-inhibition activity. Whey proteins jointly fermented with *Lb. rhamnosus* and *Kluyveromyces marxianus* have been reported to generate antihypertensive peptides such as α-lactalbumin and β-lactoglobulin (García-Tejedor et al.2013). *Lb. acidophilus* and *Lb. helveticus* applied as secondary culture in white-brined cheese have broadly reported to release BP with ACE inhibitory properties

(Okamoto et al. 2020). According to García-Tejedor et al. (2013), BP can lower blood pressure in animal clinical experiments. Despite the fact that enterococci of dairy origin have the ability to breakdown casein from milk to peptides with ACE-inhibitory property, the pathogenic potential of some *Enterococcus* strains might hinder their use in overall food manufacturing.

According to Ahtesh et al. (2018), the use of *Lb. casei* and kombucha starter cultures resulted in 21 newly developed peptides such as (LVESPPELNTVQ, VLESPPELN, WGYLAYGLD) which had ACE inhibition property. Furthermore, a 12 weeks double-blind collateral experiment in over 89 hypertensive patients who were dosed with VPP or IPP peptides obtained from milk comparatively decreased arterial hardening. Similarly, a randomized double-blinded placebo-regulated investigation in 94 hypertensive patients who were given milk fermented with *Lb. helveticus* in an increased concentration of VPP/IPP peptides two times on a daily basis for a frequency of 10 weeks lowered blood pressure in patients. Another study performed in hypertensive people who consumed fermented milk ACE inhibition peptides (VPP and IPP) every day in a 10-12 weeks' time frame resulted in reduced blood pressure. A good deal of fermented milk products is available and are utilized for hypertension therapy. However, the European Food and Safety Authority has not approved such methods to be applied exclusively as a remedy for hypertension. According to Chauhan and Kanwar (2020) BP with antihypertensive properties are a appropriate alternative for chemotherapy because they are comparatively cheaper in terms of production and have less or no side effects to consumers. Further comprehensive human involvement investigations are therefore necessary to ascertain the antihypertensive and health functions of peptides produced from milk (Ahtesh et al. 2018).

2.4.1.2 Anti-oxidant peptides

Transpiration of complex oxidative responses in biological structure is a risk factor for oxidative stress in living organism as a result of overindulgent in reactive oxygen species (ROS) which could be the cause of proteins damage, unsaturated fatty acids and nucleic acids giving rise to cell damage. This can lead to cancer, Alzheimer, cirrhosis and

coronary artery diseases (Chakrabarti et al. 2014, Chakrabarti et al. 2017). ROS and free radicals cause cell damage hence hypertension, diabetes mellitus and cardiovascular ailments (Jakubczyk et al. 2020, Tadesse and Emire 2020). It is of great concern in the food sector to eliminate toxic/carcinogenic compounds caused by oxidative reaction. For this reason, there are ongoing studies to ascertain the use of natural preservative approaches to increase shelf-life of food instead of synthetic antioxidants are underway due to consumer demand in the recent decades (Mazorra-Manzano et al. 2018).

Bioactive peptides which originate from dairy products have been reported to have preventive properties of essential fatty acids peroxidation (Chauhan and Kanwar 2020). BP have scavenging potential roles against free radicals, ROS and membrane oxidation (Sah et al. 2016, Tadesse and Emire 2020). According to the study conducted in casein, phosphorylated peptides produced upon subsequent digestion resulted in both lipophilic and hydrophilic antioxidant property attributed to metal ion hindrance and lowering reactive oxygen species (Chauhan and Kanwar 2020). Radical-scavenging activity such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,2-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH), have been proved to offer protective role against oxidation of supercoiled plasmid DNA strand and low-density lipoprotein (Mazorra-Manzano et al. 2018, Jakubczyk et al. 2020).

2.4.1.3 Anti-cancer peptides

Protein hydrolysates obtained from food have shown anticancerogenic activity. Lipopeptides have been reported to offer great impact in cancer regulation majorly in two ways: by either killing cancer cells or deterioration of cancer cell multiplication (Chauhan and Kanwar 2020). Several studies of BP produced from whey milk have shown anticancerogenic activities. In a study performed in living organism showed that peptides obtained from whey proteins have the ability eliminate tumor growth due to its superior gamma-glutamyl cysteine and cysteine di-peptides. Body immunity can be promoted by glutathione, which has ROS destruction activity, carcinogenic detoxification and hold proteins in a reduced state. According to living organism test for cytotoxic effect of BP on cancer cell experiments, peptides showed an interfering role with the various

molecular pathways of cancer formation such as; initiation, promotion, and progression without any genetic toxicity (Shivanna and Nataraj 2020). It was also reported that peptide can boost cell apoptosis, regulate cell development sequence, affect gene appearance, and hinders angiogenesis. Intake of yoghurt which contains β-casomorphins and κ-casecidin lowered tumor cell development thereby reducing intestinal cancer incidence (Vargas-Bello-Pérez et al. 2019).

BP derived from casein phosphopeptides have shown a preventative role against intestinal tumor formation and can boost apoptosis by stimulating calcium flux mediation pathway to depolarize cancer cells. β-casein such as β-casomorphin-5 has shown to growth inhibition of breast cancer cells and intestinal tumor cells. Similarly, α_{S1}-casomorphin f (158–162) peptide with series of Tyr-Val-Pro-Phe- Pro showed inhibitory property against breast cancer cells without interfering with somatostatic receptor differing from casomorphins (Chauhan and Kanwar 2020). Breast carcinogenic cells can be inhibited lactoferrin by regulating the development of nasopharyngeal carcinoma cells by lowering protein kinase B mediated pathways (Shivanna and Nataraj 2020).

2.4.1.4 Anti-diabetic peptides

Diabetes pathophysiology is thought to be caused by the non-fulfilment of the beta cells in the pancreatic islets to release insulin as a result of inadequate insulin release/insulin resistance (Vargas-Bello-Pérez et.al. 2019). Diabetes is categorized into three fundamental types (Nongonierma and FitzGerald 2018). Firstly, type-I diabetes, which results from the lack of the b-cells in the pancreas to discharge insulin as a consequence of autoimmune disease state. The second class is type-2 diabetes which is caused by failure of insulin generation as a caused by impairment of the b-cells in the pancreas intransigence (Guney et al. 2020) and lastly, gestational diabetes mellitus which occurs in pregnant mothers (Acquah et al. 2022).

Bioactive peptides have widely depicted therapeutic influence in blood sugar reduction in diabetic patients. Inhibitory enzymes particularly the dipeptidyl peptidase IV (DPP-IV), a-amylase, a-glucosidase which serve as receptor agonists of glucagon-like peptide-

1 (GLP-1) can regulate blood sugar (Mudgil et al. 2019). DPP-IV enzyme which takes part in intestinal food digestion of and absorption has been studied for its inactivation effect of incretins glucagon-looking peptide-1 (GLP-1) and gastric inhibition polypeptide activity (Tadesse and Emire 2020). GLP-1 and GIP play a role in insulin and mass of β-cells release, these compounds have shown decrease glucagon secretion. Furthermore, prevention of DPP-IV, amylase, and glucosidase, peptides with antidiabetic properties also leads to sensitivity of insulin and reduces blood sugar (Shivanna and Nataraj 2020).

Holding back gastric emptying is among is an important mechanism responsible for over 40-50% influence in the decrease of postprandial glycemia in type-2 diabetic sufferers. It is suggested that bioactive peptides play a big role providing satiety and gastric emptying and it is stimulated BP in the lumen where intestinal hormones and incretin hormones are secreted (Nongonierma and FitzGerald 2018). β-casomorphins, which are short sequence proteolytic peptides produced from b-casein, have been reported to control satiety and gluconeogenesis in children. Dietary intake of Gouda cheese peptide, Leu-Pro-Gln-Asn-Ile-ProPro-Leu resulted in a decrement levels of peripheral plasma glucose in the experiments performed in rats (Acquah et al. 2022). An investigational study performed in diabetic rats administered with milk-derived peptides also confirmed a decrease in the total cholesterol, blood plasma lipids concentration, low-density lipoprotein, and plasma glucose. Giving oral β-casomorphin-7 to rats in diabetic state resulted to an increased the plasma insulin level with lowered level of blood sugar. Peptides from milk protein was reported to have an *in vitro* DPP-IV inhibition activity and experiments reduced blood sugar concentration (Chauhan and Kanwar 2020).

2.4.1.5 Opioid peptides

Dairy products are considered a rich in opioid peptides from three precursor proteins of proenkephalin, prodynorphin and proopiomelanocortinse that provide essential roles in the functionality of the nervous system (Akkurt and Tomasula 2022).

Opioid BP obtained from milk protein serve as pheromones or exorphins. Opioid BP correlate with opioid receptors available in the gastro-intestinal system, hormonal, and

nervous system of mammals. They affect the functionality of several organs. The hydrolysis of casein by enzymes into different casein molecules leading in the release of peptides with opioid activity (Chauhan and Kanwar 2020). Opioid peptide system of the brain has essential role in behavioral, motivation, pain response and regulation for food intake. The role of opioid peptides depends on binding to target receptors of specific cell whereas the intended physiological effects rely on the individual receptors such as the s-receptor is for emotional behavior, m receptor is accountable for emotions and intestinal movement subduing and k-receptor is for food consumption. The β- and α-casomorphins is responsible for opioid agonists that might regulates social character, impact postprandial reactions which generate analgesia (Akkurt and Tomasula 2022). Opioid peptides derived from beta-casein may affect bone breakage by influencing inflammation and binding to m-opioid receptors in bone structure. There may be a positive relation between dairy intake and risk of fracture in relation to the opioid peptides got from beta-casein from milk (Akkurt and Tomasula 2022).

A number of opioid peptides considered available in dairy products can offer pharmacological properties resembling those of morphine and thus seem to have active role in the functional properties of the central nervous system. Macro, medium and low molecular weight BP such as opioid and phosphopeptides were determined in human milk by tandem mass spectrometry and high-performance liquid chromatography-mass spectrometry (HPLC-MS) methods (Chauhan and Kanwar 2020).

2.4.1.6 Antimicrobial peptides

Antimicrobial activity peptides have been applied as preventive agents against different pathogenic bacteria (Jakubczyk et al. 2020). Antimicrobial activity depends largely on their structure. Antimicrobial peptides are mainly categorized into three approaches namely: (i) open-ended cyclic peptides and disulfide-bridged cyclic; (ii) peptides with vast quantity of glycine, proline/histidine in the initial matrix and (iii) a-Helical linear peptides (Chauhan and Kanwar 2020). Antimicrobial activity of different peptides obtained from milk proteins have inhibition activity some pathogens have been studied by (Folliero et al. 2022). Among the antibacterial peptide's activity mechanism include

the binding to the bacterial cell or mitochondrial surface thereby causing cell breakdown and there after cell death. This approach is dependent on the electrostatic interlinkage between the positively charged peptides and negatively charged surface of the cell surface. The peptides then distort the progress and the membrane cell membrane structure hence inhibiting pathogen growth (Jakubczyk et al. 2020). Haque and Chand (2008) reported that milk has bioactivity that inhibits the development of streptococcal cells mainly attributed to immunoglobulins, lactoferrin, lactoperoxidase and lysozyme. Whereas lactoferrin obtained from milk whey protein released peptides with antimicrobial action against some viruses such as adenovirus enterovirus and rotavirus, casein-derived antibacterial peptides offered inhibitory role to pathogenic microbes such as *E. coli*, *Salmonella*, *St. sangui*, and *St. aureus* (Chauhan and Kanwar 2020). The effectiveness of antibacterial peptides against a wide range of microbes to combat the increasing incidences of antibiotic-resistant bacteria and multidrug-resistant infections providing natural bio preservatives (Dullius et al. 2020). Some bioactive peptides with antimicrobial effects are illustrated in table 2.1.

Table 2.1 Some bioactive peptides in dairy products with antibacterial effects

Milk peptide fragment	Release protease	Yeast and fungi*	Gram (+)	Gram (-)
lactoferricin B lactoferrin (f17-41)	Pepsin	*Candida albicans Dermatophytes: *Cryptococcus unigulattulus *Penicillum pinophilum *Trichophyton mentagrophytes*	*Bacillus Listeria Streptococci Staphylococci*	*E. coli 0111 E. coli 0157H:7 Klebsiella Proteus Pseudomo- nas* Salmonella
lactoferrampin lactoferrin (f265-284)	Pepsin	*Candida albicans*	*Streptococcus mutans*	*E. coli*
Kappacin *κ*-cN (f106-169)	Chymosin		*Streptococcus mutans*	*E. coli*

Table 2.1 Some bioactive peptides in dairy products with antibacterial effects (continue)

Milk peptide fragment	Release protease	Yeast and fungi*	Gram (+)	Gram (-)
Casecidin κ-cN (f 17-21) αs1-cN	Chymosin and Tripsin		*Staphylococcus aureus Sarcina Bacillus subtilis Diplococcus pneumoniae Streptococcus pyogenes*	
Caseicin αs1-cN A (f 21-29) B (f 30-38) c (f 195-208)	Synthetic peptide Synthetic peptide Synthetic peptide		*Listeria innocua*	*E. coli, E. sakazakii E. coli, E. sakazakii*
Casocidin-I αs2-cN (f 165-203)	Synthetic peptide		*Staphylococcus carnosus*	*E. coli*
Isracidin αs1-cN (f 1-23)	Chymosin and Tripsin	*Candida albicans*	*Staphylococcus aureus*	

Data from (Flavio et.al. 2009)

2.4.1.7 Immunomodulatory peptides activity

Peptides pose immunomodulatory dietary components which have a positive health impact. Studies have reported immunomodulatory activity of protein hydrolysates derived from food proteins like whey proteins (Chauhan and Kanwar 2020). Immunomodulatory peptides mechanisms involve the activation of the immune system such as lymphocyte growth, antibody synthesis, natural killer cell activity and cytokinin regulation (Jemil et al. 2017, Zaky et al. 2022).

Peptides from milk caseins serve as stimulates macrophages, phagocytosis, promotes leukocytes, induces cytokines and immunoglobulins and stimulates natural killer cells. Casein and whey proteins are the most fundamentally researched protein sources as far as immunomodulatory peptides are concerned (Dullius et al. 2020).

Bioactive peptides of β-casomorphin, β-casokinins and lactoferricin B are known for their ability to stimulate immune responses (Cheison et al. 2003). Peptides obtained from casein modulates the rapid increase of lymphocytes and phagocytic activity of macrophages in humans (Korhonen and Pihlanto 2007). They are responsible for the prevention against infections resulting from viruses, bacteria and parasites, decrease autoimmune inflammatory processes and hinders objection of transplants. Lactoferrin have bactericidal and bacteriostatic properties that prevents damaging of the outer surface membrane of Gram-negative bacteria and hence influencing the production of lipopolysaccharides which then stimulates the body immune response against bacterial disorders. Bioactive peptides got from milk can lower monocyte attachment to swollen endothelia, decreasing damage and contributing to the prevention of heart vessel hardening. Comparatively, β-casomorphin inhibition activity of ACE, which results in stimulation of bradykinin, a potent vasoactive peptide that cause high blood pressure (Vargas-Bello-Pérez et al. 2019). Furthermore, lactoferrin has the ability to chelate iron because it acts as a nuclear property of kappa-light-chain-enhancer, which activates B cells (Marcone et al. 2017). A study determined that cytomodulatory peptides could inhibit not only cancer cell proliferation but also activates activity of newborn intestinal and immune competent cells. BP is also known to stimulate mucosal immunity of the gastrointestinal structure and can regulate allergic effects in atopic humans (Chauhan and Kanwar 2020).

2.4.1.8 Coronavirus (Covid-19) preventive activity of bioactive peptides

The coronavirus (COVID-19) pandemic is arguably regarded as the greatest public health challenges in the recent years with over 240 million registered cases and 4 million deaths world over. Despite the positive impact of the COVID-19 vaccines in symptomatic reduction, there is no effective COVID-19 drug (Chamata et al. 2021). However, it is crucially reported that BP have a preventive role against COVID-19. The critical respiratory syndrome coronavirus 2 (SARS-CoV-2) applies ACE- 2 as a receptor to enters the host cells (Ni et al. 2020) by interaction with viral glycoprotein spikes (Behl et al. 2020). Despite some irregularities, several studies have cited a significant relation

between the application of ACE inhibitors and COVID-19 reduction (Savastano et al. 2019, Ni et al. 2020, Pradeep et al. 2021, Ashokbhai et al. 2022).

In a study performed on 8.3 million individuals, ACE inhibition were not seen to be significantly linked with an increase COVID-19 risk, nor required ICU care (Hippisley-Cox et al. 2020, Chamata et al. 2021). Similarly, a meta-analysis investigation highlighted that the use of ACE inhibitors didn't necessitate the use of mechanical ventilation, intensive care, advancement to severe disease, and increased death risks. A great deal of researchers have reported a 16% decrease in the risk of COVID associated with mortality with a credit attributed to ACE inhibitory property (Barochiner and Martínez 2020). A number of experimental evidences indicate that ACE inhibitions can decrease the stimulative action of Ang II and thus attenuate Ang II-driven acute lung damage (Dapkevicius et al. 2021). Therefore, ACE inhibition potentially significant in COVID-19 therapy. IPP, LIVTQ, LVYPFP, and IIAE BP obtained from whey proteins have been suggested to offer ACE inhibitory effect with no toxic side effects compared to the synthetic ACE-inhibitory chemotherapeutic drugs (Chamata et al. 2021).

2.4.1.9 Mineral-binding peptides activity

Food proteins produced as a result of enzymatic hydrolysis have ability to trap mineral and trace elements. These peptides can facilitate the absorption of calcium which is responsible for osteoporosis treatment, anemia and dental cavities. Peptides released from milk and whey protein hydrolysates have been reported to have potential mineral-binding influences. For instance, phosphopeptides derived from casein form salts with calcium by their phosphorylated serine metabolites (Mora et al. 2019).

According to Behl et al. (2020), it was specified that various milk proteins can bind to some metal ions particularly transitional metal ions, such as Mn^{2+}, Cu^{2+}, and $^{+}$, Fe^{3+}, and the main group ions Mg^{2+}, Ca^{2+}, Pb^{2+}, Al^{3+}, and In^{3+}.$^{42-45}$. Intake of milk whey protein (β-lactoglobulin) in a state of an amyloid- carbon hybrid membrane is significantly in binds and the separates uranyl ions from aqueous liquid.

It was investigated that peptides with series of Ser (P)-Ser (P)-Ser(P)-Glu-Glu can be attached to minerals and keep it composed in the liquid state at stomach pH, which contributing to the bioavailability of the minerals (Gagnaire et al. 2001). Whey protein such as Tyr-Asp-Thr provide a calcium-holding ability of about 79.5 μg/mg. Peptides with mineral binding properties can be exploited for generation of functional foods essential specific in the elimination of mineral deficiencies such as rickets and osteoporosis (Shivanna and Nataraj 2020).

2.5 Dairy Products as Carriers of Bioactive Peptides

2.5.1 Peptide occurrence in dairy products

Consumer demands for functional foods promoted the manufacturing of food with particular health benefits (Peredo-Lovillo et al. 2022). Food macronutrients such as proteins and peptides are importance as a result, they offer the required raw matter for protein biosynthesis (Sánchez and Vázquez 2017). Dairy products like yoghurt, kefir and cheese are rich bioactive in protein among peptides derived from food structure (Banihashemi et al. 2020) released by fermentation of milk under controlled conditions with suitable bacterial culture (Shivanna and Nataraj 2020). Some biological properties of some selected milk metabolites from dairy fermented products produced with LAB are shown in table 2.2.

Milk fermentation by LAB is a dominant approach for the generation functional foods with BP with a relatively cheaper cost. Moreover, fermented milk product have vast nutritional advantages (Sánchez and Vázquez 2017). Most of the LAB have solely one CEP, such as PrtP of *St. thermophilus, Lc. lactis* and *Lb. rhamnosus* whereas as some LAB such as *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus* possess two or more CEPs. However, *Lb. bulgaricus* and *S. thermophilus* are the most jointly exploited starter cultural strains milk fermentation in the food sector (Kinariwala et al. 2020, Yu et al. 2021).

There are three ACE- inhibition peptides namely; VPP, NIPPLTQTPV, and RY which were reported in a commercial-brand fermented milk by RP-HPLC–ESI-IT with known active fragments. A study by Quirós et al. (2005) noticed the presence of two ACE-inhibition peptides of PYVRYL and LVYPFTGPIPN series in kefir made commercially from caprine milk by analogous MS analysis. Lactoferroxin A, β-CM-7 opioid peptides, casoxin C, and casoxin 6 were also detected in two commercial yoghurts and kefir sample respectively (Jarmołowska and Krawczuk 2012).

Dallas et al. (2013) singled out over 300 peptides in milk from humans produced by nano LC–ESI-QTOF. In the same study, they reported that a broad spectrum of these peptides were reported to have antimicrobial activities and immunomodulatory effect. In addition, MALDI-TOF and nano LC–ESI-linear IT approaches were applied for peptide profiling analyses of drying-off cow milk during *in-situ* proteolysis. In a range of 202 recognized peptides, five of which possessed bioactivity namely: ACE-inhibitory, opioid and or immunomodulatory activity with the opioid peptide β-CM-7 classified and measured with over (14 μg/ L) in milk commercially produced by optimized nano-ESI-IT approach (Sánchez-Rivera et al. 2014). Cheese is among the considerable sources of bioactive peptide (López-Expósito 2012). Peptides such as VPP and IPP present in a variety of commercial cheeses at relevant levels have been said to have ACE inhibition and antihypertensive properties (Sánchez-Rivera et al. 2014).

Table 2.2 Biological activities of selected milk hydrolysates and fermented dairy products

Fermented dairy product	**LAB strains**	**Function role of metabolites**	**Reference**
Fermented milk	*Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* (GABA producing strain) and *L. lactis* subsp. c*remoris* (non-GABA producing strain)	Anti-hypertensive, Improved sleep quality, Increased ammonia detoxification in the liver	(Hagi et al. 2016)
Fermented milk	*Lactobacillus delbruekii* ATCC 9649, *Lactobacillus casei* ATCC 335, *L. plantarum* NRC 716 and Bacillus coagulans ATCC 12425	Anti-microbial activity, Anti-proliferative and tumor-inhibitory effects	(Cukkemane et al. 2020)
Brown fermented milk	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*	Improved sensory quality (cream and coffee odours)	(Jia, Liu, and Shi 2021)

Table 2.2 Biological activities of selected milk hydrolysates and fermented dairy products (continue)

Fermented dairy product	**LAB strains**	**Function role of metabolites**	**Reference**
Fermented milk	*L. plantarum* AF1	Investigating the effect of ultrasound	Improved lactose tolerance and anti-oxidant activity
Yoghurt	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophiles*	Improved MCFAs, improved protein absorption	(Sharma et al. 2021)
Yoghurt	*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*	Anti-stress, anti-diabetic, anti-hypertensive and anti-microbial	(Murgia et al. 2019)
Yoghurt	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*	Improved vasoconstriction, glycolysis and glycosylation but decreased fatty acid synthesis	(Sharma and Ramanathan 2021
Yoghurt	*Lactobacillus bulgaricus* and *Streptococcus thermophiles*	Improved nutritional value and digestibility of yoghurt	(Yang et al. 2021)
Yoghurt	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*	Enhanced bioactive properties.	(Trimigno et al. 2020)
Ice-cream	*Lactobacillus acidophilus* LA-5 and Bifidobacterium animalis BB-12	More effective gut modulation	(Da Cruz Rodrigues et al. 2020)
Brown fermented milk	*S. thermophilus* S10 strain	Hypocholerolemic and anti-oxidant	(Li et al. 2020)
Cheese	*Lactobacillus casei*-VC199 (LC) and *Enterococcus lactis*-BT161 (EL)	Amino-acid rich cheese produced with LC strain	(Battelli et al. 2019)
Cheese	Not mentioned	Not mentioned	(Pan et al. 2018)

Data from (Sharma et al. 2021)

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Raw milk

The full fat raw milk used was obtained at 4 °C from the Dairy Pilot Plant, Department of Dairy Technology Faculty of Agriculture, Ankara University, Turkey.

3.1.2 Chemicals, mediums, standards

De Man Rogosa Sharpe (MRS), M17, ringer tablets, Anaerocult-A and Brain Heart Infusion (BHI) were purchased from Merck (Germany). Trifluoroacetic acid (TFA), acetonitrile, gallic acid, 2,2-diphenyl-1-picrylhy- drazyl (DPPH) and angiotensin converting enzyme (ACE) were sourced from Sigma-Aldrich Co., St. Louis, MO, USA.

3.1.3 Bacterial strains used for fermented milk production

The set of lactic acid bacteria (LAB) were obtained from Karaman Divle Obruğu Tulum cheese (Budak et al. 2016) and were identified from the Netherlands Culture Collection of Bacteria (NCCB) as presented in table 3.1.

Table 3.1 Lactic acid bacteria used

Species code	**Species name**
NCCB 100523	*L. plantarum*
NCCB 100526	*Lb. brevis*
NCCB 100539	*Lc. lactis subsp. lactis*
NCCB 100524	*Lb. paraplantarum*
NCCB 100528	*Lb. coryniformis*
NCCB 100527	*E. faecium*
NCCB 100525	*E. faecalis*
*11-B2**	*Lb. helveticus*
23*	*Lb. bulgaricus*
12-B2*	*Lb. helveticus*
9-B5*	*Lb. helveticus*
1-K4*	*S. thermophilus*
K16*	*S. thermophilus*

NCCB: The Netherlands Culture Collection of Bacteria
*: cultures were isolated from traditional Turkish yoghurts where the regions were as; *S. thermophilus* 1-K4 (Konya-Göynükkışla Köy), *S. thermophilus* K16 (Konya-Tepeköy), *Lb. helveticus* 11-B2 (Konya-Yukarı Kızılca Köyü), *Lb. helveticus* 12-B1 (Konya-Yukarı Kızılca Köyü), *Lb. helveticus* 9-B5 (Konya-Durayda Köyü), *Lb. bulgaricus* 23 (Konya).

3.2 Methods

3.2.1 Preparation of bacterial strains used

100 µL of lactic acid bacteria stocks (approx. 10^9 CFU/mL) stored at -80 °C were inoculated into 5 mL of MRS and M17 liquid mediums depending on the strain type. For lactobacilli, MRS broth was used and for Lactococci, Streptococci and Enterococci, M17 broth was used, respectively. Lactobacilli strains were incubated at 37 °C for 48 hours under anaerobic condition, Lactococci, Streptococci and Enterococci were incubated for 24 hours at 30 °C aerobically. Bacterial growth conditions are shown in appendix 1.

The grown cultures in broths were centrifuged at 6000 rpm for 10 min at 4 ºC to obtain

pellets after the first activation in the medium while the second activation was performed in reconstituted milk (RM) with 11% dry material (DM), which was sterilized at 108 °C/ 7 minutes. The obtained pellets were washed 2 times with distilled sterile water including 0.85% salt and the separated cell pellets were redistributed by adding them into the sterile RM after cooling. RM was incubated again 37 °C for 24 h to produce cultures.

These cultures were inoculated at a level of 2% (v/v) into bovine milk which was pasteurized at 95 °C for 10 mins. The pasteurized cow milk without any culture was used as control sample. The preparation of used strains are shown in figure 3.1.

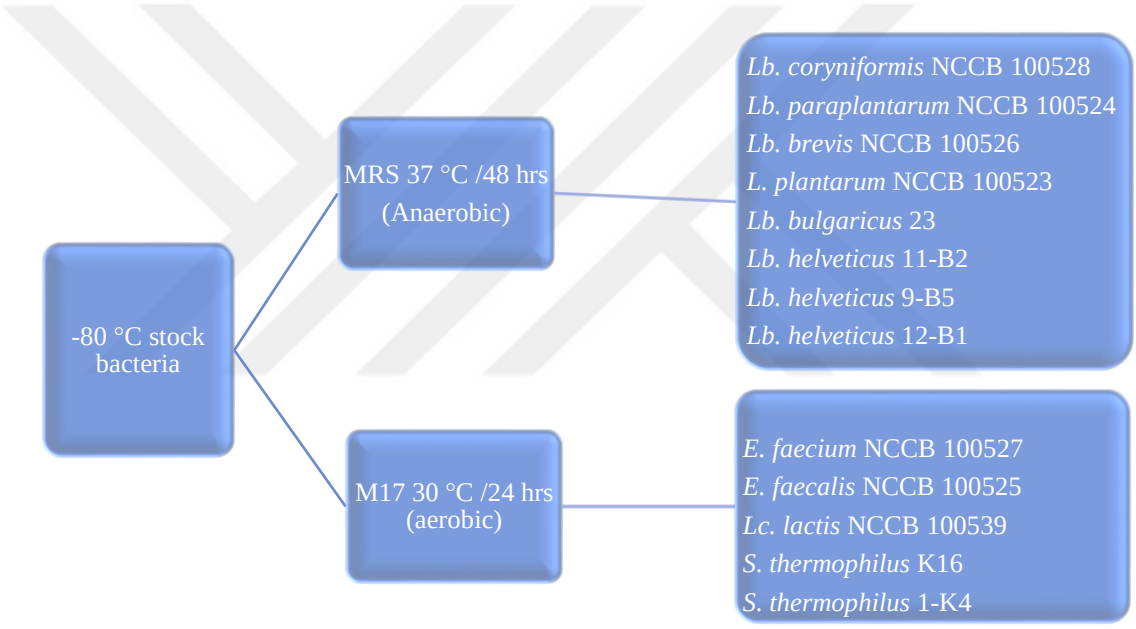


Figure 3.1 Preparation of bacteria strains used

3.2.2 Production of fermented milk

Fermented milk samples were made by adding 2% of the culture into the pasteurized and cooled RM (1L), subsequent to distribution into 80 mL sterile cups fermented at 37 °C for 24 hours. The incubation phase continued for 20-24 hours and fermentation of the milk samples was stopped at pH 4.70-4.80. The fermented milk samples were immediately cooled to about 4 ºC and stored at this temperature for a period of 28 days

for further analyses. The fermented milk production process is shown in the figure 3.2.

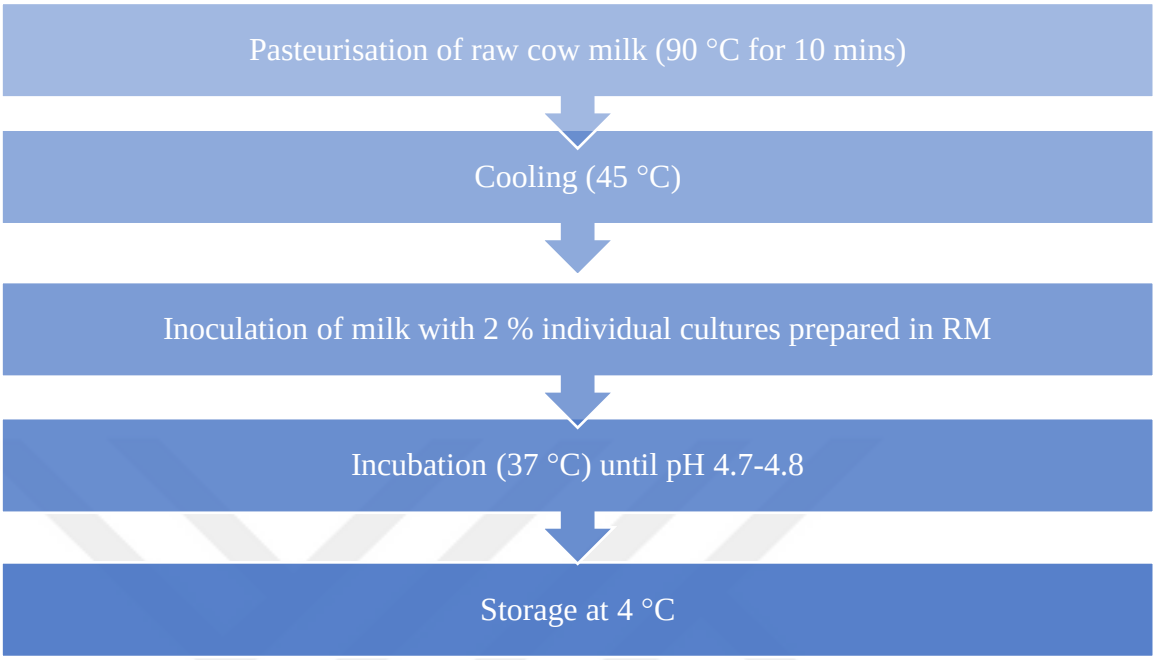


Figure 3.2 Fermented milk production process

3.3 Analyses of Fermented Milk Samples

3.3.1 pH value

The pH was conducted by a digital pH meter (WTWInoLab). The pH meter was gently put into the sample at room temperature and the pH readings were recorded in two replicates.

3.3.2 Titratable acidity value

Titratable acidity (TA) was carried out according to AOAC (2000). 10 mL of the fermented sample was weighed and put in a conical flask and 0.5 ml of 1% phenolphthalein indicator was added and followed by titration using 0.1 N NaOH until a permanent light pink color formation for about 30 seconds. Results were obtained in lactic acid percentage (% LA).

Calculation was done according to the below formula:

$$\% \, \boldsymbol{lactic\ acid} = (\boldsymbol{V} \times \mathbf{0.009} \, / \, \boldsymbol{m}) \times \mathbf{100}$$

Where; V: amount of 0.1 N NaOH solution used in titration (mL) and m: amount of fermented milk sample (mL)

3.3.3 Viable microbial number

Number of viable lactic acid bacteria cells in fermented milk samples were determined on MRS agar for *Lactobacillus* species while M17 agar for *Lactococcus, Streptococcus* and *Enterococcus* by spread plate method. MRS plates were incubated under anaerobic state at 37 °C/ 48 hours. M17 plates were incubated aerobically at 30 ºC for 24 hours. The colonies formed were enumerated and recorded as log CFU/mL (Anonymous 1998).

3.3.4 Preparation of water soluble extract (WSE) for peptide profile analysis

30 mL fermented milk sample was weighed in a beaker, ultra turrax mixed and then kept in a waterbath at 40 ºC for 60 mins. The pH of the samples was adjusted to 4.6 with 1 M HCl and the samples were subjected to centrifugation in 30 mL centrifugation tubes (SIGMA Model 3–18K, Germany) at 7500 rpm for 30 mins at 4 °C. After centrifugation, the water soluble extracts were filtered by using a Whatman 113 filter paper. The supernatants obtained were freeze dried for 24 hours at a vacuum pressure of 0.058 mBar at -58 °C with a lyophilizer (Alpha 1-2 LD plus, Germany). The freeze dried samples were kept at -20 °C for future analysis.

