DEPTHS OF SCIENCE

EDITOR
Cenk YÜCEL

AUTHORS
Ali Fazıl YENİDÜNYA
Aydemir BARIŞ
Betül AYDIN
Cenk YÜCEL
Meryem Burcu KÜLAHCI
Nebahat Aytuna ÇERÇİ
Neslihan BAL
Özgür KEBABCI
Pınar CAGLAYAN
Serap ÇETİNKAYA

İKSAD
Publishing House

DEPTHS OF SCIENCE

EDITOR

Cenk YÜCEL

AUTHORS

Ali Fazıl YENİDÜNYA

Aydemir BARIŞ

Betül AYDIN

Cenk YÜCEL

Meryem Burcu KÜLAHCI

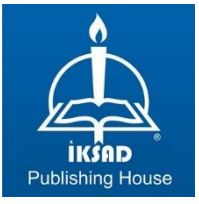
Nebahat Aytuna ÇERÇİ

Neslihan BAL

Özgür KEBABCI

Pınar CAGLAYAN

Serap ÇETİNKAYA



Copyright © 2022 by iksad publishing house
All rights reserved. No part of this publication may be reproduced, distributed or transmitted in any form or by
any means, including photocopying, recording or other electronic or mechanical methods, without the prior written permission of the publisher, except in the case of brief quotations embodied in critical reviews and certain other noncommercial uses permitted by copyright law. Institution of Economic Development and Social Researches Publications®
(The Licence Number of Publicator: 2014/31220)
TURKEY TR: +90 342 606 06 75
USA: +1 631 685 0 853
E mail: iksadyayinevi@gmail.com
www.iksadyayinevi.com

It is responsibility of the author to abide by the publishing ethics rules.
Iksad Publications – 2022©

ISBN: 978-625-8405-83-5
Cover Design: İbrahim KAYA
March / 2022
Ankara / Turkey
Size = 16x24 cm

CONTENTS

PREFACE

Dr. Cenk YÜCEL …… 1

CHAPTER 1

TURKISH CHRYSOMELIDAE SPECIS WORKED IN BOLD SYSTEM

Dr. Neslihan BAL Dr. Cenk YÜCEL & Dr. Aydemir BARIŞ……*3*

CHAPTER 2

INVESTIGATION OF ANTIMICROBIAL ACTIVITY DETERMINATION METHODS OF SYNTHESIZED NANOPARTICLES

Dr. Nebahat Aytuna ÇERÇİ & Res. Assist. Dr. Meryem Burcu KÜLAHCI..*11*

CHAPTER 3

AN IMPORTANT PEST IN SAINFOIN: *BEMBECIA SCOPIGERA* (SCOPOLI, 1763) (LEPIDOPTERA: SESIIDAE)

Dr. Aydemir BARIŞ & Dr. Cenk YÜCEL ……*27*

CHAPTER 4

CRISPR/CAS IMMUNE SYSTEM WITH ITS MECHANISM, APPLICATIONS AND ETHICS ASSESSMENT

Asst. Dr. Özgür KEBABCI & Prof. Dr. Ali Fazıl YENİDÜNYA……*39*

CHAPTER 5

POSTBIOTICS AND PARAPROBIOTICS: A NEW TREND FOR FUNCTIONAL FOODS

Res. Asst. Dr. Betül AYDIN ……*75*

CHAPTER 6

BIOLOGY OF NATURAL AND SYNTHETIC LANTIBIOTICS AND THEIR CURRENT APPLICATION STATUS

Prof. Dr. Ali Fazıl YENİDÜNYA & Dr. Serap ÇETİNKAYA ……*91*

CHAPTER 7

BACTERIA SHOWING HALOVERSATILE PROPERTIES IN HYPERSALINE ENVIRONMENTS

Assoc. Prof. Pınar CAĞLAYAN ……...…..*117*

PREFACE

Biological sciences aim at the study of life and living organisms, their life cycles, adaptations, and the environment. Our aim in preparing this book for publication is to bring together academic studies and findings in different fields of science and to transfer the results obtained from these studies to the world of science. Thus, it is aimed to contribute to the emergence of new ideas by referring to future studies. It is thought that this book, which consists of studies in interdisciplinary fields, will contribute to the development and studies of students, academicians and researchers. each of which has been contributed by highly qualified professionals in the respective fields of research.

I am proud to present this book, which contains recently updated information on various research areas and techniques in the biological sciences, which will benefit many researchers from different life science institutions around the world.I would like to thank with all my heart to all my professors who contributed to this meticulously prepared book, and to the valuable managers and employees of the **ISPEC Publishing Agency** family, who gave the opportunity to convey these carefully prepared studies to the readers.

With my regards

Dr. Cenk YÜCEL

CHAPTER 1

TURKISH CHRYSOMELIDAE SPECIS WORKED IN BOLD SYSTEM

Dr. Neslihan BAL[1], Dr. Cenk YÜCEL[2], Dr. Aydemir BARIŞ[2]

[1] Gazi University Faculty of Science, Department of Biology, Ankara, Türkiye, neslihansilkin@gmail.com,ORCID ID: 0000-0002-8122-7914

[2] Plant Protection Central Research Institute, Ankara, Türkiye. cenkyucel@gmail.com, Orcid ID: 0000-0001-5223-9257

[2] Plant Protection Central Research Institute, Ankara, Türkiye. aydemir.baris@tarimorman.gov.tr, Orcid ID: 0000-0003-3509-5700

INTRODUCTION

Insects are the most diverse creatures on earth, evolving in a variety of forms. It took taxonomists almost 200 years to describe only 10% of the species, and there are still many insect species waiting to be described. (Jalali et al., 2015). Species identification is an essential part of recognizing and describing biodiversity. With the growing interest in biodiversity in the fields of ecology, evolutionary biology and agriculture, reliable identification of species has become increasingly important. Traditionally, species identification has been based on morphological diagnoses. However, the number of taxonomists and other definition experts is gradually decreasing (Page 2016). With the developments in DNA sequencing technologies in recent years, it is possible to identify species accurately, quickly and easily. DNA barcoding, known as the DNA-based taxonomy method, is now widely used in the identification of species and the discovery of new species (Hajibabaei et al. 2016).