3.3.5 Water soluble extract (WSE) protein quantity

A Qubit™ 4 Fluorometer method was applied to evaluate the protein concentration in WSE obtained from fermented milk by different lactic acid bacterial strains. WSE were used for protein measurement. The equipment was calibrated with protein standardized

according to the manufacturer's directions. The needed solution was made by serial dilution with QubitTM reagent at 1:200 in the QubitTM buffer. Then, 200 µL of the used solution was prepared for individual standard and sample.

The tubes were mixed by vortexing for 2 to 3 sec, incubated at normal room temperature for approximately 15 min, and were prevented from the dark. The tube was then put in the Qubit 4 Fluorometer for analysis and the read values were recorded (Bickmore and Miklavcic 2020, Muñoz-Salinas et al. 2022).

3.3.6 Determination of peptide profile

Peptide profile was conducted by RP-HPLC from the WSE. The lyophilized extracts were dissolved in deionized water (0.1 g sample in 2 mL deionized water) and the mixture was vortexed for 2 mins. The obtained supernatant was filtered with the help of 0.45 µm membrane (Merck Milipore, Durapore, Hydrophilic, PVDF) and then 50 µL of the filtered sample was injected in the RP-HPLC coupled with C18 column (4.6 mm × 250 mm × 5 µm, 300ºA) A gradient program was used with two mobil phases. Mobile phase A consisted of deionized water with 0.1% TFA (v/v) and mobil phase B consisted of UV grade acetonitrile (v/v) with 0.1% TFA. The gradient program that was used is shown in Table 3.2. Measurements were conducted at a wavelength of 214 nm, temperature of 30 °C and flow of 0.800 mL/min (Torres-Llanez 2011, Rana 2018, Kinariwala 2020).

Briefly, the measurement conditions were as follows:
Column: C18 (4,6 cm x 250 mm x 5 µm, 300˚A)
Column Temperature: 30 ^{o}C
Mobile phase B: Deionized water containing 0.1% TFA
Mobile phase C: Acetonitrile containing 0.1% TFA
Wavelength: 214 nm

Table 3.2 RP-HPLC Gradient Program

Minutes	Mobile Phase B	Mobile Phase C	Flow Rate (mL/min)
0	100	0	0.800
10	100	0	0.800
90	50	50	0.800
95	40	60	0.800
100	40	60	0.800
105	5	95	0.800
110	5	95	0.800
115	100	0	0.800
120	100	0	0.800

3.3.7 Obtaining peptides with molecular size below 3kDa by centrifugation

Small molecular weight peptide fractions (<3 kDa) containing potential bioactive peptides in WSE was obtained by Amicon Ultra-15 centrifugal filters. A 500 mg of the lyophilized samples was weighed and 10 mL of deionized water was added and vortexed for 2 mins before centrifugation at 8000 rpm for 20 mins interval (for 1 hour) at 4 °C using 3 kDa cut-off centrifugal filter units (Amicon Ultra-15 centrifugal filters, Merck Millipore Ltd., Germany) (Torres-Llanez 2011; Abdel-Hamid et al. 2019). The obtained supernatant of the peptide extracts was later used for antioxidant activity, antimicrobial activity, phenolic activity and ACE-inhibitory activity analyses.

3.4 Determination of Peptide Bioactivities

Small molecular weight peptide fraction (<3 kDa) was analyzed in terms of bioactivity analyses including antioxidant activity, antibacterial activity, phenolic content and ACE-inhibitory activity.

3.4.1 Determination of antioxidant activity

The antioxidation activity of the fraction containing small molecular weight (<3 kDa) peptides was determined by 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging method (Wali et al. 2020, Abdel-Hamid et al. 2020). In this method, the DPPH radical is used and the antioxidant activity is determined by measurement of the antioxidant's ability to apprehend this free radical. DPPH was prepared in methanol (0.2 mM in 95% methanol) mixed with 100 µL of small molecular weight (<3 kDa) peptide fraction, subsequently 2 mL of DPPH solution was added and the mix was kept at 4 °C for 30 mins in the dark environment. At the end of the incubation, the absorbance of the solution was determined at 517 nm by UV/VIS spectrophotometer (PerkinElmer, Lambda 25, USA. Distilled water was used as a blank. 5.00, 2.50, 1.00, 0.50, 0.25, 0.10 and 0.05 mM were the concentrations of trolox solution which were used for plotting the standard trolox curve. The measured absorbances were converted to µM Trolox equivalent antioxidant capacity (µM TEAC).

Antioxidant activity of samples were computed using the following formula;

$$\% \text{ Inhibition} = \frac{A_{Blank-} - A_{Sample}}{A_{Blank}} \times 100$$

A_{blank}: Absorbance of DPPH solution with water instead of sample.
A_{sample}: Absorbance of DPPH solution with sample.

The inhibition percentage values for each milk sample fermented with different LAB were compared with the trolox standard. The formula was obtained from the trrolox standard curve (appendix 2-A) which was used to calculate antioxidant capacity (µM TEAC).

$$y = 17.465x + 0.2188$$

$$x = y - 0.2188 \,/\, 17.465$$

3.4.2 Determination of phenolic content

The phenolic activity was determined by the Folin-Ciocalteu approach as defined by (Abdel-Hamid et al. 2020). 100 µL of the supernatant was combined with 500 µL of folin reagent, 1.5 mL of 20% sodium carbonate (Na_2CO_3) and 7.9 mL of distilled water, after vortexing, mixtures were kept in the dark for incubation at room temperature for 60 min. The absorbance was determined at 760 nm by UV/VIS spectrophotometer (PerkinElmer, Lambda 25, USA). Preparation of gallic acid concentration was done at 5.00, 4.00, 3.00, 0.75, 0.50, 0.25, 0.05 mg/mL and this concentration were used to obtain the standard curve. Total phenolic contents of samples were calculated in mg gallic acid equivalents (GAE). This equation was obtained from the gallic acid curve, appendix 3-A.

$$Y=(1.3356x - 0.0413)$$

Y: The absorbance.

X: Concentration value to reach this absorbance

3.4.3 Determination of antibacterial activity

Antibacterial activity was tested against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella enterocolitica* (ATCC 14028), *Bacillus cereus* (ATCC 14579) *Mycobacterium tuberculosis* (ATCC 7644), *Enterococcus faecalis* (ATCC 29212) by agar well diffusion method (Sah et al. 2016a). The bacteria to be tested were first activated overnight in Brain Heart infusion (BHI) broth medium at 37 °C. 100 µL of the activated pathogenic bacteria were spread (6.0 log CFU/mL) on BHI agar medium, on which the wells with a diameter of 6 mm were created with a sterile steel drill. The fraction containing small molecular weight (<3 kDa) peptides obtained by Amicon Ultra-15 centrifugal filters was added in the wells with 50 µL and sterile distilled water was utilized as negative control. Petri dishes were then incubated at 37 °C for 24 hours. Antibacterial activity was determined by evaluation of the diameters of the inhibition zone in millimeters (mm) on mediums.

3.4.4 Determination of ACE inhibitory activity

ACE inhibitor activity was determined by the RP-HPLC (Pihlanto et al., 2010). Shortly, N hippuryl-Her-Leu tetrahydrate (HHL) was dissolved (5 mM) in phosphate buffer (0.1 M, pH 8.3, 0.4 M NaCl) for the activation of enzyme. During analysis, 25 µL was taken from the samples and 60 µL of HHL was added. The mixture was incubated at 37 °C for 5 minutes, followed by the addition of 50 µL of ACE. After that, the prepared samples were incubated at 37 °C for 30 minutes. Distilled water was used as a blank. For the control sample, 25 µL of phosphate buffer (0.1 M, pH 8.3, 0.4 M NaCl) was used instead of the sample. At the end of the incubation, 10 µL of 5 M HCl was added (the pH of the environment was lowered below pH 4) to stop ACE enzyme activity. The prepared mixture was filtered using a 0.45 µm membrane filter (PVDF) and then transferred into the vials.

The amount of hippuric acid separated from HHL was determined using a C18 column (4.6 × 250 mm, 5 µm). Mobile phase A used contained 75% acetonitrile mixed with 0.1 % TFA at a flow rate of 1.5 mL/min. Measurements were made at a wavelength of 228 nm and a column temperature of 30 °C.
ACE inhibitor activity was computed according to the formulation below:

$$\% \text{ ACE inhibitor activity} = [(Cc-Cs) \div (Cc-Cb)] \times 100$$

Cc: Hippuric acid concentration in the control group (without the tested sample),
Cb: Hippuric acid concentration in the evidence group (without ACE),
Cs: Hippuric acid concentration in the experimental group (with both ACE and the tested sample).

The IC50 value for ACE inhibition in 3 kDa WSE was outlined as the protein concentration (mg/mL) necessary for a 50% decrease in the quantity of hippuric acid produced by ACE inhibition percentage.

3.5 Statistical Analyses

All the test measurements were performed in two replicates. Analysis of variance (ANOVA) was performed on data produced from fermented milk samples during the storage period on days 1., 14., and 28 using Minitab software version 17 (State College, USA). Results were provided as mean ± standard deviation. Significant differences among the storage days and different LAB starter species were measured using Tukey test ($p \leq 0.05$) at 95% confidence level.

4. RESULTS AND DISCUSSION

4.1 Properties of Pasteurized Milk Used in Fermented Milk Production

The analysis results of the chemical properties of pasteurized milk used in production are shown in table 4.1.

Table 4.1 Properties of pasteurized milk used in production (n=2)

Properties	**Mean values**
pH value	6.67±0.01
Titratable acidity (% LA)	0.160±0.004
Water soluble extract (WSE) protein amount (mg/mL)	0.315±0.005
Microbial count (log cfu/mL)	0.00±0.00
Antioxidant capacity (μM, TEAC)	235.20±0.20
Phenolic content (mg GAE/g)	0.06±0.00
ACE-inhibitor activity (%)	5.00±0.80

4.2 pH Values of the Fermented Milk Samples

Milk pH is a critical factor in the production of fermented dairy products. It determines dissociation of milk acidity, proteins conformation and enzymes activity. pH is also an essential factor for the rate of proteolysis and production of peptides (Soleymanzadeh et al. 2016). Lactobacilli strains were incubated at 37 °C, Lactococci, Streptococci and Enterococci bacteria were incubated at 30 °C. The pH value of fermented milk samples produced with different LAB are shown in table 4.2.

The obtained results indicate that there were significant differences ($P \leq 0.05$) in the pH values among the fermented milk samples produced with different LAB strains and among the storage days. The pH values among the milk fermented from different LAB strains ranged between 4.81-3.88.

Table 4.2 pH values of the samples during the storage days (n=2)

Samples	Storage days		
	1	**14**	**28**
Lb. coryniformis NCCB 100528	4.78 ± 0.02^{aA}	4.65 ± 0.01^{aB}	4.71 ± 0.08^{aAB}
Lb. paraplantarum NCCB 100524	4.75 ± 0.02^{aA}	4.71 ± 0.06^{aA}	4.77 ± 0.03^{aA}
Lb. brevis NCCB 100526	4.80 ± 0.01^{aA}	4.70 ± 0.10^{aA}	4.72 ± 0.03^{aA}
L. plantarum NCCB 100523	4.74 ± 0.14^{aA}	4.68 ± 0.18^{aA}	4.64 ± 0.17^{bA}
Lb. bulgaricus 23	4.45 ± 0.06^{bA}	4.27 ± 0.07^{bA}	3.88 ± 0.13^{bB}
E. faecium NCCB 100527	4.76 ± 0.02^{aA}	4.74 ± 0.05^{aA}	4.74 ± 0.07^{aA}
E. faecalis NCCB 100525	4.72 ± 0.03^{aA}	4.74 ± 0.02^{aA}	4.77 ± 0.06^{aA}
Lc. lactis NCCB 100539	4.80 ± 0.07^{aA}	4.76 ± 0.04^{aA}	4.81 ± 0.06^{aA}
Lb. helveticus 11-B2	4.16 ± 0.06^{cA}	3.93 ± 0.05^{cB}	3.88 ± 0.04^{bB}
Lb. helveticus 9-B5	4.16 ± 0.04^{cA}	4.09 ± 0.13^{bcA}	4.07 ± 0.04^{bA}
Lb. helveticus 12-B1	4.07 ± 0.18^{cA}	4.01 ± 0.05^{cA}	3.98 ± 0.03^{bA}
S. thermophilus K16	4.68 ± 0.07^{aA}	4.70 ± 0.09^{aA}	4.76 ± 0.13^{aA}
S. thermophilus 1-K4	4.68 ± 0.08^{aA}	4.59 ± 0.02^{aA}	4.58 ± 0.02^{aA}

Significant differences were determined with the Tukey test ($P \leq 0.05$). Different lowercase letters in the same column represent differences between samples while uppercase letters in the same row indicates differences between storage days.

It was determined that fermented milk sample made with both *Lc. lactis* NCCB 100539 and *Lb. brevis* NCCB 100526 presented the highest pH value among all the samples during the storage day 1. However, samples with *Lb. bulgaricus* 23 and *Lb. helveticus* 11-B2 produced the lowest pH on day 28. The low pH observed in the fermented milk samples mentioned above is thought to be as a result of a stronger acidifying capacity of those strains (Beal et al. 1999, Skryplonek et al. 2019). Sample made with *Lb. bulgaricus* 23 resulted a high acidity on day 1, and its pH value sharply dropped at the end of storage. Fermented milk sample produced with *Lb. helveticus* 11-B2 had similar pH values on day 14 and 28th day of storage.

Fermented milk samples made with *L. plantarum* NCCB 100523, *Lb. bulgaricus* 23 and *Lb. helveticus* 11-B2 showed gradual drop in the pH from the first day up to 28th day of storage period figure 4.1. A drop pattern in pH during the storage days in our samples is in agreement with a study conducted by Li et al. (2020) who reported that pH of fermented milk decrease with increase in storage time and it is based on the metabolic activity and the growth requirement of the particular bacteria strain (Wansutha et al. 2018, Li et al. 2020). A higher lactose hydrolysis during fermentation results in higher production of organic acids, such as lactic acid, propionic acid and acetic acid as the main end products of microbial activity and their release mainly depends on the type of microbial strain (Ayivi et al. 2020, Rosa et al. 2023). Casarotti et al. (2014) also noted that pH value in fermented milk samples were related to proteolysis and appeared to be dependent on storage time and acid formation capacity of the starters used.

However, there is a fluctuation in pH values of samples produced with *Lb. coryniformis* NCCB 100528 exhibited arise in the pH at the end of the storage days. This could be as a result of fungal strain development which causes the absorption of the lactic acid and the deamination of the amino acids (Schlesser et al.1992).

In a study conducted by Panayotova et al. (2018), they obtained lower pH values (4.30-4.62) on day 1 from fermented milk samples made with *Lb. bulgaricus* and *S. thermophilus* strains compared to the pH results we obtained in our fermented milk samples made with similar strains. However, we obtained much lower pH value for

fermented milk samples produced with *Lb. helveticus* 11-B2, *Lb. helveticus* 9-B5, *Lb. helveticus* 12-B1and *Lb. bulgaricus* 23 strains at the end of storage period except for the fermented milk samples with S. *thermophilus* K16 compared to the pH values they obtained. In the study conducted by Rosa et al. (2023) in probiotic fermented whey-milk beverages, the pH value of sample produced with *Lc. lactis* on day 1 was lower than the results we obtained for the fermented milk sample with similar strain on storage but on day 14, we obtained similar pH values for the same strain. The pH value for the fermented milk samples made with *Lb. bulgaricus* 23 and *S. thermophilus* strains in our study on day 1 were higher than the pH values obtained in yoghurt samples produced with combined strains of *Lb. bulgaricus* and *S. thermophilus* by (Zainoldin and Baba 2009). The low pH was most likely due to a mutual protocooperation of both *S. thermophilus* and *Lb. bulgaricus* strains used in their study (Brabandere and Baerdemaeker 1999).

Milk samples fermented with *Lb. helveticus* strains in our study produced a remarkably much lower pH values than the pH value from fermented donkey milk samples registered with Aspri et al. (2018), on the other hand, they produced lower pH values for *E. faecium* and *Lc. lactis* strains than in our samples. Ayyash et al. (2018) obtained higher pH values from camel milk fermented with *L. plantarum* strain on day 1 compared to our sample. Dan et al. (2019) reported a much lower pH values for the samples produced with *L. plantarum* and *S. thermophilus* on day 1 and 14 than the pH results found in our study for the mentioned LAB strains. In another study by Abd El-Fattah et al. (2017), sterilized skim milk samples with *Lb. brevis* and *L. plantarum* presented significantly lower pH values than in our samples on day 1. However, samples produced with *Lb. helveticus* strains in our study produced lower pH values. pH values obtained in the Aloe vera juice fermented with *E. faecium* and *Lc. lactis* on day 1 were lower than the pH values that we obtained in samples made with similar strains (Cuvas-Limón et al. 2022). Camel milk fermented with *L. plantarum* produced a lower pH value (pH 4.1) Algboory and Muhialdin (2021) than the result we obtained in our fermented milk sample with a similar strain on storage day 1. Our pH values in samples produced with *L. plantarum* NCCB 100523 on day 1 is in agreement with the pH values obtained in Cheddar cheese made with *L. plantarum* as an adjunct culture (Duan et al. 2019).

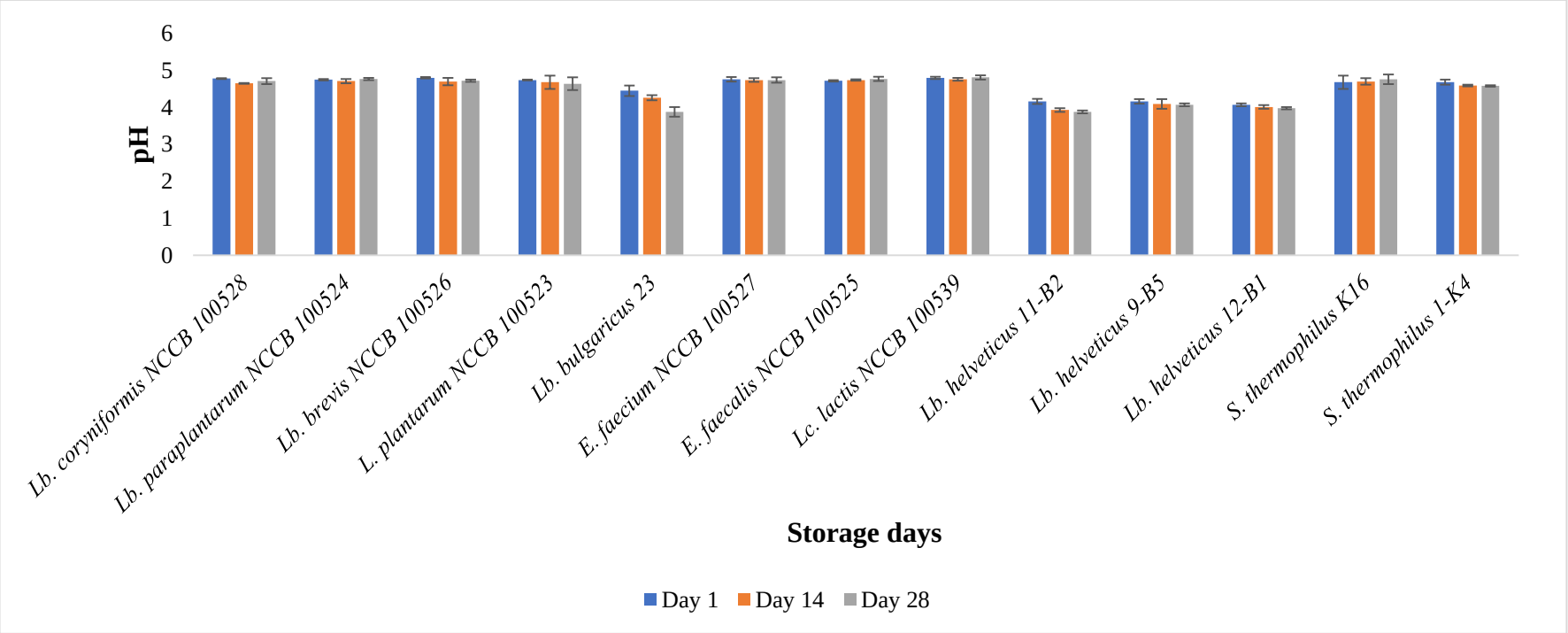


Figure 4.1 pH values of the samples during storage days (n=2)

4.3 Titratable Acidity (% Lactic acid) of Fermented Milk Samples

Titratable acidity values measured in terms of lactic acid percentages (% LA) for fermented milk during storage days are given in table 4.3. The results illustrated that there were significant differences in titratable acidity among the fermented milk samples and the storage days ($P \leq 0.05$). Titratable acidity for our fermented milk samples from different bacteria ranged from 0.61-1.52 % LA.

Fermented milk sample produced with *Lb. bulgaricus* 23 registered the highest titratable acidity values at the end of the storage days followed by sample made by *Lb. helveticus* strains. A higher titratable acidity exhibited in the fermented milk sample made by the above bacteria might be due to the high acid formation ability (Skryplonek et al. 2019). In addition, it took less than 4 hours during incubation to get the pH between 4.06-4.16 among the samples with *Lb. helveticus* strains. However, fermented milk samples produced by *Lb. coryniformis* NCCB 100528, *Lb. paraplantarum* NCCB 100524, *Lc. lactis* NCCB 100539, *S. thermophilus* 1-K4 and *S. thermophilus* K16 produced the lowest titratable acidity. The low acidity values produced by these strains is thought to be due to the poor activity of these bacterial strains to breakdown lactose to generate lactic acid (de Castro et al. 2009, Skryplonek et al. 2019). Despite the low acidity observed in sample produced by *Lb. bulgaricus* 23, this strain showed a sharp increase and dominated titratable acidity on day 14 and 28. This probably reflects that *Lb. bulgaricus* strain has a high metabolic activity of lactic acid formation from milk lactose (Zainoldin and Baba 2009). Samples with *Lb. coryniformis* NCCB 100528, *Lb. brevis* NCCB 100526, *L. plantarum* NCCB 100523, *E. faecalis* NCCB 100525, *Lc. lactis* NCCB 100539, *Lb. helveticus* 12-B1, *S. thermophilus* K16 and *S. thermophilus* K16 produced similar pH values during the storage days, figure 4.2.

Table 4.3 Titratable acidity (% LA) values of the samples during storage days (n=2**)**

Samples	Storage days		
	1	**14**	**28**
Lb. coryniformis NCCB 100528	0.68 ± 0.02^{cdeA}	0.74 ± 0.01^{cdeA}	0.68 ± 0.06^{dA}
Lb. paraplantarum NCCB 100524	0.64 ± 0.04^{deB}	$0.73\pm0,01^{deA}$	0.73 ± 0.04^{dA}
Lb. brevis NCCB 100526	0.72 ± 0.0^{cdA}	0.76 ± 0.04^{cdeA}	0.75 ± 0.05^{dA}
L. plantarum NCCB 100523	0.77 ± 0.01^{bcA}	0.79 ± 0.03^{bcdeA}	0.85 ± 0.15^{cdA}
Lb. bulgaricus 23	0.61 ± 0.02^{eC}	1.06 ± 0.21^{aB}	1.52 ± 0.18^{aA}
E. faecium NCCB 100527	0.72 ± 0.02^{cdB}	0.76 ± 0.00^{cdeA}	0.76 ± 0.02^{cdA}
E. faecalis NCCB 100525	0.70 ± 0.02^{cdeA}	0.71 ± 0.03^{eA}	0.72 ± 0.02^{dA}
Lc. lactis NCCB 100539	0.65 ± 0.02^{deA}	0.68 ± 0.05^{eA}	0.70 ± 0.05^{dA}
Lb. helveticus 11-B2	0.89 ± 0.08^{aB}	0.97 ± 0.01^{abB}	1.15 ± 0.05^{bA}
Lb. helveticus 9-B5	0.85 ± 0.03^{abB}	0.92 ± 0.04^{abcAB}	0.99 ± 0.05^{bcA}
Lb. helveticus 12-B1	0.91 ± 0.07^{aA}	0.91 ± 0.02^{abcdA}	1.09 ± 0.11^{bA}
S. thermophilus K16	0.63 ± 0.03^{deA}	0.64 ± 0.03^{eA}	0.67 ± 0.02^{dA}
S. thermophilus 1-K4	0.66 ± 0.00^{deB}	0.70 ± 0.03^{eAB}	0.73 ± 0.03^{dA}

Significant differences were determined with the Tukey test ($P \leq 0.05$). The means denoted by different lowercase letters in the same column represent differences between samples while uppercase letters in the same row indicates differences between storage days.

Whereas majority of our fermented milk samples from different bacterial strains showed steady increase in titratable acidity with increase in storage days, some fermented milk samples with some bacterial strains demonstrated fluctuations in acidity values during the storage days. The increasing trend for titratable acidity during the storage days is in support with results by (Marth and Steele 2001, Salwa and Galal 2002). A continuous increase of titratable acidity during the storage period could have been caused by an increase in metabolic actions and other biochemical alterations made by LAB at refrigeration temperature (Brabandere and Baerdemaeker 1999, Antunes et al. 2005, Yadav et al. 2018). In addition to that, the β-galactosidase activity in LAB for milk sugar hydrolysis occurs despite the lower temperature of about (0–5 °C) storage, hence resulting in the increase of lactic acid, formic acid, citric acid, acetic acid, acetaldehyde, and butyric acid (Thakur et al.2022).

Zainoldin and Baba (2009) obtained higher acidity values on day 1 for the yoghurts enriched with white and red dragon fruits produced with *Lb. bulgaricus* and *S. thermophilus* strains compared to the acidity results we got in our fermented milk samples with the similar bacterial strains. Similarly, Casarotti et al. (2014) reported a higher titratable acidity (0.82-1.14) and lower pH throughout the storage days for fermented milk beverages produced with *S. thermophilus* and *Lc. lactis* compared to the results we obtained from our fermented milk samples produced with similar strains. It is thought that the higher titratable acidity observed in their study was because they supplemented their fermented beverage with quinoa flour. Titratable acidity values for fermented milk samples made by *Lb. bulgaricus* 23 and *S. thermophilus* strains in our study produced lower titratable acidity values than that observed for the same bacterial strains studied by (Thakur et al. 2022). The higher titratable acidity value in that study was obtained in yoghurt enriched with microencapsulated phenolic extract powder obtained from pomegranate fruit lyophilized peels probably was due to the high amounts of organic acids produced during fermentation. Therefore, it is possible that the starter culture could have utilized the residual carbohydrates during storage days hence increase in acidity. In another study by B El-Alfy (2021), symbiotic-drinkable yoghurt fortified with oat and produced with *L. plantarum* strains recorded lower titratable acidity values on day 1 and 14 compared to the results for the same species in our study. In our study, fermented milk

samples made with *Lb. helveticus* strains produced higher titratable acidity values than the titratable acidity results obtain in sterilized milk fermented with similar strains by (Abd El-Fattah et al. 2017). However, samples with *Lb. brevis* NCCB 100526 and *L. plantarum* NCCB 100523 produced lower titratable acidity compared to their study. We obtained higher titratable acidity for samples produced with *Lb. helveticus* strains on both day 1 and 14 compared to the results obtained in fermented camel milk samples produced with *Lb. helveticus* strains (El-Sayed et al. 2021). In a study where camel milk was fermented with *L. plantarum* produced a higher titratable acidity on storage day 1 than the titratable acidity result we obtained in fermented milk sample made with *L. plantarum* NCCB 100523 (Algboory and Muhialdin 2021).

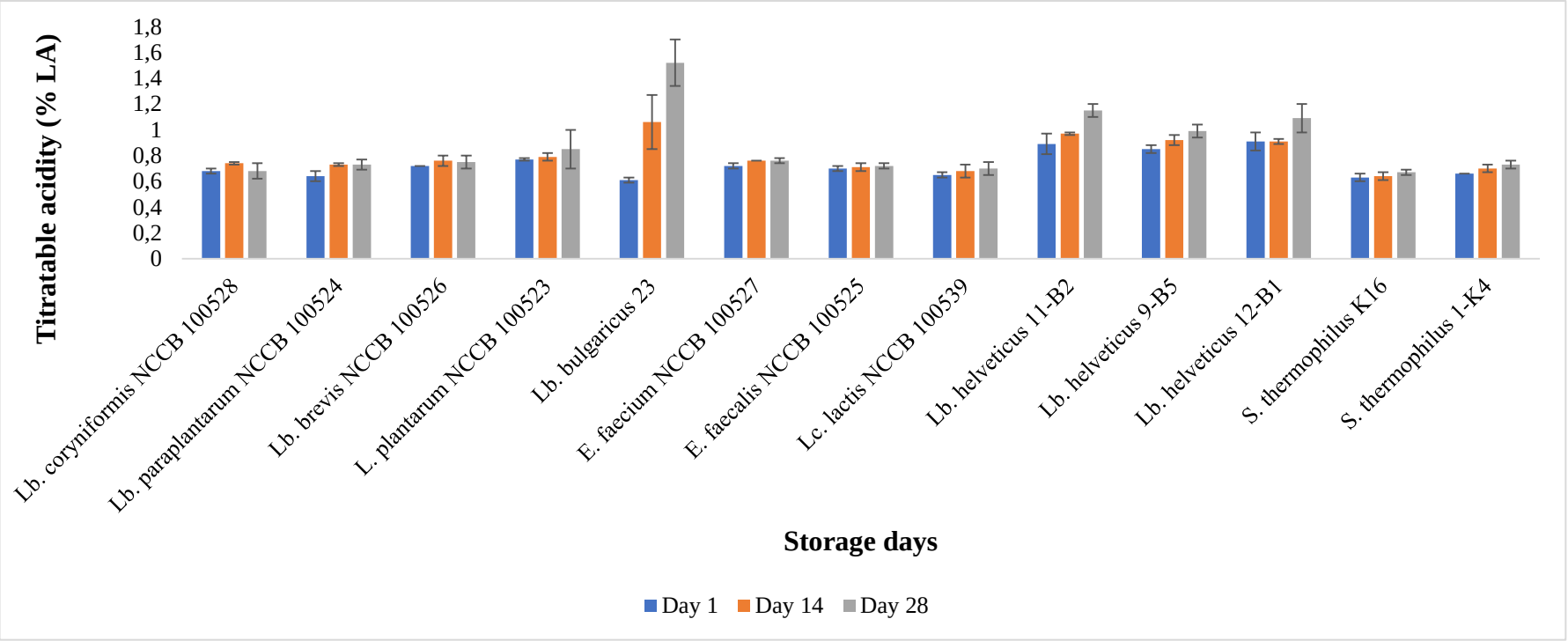


Figure 4.2 Titratable acidity values for the samples during storage days (n=2)

4.4 Viable Microbial Count of the Fermented Milk Samples

The results for viable lactic acid bacteria count (log CFU/mL) for all the fermented milk samples produced individual bacterial strains during the storage period are given in Table 4. 4. The results determined that there were significant differences among the fermented milk samples made with different bacterial strains and among the storage days ($P \leq 0.05$). The bacterial counts from all the samples produced with different strains ranged from 8.21-11.10 log CFU/mL, this justifies strain presence in fermented products. Despite the fact that there is no obvious agreement in the average quantity of cells to attain health functional advantages, these values are higher than the inception of 6.0–8.0 log CFU/mL proposed by (Aspri et al. 2018). Fermented milk samples produced with *S. thermophilus* K16 produced the highest microbial count on the first day of storage. On the contrary, samples with *Lb. coryniformis* NCCB 100528, *Lb. paraplantarum* NCCB 100524 and *Lb. bulgaricus* 23 produced the least. The high microbial count of fermented milk with S. *thermophilus* K16 could be as a result of the high pH which was observed in that sample. Bacteria could have survived in the high pH environment, so the number of bacteria increases (Jaster et al. 2018).

Fermented milk samples produced with *Lb. helveticus* 12-B1 and *S. thermophilus* K16 showed a steady decrease in microbial count during the storage days. On the contrary, the other samples expressed fluctuations of microbial counts during the storage days figure 4.3. A reduction in the bacterial counts in yoghurt during refrigeration storage was highlighted by other researchers (Vasiljevic et al. 2007, Ayyash et al. 2018, Hanlon et al. 2021). Decreasing viable microbial count during storage days is thought to be related to the decreased in pH and consequential acidic environment which inhibits the growth of LAB (Jaster et al. 2018, Li et al. 2023). Furthermore, the decrease in the growth of viable microorganism during storage is probably due to the inhibitory factors, such as lactoferrin, lactoperoxidase and lysozyme IgA, and IgG, which adversely affect bacterial growth with storage time (Aleman et al. 2023). On the other hand, fluctuation of microbial counts during the storage days is thought to be caused by acidity alteration in the fermented milk (Ozturkoglu-Budak et al. 2016b).

Table 4.4 Viable microbial counts (log CFU/mL) values of the samples during storage days (n=2)

Samples	Storage days		
	1	**14**	**28**
Lb. coryniformis NCCB 100528	8.58 ± 0.08^{efgB}	8.84 ± 0.04^{fA}	8.96 ± 0.04^{gA}
Lb. paraplantarum NCCB 100524	8.34 ± 0.02^{fgB}	9.28 ± 0.08^{eA}	9.33 ± 0.18^{efgA}
Lb. brevis NCCB 100526	9.00 ± 0.01^{deB}	9.20 ± 0.10^{eB}	9.90 ± 0.10^{abcdA}
L. plantarum NCCB 100523	8.83 ± 0.08^{efC}	9.33 ± 0.01^{eB}	9.70 ± 0.10^{cdefA}
Lb. bulgaricus 23	8.21 ± 0.10^{gC}	9.10 ± 0.20^{efB}	$9.80\pm0.10b^{cdeA}$
E. faecium NCCB 100527	9.60 ± 0.10^{bcB}	10.15 ± 0.15^{bA}	$9.85\pm0.15a^{bcdeAB}$
E. faecalis NCCB 100525	9.40 ± 0.10^{cdA}	9.70 ± 0.10^{cdA}	9.55 ± 0.25^{cdefA}
Lc. lactis NCCB 100539	9.00 ± 0.20^{deB}	10.15 ± 0.22^{bA}	9.15 ± 0.15^{fgB}
Lb. helveticus 11-B2	9.95 ± 0.05^{aA}	9.38 ± 0.03^{efB}	$9.40\pm0.20d^{efgB}$
Lb. helveticus 9-B5	9.60 ± 0.20^{bcA}	9.08 ± 0.08^{efA}	$9.35\pm0.35d^{efgA}$
Lb. helveticus 12-B1	9.55 ± 0.25^{bcB}	10.20 ± 0.20^{bA}	$10.10\pm0.10a^{bcA}$
S. thermophilus K16	11.10 ± 0.50^{aA}	10.58 ± 0.08^{aA}	10.38 ± 0.33^{aA}
S. thermophilus 1-K4	9.45 ± 0.0^{cbcdC}	9.75 ± 0.05^{cB}	10.33 ± 0.13^{abA}

Significant differences were determined with the Tukey test ($P \leq 0.05$).
The means denoted by different lowercase letters in the same column represent differences between samples while uppercase letters in the same row indicates differences between storage days

On storage day 1, there were significant differences in microbial count ($P \leq 0.05$). Samples with *Lb. coryniformis* NCCB 100528, *Lb. paraplantarum* NCCB 100524, *Lb. brevis* NCCB 100526, *E. faecium* NCCB 100527, *Lc. lactis* NCCB 100539, *Lb. helveticus* 12-B1; *L. plantarum* NCCB 100523, *Lb. bulgaricus* 23, *S. thermophilus* 1-K4 and samples with *E. faecalis* NCCB 100525, *Lb. helveticus* 11-B2, *Lb. helveticus* 9-B5, and *S. thermophilus* K16 produced similar microbial count. The lowest microbial count was observed in sample made with *Lb. bulgaricus* 23 on day 1 while the highest was registered by sample made with *S. thermophilus* K16 on storage day 1. Samples made with *Lb. coryniformis* NCCB 100528, *Lb. helveticus* 11-B2 and *Lb. helveticus* 9-B5 produced the least microbial count on day 14 and 28 among all the other samples. However, samples with *S. thermophilus* K16 produced the highest microbial count on day 14 and 28.

The microbial counts in fermented milk samples produced with *Lb. bulgaricus* 23, *S. thermophilus* and *Lb. helveticus* bacteria in our study were higher comparatively to that observed by Panayotova et al. (2018) in fermented milk. However, we obtained similar bacterial counts varied from 8.97 to 9.12 log CFU/mL on day 1. Whereas in the investigation by Wansutha et al. (2018) in almond fermented milk presented slightly higher microbial counts than in our fermented milk samples except for the samples produced with *S. thermophilus* K16, *E. faecium* NCCB 100527 and *Lb. helveticus strains.* Furthermore, microbial counts for fermented milk samples produced with *L. plantarum* NCCB 100523, *Lc. lactis* NCCB 100539, *Lb. bulgaricus* 23 and all *S. thermophilus strains* in our study were found to be higher than the results obtained from cashew milk-based yoghurt for the similar species throughout the storage period (Shori et al. 2022). Ayyash et al. (2018) obtain a higher microbial count in fermented camel and milk from bovine made with *Lc. lactis* counts at the beginning of storage, however, we achieved a much higher result at the 28th day of storage.

Microbial count for the fermented milk sample made with *S. thermophilus* 1-K4 on day1 is in agreement with the results obtained in fermented milk samples for a similar strain (Casarotti et al. 2014). However, fermented milk sample with *S. thermophilus* 1-K4 produced higher microbial count on both14th and 28th day of storage. Paseephol and Sherkat (2009) detected a similar microbial count for *Lb. bulgaricus* in yoghurts containing

Jerusalem artichoke inulin on day 1. However, we obtained a higher microbial count in fermented milk samples produced by *Lb. bulgaricus* on day 14 and 28. In addition, yoghurts made with *S. thermophilus* produced higher microbial counts throughout the storage days compared to the results obtain with similar strain in our study. Microbial count for fermented milk samples produced with *L. plantarum* NCCB 100523 and *S. thermophilus* strains on day 1 corresponds with the results obtained in fermented milk for the same bacteria strains in an investigation by Dan et al. (2019), however, we obtained higher microbial count in our fermented milk samples for the above strains on day 14. Microbial counts for fermented milk sample with *L. plantarum* on day 1 is in agreement with the results obtained from milk samples fermented with the similar bacterial strain Gonzalez-Gonzalez et al. (2011), however, we obtained a slightly higher bacterial count in fermented milk samples made by *Lb. helveticus* strains on the 1st day of storage compared to the results obtained for the similar strains.

According to B El-Alfy (2021), symbiotic-drinkable yoghurt fortified with oat and produced with *L. plantarum* and *S. thermophilus* strains obtained lower microbial count on day 1 and 14 during storage compared to the higher microbial counts registered in our samples with the same strains. Sample produced with *Lb. bulgaricus* 23 at the beginning of storage is in agreement with the results for the same species for the study of Li et al. (2020), however, we obtained higher microbial count at the end of storage days. Moreover, microbial count results on day 1 for our samples with *L. plantarum* NCCB 100523 is in agreement with the results obtained from fermented soy milk by Martinez-Villaluenga et al. (2012). However, we got higher microbial count for samples with *Lc. lactis* NCCB 100539 and *S. thermophilus* strains. Camel milk fermented with *L. plantarum* produced a lower microbial count (Algboory and Muhialdin, 2021) than the result we obtained in our fermented milk samples with a similar strain on day 1.

Microbial count for our samples made with *Lb. helveticus* 9-B5 and *Lb. helveticus* 11-B2 were in agreement with the results of Mo et al (2019) for fermented milk products in Xinjiang, China using *Lb. helveticus* species, however, we obtained higher microbial count in fermented milk with *Lb. bulgaricus* 23 and *L. plantarum* NCCB 100523.

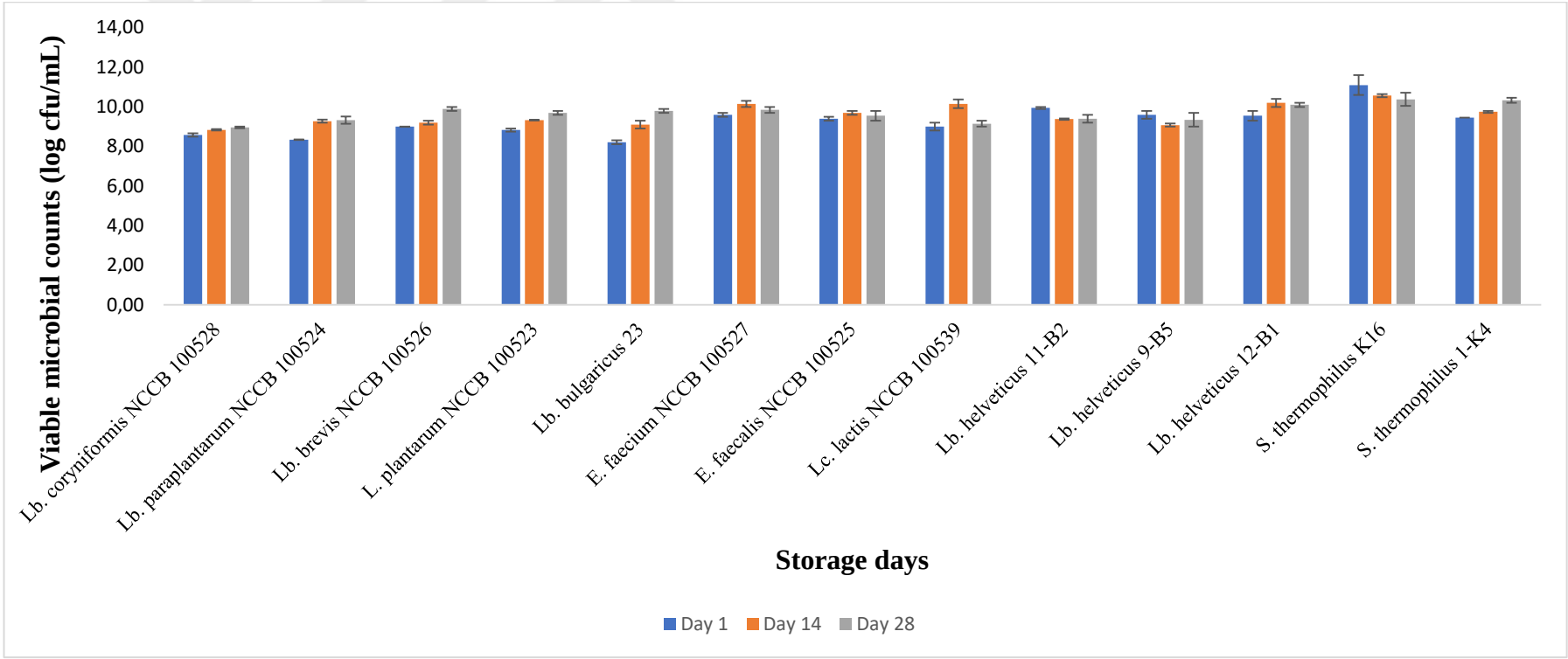


Figure 4.3 Viable microbial counts of the samples during storage days (n=2).

4.5 Water Soluble Extract Protein Quantity of the Fermented Milk Samples

Milk protein is known as a significant origin of biologically active peptides and has shown differences in bioactive peptides among dairy starter cultures (Kim et al.2021). The measurement of WSE is largely used as an indicator for proteolysis of fermented milk (Dimitrellou et al. 2010, Beirami-Serizkani et al. 2021).

The protein quantity in WSE samples were expressed in mg/mL (Table 4.5). The results confirmed that there were significant differences ($P \leq 0.05$) in terms of WSE protein quantities among the fermented milk samples produced with different LAB strains and among the storage days. In overall, protein quantity ranged from 0.106 – 0.390 mg/mL. Fermented milk samples produced with *Lb. helveticus* 9-B5 generated the highest protein quantity on day 1 and sample with *Lb. helveticus* 11-B2 fermented milk produced the highest protein content at the end of storage. Samples made by *Lb. helveticus* strains and *Lb. coryniformis* NCCB 100528 resulted in much higher protein quantity during the storage days. Higher protein quantity determined in the samples with the relative bacterial strains is thought to be as a result of a high proteolytic activity which might be connected to a large number of proteases and peptidases enzymes available in those strains (Reyes-Díaz et al. 2018).

At the beginning of storage, fermented milk with *Lb. coryniformis* NCCB 100528, *Lb. brevis* NCCB 100526, *E. faecium* NCCB 100527, *S. thermophilus* K16 and *E. faecalis* NCCB 100525 produced similar protein quantity in WSE. Similarly, samples with *Lb. paraplantarum* NCCB 100524 and *Lc. lactis* NCCB 100539 as well as *L. plantarum* NCCB 100523 and *Lb. bulgaricus* 23 had approximately the same amounts of protein. Also, fermented milk samples produced with *Lb. helveticus* 12-B1 and *S. thermophilus* 1-K4 presented a similar protein quantity. In the same way, fermented milk samples made by *Lb. paraplantarum* NCCB 100524 produced a similar protein quantity on day 14 and 28, which was different on the first day of storage. Fermented milk sample with *Lb. brevis* NCCB 100526 had similar protein quantity on day 1 and 14 but differed at the end of storage. Samples produced with *E. faecium* NCCB 100527 generated a similar protein quantity on day 14 and 28 but was different on day 1.

Table 4.5 Water soluble extract (WSE) protein quantity of the samples during storage days (n=2)

Samples	Storage days		
	1	**14**	**28**
Lb. coryniformis NCCB 100528	0.232 ± 0.018^{cdA}	0.220 ± 0.000^{bcdA}	0.251 ± 0.031^{bA}
Lb. paraplantarum NCCB 100524	0.106 ± 0.003^{gB}	0.118 ± 0.002^{jA}	0.116 ± 0.004^{eA}
Lb. brevis NCCB 100526	0.212 ± 0.001^{cdeA}	0.203 ± 0.004^{fA}	0.183 ± 0.006^{dB}
L. plantarum NCCB 100523	0.164 ± 0.007^{fC}	0.221 ± 0.001^{bcdA}	0.194 ± 0.010^{dB}
Lb. bulgaricus 23	0.157 ± 0.001^{fB}	0.130 ± 0.000^{iC}	0.187 ± 0.004^{dA}
E. faecium NCCB 100527	0.237 ± 0.027^{cA}	0.168 ± 0.007^{gB}	0.191 ± 0.007^{dB}
E. faecalis NCCB 100525	0.212 ± 0.002^{cdeA}	0.207 ± 0.003^{efA}	0.210 ± 0.002^{cdA}
Lc. lactis NCCB 100539	0.110 ± 0.002^{gC}	0.153 ± 0.010^{hB}	0.199 ± 0.001^{cdA}
Lb. helveticus 11-B2	0.308 ± 0.006^{bB}	0.252 ± 0.000^{aC}	0.358 ± 0.000^{aA}
Lb. helveticus 9-B5	0.390 ± 0.008^{aA}	0.216 ± 0.000^{cdeC}	0.253 ± 0.015^{bB}
Lb. helveticus 12-B1	0.198 ± 0.003^{eC}	0.232 ± 0.000^{bB}	0.253 ± 0.001^{bA}
S. thermophilus K16	0.204 ± 0.005^{deA}	0.209 ± 0.007^{defA}	0.206 ± 0.004^{cdA}
S. thermophilus 1-K4	0.199 ± 0.005^{eB}	0.226 ± 0.000^{bcA}	0.227 ± 0.013^{bcA}

Significant differences were determined with the Tukey test ($P \leq 0.05$).
The means denoted by different lowercase letters in the same column represent differences between samples while uppercase letters in the same row indicates differences between storage days

Protein quantity for WSE samples with *Lb. brevis* NCCB 100526 decreased significantly after the 14th day. However, samples with *Lb. helveticus* 12-B1 and *Lc. lactis* NCCB 100539 showed a steady increase in protein quantity throughout the storage days. The increment in protein quantity throughout the storage days could be due to an increase in proteolysis (Mudgi et al. 2023). However, fluctuations in protein quantity were observed in most fermented milk samples. These fluctuations could be related to the differences inability to hydrolyze milk protein by different LAB strains (Chen et al 2021). In addition, the variation in their proteolytic activity against milk protein could be attributed to their differences in the capacity to release proteases by the individual LAB (Rubak et al. 2022). In the present study, protein contents in WSE provide information about the activities of microorganisms in protein synthesis and enzyme activities during the storage. In addition, the variety and amounts of microorganisms may influence the nutritional value and quality of fermented milk. In our study, for instance, protein content in WSE of fermented milk sample with *Lb. brevis* NCCB 100526 decreased at the end of the storage, indicating that the efficiency of this LAB strain in protein synthesis during storage decreases over time. On the other hand, samples with *Lc. lactis* NCCB 100539 and *Lb. helveticus* 12-B1samples presented an increasing amount of protein over time, which indicates that the efficiency of these microorganisms in protein synthesis increases over time, figure 4.4. These findings are important in terms of determining strategies for the selection and use of cultures in milk products depending on the required protein characteristics.

In a previous study done by Kim et al. (2021), yoghurt produced with *L. plantarum* and *Lb. bulgaricus* showed higher protein content on day 1 compared to the results obtained in our samples made with the similar bacterial strains. We found remarkably higher protein quantity in our fermented milk samples with *L. plantarum* NCCB 100523 than in the Iraqi camel fermented milk with *L. plantarum* (Muhialdin and Algboory 2018). According to Skrzypczak et al. (2020), milk beverages fermented *with Lb. helveticus* strains produced a higher protein quantity than all our samples with *Lb. helveticus* strains. In another study conducted in Beyaz cheese produced with a combination of *L. plantarum*, *Lb. paraplantarum* and *Lc. lactis* strains, the protein contents in WSE varied from 0.180 to 0.190 mg/mL (Karahan et al. 2010), which was slightly higher than the protein contents in fermented milk samples made with similar strains in our study. Protein

content in WSE from cheese produced with *Lc. lactis* subsp. *lactis* resulted in higher protein quantity than those of our study because they used combined culture (Novella-Rodríguez et al. 2002). In another study regarding symbiotic-drinkable yoghurt fortified with oat, samples with *L. plantarum* produced slightly higher protein quantity on day 1 compared to our samples made with similar strains. However, we recorded a similar protein content on day 14 (B El-Alfy 2021). Protein content of our sample made with *E. faecalis* NCCB 100525 is in agreement with the protein content results obtained from fermented milk made with a similar strain Muguerza et al. (2006), however, protein content for fermented milk samples made with *Lc. lactis, Lb. helveticus* and *Lb. brevis* was observed to be higher compared to the results we obtained in fermented milk samples made with similar strains. Donkor et al. (2007) suggested that the degree of proteolytic activity in fermented milk is greatly dependent on the LAB used and also varies with a period of time

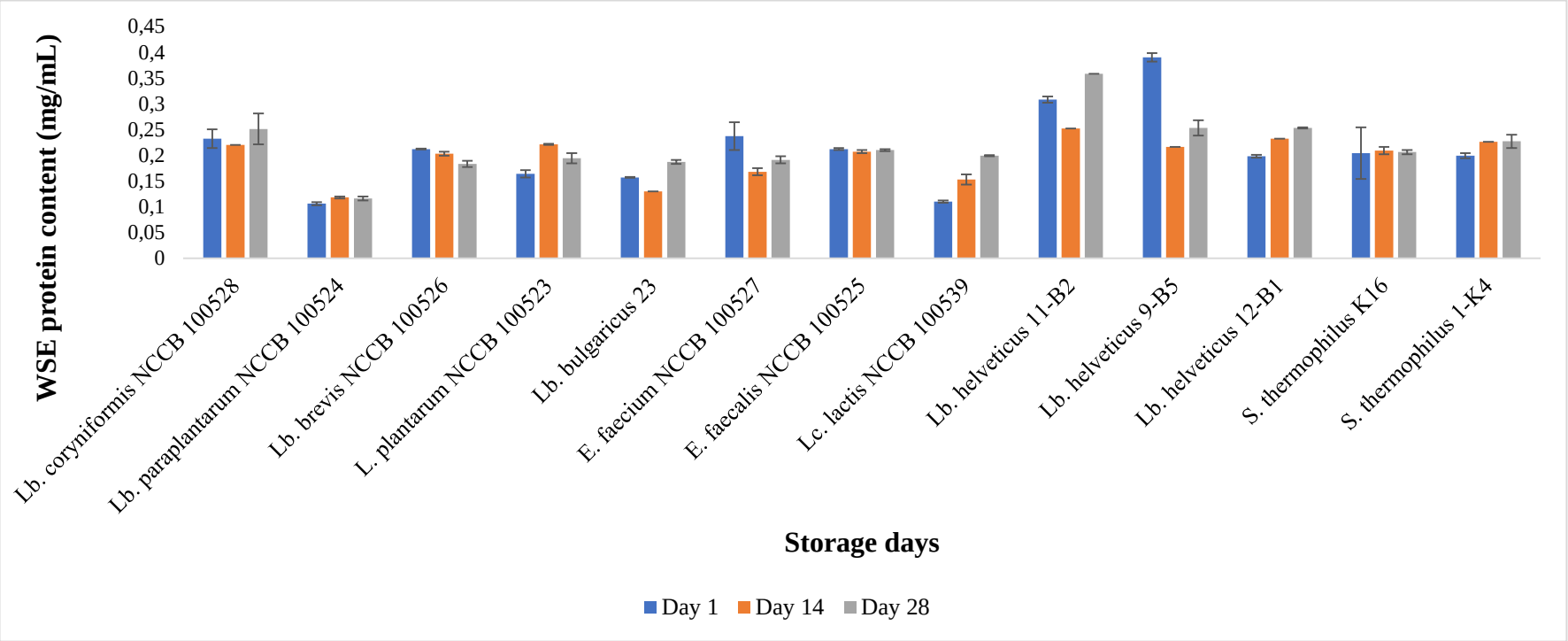


Figure 4.4 WSE protein quantity of the samples during storage days (n=2)

4.6 Peptide Profile of Fermented Milk Samples

Temperature, incubation time, pH, gastrointestinal digestion and enzyme preparation conditions prevailing during gastrointestinal digestion significantly influence the extent of proteolysis and bioactivity of proteins (Rafiq 2015, Abd El-Fattah et al. 2017). Moreover, the level of proteolysis greatly depends on bacterial strains (Penna et al. 2015). Therefore, peptides need to reach their target sites without any alteration or inactivation by digestive enzymes and/or pH conditions (Lopez-Fandino et al. 2006, Rafiq 2015). The peptide profiles for fermented milk samples were determined by RP-HPLC on day 1.,14., and 28 as shown in Fig. 4.5- 4.18.

The number of peaks that occur, peak heights and the regions where they occur (hydrophilic or hydrophobic region) can provide important information about proteolysis in fermented milk (Öztürk and Akin 2017, Altin et al. 2023). In reference to these RP-HPLC chromatograms, the eluted peptide peaks in the earlier and later retention times are a representation of hydrophilic and hydrophobic peptides, respectively. The hydrophobic peptides have a lower molecular weight. However, the degradation of macro peptides to smaller amino acids results in a decrease in peak heights and peak area in the hydrophobic zone (Hayaloğlu et al. 2004, Pavia et al 2000).

Differences were observed in our samples made with different bacteria in terms of peak numbers and peak heights in WSE during the storage time. Each peak signifies at least one peptide and the peak area formed corresponds to the quantity of the peptides. In the control sample made with pasteurized milk without any bacteria, a peak eluted at around 45-55 min of absorption time and there was no peak fragmentation since proteolysis did not take place.

Some qualitative differences were observed in RP-HPLC peptide chromatograms for WSE during the storage days. It was indicated that the peaks in fermented milk samples with *Lb. helveticus* 9-B5 (Fig. 4.15**)**, *Lb. helveticus* 11-B2 (Fig. 4.16) and *Lb. helveticus* 12-B1 (Fig. 4.17) samples were much smaller and less than in the other samples. This is thought to be due to the lower proteolysis activity in these samples. The most intense

peaks were observed in samples made with *E. faecium* NCCB 100527 (Fig. 4.9), *E. faecalis* NCCB 100525 (Fig. 4.10), *Lb. brevis* NCCB 100526 (Fig. 4.7), *Lb. bulgaricus* 23 (Fig. 4.11) and *Lb. paraplantarum* NCCB 100524 (Fig. 4.12). These peaks have shown that the cultures utilized in these fermented milk samples probably have higher proteolytic activity. As seen in the chromatograms, samples made with *Lc. lactis* NCCB 100539 (Fig. 4.5), *Lb. coryniformis* NCCB 100528 (Fig. 4.6), *L. plantarum* NCCB 100523 (Fig. 4.8), *E. faecium* NCCB 100527 (Fig. 4.9), *Lb. paraplantarum* NCCB 100524 (Fig. 4.12, *S. thermophilus* K16 (Fig. 4.13), and *Lb. helveticus* 9-B5 (Fig. 4.15), the large peak that eluted in the hydrophilic region around the 45th minute tended to decrease later on. This may be due to the peptidase/protease enzyme activity of the bacterial strains (Michaelidou et al. 1998).

Some peptides showed an increasing trend until the 14th day and decreasing way later on. It has been reported that enzymes found in LAB lysates probably have an effect on the degradation of peptides (El Soda 1995, Urbach 1995). Variability in form of increase/decrease in the peak height during storage was detected in the peaks coming at approximately 67th and 75th minutes in the hydrophobic region in the fermented milk samples. It was also observed in the chromatograms that the numbers of peptides occurring in the hydrophilic region did not change much and their concentrations increased. Furthermore, there was an increase in the numbers and concentrations of those peaks formed in the middle of hydrophobic region. However, no significant peak increase was detected in the hydrophobic region for the samples with *S. thermophilus* 1-K4 (Fig. 4.14), *Lb. helveticus* 9-B5 (Fig. 4.15), *Lb. helveticus* 11-B2 (Fig. 4.16**)** and *Lb. helveticus* 12-B1 (Fig. 4.17). In the fermented milk samples with *Lb. bulgaricus* 23 (Fig. 4.11), *Lb. paraplantarum* NCCB 100524 (Fig. 4.12) and *S. thermophilus* K16 (Fig. 4.13), the peaks in the hydrophobic region are fluctuating. This fluctuation could again be attributed to enzymatic activity.

In general, fermented milk sample made with *Lb. coryniformis* (Fig. 4.6) produced the longest peptide heights on day 28 followed by *Lc. lactis* (Fig. 4.5). Sample with *Lb. brevis* (Fig. 4.7) had the most numbers of peaks throughout the storage period. However, samples with *S. thermophilus* K16 (Fig. 4.13) produced the least peak heights although

the heights increased on the 28th day of storage time. On the 28th day, the sample with *L. plantarum* NCCB 100523 (Fig. 4.8) produced the least numbers of peaks and peak height followed by samples produced with *E. faecium* (Fig. 4.9) and *S. thermophilus* K16 (Fig. 4.13), respectively.

The quantity of water-soluble peptides of all the fermented milk samples on day 1 of storage period were on a great extent poor but increased with the progress of storage period. Diezhandino et al. (2015) evaluated a progressive rise in the peptide number throughout the storage period. The increase of peak numbers during the storage period was due to proteolysis and peptides formation. In regard to the number of storage days, fractions of large and inter-mediate size peptides obtained from caseins start to appear as a result of proteolytic enzymes such as plasmin, microbial enzymes, and remaining coagulant activity involved. Consequently, these components are turned into shorter chain peptides and amino acids by microbial enzymatic activity (Öztürk and Akin 2017).