DNA barcoding can be defined as a taxonomic method that enables organisms to be identified using a short and standardized fragment of genomic DNA. The resulting standard DNA sequence is called the DNA barcode. In DNA barcoding technique, only the differences/similarities in the sequence of one or more gene regions are based, not the genome of the species, in order to reveal the unique nucleotide sequence of each species. The most commonly used gene regions for DNA barcoding are nuclear DNA (ITS), chloroplast DNA (rbcL, trnL-F, matK, psbA, trnH, psbK), and mitochondrial DNA (COI). The COI region, the standard DNA barcode region, is a mitochondrial gene and is very effective for species identification. This region has good discrimination power for most animal groups, so the COI gene has been identified by the International Barcode of Life Project team as the official barcode marker for animals (Hebert et al. 2003). The COI gene region is also widely used as an ideal species identification marker in entomology studies (Yazgın & Güz, 2018).

The first task of DNA barcoding is to associate the species names of sequences. (Floyd et al. 2009). These sequences from "correctly" identified individuals, summarized by external information and classical morphological methods, are then included in the reference barcode library the Life Barcode Data System (BOLD) (Ratnasingham and Hebert, 2007). Campaigns charged

with the goal of populating BOLD have embraced different approaches: large-scale regional inventories using freshly caught specimens (Janzen et al., 2009) or barcode "blitzes" of national parks (Xhou et al., 2011) or museum collections (BOLD, www.boldsystems.org).

Species diverge in ecology, morphology and behavior, as well as DNA sequences. Therefore, at least in principle, it can be used to describe a particular gene or gene part (fragment) in the same way that retail barcodes uniquely describe each consumer product. In practice, we do not expect DNA barcoding to work, so actual DNA sequences are subject to all the inherent complexities of molecular evolution and can vary significantly in species (Mallet and Willmott 2003). They are not systematically 'designated ' to individual assets such as retail barcodes. However, if successful, DNA barcoding, barcode by determining the sequence of the region, ultimately avoiding the complexities native in the morphological description and advocates the establishment of a viable system by encouraging the ability to automate the entire life promises to defend identification of samples. (Tautz et al. 2003, Blaxter 2004, Savolainen et al. 2005).

1.CHRYSOMELIDAE SPECIES WITH BARCODE STUDY

The order Coleoptera is the fourth most important insect order studied after Diptera (4.652.548), Lepidoptera (2.001.858) and Hymenoptera (1.677.466), with barcodes of 855,205 species among the insect orders. The most studied family among Coleoptera is Chrysomelidae with 93,600 specimens.

Bruchus hamatus Miller, 1881 and ***Bruchus libanensis*** Zampetti 1993 species belonging to the subfamily Bruchinae; ***Chrysolina reitteri*** (Weise, 1884), ***Leptinotarsa decemlineata*** (Say, 1824) ve ***Gonioctena sp.*** (2 examples from Antalya) species belonging to the subfamily Chrysomelinae; ***Cryptocephalus duplicatus*** Suffrian, 1845 (2 examples from Konya, Beyşehir, 2 examples specified as Turkey), ***Cryptocephalus paphlagonius*** (Sassi et Kismali, 2000)**,** ***Cryptocephalus virens*** **Suffrian, 1847** (2 examples specified as Turkey), ***Cryptocephalus albzorensis*** (2 examples from Antalya, 1 example from Kahramanmaraş), ***Cryptocephalus turcicus*** Suffrian, 1847 (1 example from Antalya), ***Cryptocephalus rugicollis*** Olivier, 1791 (2 examples from Balıkesir) species belonging to the subfamily Cryptocephalinae; ***Cassida***

rubiginosa Müller, O.F., 1776 (1 example from Antalya, 1 example from Balıkesir) species belonging to the subfamily Cassidinae; *Clytra* sp. (1 example from Uşak), ***Clytra atraphaxidis*** Pallas, 1773 (1 example from Kahramanmaraş), ***Smaragdina limbata*** (Steven, 1806) (1 example from Diyarbakır, 2 examples from Antalya, 1 example from Marmara), ***Labidostomis* sp**. (2 examples from Ankara, 2 examples from Antalya and 2 examples from Diyarbakır), ***Tituboea macropus*** (Illiger, 1800) species belonging to the subfamily Clytrinae***; Nymphius sp.*** (2 examples from Antalya and 2 examples from Diyarbakır) ve ***Exosoma sp.*** (4 examples from Muğla) species belonging to the subfamily Galerucinae; ***Chaetocnema concinna*** (Marsham, 1802) (1 example from Bartın), ***Chaetocnema coyei*** (Allard, 1863) (2 examples from Kayseri), ***Chaetocnema arenacea*** (Allard, 1860) (2 examples from Kayseri), ***Chaetocnema tibialis*** (Illiger, 1807) (2 examples from Kayseri, 1 example from Zonguldak, 1 example from Bartın and 1 example from Ankara), ***Psylliodes sp.***, ***Phyllotreta atra*** (Fabricius, 1775) 2 examples from Zonguldak and 2 examples from Bartın'dan), ***Phyllotreta astrachanica*** Lopatin, 1977 (1 example from Zonguldak), ***Phyllotreta corrugata*** Reiche, 1858 (1 example from Şanlıurfa), ***Phyllotreta cruciferae*** (Goeze, 1777) (4 examples from Kayseri and 3 examples from Ankara), ***Phyllotreta diademata*** Foudras, 1860 (1 example from Bartın), ***Phyllotreta erysimi erysimi*** Weise, 1900 (1 example from Ankara), ***Phyllotreta nemorum*** (Linnaeus, 1758) (1 example from Bartın and 1 example from Zonguldak), ***Phyllotreta nigripes*** (Fabricius, 1775) (6 examples from Ankara and 3 examples from Kayseri), ***Phyllotreta pallidipennis*** Reitter, 1891 (1 example from Kayseri), ***Phyllotreta procera*** (Redtenbacher, 1849) (3 examples from Kayseri), ***Phyllotreta striolata*** (Illiger, 1803) (2 examples from Zonguldak), ***Phyllotreta undulata*** Kutschera, 1860 (1 example from Bartın), ***Phyllotreta variipennis*** (Boieldieu, 1859) (1 example from Bartın and 1 example from Zonguldak) species belonging to the subfamily Alticinae Barcodes of a total of 83 samples belonging to 7 subfamilies from our country were entered into the system (BOLD, www.boldsystems.org). Species were collected from Ankara, Antalya, Balıkesir, Bartın, Diyarbakır, Kahramanmaraş, Kayseri, Kahramanmaraş, Konya, Muğla, Şanlıurfa, Uşak and Zonguldak provinces. In terms of such studies, there are quite large gaps

in our country. Studies in this direction are very important and necessary for our country.