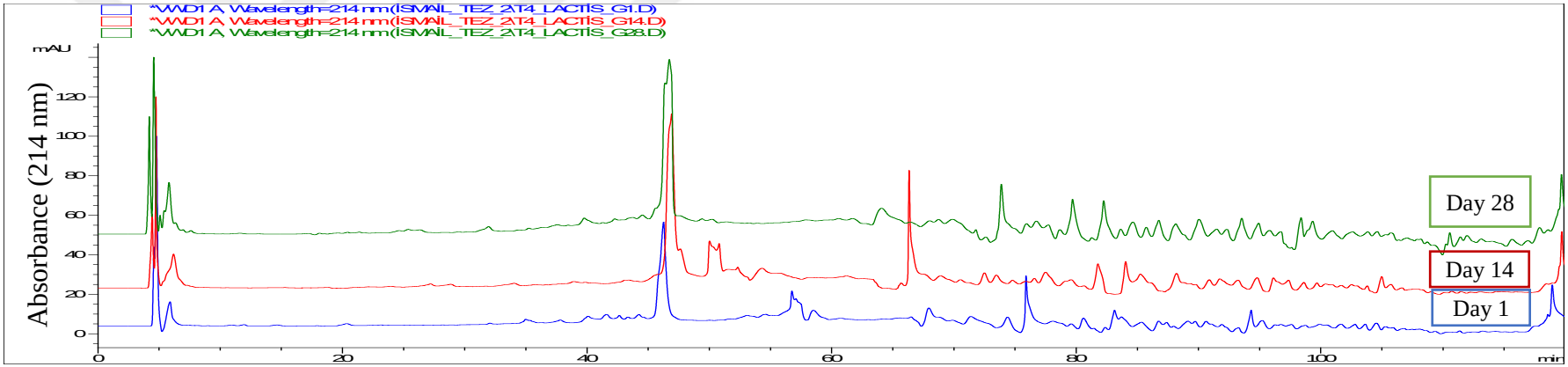


Figure 4.5 *Lc. lactis* NCCB 100539 peptide profile during storage days

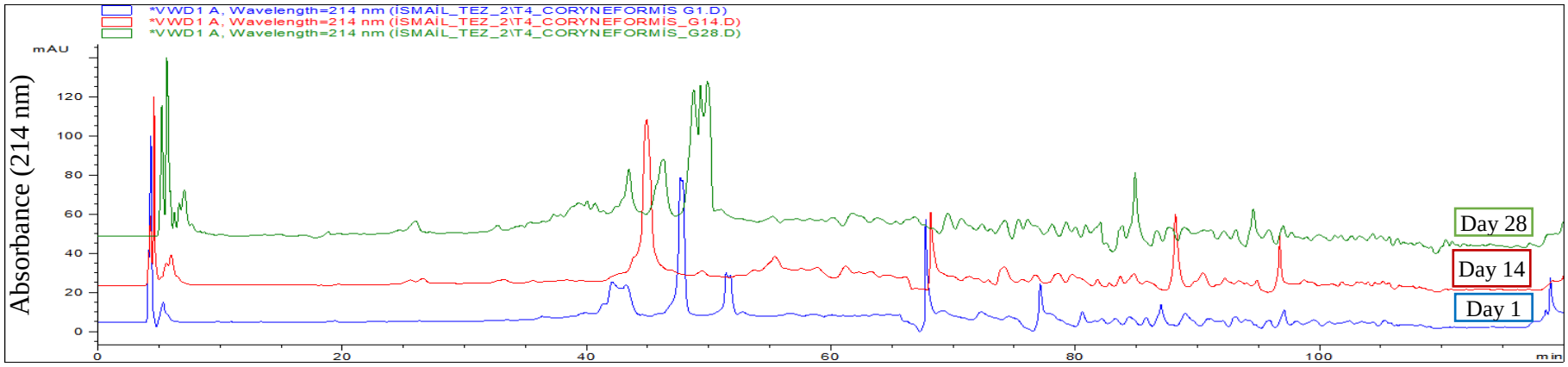


Figure 4.6 *Lb. coryniformis* NCCB 100528 peptide profile during storage days

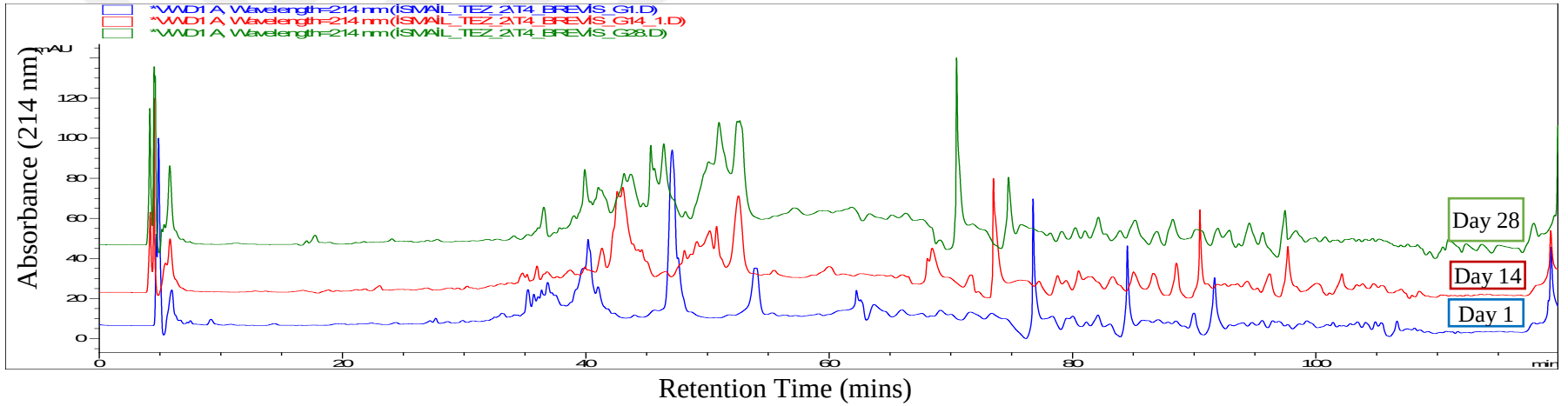


Figure 4.7 *Lb. brevis* NCCB 100526 peptide profile during storage days

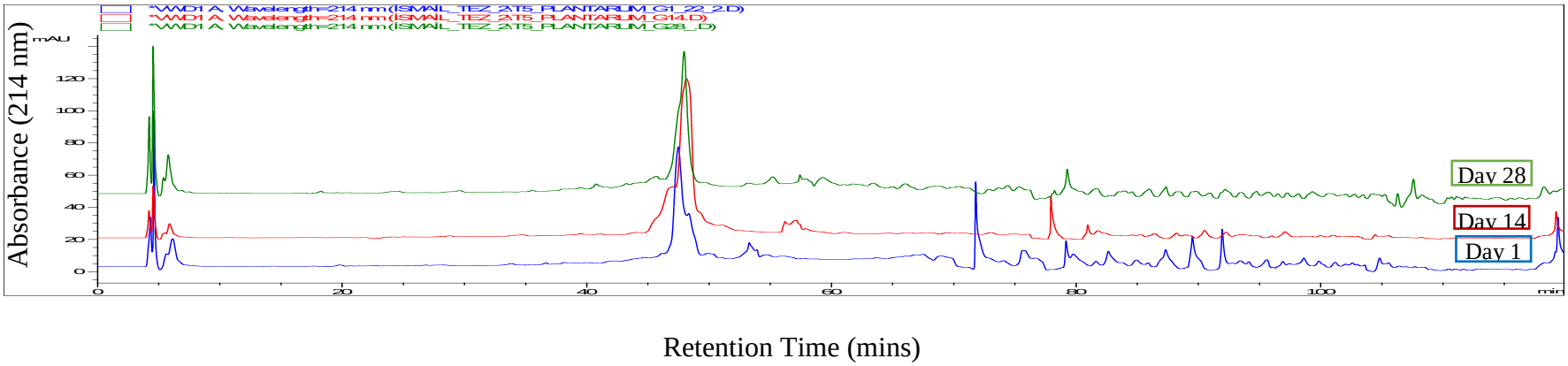


Figure 4.8 *L. plantarum* NCCB 100523 peptide profile during storage days

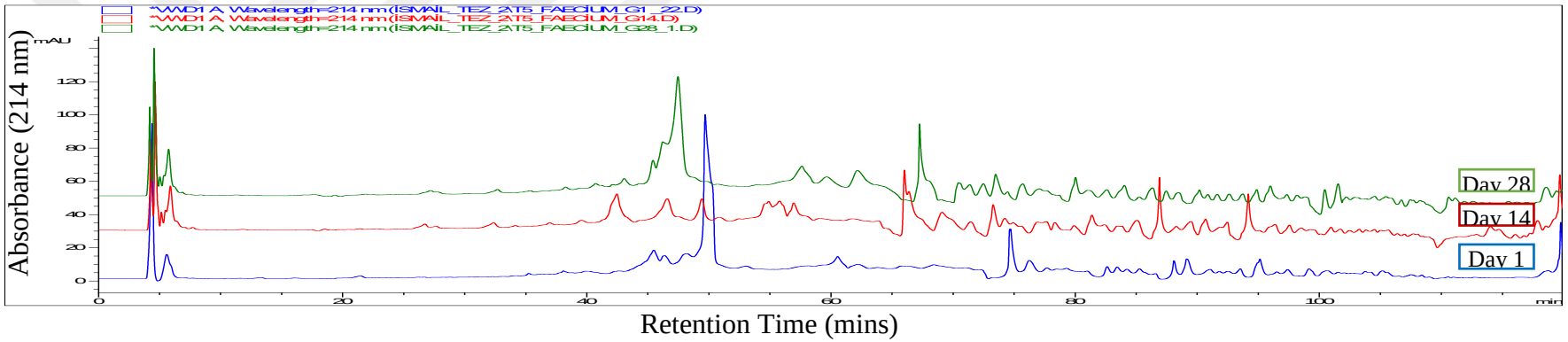


Figure 4.9 *E. faecium* NCCB 100527 peptide profile during storage days

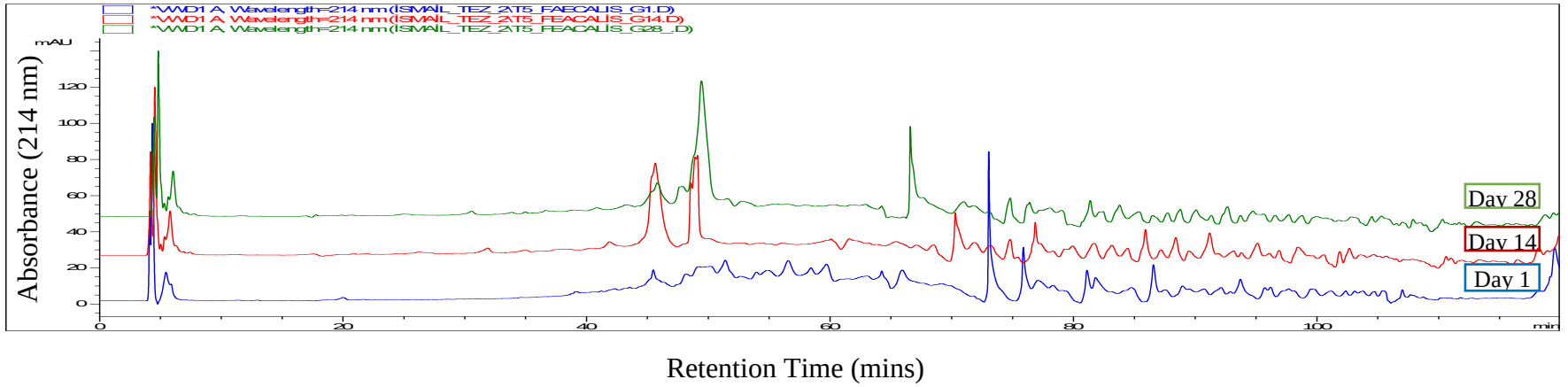


Figure 4.10 *E. faecalis* NCCB 100525 peptide profile during storage days

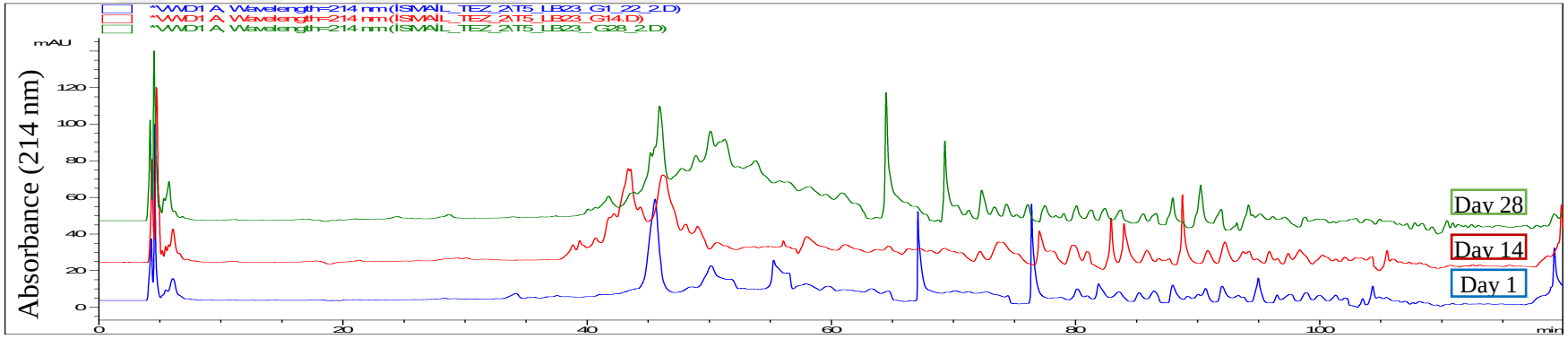


Figure 4.11 *Lb. bulgaricus* 23 peptide profile during storage days

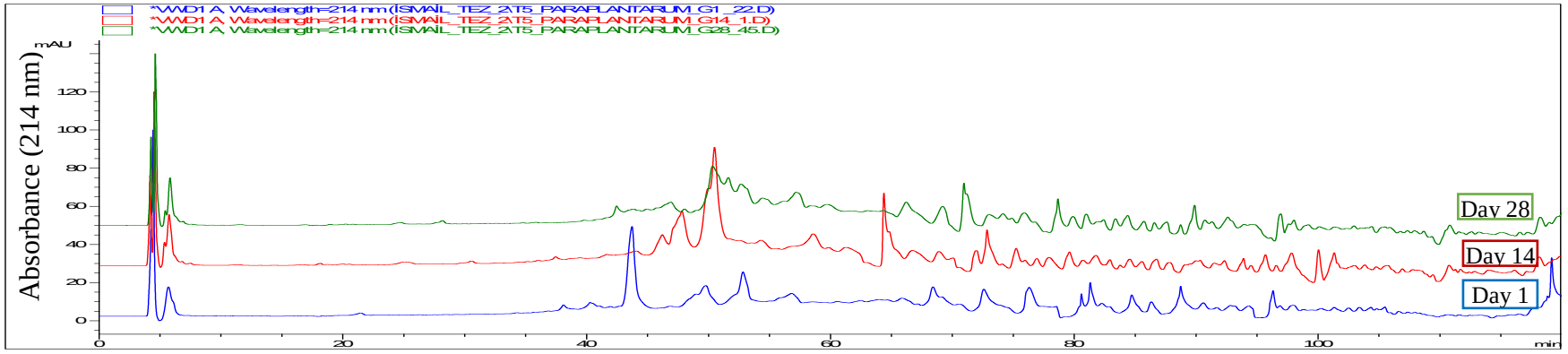


Figure 4.12 *Lb. paraplantarum* NCCB 100524 peptide profile during storage days

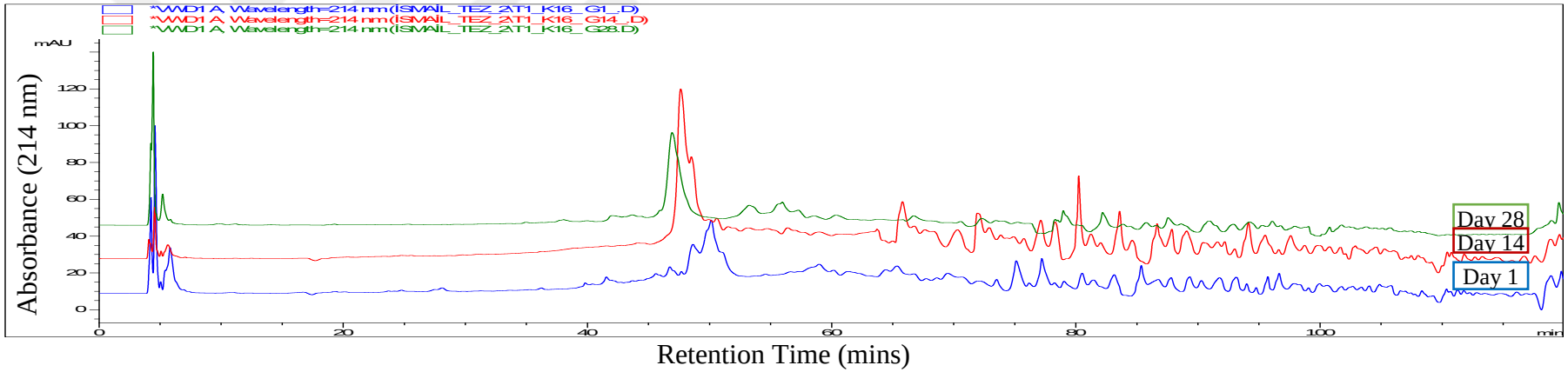


Figure 4.13 *S. thermophilus* K16 peptide profile during storage days

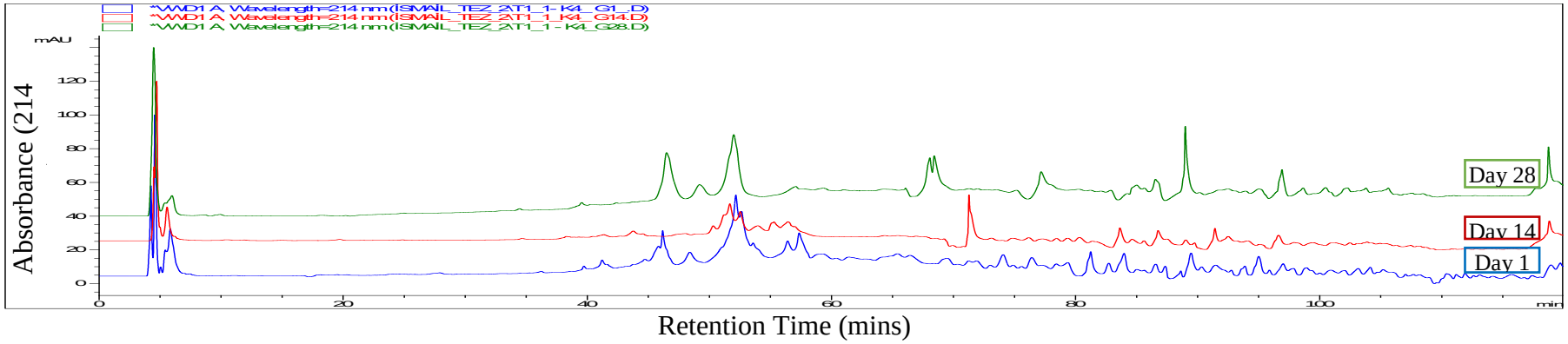


Figure 4.14 *S. thermophilus* 1-K4 peptide profile during storage days

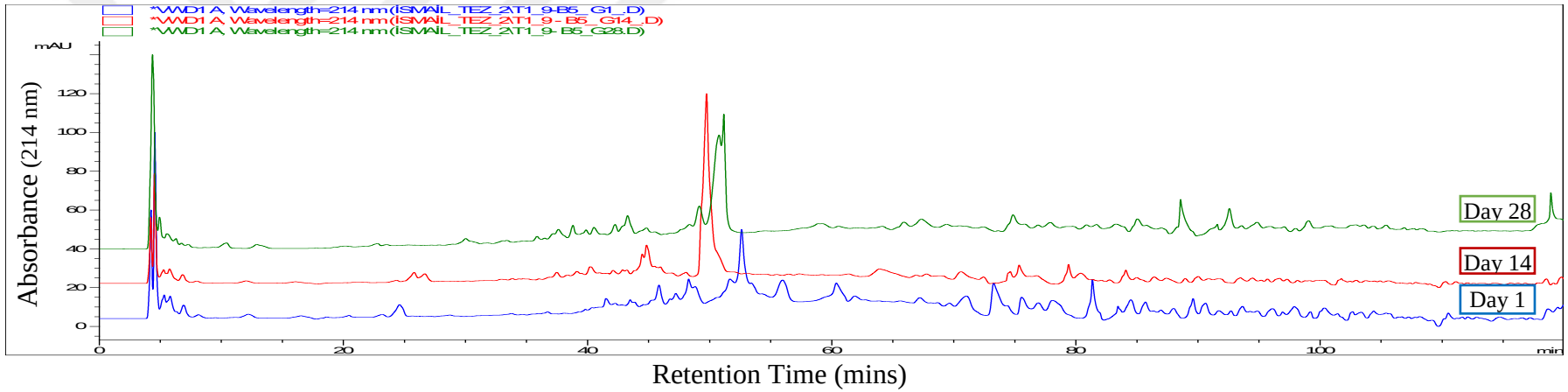


Figure 4.15 *Lb. helveticus* 9-B5 peptide profile during storage days

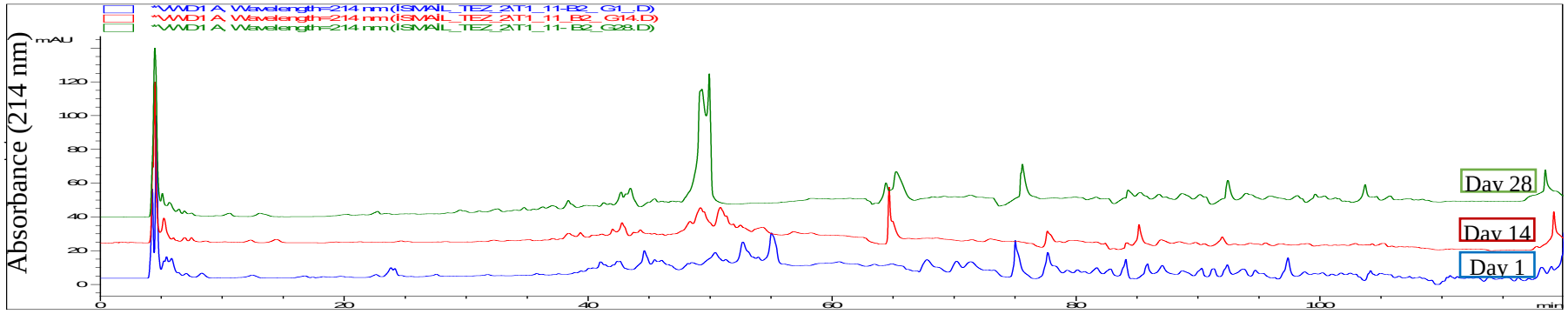
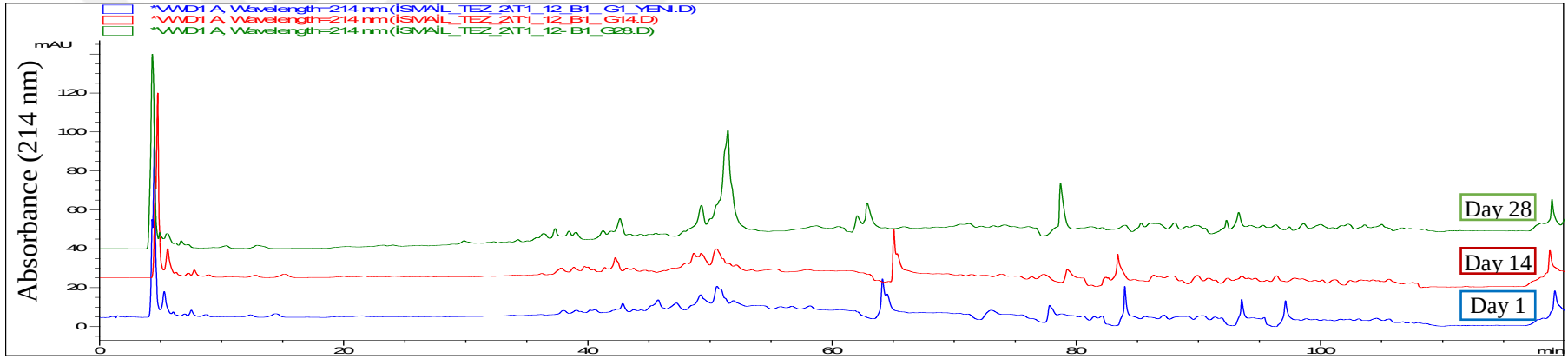
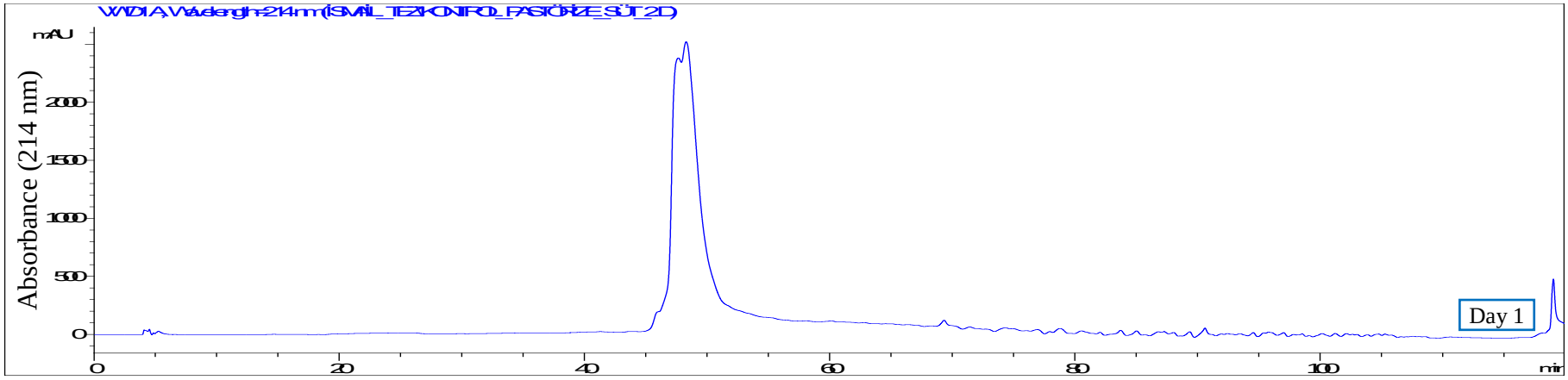


Figure 4.16 *Lb. helveticus* 11-B2 peptide profile during storage days



Retention Time (mins)

Figure 4.17 *Lb. helveticus* 12-B1 peptide profile during storage days



Retention Time (mins)

Figure 4.18 Pasteurized milk (control) peptide profile during storage day

4.7 Antioxidant Activity (DPPH) of the Fermented Milk Samples

There has been an emerging interest focused on the potential role of milk products as fundamental origins of antioxidants depending on bioactive compounds it contains (Li et al. 2023). The production of peptides with antioxidant activity by enzymatic breakdown or microbial fermentation of proteins has been widely reported in many studies in the recent decade (Tadesse et al. 2020, Cruz-Casas et al. 2021). These protein hydrolysates that carry antioxidant peptides which can preserve human body by getting hold of free radicals like reactive oxygen species. Antioxidant activity of BP in fermented milk products have been reported to prevent enzymatic and non-enzymatic peroxidation of fatty acids (Pihlanto and Korhonen 2014, Aspri et al. 2018). Amino acid composition of peptides along with their structure determines their antioxidative potentials. For instance, low molecular weight peptides tend to have strong antioxidant activity (Wali et al. 2020).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was analyzed to determine antioxidant activity of fermented milk samples made with different LAB strains. The mean antioxidant activity (μM TEAC) values for the fermented milk samples produced with different bacterial strains during storage days are illustrated in Table 4.6**.** The results in this present study show that there were significant differences among the fermented milk samples produced with different bacterial strains and among the storage days ($P \leq 0.05$).

The DPPH radical scavenging activity of samples ranged between 118-1935 μM TEAC which generally showed a decreasing value in almost all the samples. Samples produced with *S. thermophilus* K16 exhibited the highest whereas samples made with *Lb. brevis* NCCB 100526 registered the least antioxidant activity among the samples during the storage days. The high antioxidant activity could be attributed to the peptide formation due to proteolysis, extracellular metabolites and products of cell disintegration (Osuntiki and Korie 2010, Abubakr et al. 2012, Shori 2020, Dharmisthaben et al. 2021, Rosa et al. 2023). In addition, a high antioxidant activity in fermented milk might again be ascribed from pressure stimulated alterations in the casein-protein micelles matrix, which makes proteins more vulnerable to enzymes action (Guimaraes et al. 2019).

Table 4.6 Antioxidant activity (TEAC, μM) values of the samples during storage days (n=2)

Samples	Storage days		
	1	**14**	**28**
Lb. coryniformis NCCB 100528	621.0 ± 61.5^{deA}	272.0 ± 0.6^{gC}	478.5 ± 13.8^{eB}
Lb. paraplantarum NCCB 100524	262.0 ± 58.7^{gB}	384.3 ± 36.6^{fA}	207.3 ± 13.2^{gB}
Lb. brevis NCCB 100526	263.6 ± 61.7^{gA}	156.3 ± 4.5^{hB}	152.3 ± 33.5^{gB}
L. plantarum NCCB 100523	465.3 ± 26.8^{fA}	353.6 ± 5.1^{fB}	375.4 ± 8.3^{fB}
Lb. bulgaricus 23	599.0 ± 45.2^{deA}	507.9 ± 0.5^{eB}	495.3 ± 37.8^{deB}
E. faecium NCCB 100527	1087.1 ± 33.5^{bA}	1014.1 ± 22.3^{bB}	936.2 ± 5.7^{bC}
E. faecalis NCCB 100525	733.1 ± 12.9^{cA}	649.7 ± 16.3^{dB}	486.5 ± 29.5^{deC}
Lc. lactis NCCB 100539	810.8 ± 81.7^{cA}	830.3 ± 33.7^{cA}	749.9 ± 44.4^{cA}
Lb. helveticus 11-B2	687.7 ± 17.3^{cdC}	816.6 ± 0.03^{cB}	880.7 ± 12.0^{bA}
Lb. helveticus 9-B5	649.4 ± 31.5^{deA}	615.4 ± 4.0^{dAB}	563.2 ± 27.2^{dB}
Lb. helveticus 12-B1	536.7 ± 28.9^{efA}	394.5 ± 4.8^{fB}	489.9 ± 46.7^{deA}
S. thermophilus K16	1935.9 ± 52.7^{aA}	1882.8 ± 0.7^{aA}	1910.7 ± 18.9^{aA}
S. thermophilus 1-K4	626.2 ± 1.2^{deA}	511.6 ± 0.5^{eB}	436.1 ± 8.7^{efC}

Significant differences were determined with the Tukey test ($P \leq 0.05$).

The means denoted by different lowercase letters in the same column represent differences between samples while uppercase letters in the same row indicates differences between storage days

Apart from the samples made with *Lb. paraplantarum* NCCB 100524 and *Lb. helveticus* 11-B2, all the remaining samples presented similar antioxidant activity on day 1. On the 14th day of storage, samples made with *Lb. paraplantarum* NCCB 100524, *L. plantarum* NCCB 100523, and *Lb. helveticus* 12-B1 presented a similar antioxidant activity. Samples with *Lb. bulgaricus* 23 and *S. thermophilus* 1-K4 also were determined with similar antioxidant activity. In the same manner, the samples including *E. faecalis* NCCB 100525 and *Lb. helveticus* 9-B5 also present similar antioxidant activity. However, the sample made with *Lb. coryniformis* NCCB 100528 presented in the lowest antioxidant activity on the storage day 14.

On the 28th day of storage, fermented milk samples with *Lb. paraplantarum* NCCB 100524 and *Lb. brevis* NCCB 100526 produced similar antioxidant activity. The samples made with *Lb. bulgaricus* 23, *E. faecalis* NCCB 100525 *Lb. helveticus* 9-B5, *Lb. coryniformis* NCCB 100528 and *Lb. helveticus* 12-B1 presented similar antioxidant activity. A similar situation was observed with sample produced with *E. faecium* NCCB 100527 and *Lb. helveticus* 11-B2 which also showed similar anti-oxidation activities.

It was observed that samples produced with *Lc. lactis* NCCB 100539 and *S. thermophilus* K16 showed similar antioxidant activity throughout the storage periods. In the same way, samples with *Lb. brevis* NCCB 100526, *L. plantarum* NCCB 100523, *Lb. bulgaricus* 23, *Lb. helveticus* 9-B5 and samples with *E. faecium* NCCB 100527, *E. faecalis* NCCB 100525 and *S. thermophilus* 1-K4 produced similar antioxidant activity throughout the storage days. However, samples with *Lb. paraplantarum* NCCB 100524 showed a similar antioxidant values on day 1 and day 28 different from day 14th day of storage.

Fermented milk samples with *Lb. brevis* NCCB 100526, *Lb. bulgaricus* 23, *E. faecium* NCCB 100527, *E. faecalis* NCCB 100525, *Lb. helveticus* 9-B5, and *S. thermophilus* 1-K4 showed a steady decrease in antioxidant activity throughout the storage days. However, all the other samples showed fluctuations during the storage period (Fig. 4.19). Moreover, a decrease in the antioxidant activity during storage can be justified by starter cultures used which may utilize some peptides for their growth (Chai et al. 2020). In a previous study, an increasing pattern of antioxidant activity throughout the storage days

was observed by Hernández-Falcón et al. (2018) in thermo-sonicated milk samples. However, a study conducted by Yousefi et al. (2023) illustrated that antioxidant did not deliberately increase with the increase of proteolysis.

In our study, the samples made with *S. thermophilus* strains and *Lb. bulgaricus* 23 produced somewhat higher antioxidant activity than the results got by Yadav et al. (2018), except for samples with *Lb. brevis* NCCB 100526 and *Lb. paraplantarum* NCCB 100524 in our study which presented low antioxidant activities. A higher radical antioxidant activity in our sample with *E. faecalis* NCCB 100525 was more than that found by (Graham et al. 2019). We found a higher antioxidant activity in the sample with *Lc. lactis* NCCB 100539 than the data obtained from Chingshey purple sweet potato milk fermented with *Lb. delbrueckii* subsp. *lactis* (Wu et al. 2012). Our samples produced with *Lb. helveticus* strains, *Lb. brevis* NCCB 100526 and *L. plantarum* NCCB 100523 produced higher antioxidant activity compared to the results of Abd El-Fattah et al. (2017) regarding sterilized skim milk. El-Sayed et al. (2021) found higher amount of antioxidant activity in fermented camel milk samples produced with *Lb. helveticus* both on day1 and 14 of storage period compared to our data.

Chen et al. (2015) obtained higher antioxidant activity in fermented goat milk with *Lb. bulgaricus* and *L. plantarum* isolates than in our samples with similar species. We produced a higher antioxidant activity in our samples produced with *Lc. lactis* NCCB 100539 than the results obtained by Yerlikaya (2019) in cow and goat milk fermented with kefir grains. Tomovska et al. (2016) obtained higher antioxidant property in fermented milk on day 1 and 14 in which they used combined culture of *S. thermophilus* and *Lb. bulgaricus* strains. Fermented milk samples made with *S. thermophilus* strains and *L. plantarum* NCCB 100523 in this study presented higher antioxidant activity than fermented goat milk produced with single strain of *S. thermophilus* and combined strains of *S. thermophilus* and *L. plantarum* (Moreno-Montoro et al. 2017). Likewise, Singh et al. (2020) determined higher antioxidant activity in soy milk fermented with *L. plantarum* than in our fermented milk samples with *L. plantarum* NCCB 100523. Milk protein preparation fermented by *Lb. helveticus* strains produced higher antioxidant activity than all the samples with *Lb. helveticus* strains in our study (Skrzypczak et al. 2017). The

antioxidant activity of fermented milk sample made with *Lb. helveticus* 11-B2 in our study is in agreement with the activity of fermented buffalo milk with *Lb. helveticus* strain (Tak et al. 2018). Liu et al. (2018) determined higher antioxidant activity in tomato juice fermented by *L. plantarum* than our fermented milk with *L. plantarum* NCCB 100523 at the beginning of storage. We obtained lower antioxidant activity in samples produced with *L. plantarum* than the results of Kantachote et al. (2017) who found higher antioxidant activity in coconut water fermented with *L. plantarum.* The higher antioxidant activity was thought to be caused by phenolic compound (Fu et al. 2011). In yoghurt produced with *L. plantarum*, Zhao and Liang (2022) found high antioxidant activity compared to our samples. Rahnama Vosough et al. (2021) obtained higher antioxidant activity in traditional Iranian Kishk (milk by-products) made with *E. faecium* strains compared to our study.

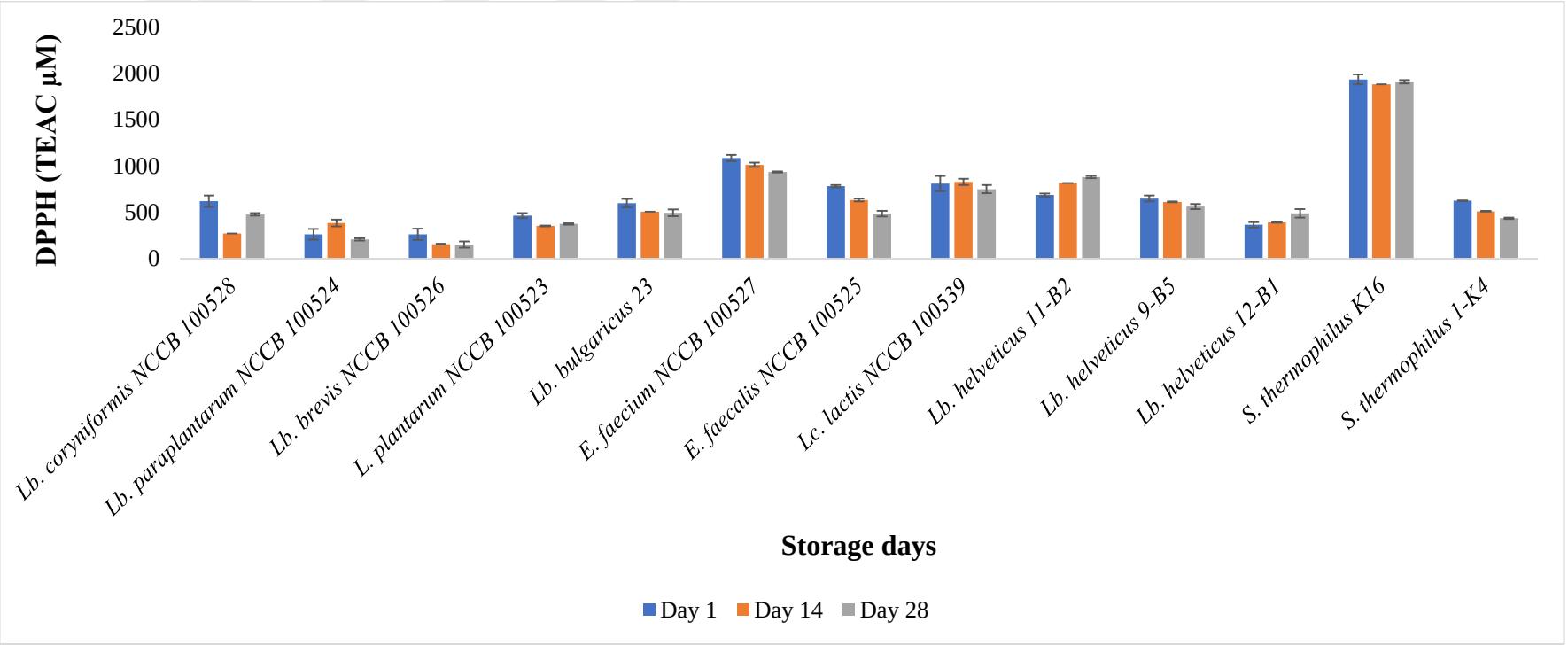


Figure 4.19 DPPH (µM TEAC) values of the samples during storage days (n=2)

4.8 Phenolic Content in Fermented Milk Samples

Similar with antioxidants, phenolic compounds have a preventive or regulatory role in the formation of free radicals with deleterious health effects and hence paramount in chronic diseases risk reduction (Graham et al. 2019, Munir et al. 2020). The phenols and flavonoids in fermented milk are indicators of antioxidant activity (Munir et al. 2020). The fact that phenolic presence in milk has rarely been investigated, some studies have reported that milk is a good source of phenolic compounds (Kuhnen et al. 2014, Oh et al. 2016). Total phenolic content of fermented milk samples produced from different bacteria was determined by Folin-Ciocalteu method. Total phenolic content (GAE/g) values for the fermented milk made from different bacteria strains during storage days are illustrated in Table 4.7. The results in this study show that there were significant differences of phenolic content among the fermented milk samples made with different LAB strains and among the storage days ($P \leq 0.05$).

The phenolic content in our fermented milk samples by different bacterial strain ranged from 10.68-35.61 mg GAE/g. Samples with *Lb. brevis* NCCB 100526 produced the most dominant phenolic content while samples made with *Lb. paraplantarum* NCCB 100524 produced the least. The high phenolic content in samples with *Lb. brevis* is thought to be as a result of a great tolerance to phenolic compounds compared to other LAB (Guo et al. 2010, Winkler and Kao 2011). A previous study by Hassan et al. (2014) suggested that phenolic compounds are made stable via hydrophobic bonding with the proline in casein and can be distorted during high-pressure treatment. Similarly, it is reported that sonication could hydrolyze hydrophobic interlinkages between phenols and proteins leading to the release and solubilization of phenols (Tsai et al. 2018, Munir et al. 2020).

Table 4.7 Phenolic content (mg GAE/g) values of the samples during storage days (n=2)

Samples	Storage days		
	1	**14**	**28**
Lb. coryniformis NCCB 100528	12.47 ± 0.05^{fgB}	13.52 ± 0.00^{hA}	12.63 ± 0.25^{fB}
Lb. paraplantarum NCCB 100524	10.68 ± 0.15^{hC}	12.85 ± 0.02^{jA}	12.02 ± 0.15^{fB}
Lb. brevis NCCB 100526	30.97 ± 0.13^{aB}	34.86 ± 0.12^{aA}	35.61 ± 2.43^{aA}
L. plantarum NCCB 100523	16.97 ± 0.45^{eB}	17.56 ± 0.00^{fB}	20.79 ± 0.07^{bcA}
Lb. bulgaricus 23	18.69 ± 0.27^{dA}	13.52 ± 0.00^{hB}	19.52 ± 0.55^{cdA}
E. faecium NCCB 100527	15.77 ± 0.15^{eA}	13.08 ± 0.30^{ijC}	14.57 ± 0.45^{efB}
E. faecalis NCCB 100525	11.76 ± 0.46^{ghB}	13.37 ± 0.15^{hiB}	19.74 ± 1.02^{cdA}
Lc. lactis NCCB 100539	13.67 ± 0.60^{fC}	16.44 ± 0.08^{gB}	23.48 ± 1.12^{bA}
Lb. helveticus 11-B2	20.26 ± 0.64^{cB}	21.16 ± 0.03^{cB}	23.40 ± 0.14^{bA}
Lb. helveticus 9-B5	21.16 ± 0.74^{bcA}	19.96 ± 0.01^{dAB}	18.62 ± 0.90^{cdB}
Lb. helveticus 12-B1	15.92 ± 0.30^{eC}	18.62 ± 0.02^{eA}	17.04 ± 0.22^{deB}
S. thermophilus K16	22.58 ± 1.12^{bB}	31.50 ± 0.05^{bA}	19.36 ± 1.24^{cdC}
S. thermophilus 1-K4	12.70 ± 0.08^{fgA}	13.08 ± 0.04^{ijA}	12.40 ± 0.52^{fA}

Significant differences were determined with the Tukey test ($P \leq 0.05$).

The means denoted by different lowercase letters in the same column represent differences between samples while uppercase letters in the same row indicates differences between storage days.

Among all samples, fermented milk sample with *S. thermophilus* 1-K4 had similar phenolic content throughout the storage days. On the 28th day of storage, the samples presented Different phenolic contents. The difference in the phenolic content levels observed among the fermented milk samples made with different LAB during the storage period could be due to the different capability of strains in formation of polymeric phenolics during fermentation (McCue and Shetty 2005).

The fermented milk samples with *Lb. brevis* NCCB 100526, *L. plantarum* NCCB 100523, *E. faecalis* NCCB 100525, *Lc. lactis* NCCB 100539 and *Lb. helveticus* 11-B2 exhibited a steady increase in phenolic content with increased in storage **(**Fig. 4.20**)**. As expected, the increase in phenolic activity during storage is not only due to enzymatic hydrolysis of amino acids but also some chemical responses which could cause the release of phenolic groups. Flavonoids and phenolic compounds fundamentally have hydrophobic and or Vander Waals associations with proteins in milk but this interaction can be suppressed by proteolytic activity. The higher values of phenols and flavonoids in the WSE could be ascribed to the breakdown of relations showed by the low molecular weight components with caseins (Hassan et al. 2013, Munir et al. 2020). However, samples with *Lb. helveticus* 9-B5 showed a gradual drop in phenolic content throughout the storage days. Moreover, other samples presented a fluctuation of phenolic content during the storage days. For example, samples with *Lb. coryniformis* NCCB 100528, *Lb. helveticus* 12-B1, *S. thermophilus* K16, *S. thermophilus* 1-K4 had a sharp increase on the 14th day of storage but later decreased on 28th day.

Fermented milk samples made with *S. thermophilus* and *Lb. bulgaricus* strains produced higher total phenolic content more than the results obtained by Yadav et al. (2018) which had (5.8 mg GAE/g) as the highest for the above similar strains used in the fermented yoghurt encapsulated with grape seed extract. The phenolic activity results for fermented milk samples with *L. plantarum* NCCB 100523 on day 1 in our study were somewhat higher than that showed by Shori et al. (2022) who examined cashew milk-based yoghurt. However, their results were comparatively higher than ours at the end of the storage. In another study performed by Graham et al. (2019), total phenolic of milk fermented with *E. feacalis* was comparatively higher than the result we obtained in fermented milk

sample made with similar strain. Thakur et al. (2022) obtained an arguably higher phenolic content throughout the storage days for yoghurt samples with *Lb. bulgaricus* and *S. thermophilus* probably due to the enrichment of yoghurts with lyophilized microencapsulated phenolic powder from peels of wild pomegranate fruit. According to the study by Virtanen et al. (2007), fermented whey samples with *Lb. brevis*, *Lc. lactis* and *Lb. helveticus* strains produced less total phenolic content activities than the results we obtained in fermented milk samples with similar species. El-Sayed et al. (2021) produced higher phenolic content in fermented camel milk produced with *Lb. helveticus* on day 1, however, we obtained higher phenolic content on day 14.

An investigation by Wu et al. (2012) in Chingshey purple sweet potato fermentation milk with *L. delbrueckii subsp. lactis*, had higher total phenolic activity compared to the results we obtained in our fermented milk sample made with *Lc. lactis* NCCB 100539. The higher total phenolic content in their study could have been caused by the evolution of anthocyanins and glycosides of fermented purple sweet potato milk during the fermentation processes, which resulted in the formation of ester-type phenolic acids (Ju et al. 2009).

Samples produced by *L. plantarum* NCCB 100523 generated higher phenolic content than the results got by Michalak et al. (2020) in curly kale juice fermentation by *L. plantarum* strain on day 1. Total phenolic content obtained in the fermented Aloe vera juice with *E. faecium* and *Lc. lactis* on day 1 were lower than the phenolic content we obtained in samples made with similar species (Cuvas-Limón et al. 2022). Samples with *L. plantarum* NCCB 100523 produced lower phenolic content on day 1 than that of curly kale juice fermented by *L. plantarum* strain (Michalak et al. 2020).

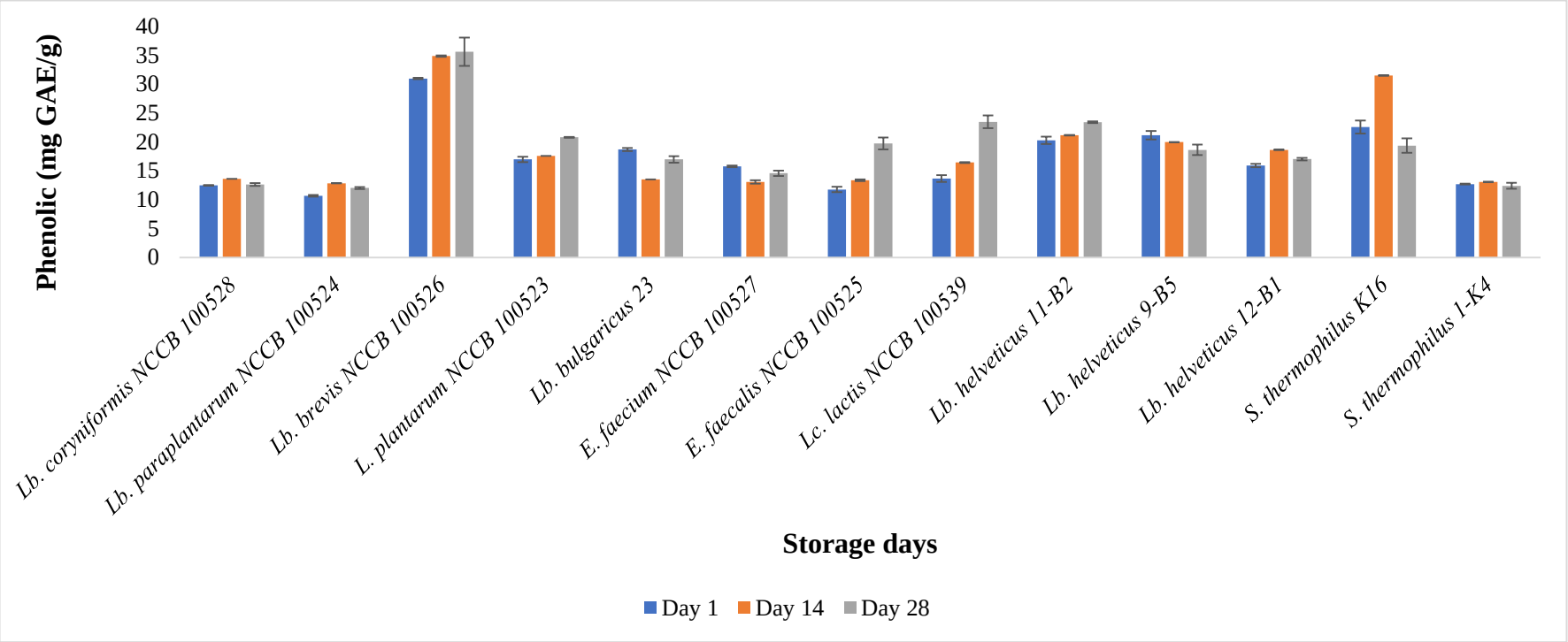


Figure 4.20 Phenolic content of the samples during the storage days (n=2)

4.9 Antibacterial Activity of the Fermented Milk Samples

Antibacterial peptides have become an alternative to the traditional antibiotics (Guo et al. 2023). A deliberate use of natural antibacterial components in food products have gained attention in the recent years due to minimal processed food demands by consumers. Fermented milk contains a number of metabolites and inhibitors, such as organic acid, antibacterial peptides and other metabolites which are derived from LAB (Teneva et al. 2017). This antibacterial property of LAB in fermented milk is partially formed by high amounts of lactoferrin and lysozyme compounds. Lysozyme shows resistance to degradation by gastrointestinal enzymes. The results from this current research shows that milk fermented by LAB showed different antibacterial properties. Furthermore, antibacterial peptides derived from milk proteins in particular present a great advantage of being manufactured from not only non-toxic but also cheaper starter culture sources. This, therefore, means that application of these peptides could serve as bio-preservatives in food and exert health functionality as food supplements in the food industrial sector (Aspri et al. 2018). Antibacterial activity for our fermented milk samples produced with different LAB strains are shown in Table 4.8.

In the present study, antibacterial activity of lyophilized WSE fractions (<3 kDa) of fermented milk produced with LAB strains were tested against the following pathogenic bacteria; *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella enterocolitica* (ATCC 14028), *Bacillus cereus* (ATCC 14579), *Mycobacterium tuberculosis* (ATCC 7644) and *Enterococcus faecalis* (ATCC 29212) by agar well diffusion method. Fermented milk samples produced with *Lb. paraplantarum* NCCB 100524 showed a broader spectrum inhibition against *Escherichia coli* (ATCC 25922) on day 28 of storage, followed by samples made with *Lb. brevis* on the same pathogen both on day 1 and 28. It was reported that kefir or fermented milk possessed antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* (Kivanc and Yapici 2018). Fermented milk samples with *L. plantarum* NCCB 100523 showed inhibition against *E. coli* (ATCC 25922) on day 1. A study in kefir fermented with *L. plantarum* also presented inhibition activities against *E. coli* Monteiro et al. (2019).

Antibacterial activity of *L. plantarum* could be due to its ability to produce antibacterial components with pathogenic inhibition properties (Monteiro et al. 2019).

It was also revealed from our study that the antibacterial property was higher in fermented milk samples made with *Lb. paraplantarum* NCCB 100524 against *Escherichia coli* (ATCC 25922) and it showed a slight inhibition with 1 mm inhibition zone for *Salmonella enterocolitica* (ATCC 14028) on the 28th day of storage. However, there was antibacterial inhibition for samples with *Lb. paraplantarum* NCCB 100524 regarding other pathogenic bacteria. Sample with *Lb. coryniformis* showed inhibition against *Bacillus cereus* (ATCC 14579) on the first day of storage only.

Fermented milk sample with *Lb. brevis* NCCB 100526 showed inhibition effect against *Escherichia coli* (ATCC 25922) on both day 1 and day 28 but showed no effect with other pathogenic bacteria throughout the storage days. The samples made with *L. plantarum* NCCB 100523 was able to inhibit *Bacillus cereus* (ATCC 14579) on the 28th day of storage but inhibition was not detected for the other pathogenic bacteria at the same storage time. The samples produced with *S. thermophilus* 1-K4 showed inhibition against *Mycobacterium tuberculosis* (ATCC 7644) both on day 1 and 28, however, it was detected as positive against *Bacillus cereus* (ATCC 14579), *Enterococcus faecalis* (ATCC 29212), *Salmonella enterocolitica* (ATCC 14028) at the beginning of the storage. Fermented milk samples with *Lb. helveticus* 12-B1 showed some inhibition for almost all the pathogenic bacteria except for *Staphylococcus aureus* (ATCC 25923). Samples produced with *Lb. bulgaricus* 23 was detected as inhibitor for *Bacillus cereus* (ATCC 14579), *Salmonella enterocolitica* (ATCC 14028), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 25923) at the end of storage. This inhibition is thought to be the result of releasing some inhibiting substances, such as bacteriocins (Anas et al. 2008).

The samples made with *E. faecium* NCCB 100527 was detected as inhibitor for all the bacteria except for *Bacillus cereus* (ATCC 14579). In addition, the sample with *Lb. helveticus* 11-B2 showed inhibitions against *Enterococcus faecalis* (ATCC 29212),

Staphylococcus aureus (ATCC 25923) and *Salmonella enterocolitica* (ATCC 14028) but did not show any inhibition against *Escherichia coli* (ATCC 25922). This shows that the Gram-positive bacteria, such as *S. aureus*, is sensitive to the inhibitory materials produced by the lactobacilli in comparison with the Gram-negative bacteria such as *Escherichia coli* (Anas et al. 2008). Fermented milk samples with *L. plantarum* NCCB 100523 showed inhibition activity against *Escherichia coli* (ATCC 25922) on the day 1 which is in agreement with the results of Kostelac et al. (2021). However, our sample showed a relatively weaker inhibition rate.

Hajikhani et al (2021) determined a higher inhibition of *E. faecalis* and *E. faecium* isolated from white cheese against *Bacillus cereus, Salmonella enterocolitica* and *Staphylococcus aureus* than our samples with similar species. Fermented camel milk with *L. plantarum* demonstrated a higher antimicrobial inhibition against *Escherichia coli* and *Staphylococcus aureus* than our samples with similar species. (Algboory and Muhialdin 2021). Mufandaedza et al. (2006) reported a higher inhibition against *Escherichia coli* and *Salmonella enteritidis* strains in traditional fermented milk with *Lc. lactis* as compared to our study. Whereas our samples made with *Lb. bulgaricus* 23, *Lb. helveticus* 11-B2 and *Lb. brevis NCCB 100526* demonstrated similar inhibition effect against *Escherichia coli* in agreement with Eid et al. (2016) for the similar bacterial strains isolated from raw milk, they determined a higher inhibition effect against *Staphylococcus aureus* than in our samples made with similar strains. Fermented milk sample made with *Lb. paraplantarum* NCCB 100524 presented a higher inhibition against *Escherichia coli* than in kimchi (Korean traditional food) (Kim et al. 2009). Their study also had a positive inhibition against *Staphylococcus aureus* than our samples. Michalak et al. (2020) detected more inhibition against *Staphylococcus aureus, Escherichia coli* and *Salmonella enterocolitica* in fermented curly kale juice with *Lb. paraplantarum* NCCB 100524 and *L. plantarum* NCCB 100523 than our samples with similar bacterial strains.

Table 4.8 Antibacterial activity of the samples during storage days (n=2)

Bacteria sample	**Storage days**	**Diameter of the growth inhibition zones in millimeters (mm)**					
		E. coli (ATCC 25922)	***M. tuberculosis*** (ATCC 7644)	***B. cereus*** (ATCC 14579)	***E. faecalis*** (ATCC 29212)	***S. aureus*** (ATCC 25923)	***S. enterocolitica*** (ATCC 14028)
Lb. coryniformis NCCB 100528	1	-	-	+	-	-	-
	14	-	-	-	-	-	-
	28	-	+	-	-	-	-
Lb. paraplantarum NCCB 100524	1	-	-	-	-	-	-
	14	-	-	-	-	-	-
	28	+++	-	-	-	-	+
Lb. brevis NCCB 100526	1	++	-	-	-	-	-
	14	-	-	-	-	-	-
	28	++	-	-	-	-	-
L. plantarum NCCB 100523	1	+	++	-	-	-	-
	14	-	-	-	-	-	-
	28	-	-	++	-	-	-
Lb. bulgaricus 23	1	-	-	-	-	-	+
	14	-	-	-	-	-	-
	28			+	+	+	+
E. faecium NCCB 100527	1	-	-	-	+	+	-
	14	-	-	-	-	+	-
	28	+	+	-	-	+	+
E. faecalis NCCB 100525	1	-	-	-	-	+	+
	14	-	-	-	-	-	+
	28	-	-	-	+	-	+
Lc. lactis NCCB 100539	1	-	-	-	-	-	-
	14	-	-	+	-	-	+
	28	-	-	-	+	-	+
Lb. helveticus 11-B2	1	-	-	-	+	+	+
	14	-	-	-	-	-	+
	28	-	-	-	+	+	-
Lb. helveticus 9-B5	1	-	-	-	-	-	-
	14	-	-	+	-	-	-
	28	+	-	-	-	-	-
Lb. helveticus 12-B1	1	+	-	-	+	-	+
	14	-	-	+	-	-	-
	28	+	+	+	+	-	+
S. thermophilus K16	1	-	-	-	-	-	-
	14	-	-	-	-	-	+
	28	+	-	-	-	-	-
S. thermophilus 1-K4	1	-	+	+	+	-	+
	14	-	-	-	+	-	-
	28	-	++	-	+	-	-
Control	1	-	-	-	-	-	-

(+) inhibition positive; (+) inhibition diameter 1-2 mm; (++) inhibition diameter 3-6mm; (+++) inhibition diameter 7-10 mm; (-) Inhibition negative.

4.10 Antihypertensive Activity of Fermented Milk Samples

The percentage ACE inhibition activity from fermented milk produced from different LAB strains during storage days are illustrated in table 4.9. The results demonstrate that there were significant differences in ACE inhibitory activities among samples produced with different LAB strains and among the storage days ($P \leq 0.05$). ACE inhibition activities for the fermented milk samples made with different LAB in this study ranged between 40.48-117.81%. It was reported that ACE inhibition property among the different kinds of fermented milk samples depend largely on bacterial strain (Ashokbhai et al. 2022). The observed variation in ACE inhibitory activity could also be ascribed to the differences in the kinds and quantity/amount of the generated peptides (Tagliazucchi et al. 2018). Fermented milk samples with *E. faecalis* NCCB 100525 presented the lowest ACE inhibitory activity. However, the samples produced with *Lb. paraplantarum* NCCB 100524 expressed the highest ACE-inhibition activity at the end of the storage. The high ACE-Inhibition activity might be caused by forming ACE-inhibitory peptides at the time of fermentation and storage, which shows that the enzymes in their proteolytic structure possess high specification in ACE-inhibition peptide production (Gonzalez-Gonzalez et al. 2011).

Table 4.9 ACE inhibitory activity (%) of samples during storage days (n=2)

Samples	Storage days	
	1	**28**
Lb. coryniformis NCCB 100528	76.75±0.64^{de*}	60.37±3.15^{e}
Lb. paraplantarum NCCB 100524	85.62±0.17^{c}	117.81±1.31^{a*}
Lb. brevis NCCB 100526	64.93±2.25^{fg}	80.99±7.63^{cd}
L. plantarum NCCB 100523	99.71±4.39^{b}	93.05±0.24^{b}
Lb. bulgaricus 23	59.65±4.07^{gh}	72.50±2.08^{d*}
E. faecium NCCB 100527	81.08±1.61^{cd}	81.88±4.35^{c}
E. faecalis NCCB 100525	49.92±3.59^{i}	89.49±2.39^{bc*}
Lc. lactis NCCB 100539	56.52±3.45^{hi}	81.37±3.51^{cd*}
Lb. helveticus 11-B2	85.31±1.31^{c}	93.19±2.30^{b*}
Lb. helveticus 9-B5	53.91±3.02^{hi}	56.34±0.75^{e}
Lb. helveticus 12-B1	71.64±0.56^{ef*}	40.48±0.50^{f}
S. thermophilus K16	115.80±0.72^{a*}	97.51±0.50^{b}
S. thermophilus 1-K4	55.69±0.60^{hi*}	43.16±1.00^{f}

Significant differences were determined with the Tukey test ($P \leq 0.05$).
The means denoted by different lowercase letters in the same column represent differences between samples.
Stars represent statistically significant differences between storage days according to the two-sample independent t-test ($P \leq 0.05$)

The studies by Cavalheiro et al. (2020) and Kimet et al. (2021) have shown that fermented milk with higher protein produce higher ACE inhibitory activity. This is contrary to our study observation. Whereas enzymatic digestion generally could produce ACE-inhibition peptides, progressive degradation of peptides to smaller components may result to a decline in the ACE-inhibition property (Meira et al. 2012, Tagliazucchi et al.2017).

At the beginning of the storage, samples with *S. thermophilus* K16 showed the highest ACE inhibition among all the other samples. Sample with *E. faecalis* NCCB 100525 presented the lowest ACE inhibitory activity on day 1. On the other hand, the samples with *Lb. paraplantarum* NCCB 100524 generated the highest ACE inhibition activity while sample with *S. thermophilus* 1-K4 produced the lowest on the 28th day. Fermented milk samples with *L. plantarum* NCCB 100523 and *E. faecium* NCCB 100527 produced similar ACE inhibition activity throughout the storage days.

As seen from Fig. 4.21, while fermented milk samples with *Lb. coryniformis* NCCB 100528, *Lb. helveticus* 12-B1, *S. thermophilus* K16 and *S. thermophilus* 1-K4 illustrated a decreasing ACE-inhibition activity storage. It was established in the previous studies that an increase of ACE-inhibitory activity with progressive storage days might be related to an increasing concentration of proteins, degree of proteolysis and soluble peptides during the fermentation process (Meyer et al. 2009, Nahariah et al. 2015, Rafıq 2015, Raikos et al. 2019, Du et al. 2023). It has been reported that ACE inhibitory activity increases until an equilibrium point and then it decreases as a result of extensive proteolytic degradation of ACE-inhibitory peptides (Ong et al.2007, Meira et al. 2012). However, the studies by Mudgi et al. (2023) and Yousefi et al. (2023) showed that ACE inhibitory and antioxidant activities of fermented milk samples decreased with the progress of proteolysis.

We obtained a higher ACE inhibition activity in sample with *L. plantarum* NCCB 100523 compared to the results obtained by Nahariah et al. (2015) in fermented egg albumen. Samples with *Lb. helveticus* strains showed similar ACE inhibition percentages with the ovine cheeses produced with *Lb. helveticus* strains in Southern Brazil and Uruguay regardless of the differences in manufacturing and storage time. (Meira et al. 2012). The

ACE inhibition activity for sample with *L. plantarum* NCCB 100523 was higher than the results got by Ayyash et al. (2018) that did not exceed 50% inhibition activity in bovine fermented milk samples.

Fermented milk samples with *L. plantarum* NCCB 100523 and *S. thermophilus* strains generated higher ACE inhibition activity as compared to the results found by Li et al. (2020) in fermented milk throughout the storage period. In a study performed by Graham et al. (2019) demonstrated a noticeable high level of ACE inhibition activity in fermented milk samples with *E. faecalis* compared to the results we obtained. We found higher ACE inhibitory activity in our fermented milk samples with *L. plantarum* NCCB 100523 and *Lb. bulgaricus* 23 throughout the storage days as compared to the ACE inhibition activity for the same bacteria species in yoghurt samples (Kim et al. 2021).

The percentage of ACE inhibitor activity for samples produced with *L. plantarum* NCCB 100523 in our study on day 1 supports the result recorded by (Gonzalez-Gonzalez et al. 2011). However, we obtained lower results for samples with *Lb. helveticus* strains than their data which exceeded 90%, plausibly much higher than our samples on day 1. On storage day 1, ACE inhibitory activity results for samples with *Lb. bulgaricus* 23 are similar with the results obtained by Chaves-López et al. (2014). In the same study, their samples with *E. faecalis* present higher ACE inhibition activity than in our sample. However, we obtained a higher result in the sample produced with *L. plantarum* NCCB 100523 compared to the results detected in their study. Yousefi et al. (2023) determined high percentage of ACE inhibition activity in *Lb. brevis* strain on the first day, which is slightly more than the results obtained in our fermented milk sample. Du et al. (2023) also obtained high ACE-inhibition value for *Lb. bulgaricus* sample throughout the storage period but we recorded higher values for *S. thermophilus* K16 samples during 28 days of storage than the values they obtained. Similarly, we found more ACE activity in our fermented milk samples with *Lb. helveticus* strains than the results obtained in yoghurt samples made with *Lb. helveticus* strains by (Cavalheiro et al. 2020). Samples with *L. plantarum* NCCB 100523 presented higher ACE inhibitor activity compared to the results obtained in buffalo and camel fermented milk (Khakhariya et al. 2023). ACE activity of our sample produced with *Lb. helveticus* 12-B1 and *Lb. helveticus* 11-B2 on day 1 are

similar with that of Muguerza et al. (2006), however, they recorded higher ACE inhibition activity for fermented milk samples with *Lc. lactis* NCCB 100539, *Lb. brevis* NCCB 100526 and *E. faecalis* NCCB 100525.

It was noticed that samples produced with *Lb. helveticus* 11-B2 provided similar ACE-inhibition activities with the results of Chen et al (2014). However, our other samples with *Lb. helveticus* 12-B1 and *Lb. helveticus* 9-B5 produced lower ACE inhibition activity. Fermented milk samples produced with *Lb. helveticus* 12-B1 had similar ACE inhibition activity with the results by Abd El-Fattah et al. (2017) in sterilized skim milk however, samples made with *Lb. brevis* NCCB 100526 and *L. plantarum* NCCB 100523 produced higher ACE inhibition activity compared to theirs with similar strains. Samples produced with *E. faecalis* NCCB 100525 exhibited significant ACE inhibition more than the results obtained in fermented milk samples by Muguerza et al. (2006). In our study, we obtained higher ACE inhibition activity in samples with *L. plantarum* NCCB 100523, *Lc. lactis* NCCB 100539 and *S. thermophilus* K16. However, Martinez-Villaluenga et al. (2012) detected slightly higher ACE inhibition activity in fermented soy milk with *S. thermophilus* strain than our samples fermented with *S. thermophilus* 1-K4 on day I. The differences observed in ACE inhibition activities among the different LAB during the storage time is thought to vary with the kind of bacterial strain applied for fermentation (Fuglsang et.al. 2003).

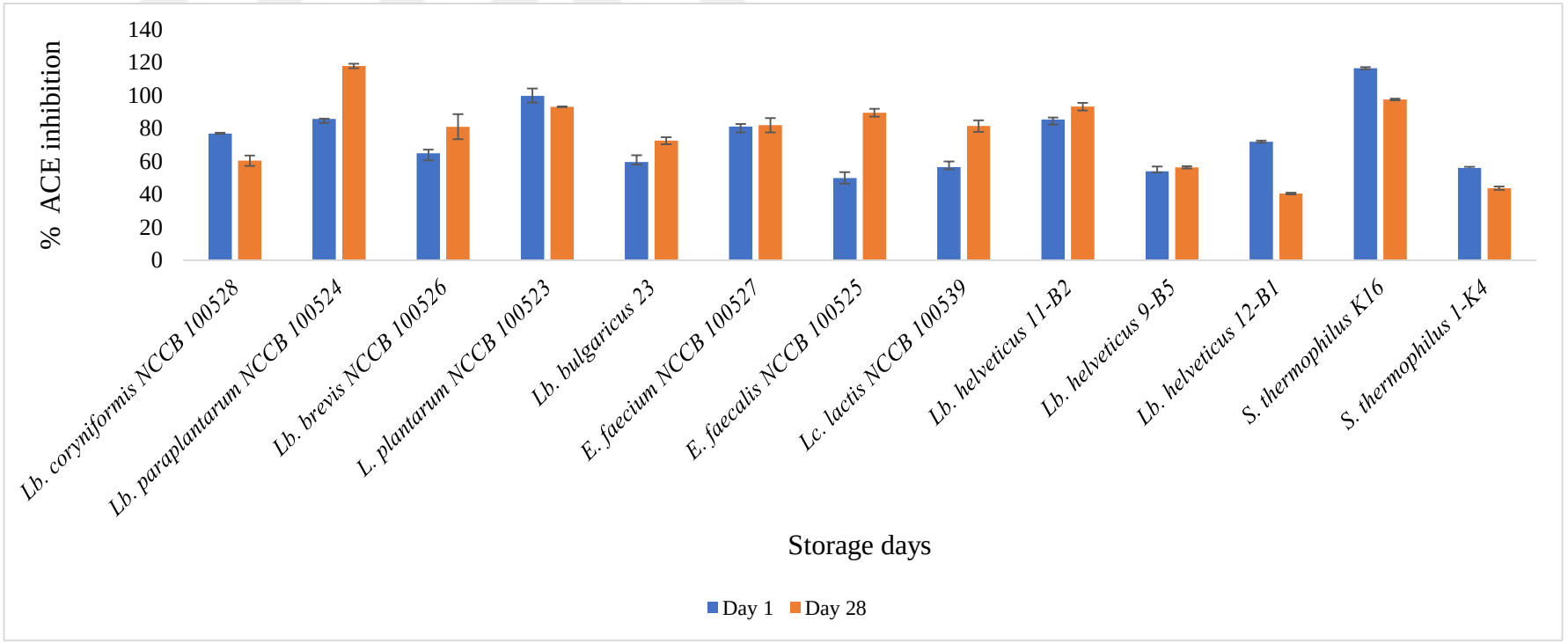


Figure 4.21 ACE inhibitory activity of the samples during storage days (n=2)

5. CONCLUSION AND FUTURE DIRECTIONS

There is a conceivably noticeable increase in several physiological health disorders such as hypertension, hypercholesterolemia, diabetes, cancer and oxidative stress among others. The recommended chemotherapeutic treatments of these ailments have limited their use since they impart some side effects. Therefore, the utilization of protein and peptides derived from fermented milk products could exhibit an alternative therapeutic potential as opposed to drug administration. Milk derived BP are produced by food processing, enzymatic hydrolysis and food fermentation by LAB.

Dairy products are considered as the main source of natural peptides, but several people are unaware of this therapeutic potential of such bioactive peptides. We produced fermented milk using natural LAB isolates. Analyses were performed to determine pH, titratable acidity, viable bacteria count, peptide profile, protein quantity in WSE obtained during the storage period on day 1, 14 and 28 of cold storage. We evaluated also the bioactive/functional properties such as antioxidant activity, phenolic content, ACE inhibitory activity and antimicrobial activity of peptide fraction smaller than <3 kDa, which obtained from WSE of fermented milk samples. The level of proteolysis (peptide profile) was also assessed via RP-HPLC.

During analysis of our fermented milk samples produced with LAB isolates, it was observed that the pH in samples was between 4.81 to 3.88 during the whole storage days. It was also noticed that there were significant differences in pH values among the different samples during storage days ($p>0.05$). The sample produced with *Lb. helveticus* 12-B1 at the beginning of storage provided the lowest pH. However, the samples with *Lb. bulgaricus* 23 and *Lb. helveticus* 11-B2 presented the lowest pH values on the 28th day. Although there was a fluctuation in the pH values for some samples, a great deal of samples generally showed a decrease in pH during the storage. However, the samples fermented with *Lc. lactis* NCCB 100539 had the highest pH values. *Lc. lactic* strains are slow acidity forming bacteria. They are mostly involved in aroma formation for dairy products, such as cheese and butter. For titratable acidity, all the samples had an acidity range of 0.61 to 1.52% LA. The sample with *Lb. bulgaricus* 23 produced the highest

titratable acidity value on the 28th day of storage, followed by sample with *Lb. helveticus* 11-B2. However, the sample made with *S. thermophilus* K16 produced the lowest acidity among all samples. Viable microbial count ranged from 8.2-11.1 log CFU/mL. The microbial count was significantly different among the LAB isolates. While the samples with *S. thermophilus* K16 presented the highest viability throughout the storage period, the samples with *Lb. bulgaricus* 23 demonstrated the least microbial count. The protein content of WSE obtained from samples were significantly different. The protein content among samples ranged from 0.106-0.358 mg/mL. The samples with *Lb. paraplantarum* NCCB 100524 produced the least while samples made with *Lb. helveticus* 9-B5 produced the highest amount of protein in WSE.

Peptide profile was investigated by RP-HPLC and the results have indicated the level of proteolysis in WSE increased progressively during the 28 days of storage. The peptide profiles showed the development from the first day until the last day of storage. Minor differences were determined in terms of peak numbers, peak area and peak heights in WSE throughout the storage period. However, few qualitative differences were determined in chromatograms among the fermented milk samples. The most intense peaks were established in fermented milk samples made with *E. faecium* NCCB 100527, *E. faecalis* NCCB 100525, *Lb. bulgaricus* 23 and *Lb. paraplantarum* NCCB 100524. As seen in the chromatograms, the large peak eluted in the hydrophilic region around the 45th minute tended to decrease later on. Fermented milk samples with *Lb. helveticus* 9-B5, *Lb. helveticus* 11-B2 and *Lb. helveticus* 12-B1 showed smaller and lesser peaks than in the other samples. The observed increase/decrease in the peak height during storage was detected in the peaks which eluted at around 67th and 75th minutes in the hydrophobic region but the numbers of peptides occurred in the hydrophilic region did not change much and instead their concentrations increased especially for the peaks which formed in the middle and hydrophobic regions. There was no significant peak increase detected in the hydrophobic region for fermented milk for samples made with *S. thermophilus* 1-K4, *Lb. helveticus* 9-B5, *Lb. helveticus* 11-B2 and *Lb. helveticus* 12-B1 samples. The peaks in samples with *Lb. bulgaricus* 23, *Lb. paraplantarum* NCCB 100524 and *S. thermophilus* K16 in the hydrophobic region were fluctuated. Whereas samples made with *Lb. brevis* NCCB 100526 produced the most

numbers of peaks throughout the storage days, the samples produced with *Lb. coryniformis* NCCB 100528 showed the highest on the 28th day of storage. However, the sample fermented with *S. thermophilus* K16 presented the least number of peaks on the 28th day of storage. Only one peak was eluted in the control (pasteurized milk) sample.

The antioxidant activity values in peptide fraction smaller than <3 kDa of WSE extracted from fermented milk samples ranged between 118–1935 μM TEAC during the storage days. The antioxidant activity showed significant differences among the fermented milk produced with different LAB strains and among the storage days ($P \leq 0.05$). Fermented milk samples with *Lb. brevis* showed the least antioxidant activity among all samples on day 28, followed by samples with *Lb. paraplantarum* NCCB 100524. However, the sample with *S. thermophilus* K16 demonstrated the highest antioxidant activity.

Total phenolic content of fermented milk samples made with LAB strains varied from 10.68 to 35.61 mg GAE/g during the storage period. Samples with *Lb. brevis* NCCB 100526 produced the highest mean value for phenolic content while samples with *Lb. paraplantarum* NCCB 100524 produced the least. Antibacterial activities were tested against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella enterocolitica* (ATCC 14028), *Bacillus cereus* (ATCC 14579), *Mycobacterium tuberculosis* (ATCC 7644) and *Enterococcus faecalis* (ATCC 29212) by agar well diffusion method. Fermented milk samples produced with *Lb. paraplantarum* NCCB 100524 showed the highest antibacterial effect against *Escherichia coli* (ATCC 25922) on day 28. Fermented milk made with *L. plantarum* NCCB 100523 had the highest antibacterial activity on both *Mycobacterium tuberculosis* (ATCC 7644) and *Bacillus cereus* (ATCC 14579).