REFERENCES

Blaxter, M. L. 2004. The promise of a DNA taxonomy. Philosophical Transactions of the Royal Society. B. Biological Sciences 359: 669–679.

Floyd R, Wilson JJ, Hebert PDN (2009) DNA barcodes and insect biodiversity. In: Footit RG, Adler PH (eds) Insect biodiversity: science and society. Blackwell Publishing, Oxford, pp 417–431.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proc Natl Acad Sci USA 103: 968-971.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003a. Biological identifications through DNA barcodes. Proceedings of the Royal Society. B. Biological Sciences 270: 313–321.

Jalali, S. K., Ojha, R. & Venkatesan, T. (2015). DNA Barcoding for Identification of Agriculturally Important Insects, A. K. Chakravarthy (ed.), New Horizons in Insect Science: Towards Sustainable Pest Management, DOI 10.1007/978-81-322-2089-3_2.

Janzen DH, Hajibabaei M, Burns JM et al (2005) Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. Phil Trans R Soc Lond B 360:1835–1845.

Mallet, J. and K. Willmott. 2003. Taxonomy: renaissance or Tower of Babel? Trends in Ecology and Evolution 18: 57–59.

Ratnasingham S, Hebert PDN (2007) BOLD: The barcode of life data system (www.barcod inglife.org). Mol Ecol Notes 7: 355–364.

Savolainen, V., R. S. Cowan, A. P. Vogler, G. K. Roderick, and R. Lane. 2005. Towards writing the encyclopedia of life: an introduction to DNA barcoding. Philosophical Transactions of the Royal Society. B. Biological Sciences 360: 1805–1811.

Tautz, D., P. Arctander, A. Minelli, R. H. Thomas, and A. P. Vogler. 2003. A plea for DNA taxonomy. Trends in Ecology and Evolution 18: 70–74.

www.boldsystems.org

Xhou X, Robinson JL, Geraci CJ, Parker CR et al (2011) Accelerated construction of a regional DNA-barcode reference library: caddisfl ies

(Trichoptera) in the Great Smoky Mountains National Park. J Nor Amer Benth Soc 30: 131–162.

Yatkın, G. & Güz, N. (2018). Entomolojide DNA Barkodlama Tekniğinin Kullanımı, YYÜ Tar Bil Derg (YYU J AGR SCI), 28(1): 126-134.

CHAPTER 2

INVESTIGATION OF ANTIMICROBIAL ACTIVITY DETERMINATION METHODS OF SYNTHESIZED NANOPARTICLES

Dr. Nebahat Aytuna ÇERÇİ[1]
Res. Assist. Dr. Meryem Burcu KÜLAHCI[2]

[1]Kırıkkale University, Scientific and Technological Research Application and Research Center, Kırıkkale, Turkey. aytunacerci@gmail.com, ORCID ID 0000-0002-7864-7213
[2]Gazi University, Department of Science, Biology, Ankara, Turkey. meryemburcu@gazi.edu.tr, ORCID ID 0000-0002-5007-5209

INTRODUCTION

In recent years, research and development studies of biomaterials in many different structures, from macrostructures to micro and nano levels, have led to the expansion of the field of nanotechnology in order to prolong human life and improve the quality of life. Nanotechnology is frequently encountered as an important area of innovative, scientific, and economic growth, with sustainable bio-based products being obtained from recyclable and biodegradable sources with commercial viability and environmental acceptability. The use of nanotechnology in the health and pharmaceutical industries has had a significant impact on the development of new systems and technologies that can interact with the body at the cellular level. To take advantage of the physical, chemical, and biological features of materials at the nanometric scale (1-100 nm), new nanotherapeutic systems have been created (Gopi et al., 2020; Shi et al., 2016).

Nanomaterials are receiving serious attention in various researches due to their size and unique properties because nanomaterials show classical mechanical properties rather than quantum mechanics. Nanomaterials are used in many biotechnological applications, including magnetic separation of biomolecules, bio labeling with fluorescent quantum dots, gene delivery, drug encapsulation, targeted and controlled drug delivery systems, bioimaging with the help of nanoparticles (Majeed et al., 2019). Nanomaterials are structures smaller than 1 micrometer created using nanoparticles. Nanoparticles (NPs), with at least one size less than 1 micrometer and potentially as small as atomic and molecular length scales (about 0.2 nm), their surfaces can operate as transporters for liquid droplets or gases, and they can be amorphous or crystalline. Nanoparticles can be classified according to their morphology, dimensionality, composition, uniformity, and agglomeration, as shown in Figure 1 (Buzea et al., 2007).

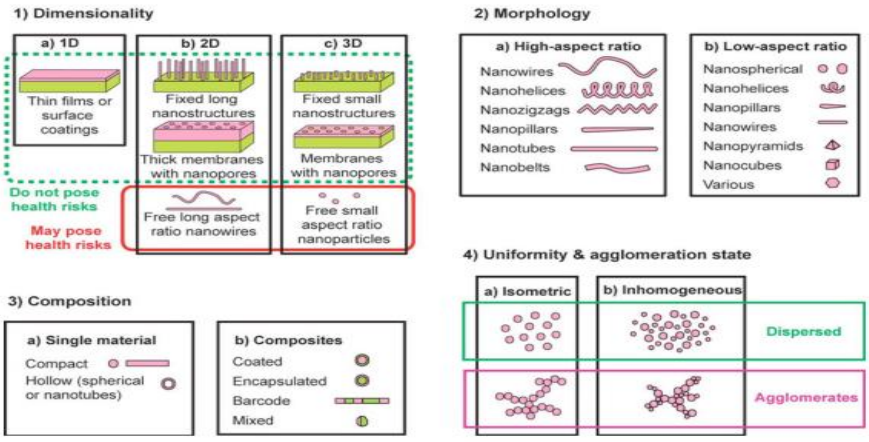


Figure 1. Nanoparticle Classification (Buzea et al., 2007).

Nanomaterials are synthesized by breaking down various materials into nanoparticles by the top-down method or by combining particles at the molecular or atomic level, with various methods using the bottom-up method (Baig et al., 2021). Nanomaterials can be produced from many different materials, especially organic or inorganic materials, polymers, and hybrid materials.

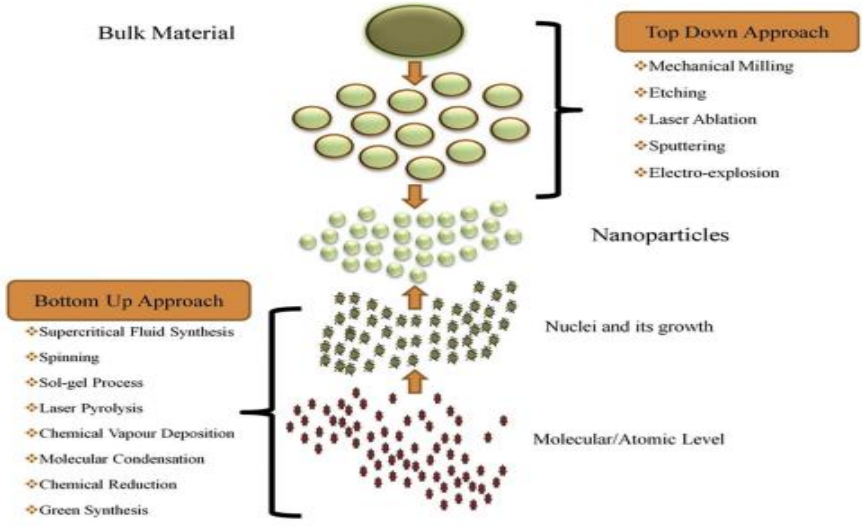


Figure 2. The Synthesis Types of Nanomaterials (Baig et al., 2021).

Microorganism infections and antibiotic resistance threaten human health and health economies all over the world. This situation leads researchers to explore alternative antimicrobial agents. Recently, bacteriophage therapy, peptide antibiotics, medicinal honey application, plant extracts, essential oils, and antimicrobial metals as well as developing nanoparticle-based materials are promising for alternative antimicrobial therapy applications (Edwards-Jones, 2013).

Nanomaterials can kill microorganisms by a variety of mechanisms, including direct cell wall or membrane destruction, the formation of reactive oxygen species (ROS), and adhering to intracellular components, and also acting as nanocarriers for treatment compounds. With their unique physicochemical properties, nanomaterials can interact with bacteria using Van der Waals forces, receptor-ligand interactions, hydrophobic interactions, and electrostatic attraction (Makabenta et al., 2021).

There are many different types of nanomaterials used in antimicrobial research, examples of which are carbon-based nanomaterials, metal-based nanoparticles, nanocomposites, nanoemulsions, polymers, liposomes, and smart nanomaterials. Metal-based nanoparticles are composed of metals of their purest form (aluminum, copper, titanium, gold, silver, zinc, etc.) or their compounds (oxides). The most commonly studied nanomaterials with good antimicrobial properties are nanomaterials containing silver and its oxides. Carbon-based nanomaterials include carbon quantum dots, nanotubes, and graphene-based two-dimensional materials. It has been determined that natural or synthetic polymers have antimicrobial properties in the polymer group, which includes polymeric micelles, dendrimers, glycopeptide dendrimers (Makabenta et al., 2021). It has been reported that nanocomposites obtained by hybridizing inorganic and organic nanoparticles have antimicrobial properties (Mei et al., 2014). Nanoemulsions are emulsions consisting of hydrophobic and aqueous layers to increase solubility and stability by encapsulating bioactive compounds such as plant extracts, essential oils, which are determined to have antimicrobial properties and antibiotics (Jaiswal et al., 2015). Liposomes are vesicles composed of an aqueous inner core and one or more layers of phospholipids, and are materials that are capable of entrapping hydrophilic and hydrophobic antimicrobial substances with good biocompatibility (Hsin-I Chang and Ming-Kung Yeh,

2012). Smart nanomaterials respond to endogenous stimuli such as pH and bacterial toxins to release their antimicrobial components into the environment, as well as external stimuli such as light, temperature and ultrasound (Chen et al., 2019).

The aim of this book chapter is to outline all existing antimicrobial testing methodologies for synthesized nanoparticles and to provide a vision for future researchers in this subject.

1. ANTIMICROBIAL ACTIVITY DETERMINATION METHODS

There are many methods in the literature using solid and liquid media or spectrophotometric measurements to evaluate the antimicrobial activities of the obtained nanoparticles. It is aimed to obtain the most accurate result by choosing the method or methods to be used depending on the microorganism feature. Parameters such as the zone of inhibition (ZOI), colony count, optical densities of the microorganism culture, minimum inhibitory concentration (MIC) are examined in the methods used in the literature to determine antimicrobial activity (Kumar et al., 2017).

1.1. Disk-Diffusion Method

In this method; microorganisms from fresh cultures whose concentrations are adjusted to the 0.5 MacFarland turbidity standard are used. Mueller Hinton Agar is used for bacteria, and Mueller Hinton Agar with 0.5µg/mL methylene blue and 2% glucose added for yeasts. The prepared inoculum is inoculated on Mueller Hinton Agar with a swab and placed on the agar surface of the nanoparticles of the desired concentration, which are adsorbed on empty discs and left for incubation. Zone diameters formed after incubation are measured and recorded (Image 1) (Balouiri, Sadiki, and Ibnsouda 2016). In a study, the researchers carried out the antimicrobial activity studies of the synthesized TiO_2 nanoparticles by disc diffusion method; It was evaluated against bacterial and fungal pathogens; *Escherichia coli* (E. coli) (MTCC-1677), *Bacillus subtilis* (*B. subtilis*) (ATCC 6051), *Pseudomonas aeruginosa* (*P. aeruginosa*) (MTCC-4030), *Enterococcus faecalis* (*E. faecalis*) (ATCC 2912), *Staphylococcus aureus* (*S. aureus*) (MTCC-3160), *Klebsiella pneumonia* (*K. pneumonia*) (NCTC 9633),

Aspergillus niger (*A. niger*) (ATCC 1015), *Aspergillus flavus* (*A. flavus*) (ATCC 10124), *Sclerotium rolfsii* (*S. rolfsii*) (ATCC 62666) and *Rhizopus oryzae* (*R. oryzae*) (ATCC 24563). Researchers have carried out microdilution method studies based on the high zone diameters obtained by the disc diffusion method. However, they determined that the concentration of 40µg/ml, where they had a high zone diameter on the bacteria they carried out, had an effect in the range of 41-59% in their MIC studies (Anbumani et al., 2022). The disk diffusion test is particularly applicable to antibacterial coatings and films, as such materials have been reported to show poor efficacy in the MIC test (Cioffi & Rai, 2012).