The ACE inhibitory activities of fractions smaller than 3kDA indicated that all samples produced with different LAB strains exhibited ACE inhibitory activity. However, the ACE percentage showed a significant difference among the samples and among the storage days ($P \leq 0.05$). The ACE inhibitory activity increased generally with progressive storage days although some fluctuations were observed. The average ACE inhibitory

activity was between 43.16-117.81%. A higher ACE inhibition activity was measured at 117.80% on day 28th day in the sample produced with *Lb. paraplantarum* NCCB 100524. However, the sample produced with *Lb. helveticus* 12-B1 showed the lowest ACE inhibition on day 28 of storage. The present findings in our study recommend the possible application BP produced from LAB fermented milk for the management of various physiological disorders and they can be effectively exploited in several functional food production.

Table 5.1 Summary of the functional properties in the samples during the storage

	Antioxidant activity (TEAC µM)	**Phenolic activity (GAE/g)**	**% ACE inhibition activity**
Lb. coryniformis NCCB 100528	++	+	++
Lb. paraplantarum NCCB 100524	+	+	++++
Lb. brevis NCCB 100526	+	++++	+++
L. plantarum NCCB 100523	+	++	++++
Lb. bulgaricus 23	++	++	++
E. faecium NCCB 100527	++++	++	+++
E. faecalis NCCB 100525	+++	++	+++
Lc. lactis NCCB 100539	+++	++	++
Lb. helveticus 11-B2	+++	+++	++++
Lb. helveticus 9-B5	++	+++	+
Lb. helveticus 12-B1	++	++	+
S. thermophilus K16	++++	++++	++++
S. thermophilus 1-K4	++	+	+

Low activity =+; Moderate activity= ++; High activity= +++; Very high=++++

5.1 Future Directions

Bioactive peptides might be used as therapeutic additives in dairy products with specific functional parameters and possibly in pharmaceutical preparations for the management of several physiological disorders. In-depth research is needed to develop and enhance the production of bioactive peptides using cultures with higher functional activities. There must be much more investigation through *in vivo* efficacy trials to establish how much concentration of bioactive peptides is effective to provide instant bioactivity. Also, an economic feasibility analysis of the production of the peptides should be conducted to provide valuable information for the food industry. Finally, there should be significant public awareness programs launched regarding the therapeutic potential of fermented dairy products produced with LAB with regards to their bioactivity.

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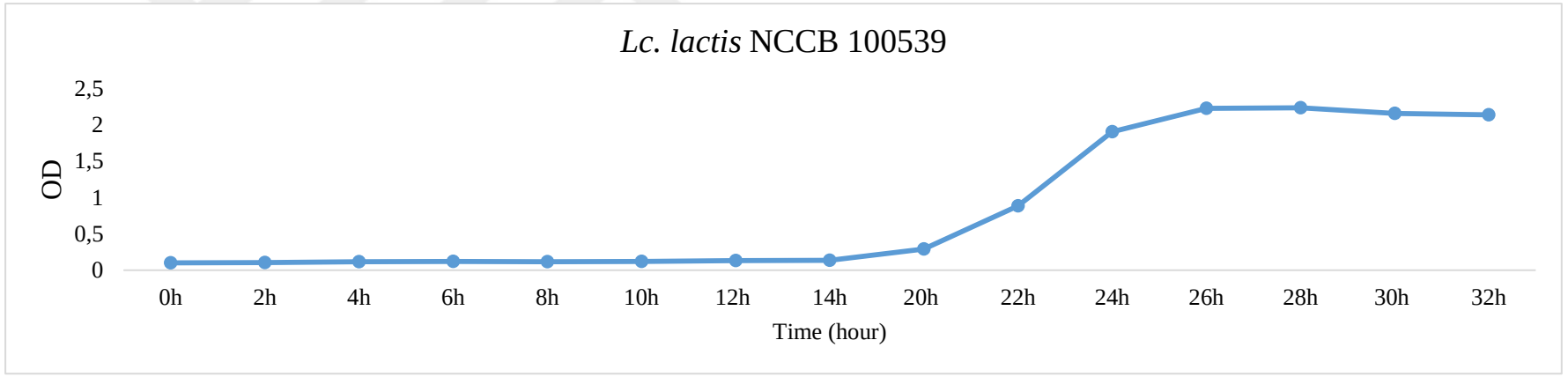
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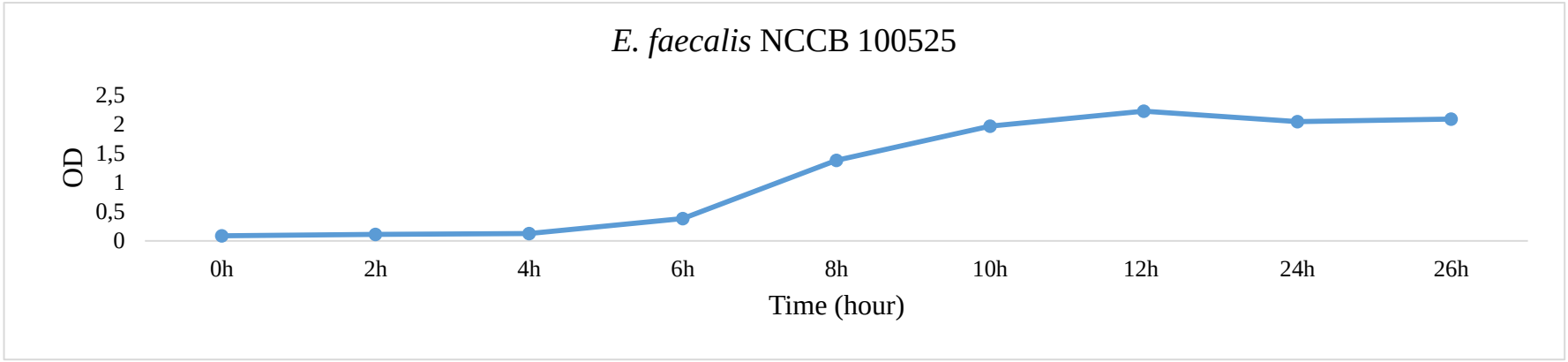
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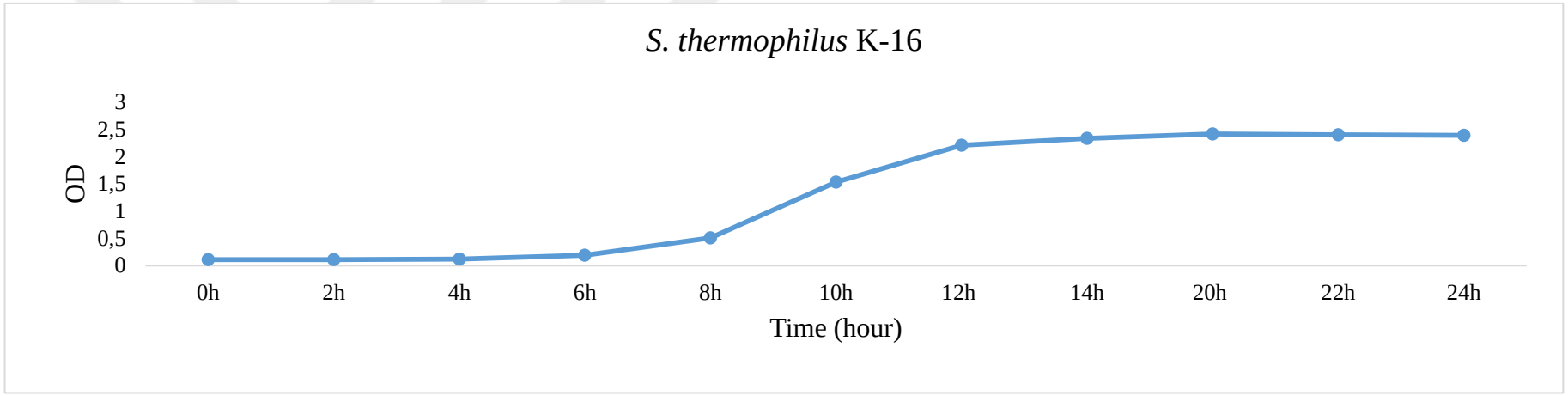
APPENDIX 1 BACTERIA GROWTH CONDITIONS



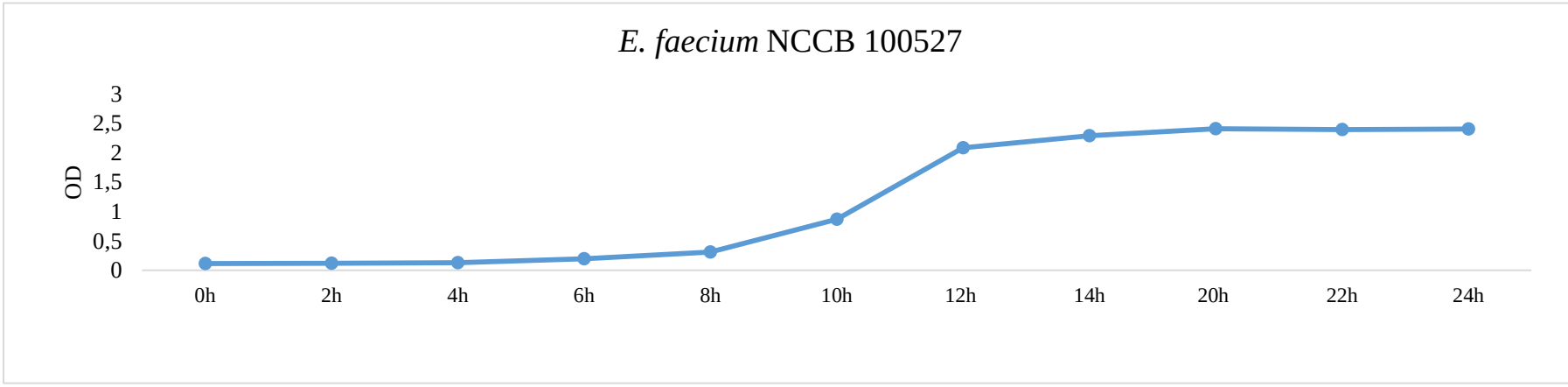
Lc. lactis NCCB 100539 (30 °C)



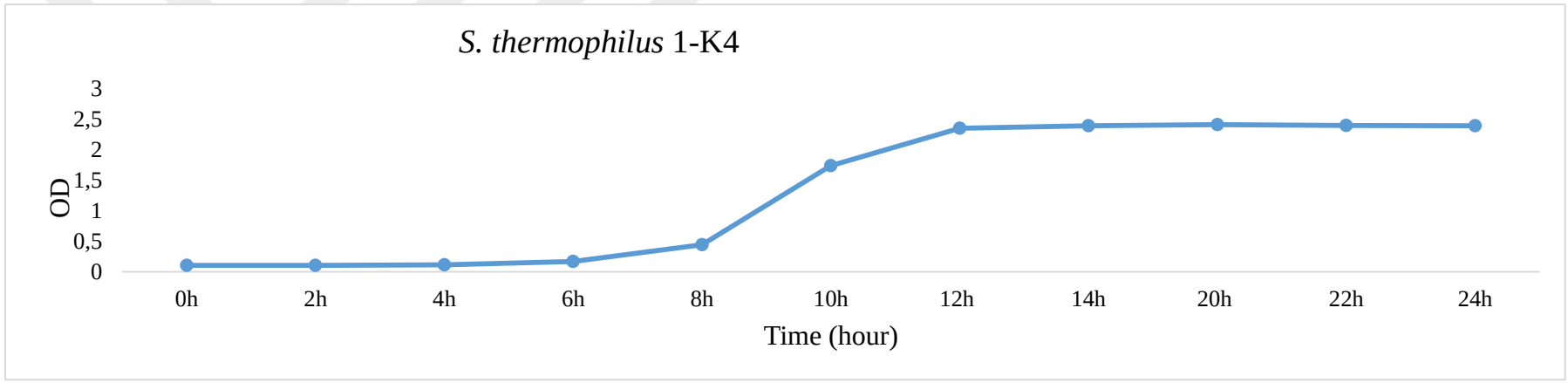
E. faecalis NCCB 10052 (30 °C)



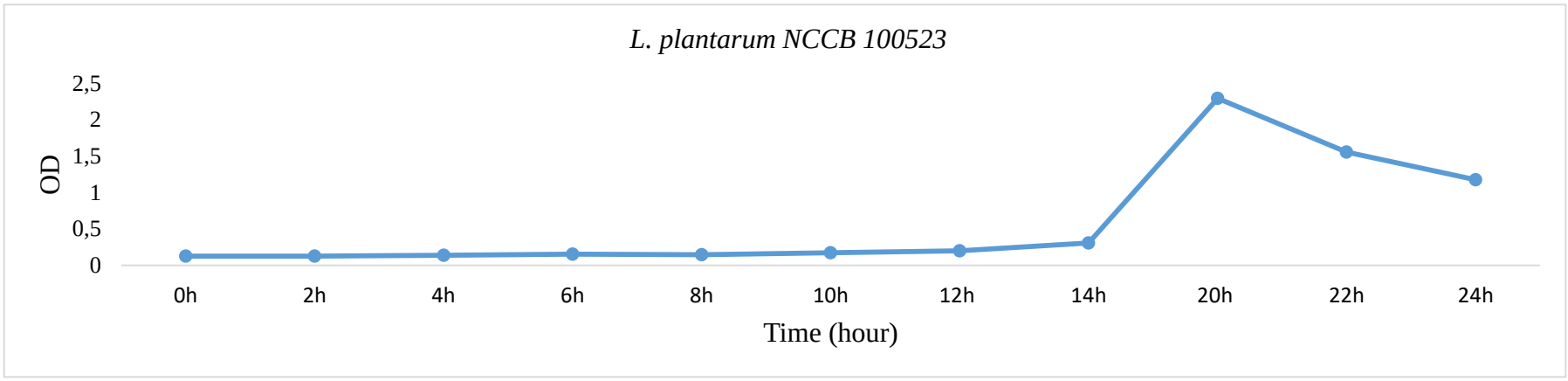
S. thermophilus K16 (30 °C)



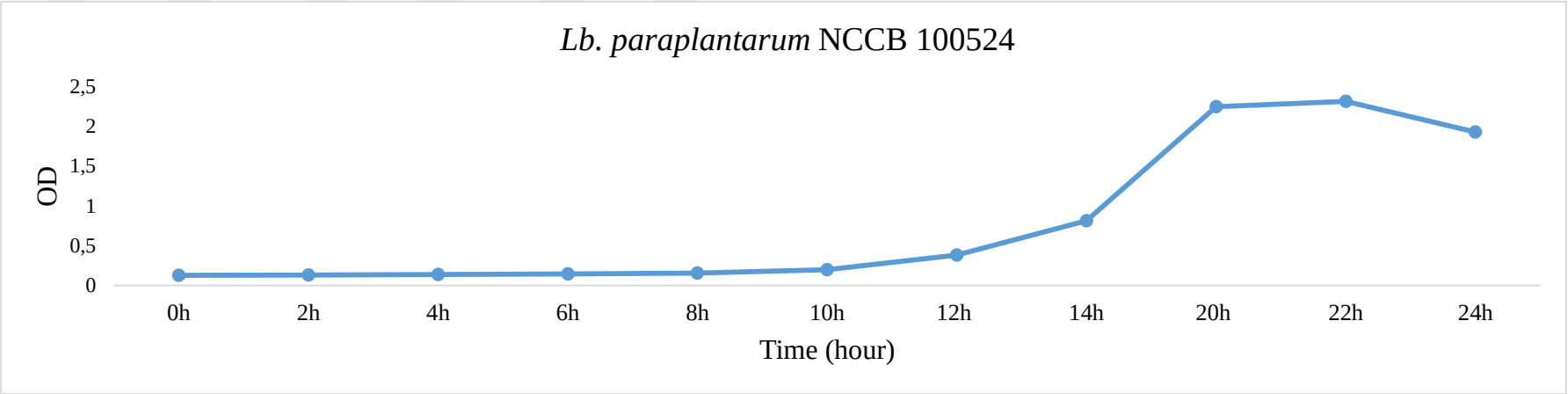
E. faecium NCCB 100527 (30 °C)



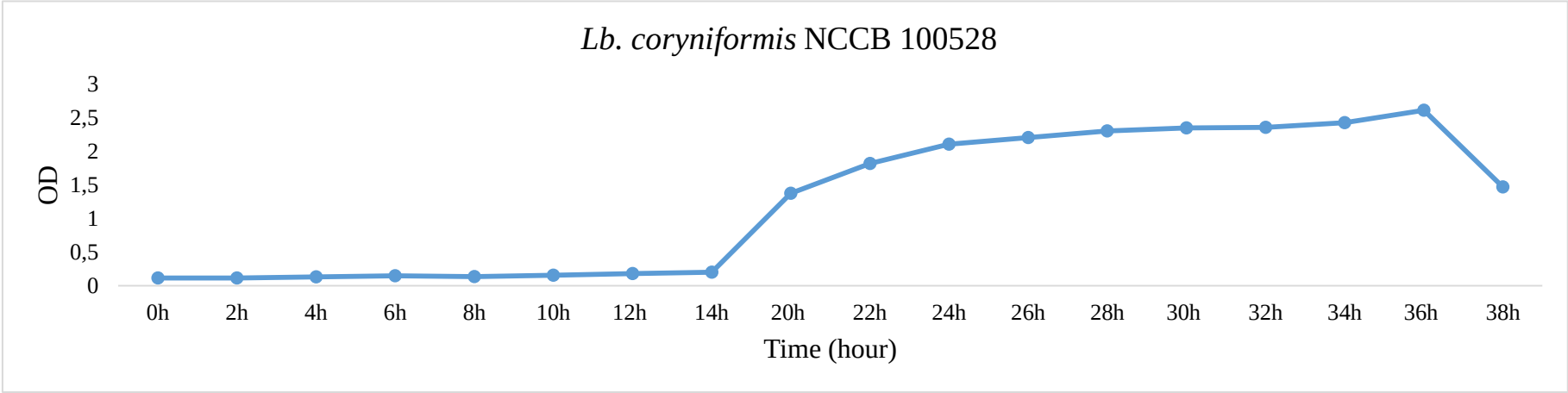
S. thermophilus 1-K4 (30 °C)



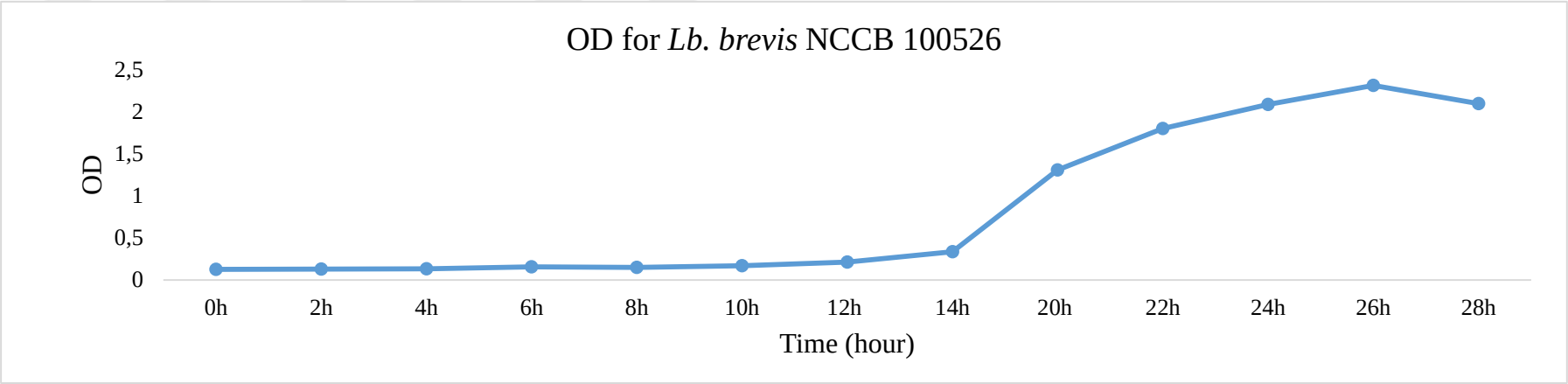
L. plantarum NCCB 100523 (37 °C)



Lb. paraplantarum NCCB 100524 (37 °C)

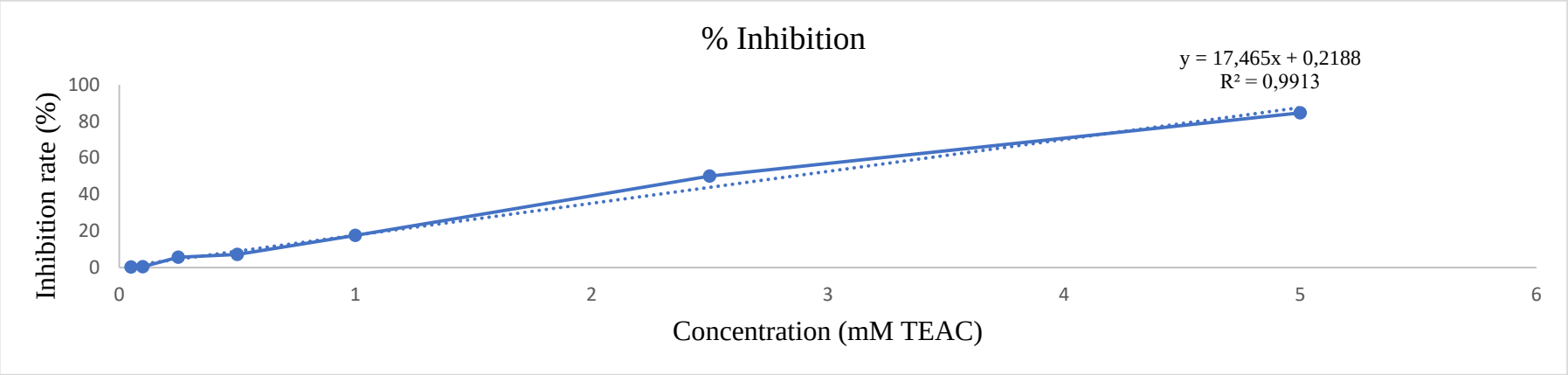


Lb. coryniformis NCCB 100528 (37 °C)

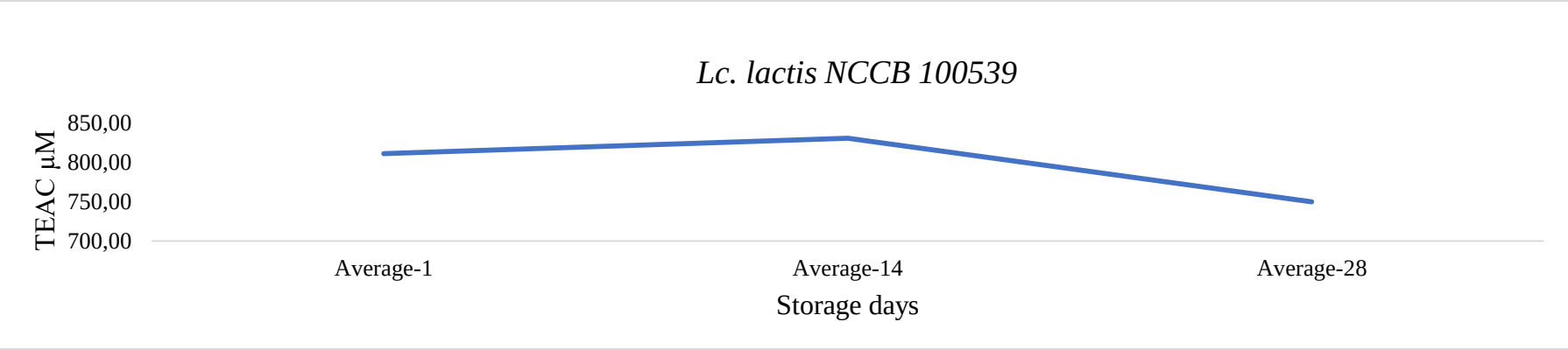


Lb. brevis NCCB 100526 (37 °C)

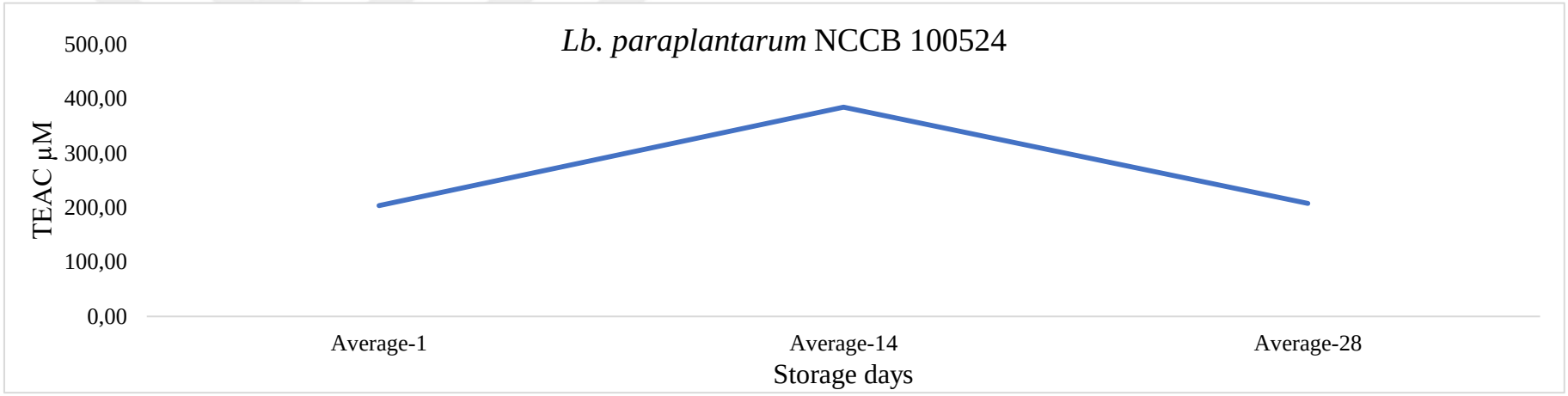
APPENDIX 2 ANTIOXIDANT ACTIVITY (µM TEAC) WITH DPPH ASSAY



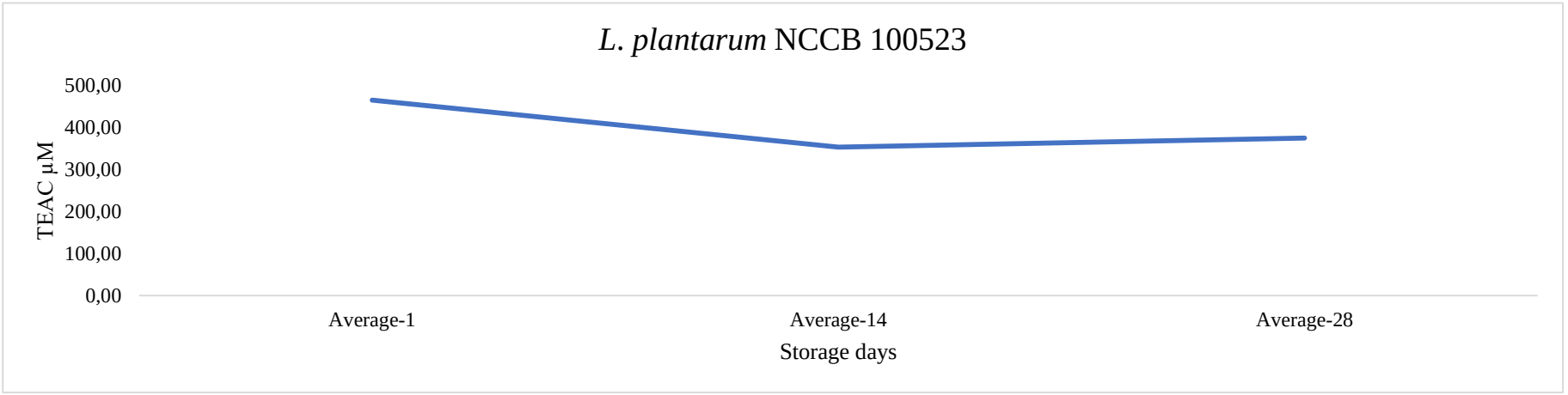
Trolox Standard Curve



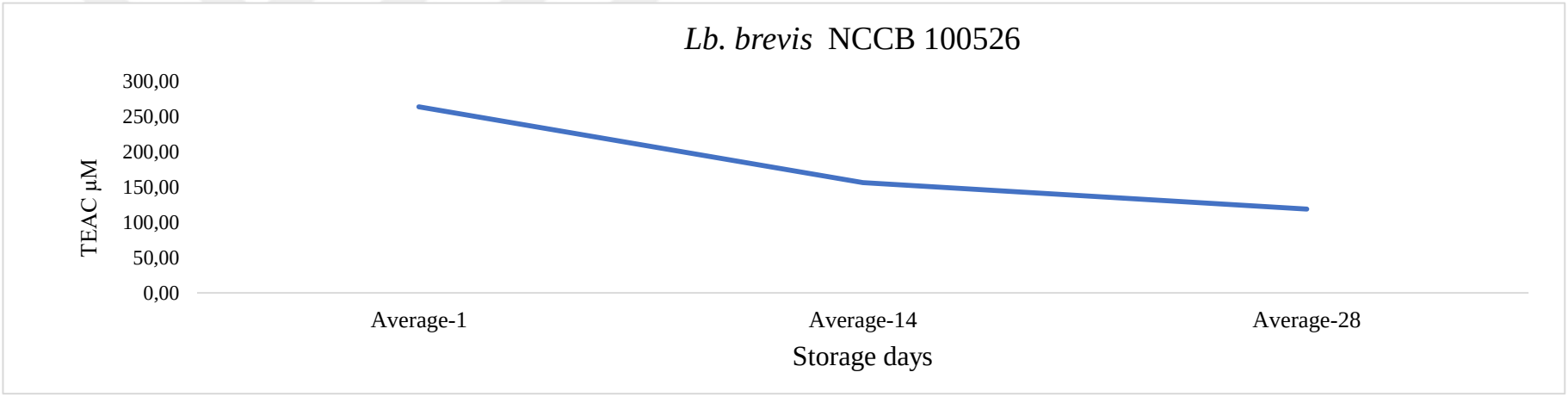
Lc. lactis NCCB 100539



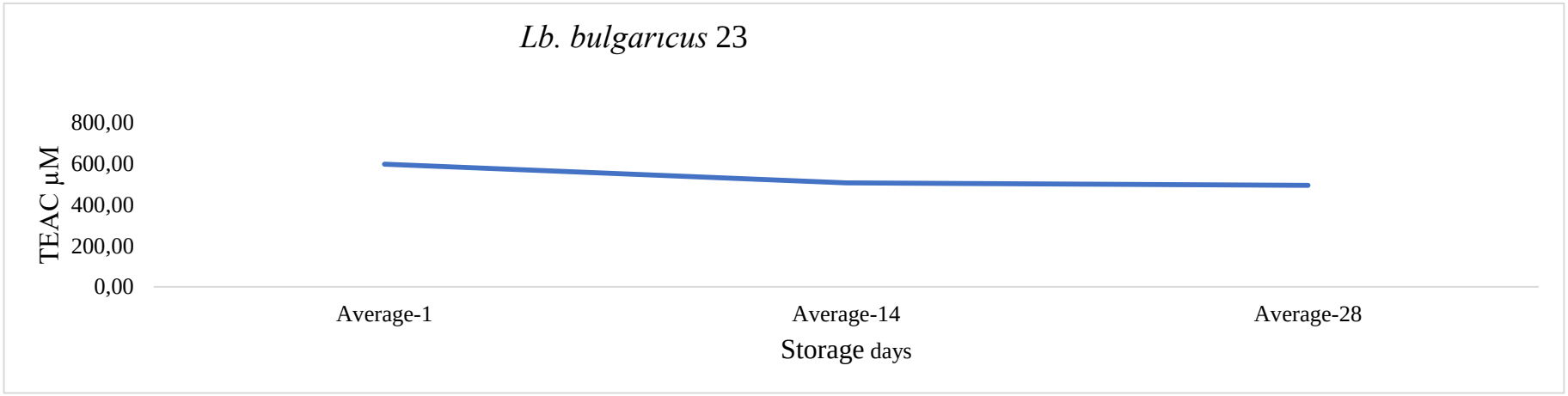
Lb. paraplantarum NCCB 100524



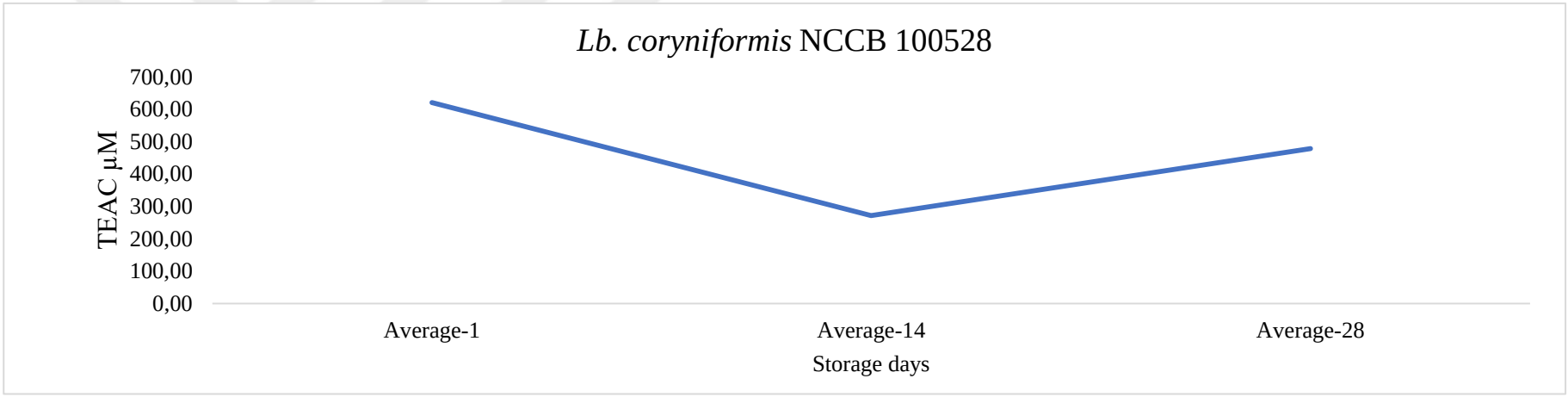
L. plantarum NCCB 100523



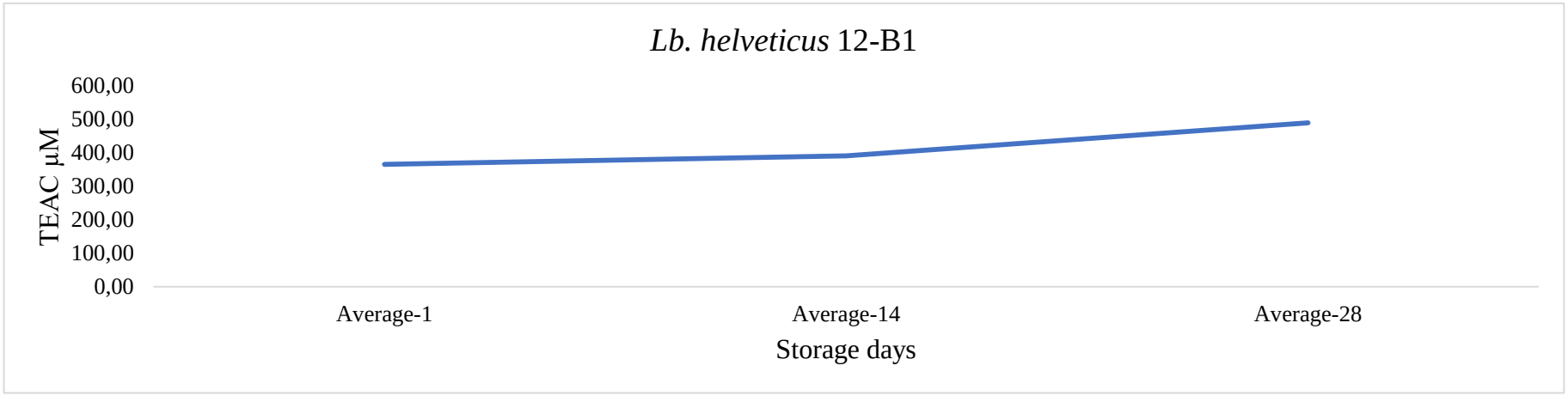
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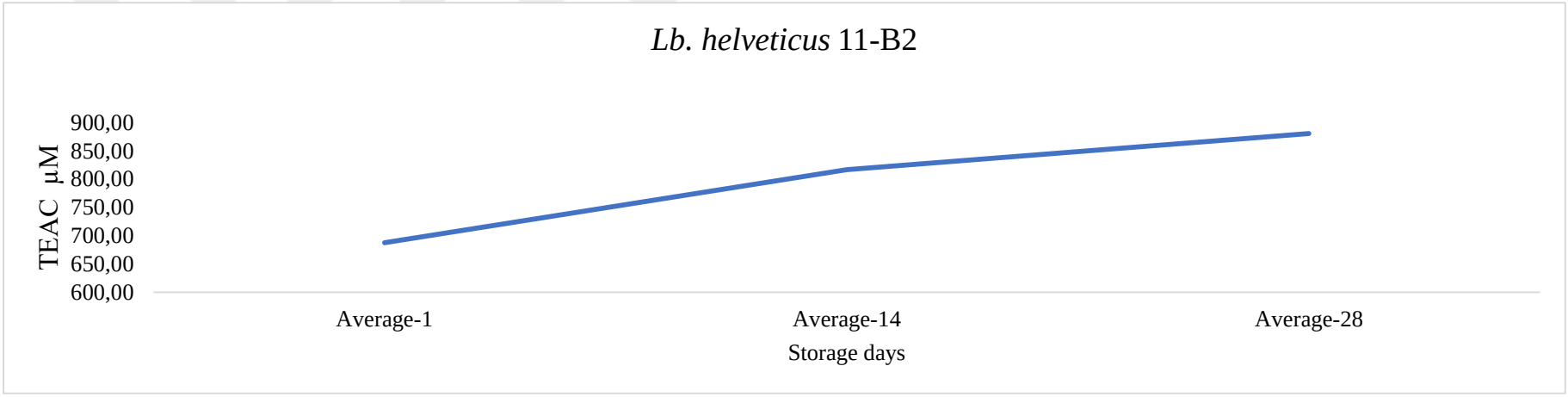
Lb. bulgaricus 23



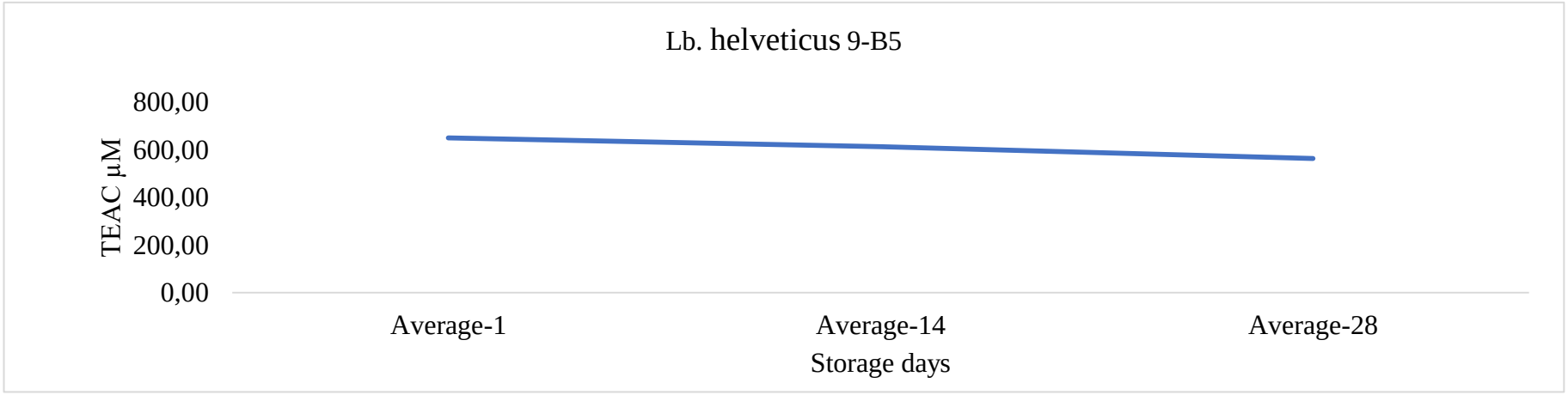
Lb. coryniformis NCCB 100528



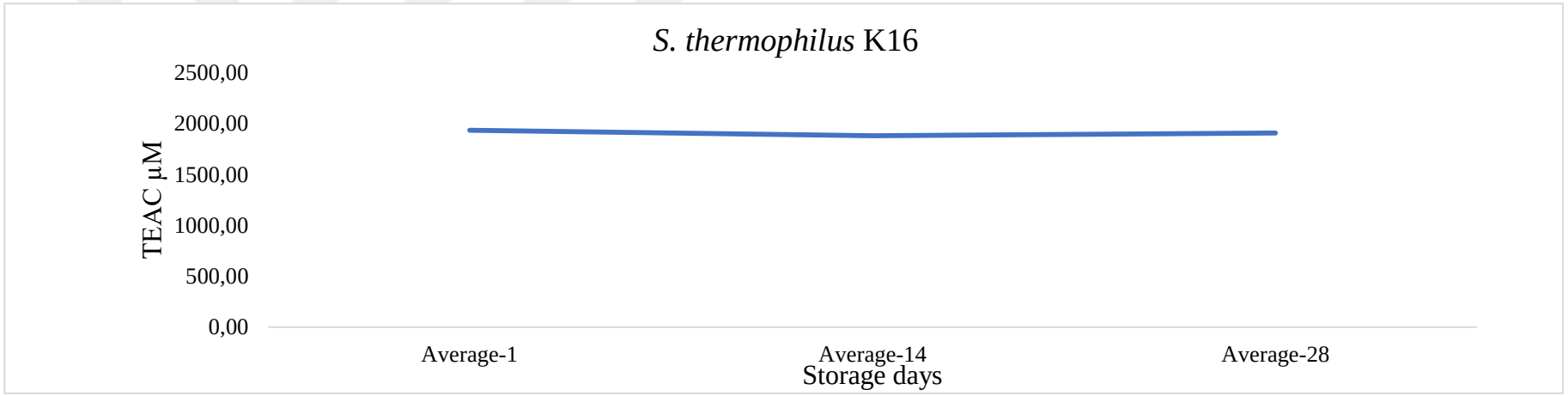
Lb. helveticus 12-B1



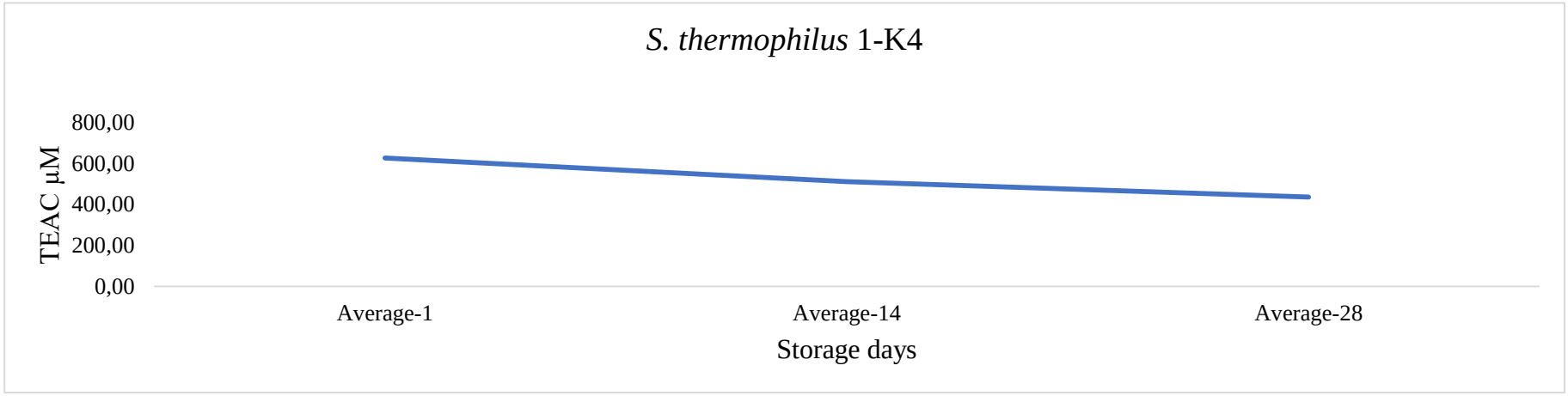
Lb. helveticus 11-B2



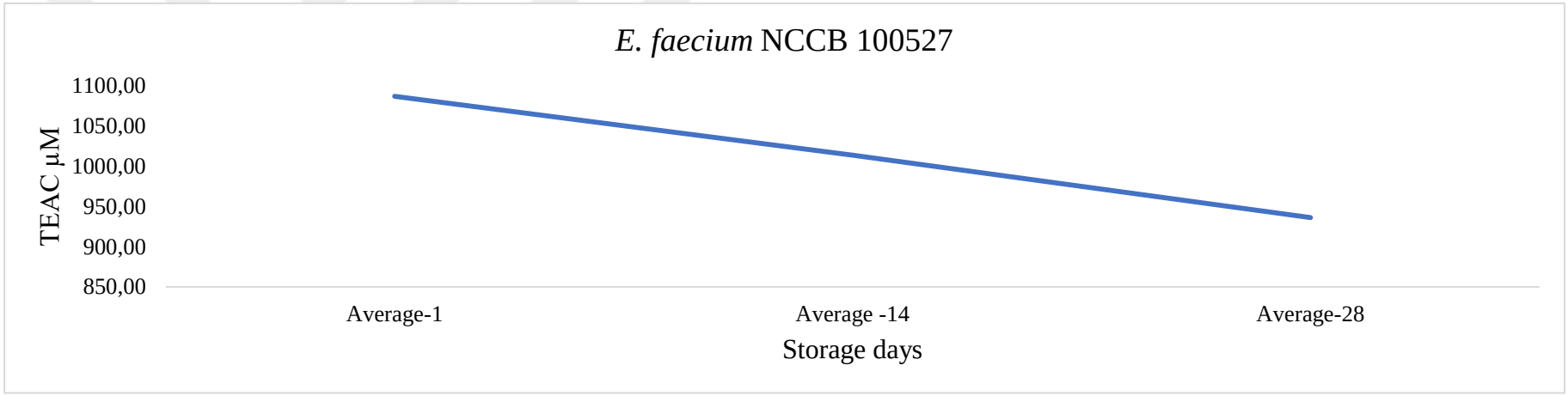
Lb. helveticus 9-B5



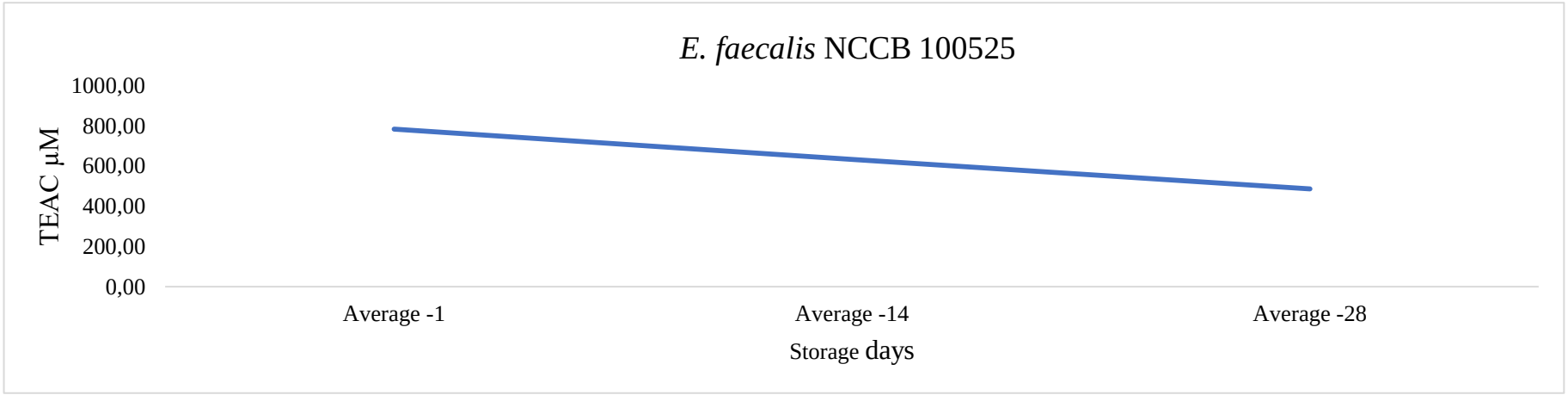
S. thermophilus K16



S. thermophilus 1-K4

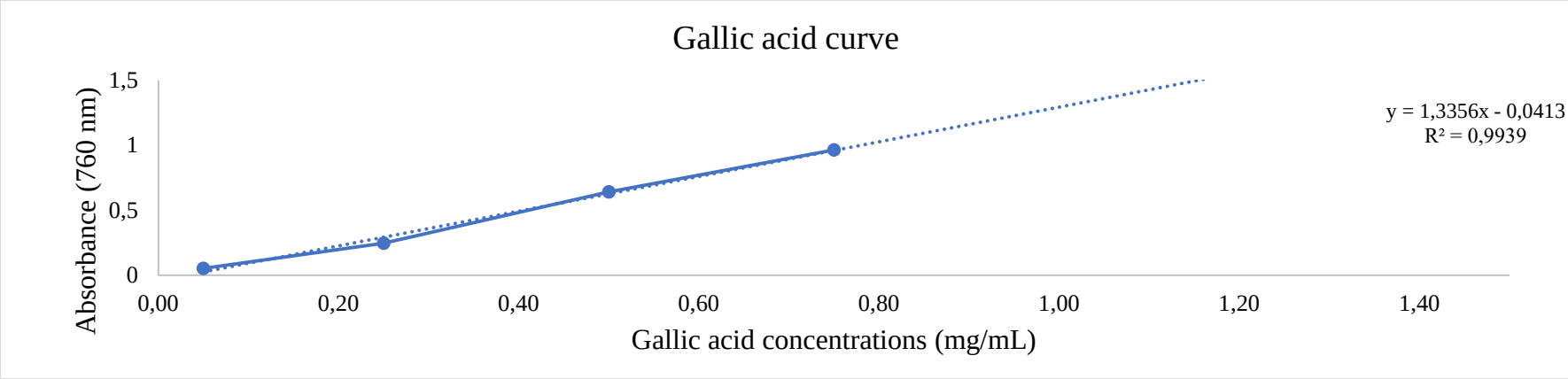


E. faecium NCCB 100527

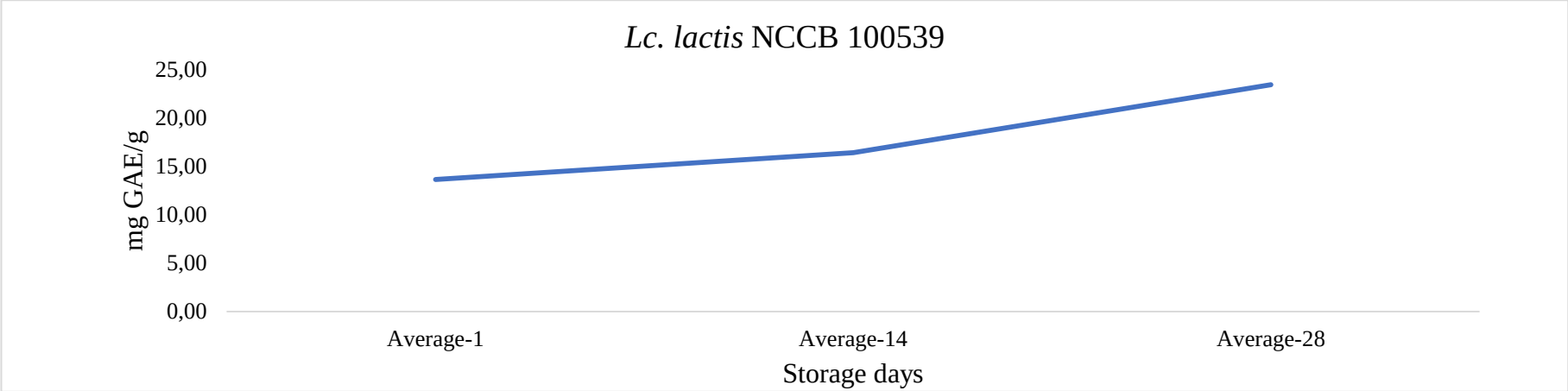


E. faecalis NCCB 100525

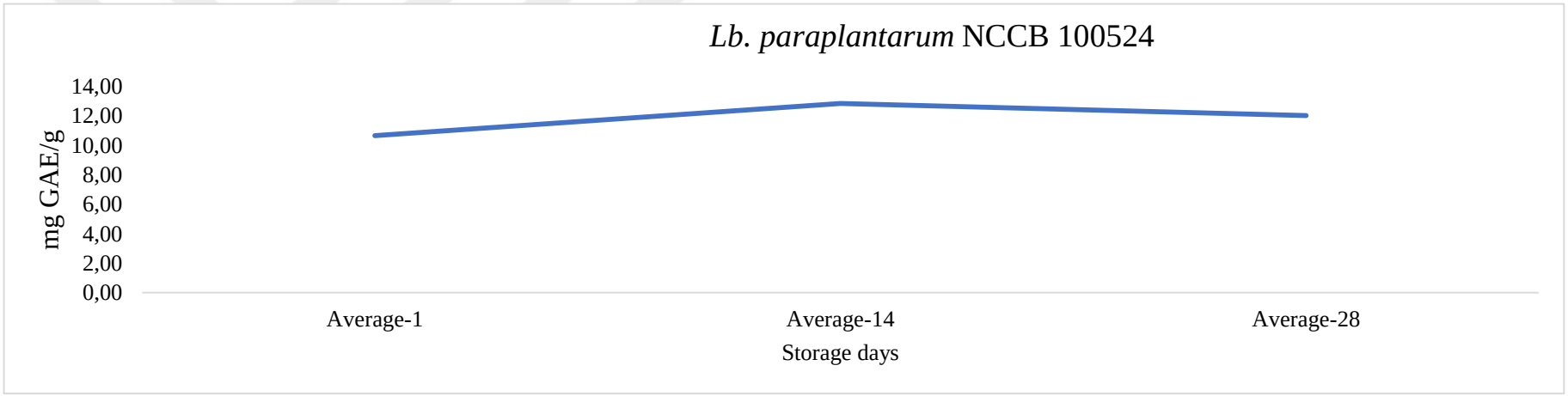
APPENDIX 3 PHENOLIC CONTENT (mg GAE/g)