1.2. Well Diffusion Method

This method is in principle the same as the disk diffusion method. Unlike the disc diffusion method, the desired concentrations of nanoparticles are placed in wells drilled on agar instead of being absorbed into blank paper discs (Image 1) (Balouiri et al., 2016; Deepakumari et al., 2022; Goyal et al., 2022). In a study in which Zirconium phosphate nanoparticles were synthesized by solution combustion method, researchers used the well diffusion method to analyze the antimicrobial activities of nanoparticles against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *K. pneumoniae.* As a result of the research, they found that various concentrations of ZrP_2O_7 nanoparticles showed moderate to good inhibitory effect against all strains tested. In general, the effectiveness of inhibition of bacterial growth primarily depends on particle size, morphology, and surface area. They reported that electrostatic interactions had a significant effect on inhibitory activity (Deepakumari et al., 2022).

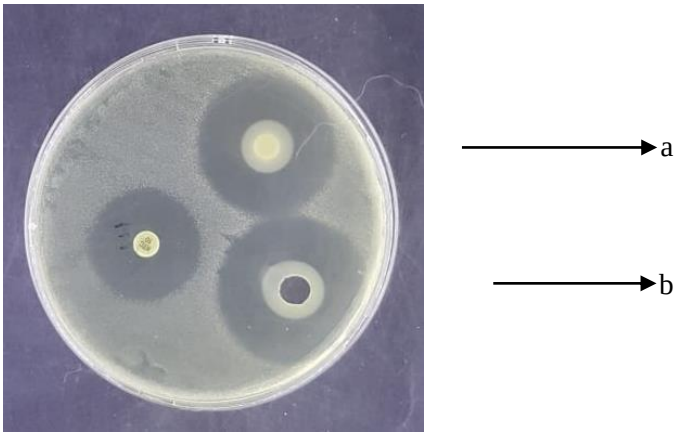


Image 1. Disk Diffusion and Well Diffusion Methods Demonstration of a Chitosan-TPP Nanoparticle Synthesized by N. Aytuna ÇERÇİ. a) Nanoparticle Impregnated on Empty Disk , b) Nanoparticle Placed in the Well.

1.3. Microdilution Method

In antimicrobial activity experiments with the microdilution method; Concentrations of fresh 24-hour cultures of microorganisms are adjusted according to the 0.5 MacFarland turbidity standard and dilutions are made to a final concentration of 10^5 cfu/ml. The inoculum obtained is added to all the wells and sowing is performed at the desired concentration. The nanoparticles dissolved with the appropriate solvent are applied to the wells at desired concentrations. Only wells with the medium are reserved for sterility control and only wells with inoculum for bacterial growth assessment. After all, applications are done, the prepared microplates are placed in a locked bag and left for incubation in a humid environment. Visual evaluation is made to determine the minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungal concentration (MFC) values, and the MIC value is determined according to the turbidity in the wells. After visual evaluation, MBC and MFC are determined by taking 10 µl from each well of the microplates and inoculating them on tryptic soy agar for bacteria and sabouraud dextrose agar for yeast (Image 2) (Balouiri et al. 2016; Zhang et al. 2020).

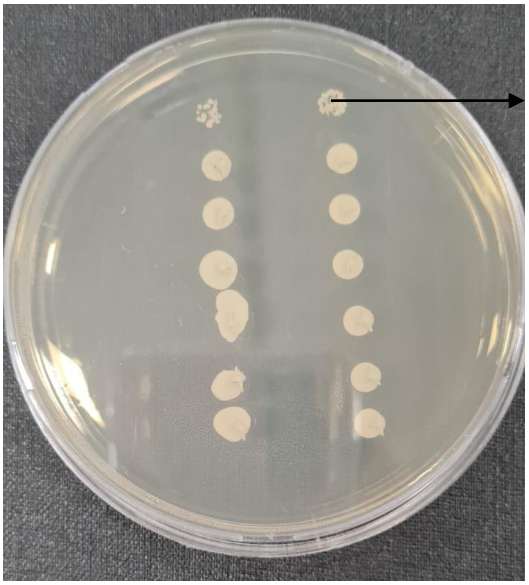


Image 2. MIC Determination of a Synthesized Nanoparticle Demonstrated by M. Burcu KÜLAHCI

1.4. Optical Density Measurement in Liquid Broth Medium

This method works with the same principle as the microdilution method. It can be performed on a micro or macro scale. The turbidity created by the microorganisms in the medium during the incubation period is essential in this method. The reduction rates of microorganisms are determined by comparing the optical densities of the media where nanoparticles and microorganisms are together and only microorganisms are present, at certain intervals during the 24-hour incubation period or at 600 nm in the spectrophotometer as a result of the 24-hour incubation period (Baldelli et al. 2022; Kumar et al. 2017). A group of researchers, encapsulating tetraaminocyclotriphosphazene derivatives into chitosan nanoparticles, studied their antimicrobial activity with disc diffusion and minimum inhibition concentration methods; Streptococcus mutans ATCC 25175, methicillin-resistant Staphylococcus aureus (MRSA) ATCC 43300, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Bacillus cereus microorganisms. They determined that the compounds they synthesized affect microorganisms by the disk diffusion method, but they could not get results from the disk diffusion method in their work with

encapsulated compounds. On the other hand, in optical density studies, they reported that encapsulated compounds showed antimicrobial activity in the range of 39-81% against microorganisms (Bozkurt et al., 2021).

1.5. Colony-Forming Units Measurement

The technique is used to count the number of viable bacterial cells. 10 microorganisms are inoculated on Plate Count Agar medium with a drigalski stick or microorganisms are added during the preparation of the agar. Similarly, while preparing agar, nanoparticles are added to the medium at the desired concentration. In another form of application, nanoparticles and microorganisms are interacted at desired times and then spread on the medium. Whichever of these treatments is chosen, colonies formed on the agar after incubation are counted. For bacterial strains incubated in the presence and absence of nanoparticles, the comparison is made by counting the CFUs. (Zhang et al., 2020).

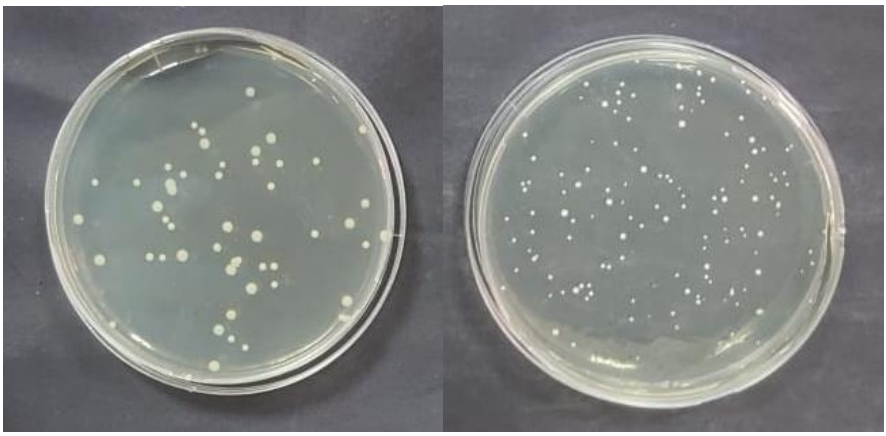


Image 3. Demonstration of Colony Forming Units Measurement Method by N. Aytuna ÇERÇİ and M. Burcu KÜLAHCI on Plate Count Agar with *Escherichia coli* (Left) and *Staphylococcus aureus* (Right).

In addition to all these known methods, there are various standard methods offered by authorities such as ASTM, ISO, EUCAST, and CLSI. By modifying these standards, it is possible to determine the antimicrobial activities of nanoparticles or nanomaterials. The ASTM 2149 standard was published to predict the antimicrobial activity of antimicrobially treated

materials under dynamic interaction conditions. It is based on Cfu, but the effectiveness of a customized non-leaching antimicrobial agent depends on ensuring direct contact of microorganisms with the active ingredient. This test allows the antimicrobial activity of the sample to be determined by agitating samples of surface bound materials in a suspension of bacteria for one hour or modified longer contact times. The suspension is serially diluted and cultured before and after contact. The number of organisms recovered from the suspension is determined and the percentage reduction is calculated by comparing the numbers of living organisms from the controls (ASTM International, 2020). Another ASTM standard, E2315 is based on the principle of measuring the changes in aerobic microorganism density of antimicrobial materials over a period of time. It offers several options for the selection of microorganisms to be studied, incubation time and temperature, sampling times, and conditions. Therefore, it provides a wide range of studies in the antimicrobial activity evaluation studies of nanoparticles or nanomaterials. The methods to be followed in the evaluation may vary depending on the areas in which the nanoparticles will be used for antimicrobial activity evaluation or the way of use. In the evaluation of a nanoparticle to be applied in the medical sector, antimicrobial activity can be evaluated by modifying the ISO 23650:2021, ISO 11930:2019, ASTM E2149, or E2315, CLSI, and EUCAST standards based on the final product (CLSI, 2022; EUCAST, 2022)

CONCLUSION

Nanoparticles have exclusive physical and chemical properties due to their shape, size, large surface area, optical properties, and reactivity. Thanks to these features, they can be diversified, as well as medical applications of nanoparticles; They are suitable candidates for a variety of commercial and individual applications, including the materials and manufacturing industry, electronics and computer technologies, the medical and healthcare industry, aerospace research, the defense industry, biotechnology, agriculture and food, imaging and environmental applications.

As a result, nanoparticles with versatile morphologies can be efficiently used as antibacterial agents against various types of microorganisms through chemical and physical interactions. The morphological features of

nanoparticles, their surface areas, their concentrations, the microorganism species for which antimicrobial activity is tested, the selected method, and cell properties are the main factors affecting biocidal and fungicidal activities. In addition to chemical interactions such as the antimicrobial action mechanism of the tested nanoparticle, the physical interactions in the experimental environment should also be taken into account in the method to be determined.

REFERENCES

Anbumani, D., Dhandapani, K. vizhi, Manoharan, J., Babujanarthanam, R., Bashir, A. K. H., Muthusamy, K., Alfarhan, A., & Kanimozhi, K. (2022). Green synthesis and antimicrobial efficacy of titanium dioxide nanoparticles using Luffa acutangula leaf extract. *Journal of King Saud University - Science*, *34*(3), 101896. https://doi.org/10.1016/j.jksus.2022.101896

ASTM International. (2020). *Standard Test Method for Determining the Antimicrobial Activity of Antimicrobial Agents Under Dynamic Contact Conditions*. https://www.astm.org/

Baig, N., Kammakakam, I., Falath, W., & Kammakakam, I. (2021). Nanomaterials: A review of synthesis methods, properties, recent progress, and challenges. *Materials Advances*, *2*(6), 1821–1871. https://doi.org/10.1039/d0ma00807a

Baldelli, A., Etayash, H., Oguzlu, H., Mandal, R., Jiang, F., Hancock, R. E. W., & Pratap-Singh, A. (2022). Antimicrobial properties of spray-dried cellulose nanocrystals and metal oxide-based nanoparticles-in-microspheres. *Chemical Engineering Journal Advances*, *10*, 100273. https://doi.org/10.1016/j.ceja.2022.100273

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Bozkurt, B., Elmas, G., Yakut, M., Okumuş, A., Çerçi, N. A., Zeyrek, C. T., Kılıç, Z., Açık, L., & Hökelek, T. (2021). Phosphorus- nitrogen compounds part 59. The syntheses of tetrachloro and tetraamino- 2-pyridylmethylspiro(N/N)ethylenediaminocyclotriphosphazenes: Structural characterization, bioactivity, and molecular docking studies. *Journal of the Chinese Chemical Society*, *December*, 1–22. https://doi.org/10.1002/jccs.202100475

Buzea, C., Pacheco, I. I., & Robbie, K. (2007). Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases*, *2*(4), MR17–MR71. https://doi.org/10.1116/1.2815690