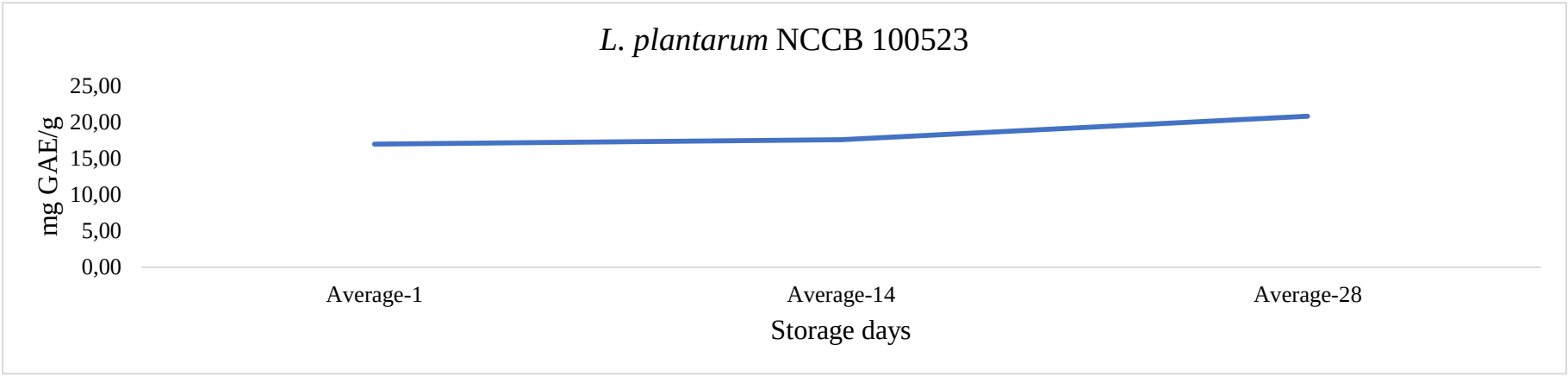
Gallic acid equivalent standard



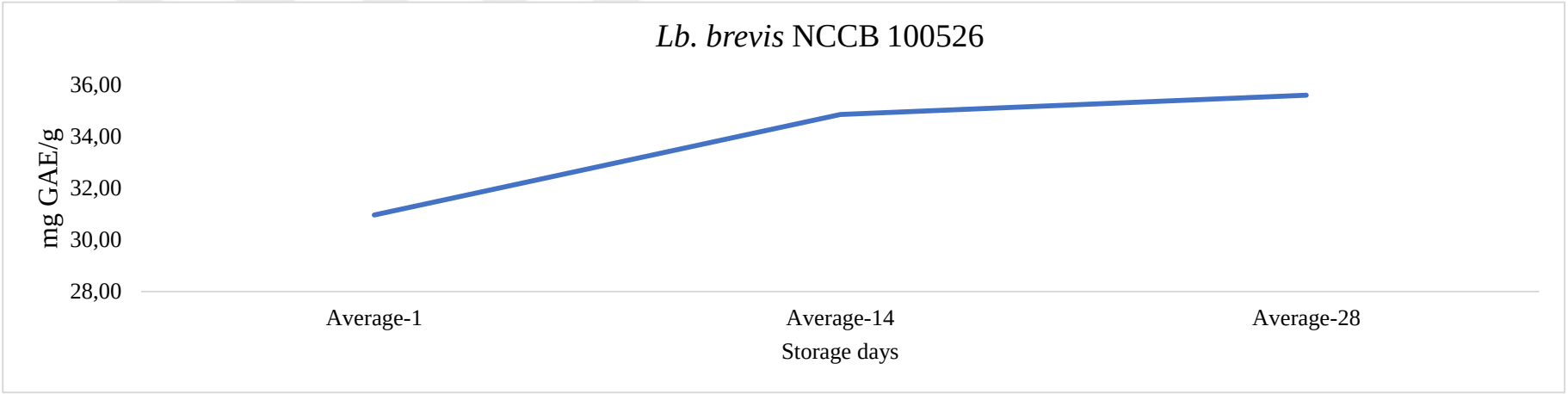
Lc. lactis NCCB 100539



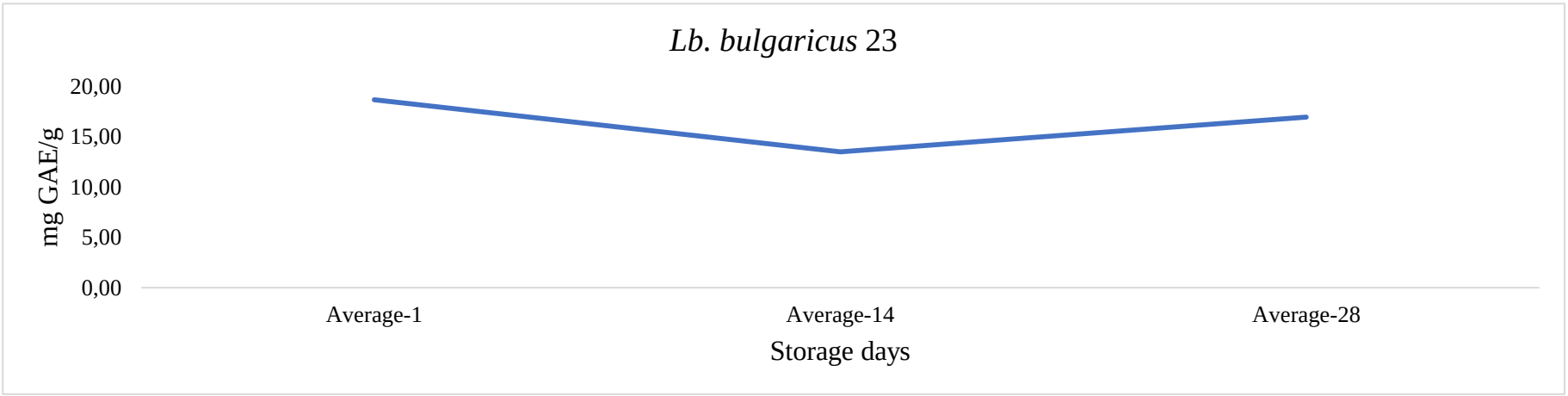
Lb. paraplantarum NCCB 100524



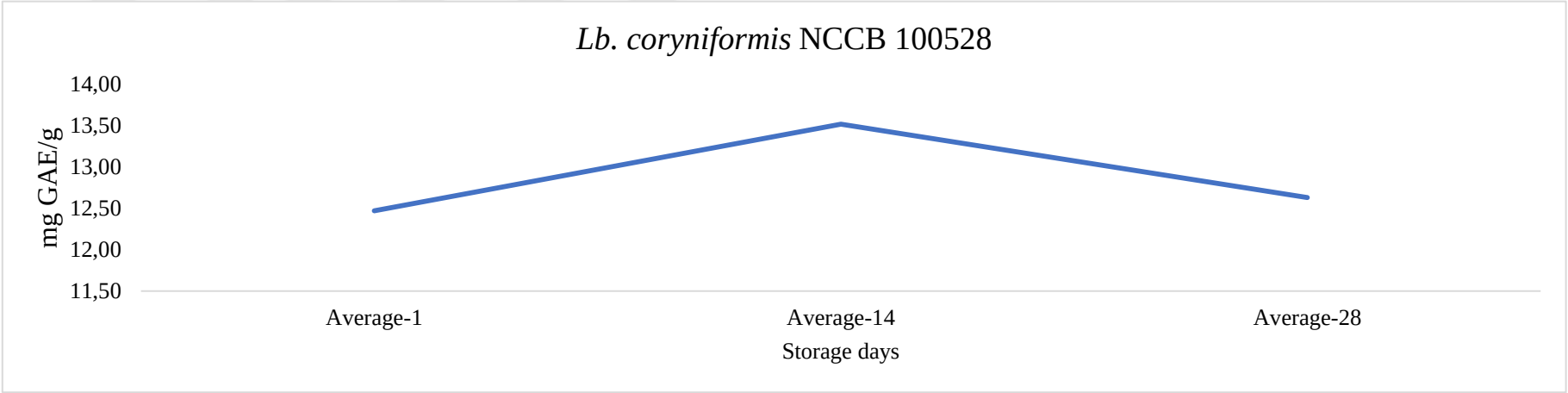
L. plantarum NCCB 100523



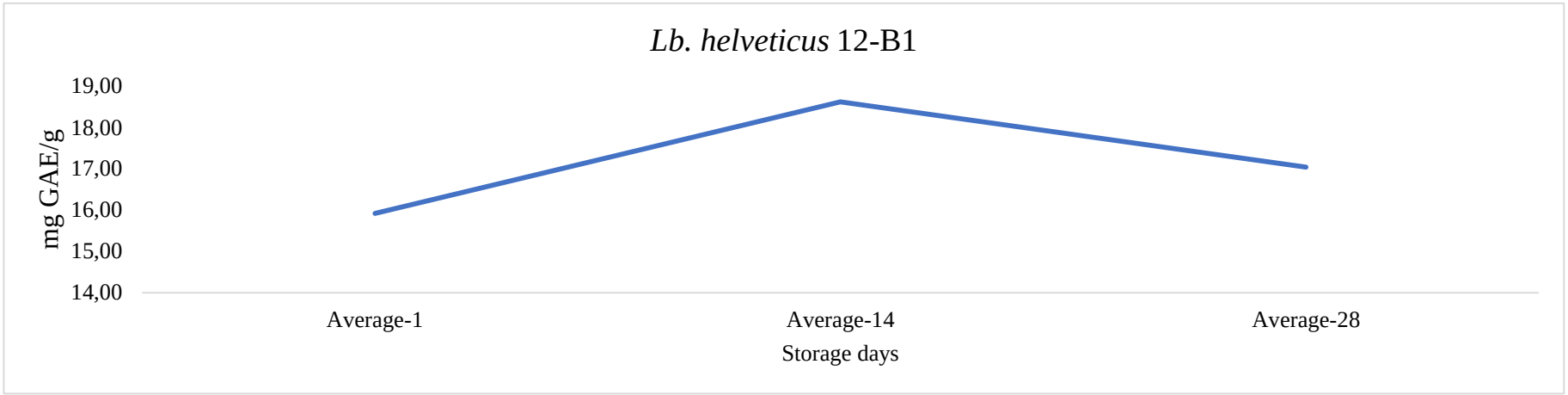
Lb. brevis NCCB 100526



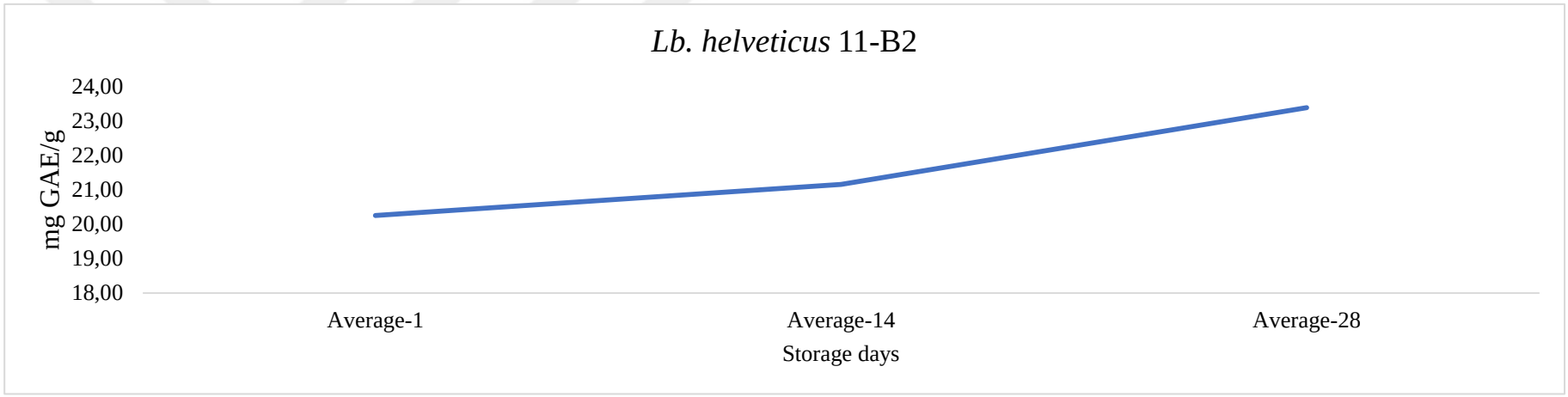
Lb. bulgaricus 23



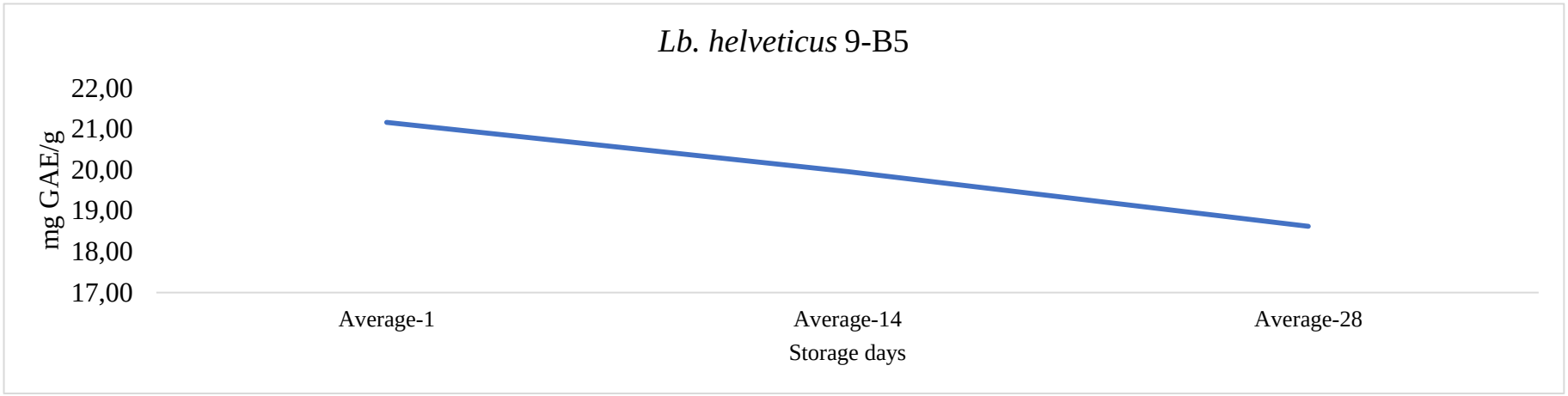
Lb. coryniformis NCCB 100528



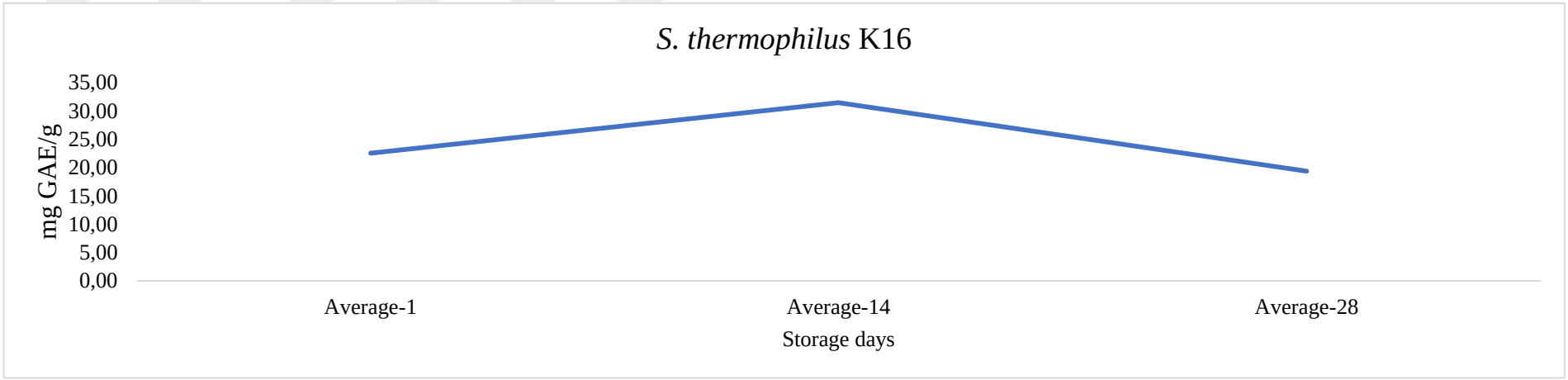
Lb. helveticus 12-B1



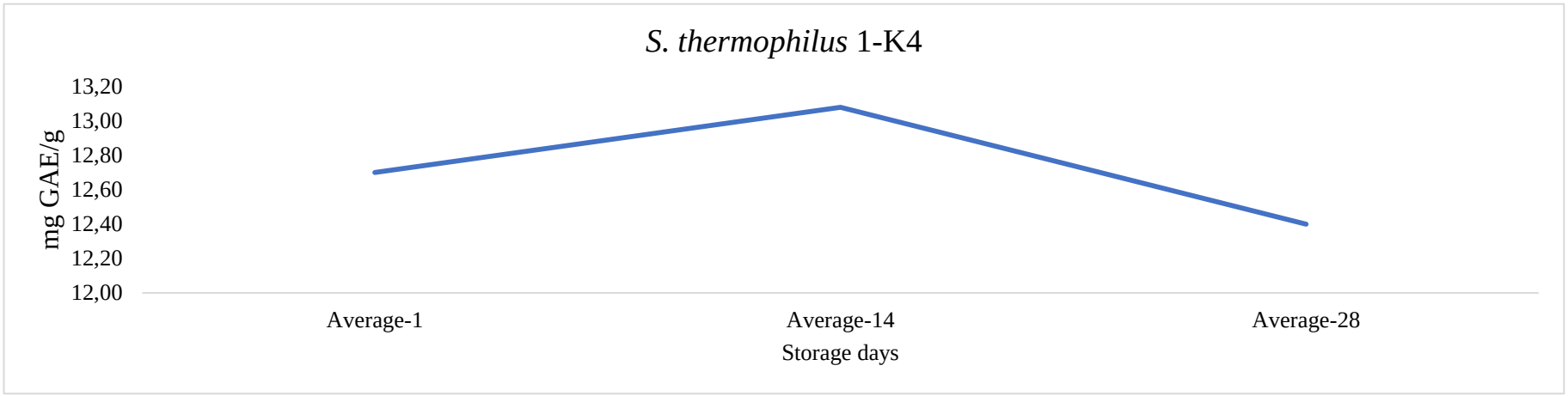
Lb. helveticus 11-B2



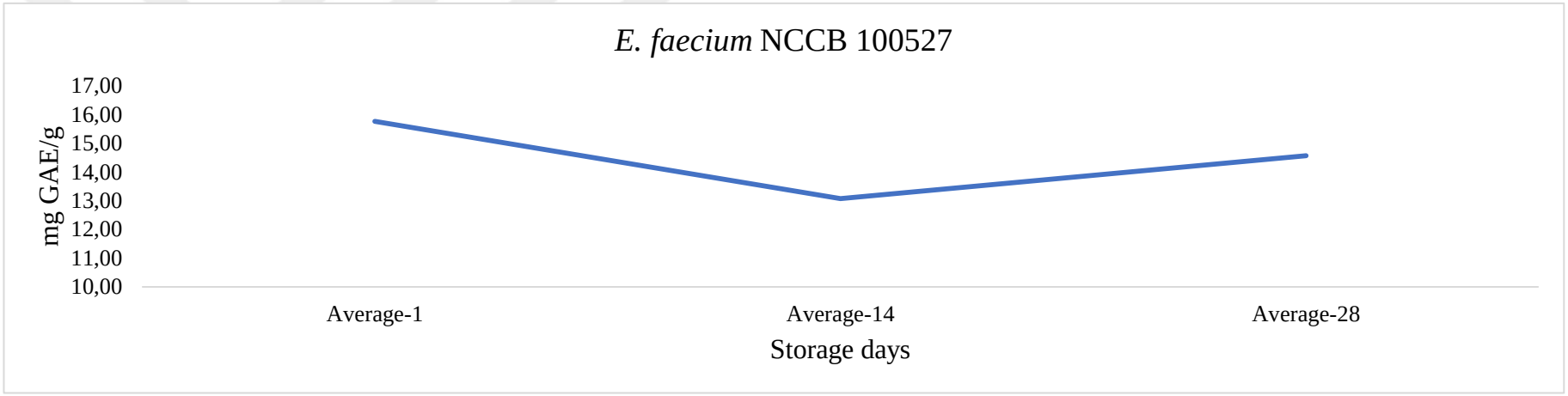
Lb. helveticus 9-B5



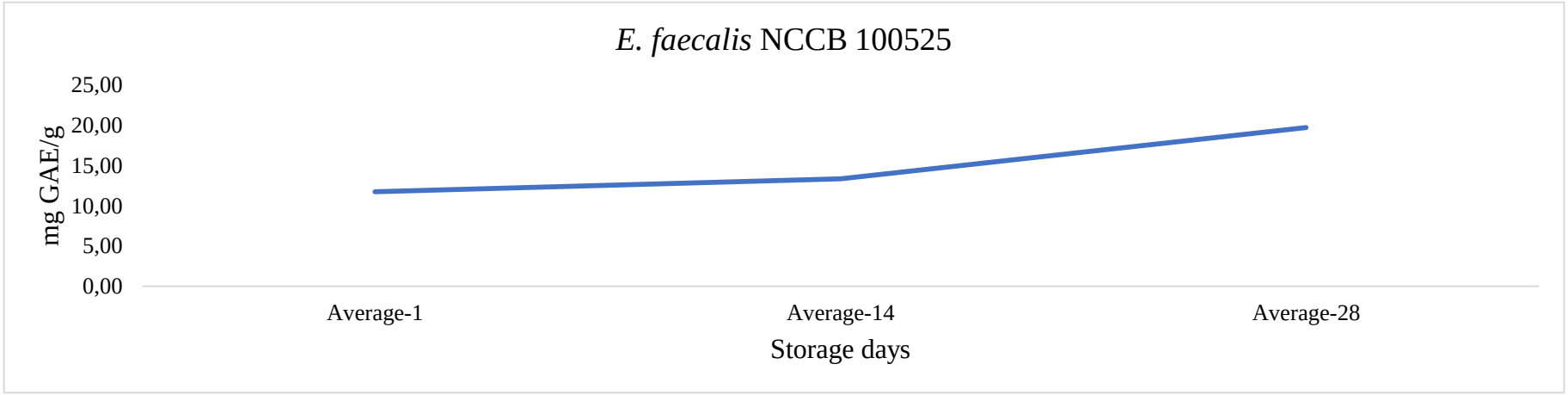
S. thermophilus K16



S. thermophilus 1-K4

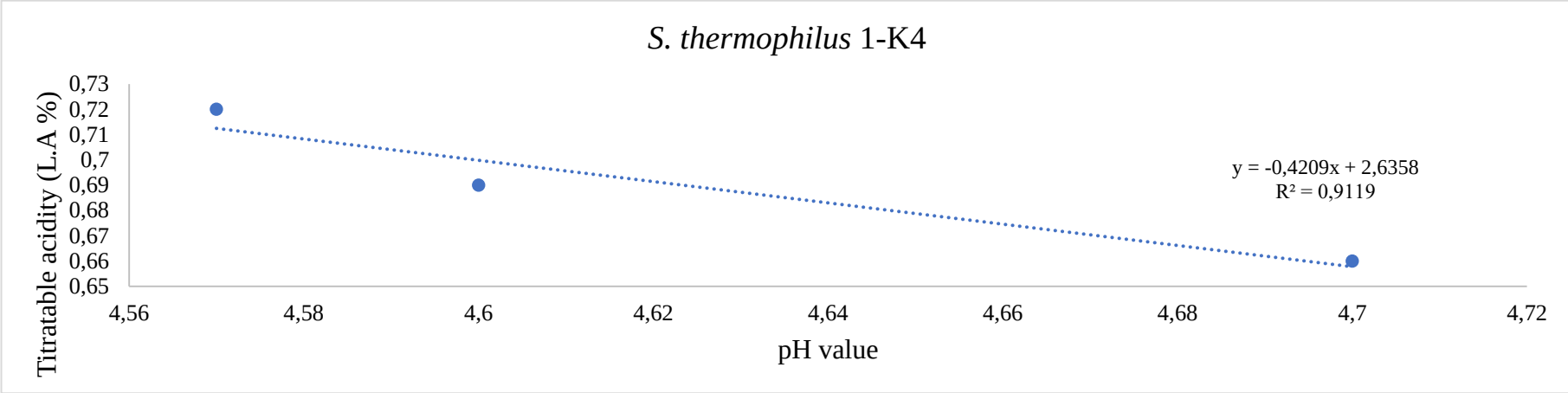


E. faecium NCCB 100527

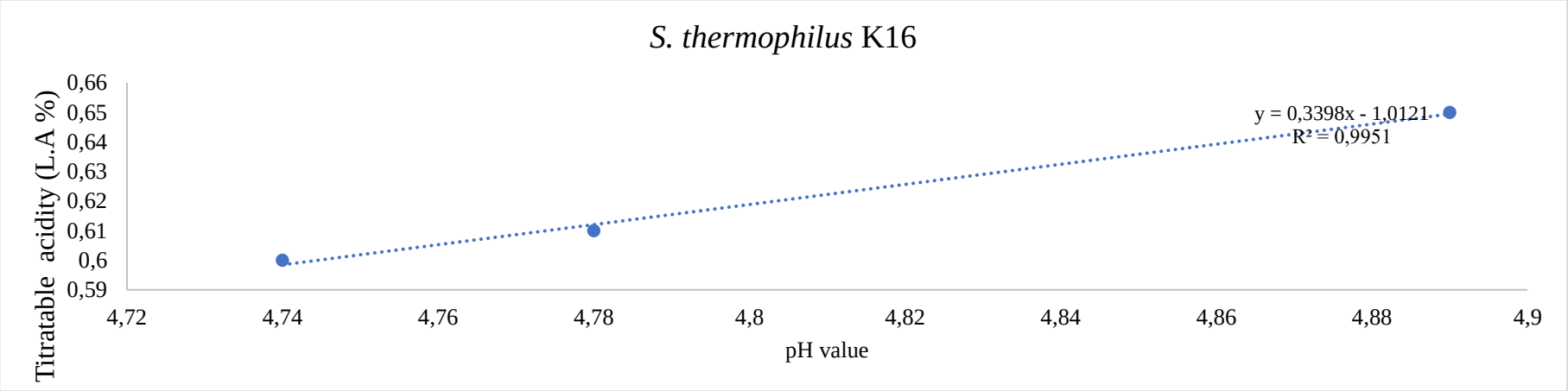


E. faecalis NCCB 100525

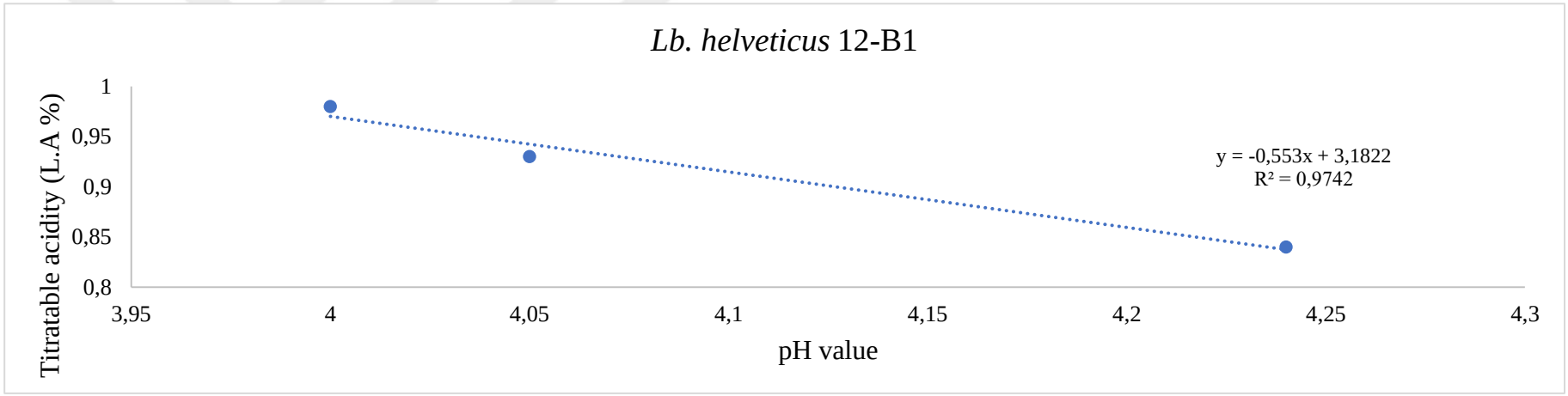
APPENDIX 4 SAMPLE PH VS TITRATABLE ACIDITY XY CURVE



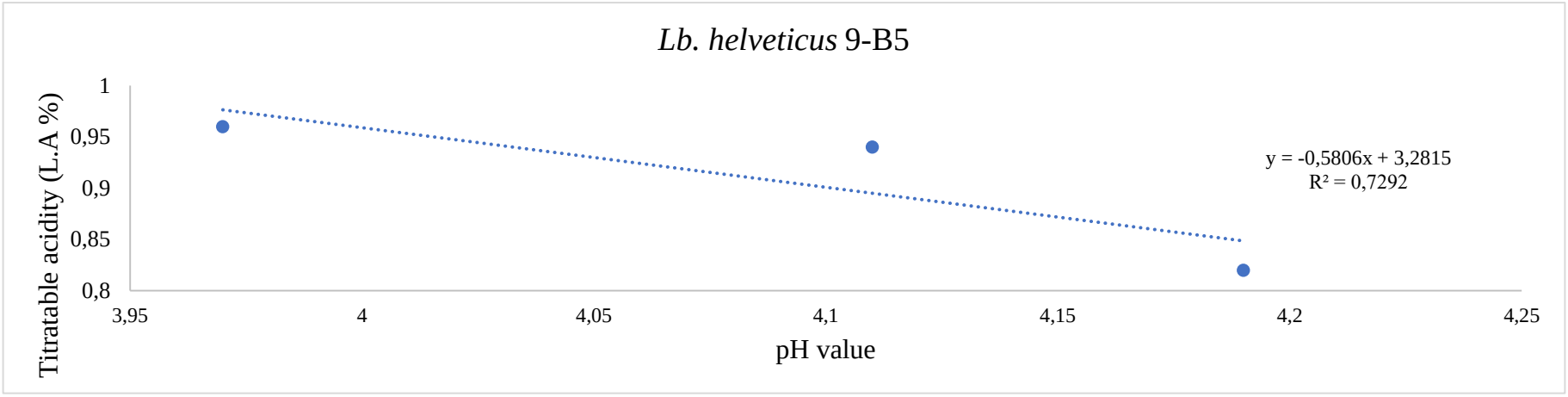
S. thermophilus 1-K4



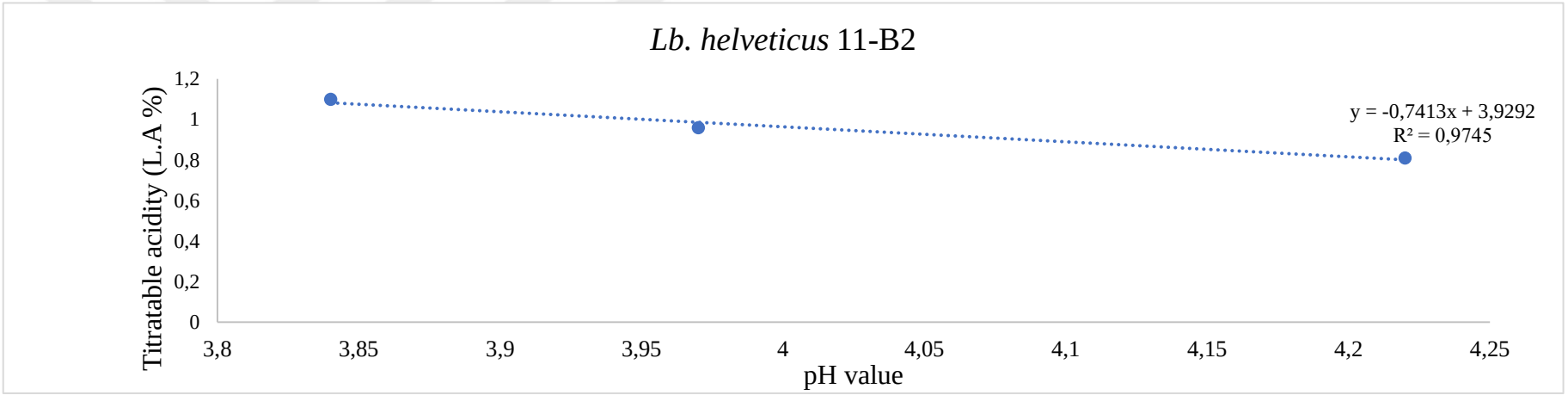
S. thermophilus K16



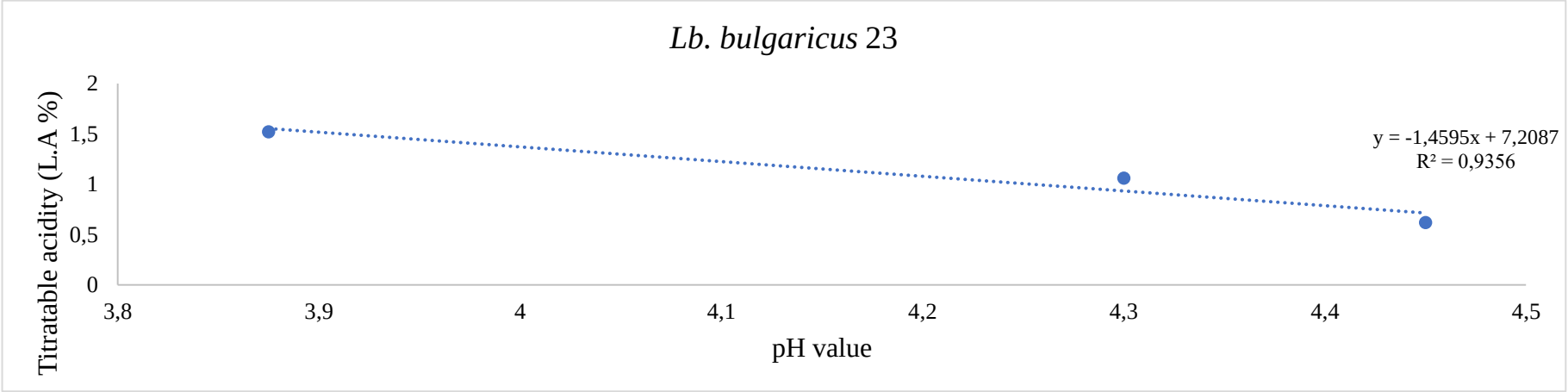
Lb. helveticus 12-B1



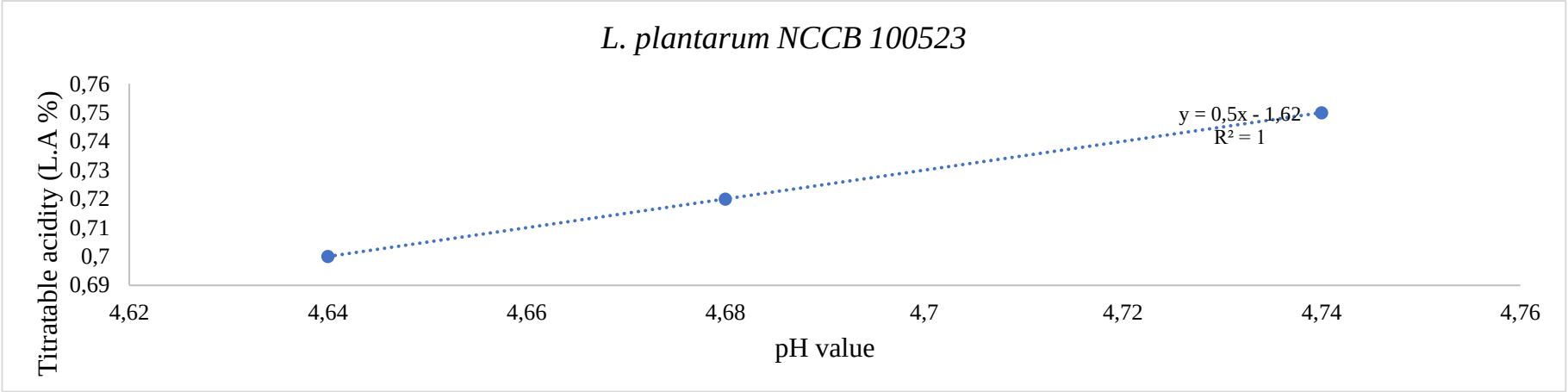
Lb. helveticus 9-B5 K4



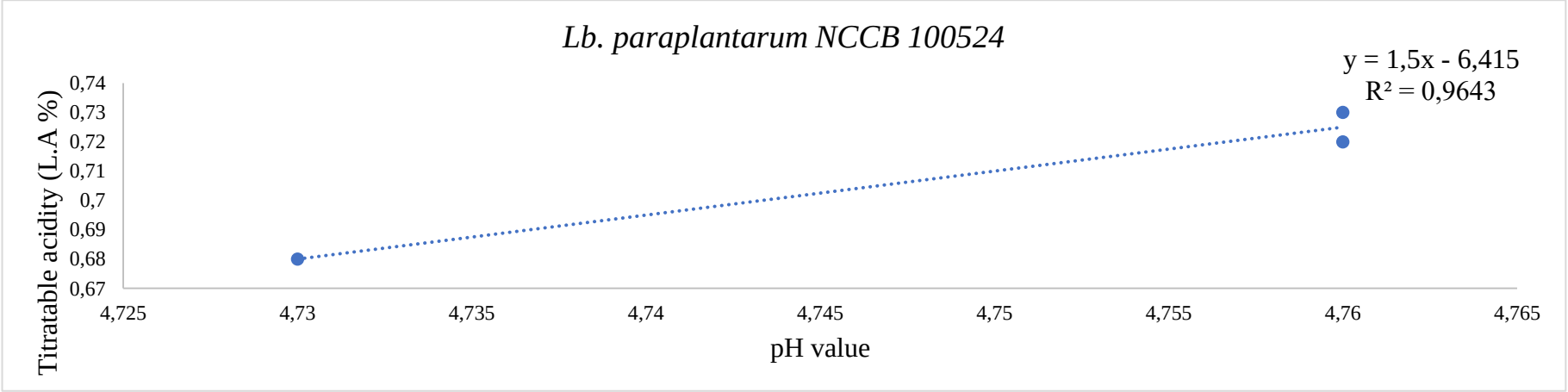
Lb. helveticus 11-B2



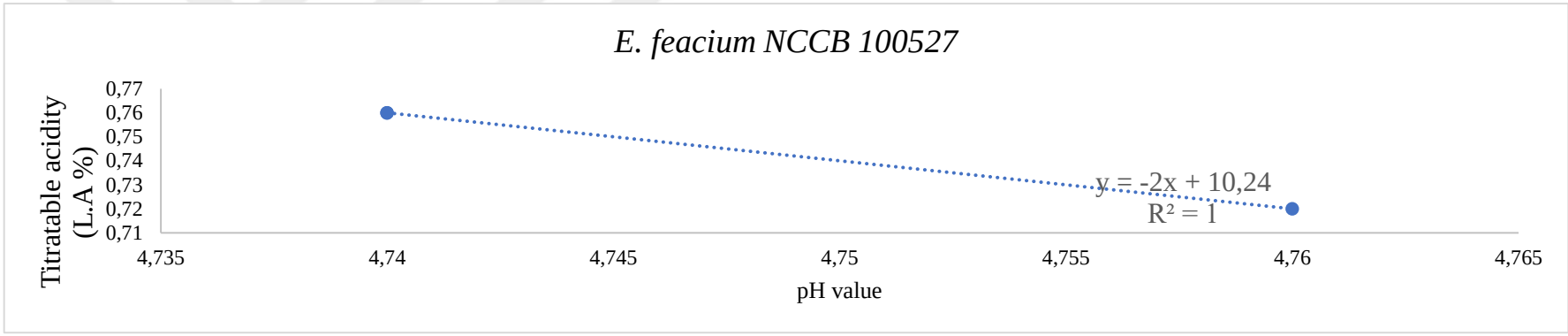
Lb. bulgaricus 23



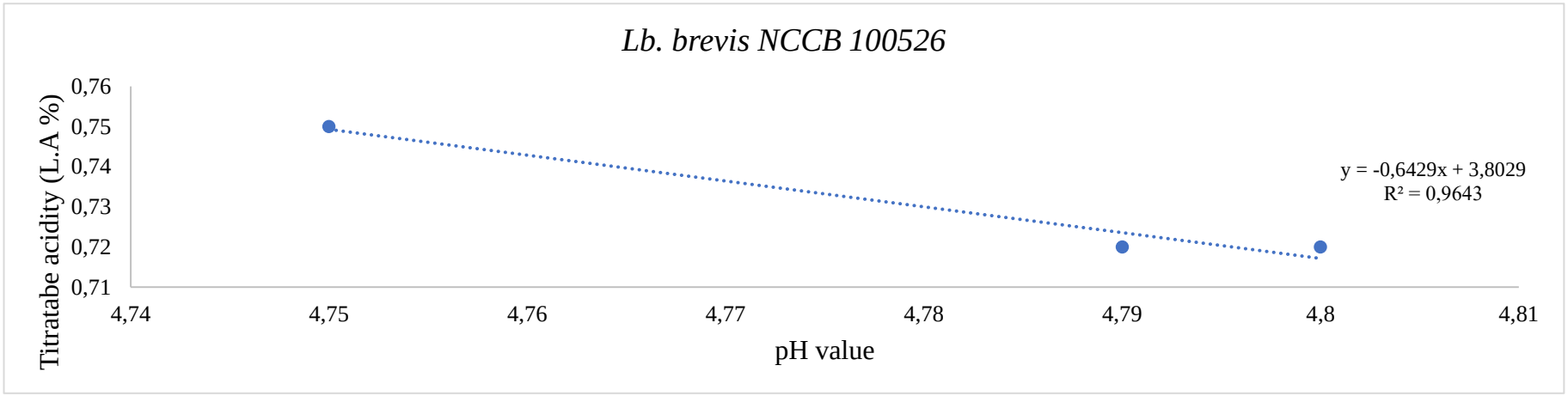
L. plantarum NCCB 100523



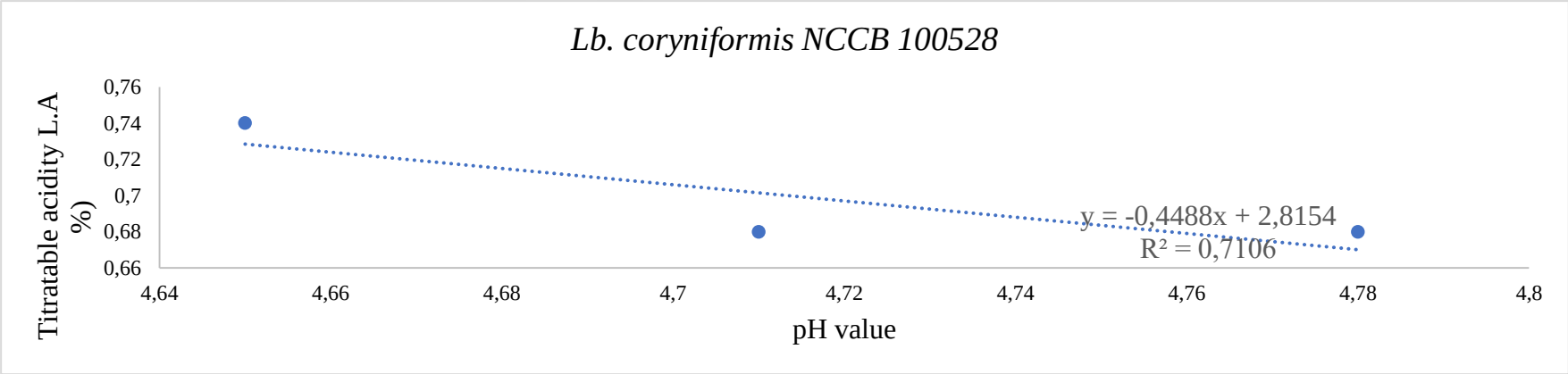
Lb. paraplantarum NCCB 100524



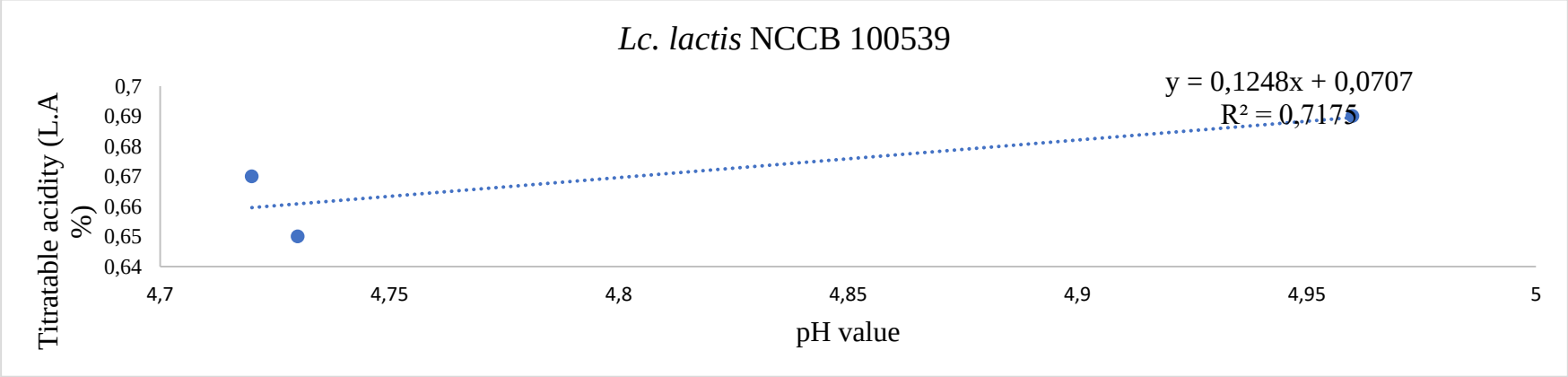
E. faecium NCCB 100527



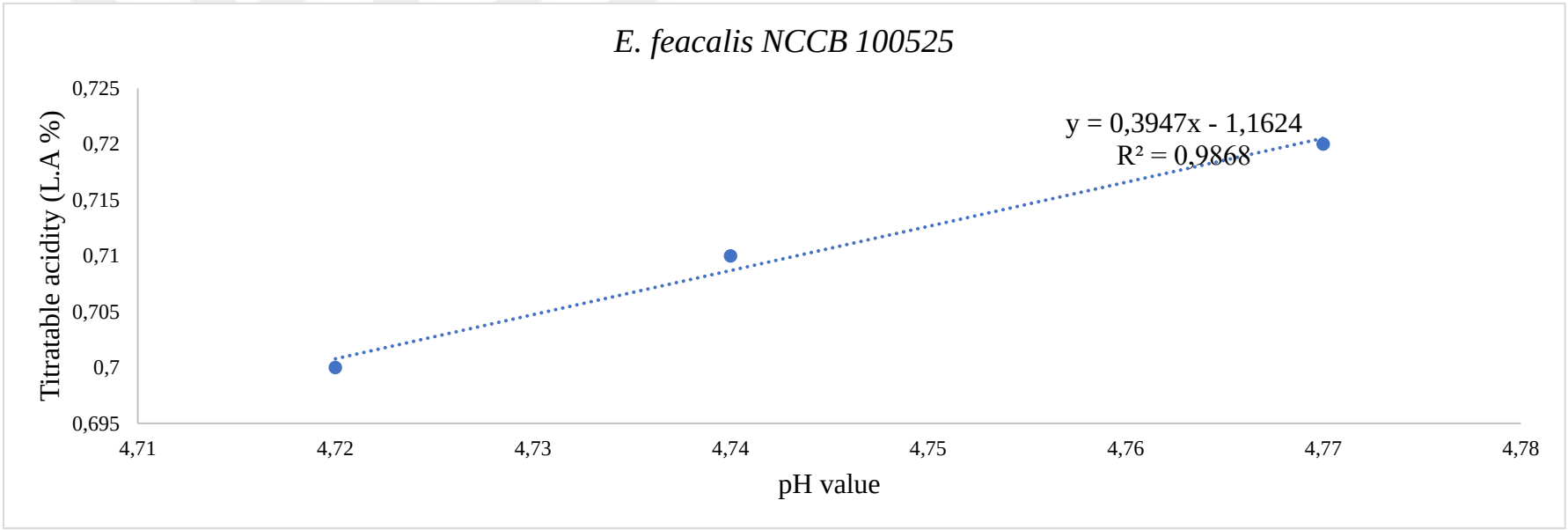
Lb. brevis NCCB 100526



Lb. coryniformis NCCB 100528



Lc. lactis NCCB 100539



E. faecalis NCCB 100525