Chen, M., Wei, J., Xie, S., Tao, X., Zhang, Z., Ran, P., & Li, X. (2019). Bacterial biofilm destruction by size/surface charge-adaptive micelles. *Nanoscale*, *11*(3), 1410–1422. https://doi.org/10.1039/c8nr05575k

Cioffi, N., & Rai, M. (2012). *Nano-Antimicrobials_ Progress and Prospects-Springer-Verlag Berlin Heidelberg (2012)*. https://doi.org/10.1007/978-3-642-24428-5

CLSI. (2022). *Performance Standards for Antimicrobial Susceptibility Testing*. https://clsi.org/standards/products/microbiology/

Deepakumari, H. N., Ranganatha, V. L., Nagaraju, G., Prakruthi, R., & Mallikarjunaswamy, C. (2022). Materials Today : Proceedings Facile green synthesis of zirconium phosphate nanoparticles using Aegle marmelos : Antimicrobial and photodegradation studies. *Materials Today: Proceedings*, *xxxx*. https://doi.org/10.1016/j.matpr.2022.02.579

Edwards-Jones, V. (2013). Alternative Antimicrobial Approaches to Fighting Multidrug-Resistant Infections. In *Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and their Components*. Elsevier. https://doi.org/10.1016/B978-0-12-398539-2.00001-X

EUCAST. (2022). *European Committee on Antimicrobial Susceptibility Testing*. http://www.eucast.org/

Gopi, S., Thomas, S., & Pıus, A. (2020). *Handbook of Chitin and Chitosan Volume 1: Preparation and Properties*.

Goyal, V., Singh, A., Singh, J., Kaur, H., Kumar, S., & Rawat, M. (2022). Biogenically structural and morphological engineering of Trigonella foenum-graecum mediated SnO2 nanoparticles with enhanced photocatalytic and antimicrobial activities. *Materials Chemistry and Physics*, 125946. https://doi.org/10.1016/j.matchemphys.2022.125946

Hsin-I Chang and Ming-Kung Yeh. (2012). Clinical Dev Liposome. *Int. J. Nanomed.*, *7*, 49–60. http://dx.doi.org/10.2147/IJN.S26766

Jaiswal, M., Dudhe, R., & Sharma, P. K. (2015). Nanoemulsion: an advanced mode of drug delivery system. *3 Biotech*, *5*(2), 123–127. https://doi.org/10.1007/s13205-014-0214-0

Kumar, R., Umar, A., Kumar, G., & Nalwa, H. S. (2017). Antimicrobial properties of ZnO nanomaterials: A review. *Ceramics International*, *43*(5), 3940–3961. https://doi.org/10.1016/j.ceramint.2016.12.062

Majeed, M. I., Bhatti, H. N., Nawaz, H., & Kashif, M. (2019). Nanobiotechnology: Applications of nanomaterials in biological research. In *Integrating Green Chemistry and Sustainable Engineering* (Issue March). https://doi.org/10.1002/9781119509868.ch17

Makabenta, J. M. V., Nabawy, A., Li, C. H., Schmidt-Malan, S., Patel, R., & Rotello, V. M. (2021). Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections. *Nature Reviews Microbiology*, *19*(1), 23–36. https://doi.org/10.1038/s41579-020-0420-1

Mei, L., Lu, Z., Zhang, X., Li, C., & Jia, Y. (2014). *against Bacterial Infection*.

Shi, J., Kantoff, P. W., Wooster, R., & Farokhzad, O. C. (2016). Cancer nanomedicine : progress , challenges and opportunities. *Nature Publishing Group*, *17*, 20–37. https://doi.org/10.1038/nrc.2016.108

Zhang, J., Cao, C., Zheng, S., Li, W., Li, B., & Xie, X. (2020). Poly (butylene adipate-co-terephthalate)/magnesium oxide/silver ternary composite biofilms for food packaging application. *Food Packaging and Shelf Life*, *24*(February), 100487. https://doi.org/10.1016/j.fpsl.2020.100487

CHAPTER 3

AN IMPORTANT PEST IN SAINFOIN: *BEMBECIA SCOPIGERA* (SCOPOLI, 1763) (LEPIDOPTERA: SESIIDAE)

Dr. Aydemir BARIŞ[1], Dr. Cenk YÜCEL[1]

[1]Plant Protection Central Research Institute, Ankara, Türkiye.
aydemir.baris@tarimorman.gov.tr, Orcid ID: 0000-0003-3509-5700
[1] Plant Protection Central Research Institute, Ankara, Türkiye. cenkyucel@gmail.com, Orcid ID: 0000-0001-5223-9257

INTRODUCTION

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Family	Seiidae
Subfamily	Sesiinae
Species	*Bembecia scopigera* (Scopoli, 1763)
Synonym(Koçak and Kemal, 2018)	*Bembecia communis* Le Cerf, 1920

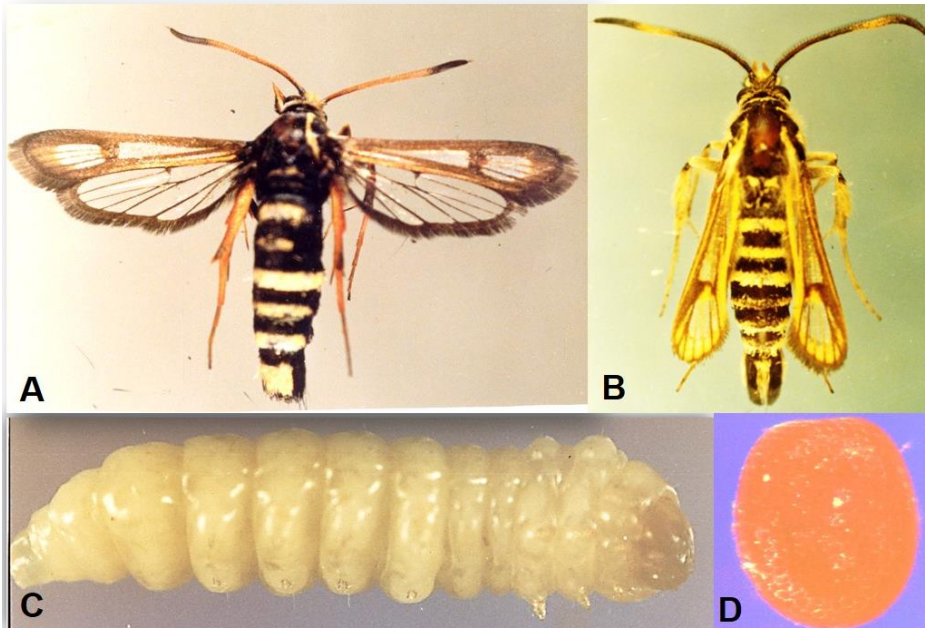


Figure. 1. A. Female, B. Male, C. Larvae, D. Egg of Bembecia scopigera (Scopoli, 1763)

Sainfoin (*Onobrychis* spp.) (Fabales: Fabaceae) is a forage plant that lives for many years, especially grown in arid areas. It is the most cultivated fodder locust in Turkey, after alfalfa and vetch, with a production of 1.546,641 tons in an area of 1,814,737 decares (Tuik, 2021). The nutritional value of sainfoin is high and its feed is rich in nitrogen-free essential substances, crude oil and crude protein. It can be grown in arid, weak, gravelly, calcareous soils where alfalfa cannot grow, and its resistance to salt

is high (Elçi, 2005). In addition, due to the tannin substance in their tissues, the ability to swell the animals seen in alfalfa is not seen in the sainfoin (Açıkgöz2001).

There are several factors affecting the production of sainfoin. Diseases and pests that are among these factors dec yield losses in sainfoin. The most important pest affecting the yield of sainfoin is *Bembecia scopigera* (Scopoli, 1763). As a result of feeding on the root of this harmful sainfoin, it protects it from year to year due to damage to the roots. In the studies conducted, 3. Starting from the year, the sainfoin fields begin to dry completely. The plant costs caused by early dismantling with the decrease in yield observed in sainfoin cause economic losses. In addition, due to wounds and openings formed by larvae in the roots, a number of fungal pathogens can cause damage by entering the roots (Tamer and Özer, 1990, Barış and Yücel, 2021).

1.Definition, Biology and Forms of Damage

When the studies related to *Bembecia scopigera* were examined, it was seen that there was very limited data. The first detection of the pest in Turkey was determined by Özer and Duran (1968) in 1958 in the sainga fields of Eskişehir province. *B. scopigera* reported that scopigera causes damage by feeding in the root of a pest in areas grown in Central Anatolia without irrigation, begins to cause significant damage to two-year-old plants, the larva that has passed into its mature period weaves a cocoon in the gallery that it opens in the root throat and passes into the pupal period. The pest is a pest of economic importance in the areas of Sainfoin then after determining that, high against the pests and Caliskaner (1970) reviewed by drug trials between the years 1964-1970. However, as a result of the studies conducted, it has been reported that the drugs tested against the pest are not effective. They reported that the biology of *Bemcecia scopigera* was not fully known in the absence of effective drugs tested, and that such trials should be conducted after the biology of the pest was first revealed. Those who were Born later, et al. (1981) conducted a study on the Lepidoptera fauna in the Eastern Anatolia Region by Erzurum province reported that the pest caused damage by opening galleries in the root throat and roots of the sainfara, gave one generation per year, and the damage increased for some years.



Figure. 2. Root damage by *Bembecia scopigera* (Scopoli, 1763)

Later, he conducted studies to determine the hymenoptera parasites detected by Doğanlar (1982) from some Lepidoptera species in Eastern Anatolia. With this study, *Chelonella nitens* (Rhd.) was identified as the natural enemy of *Bembecia scopigera*. October July (Hymenoptera: Braconidae), this species exits the pupae of the pest from July to October, parasitization is less than 5%.

Özbek (1989) gave information about the short biology of the pest and reported that it caused significant damage to sainfoin areas from time to time. Detailed studies on the biology and control of the pest in Turkey were carried out by Tamer (1990) in the sainfoin areas of Ankara province. He reported that in the years 1984-1987, the adult flights in the sainfoin areas started in July, continued until mid-October, and the adult flight duration varied between 6-13 weeks. It has been determined that the adults emerging from the pupa can mate and lay eggs, and the adult lifespan varies between 1-5 days in females and 1-4 days in males. He states that adults live for a very short time, they do not need nutrition, but their egg laying capacity varies between 219 and 730 (Figure 1). He stated that he lays his eggs on the undersides of leaves,

on flowers, and on cut sainfoin plants, singly or in groups. He states that later, the eggs are opened within 9-17 days, the larva that comes out by piercing the egg shell moves from the plant surface to the root collar, and enters the root through slits and cracks. He reported that the first larvae began to appear from the middle of July, and as the weather cooled, the larvae spent the winter in different larval stages by knitting cocoon as of the end of November and December. Later, the larva, which has completed its development, leaves the place where it feeds, opens a gallery in the plant root or in the soil, and goes into the pupa stage by knitting a cocoon. The pupal period varied between 9 and 14 days (Figure 3), and he stated that it gives one generation per year, and the damage rate can reach up to 100%. *Brocan crocatus* Schmiedeknecht, 1897; *Chelonella nitens* (Rhd.), depending on the Braconidae family as its natural enemy, stated that bacillus and some fungal agents were detected as disease agents. It has been determined that the parasitization rate can be as high as 41.37%, although it changes from year to year. He also stated that some plant protection products were tried to be used in the control of the pest, but the drugs did not show sufficient effect.

Gültekin and Güçlü (1997) conducted studies on the bioecology of the pest in Erzurum province. It was studied between 1994-1996 in order to determine the biology of the pest, its natural enemies and the rate of contamination. According to this, he stated that he spent the winter on the roots of the host plant in all larval stages, except the first period, the first pupae were seen in mid-June, the first adults were seen from the end of June, and the adult flight period ranged from 6 to 9 weeks. He reported that the first eggs were seen with the month of July, passed through the slits and cracks in the roots of the plants, fed here, and started wintering from the end of September. *Bracon* (=*Lucohracon*) *grandiceps* Thomson (Braconinae) of the family Braconidae as the natural enemy of *B.scopigera*; He stated that they detected *Ascogaste rgonocephala* Wesmeal (Cheloninae) and that the parasitism ranged between 6.09 and 7.0. In addition, some of the larvae (2-3%) showing signs of the disease Beauveria sp. and Fusarium sp. found that fungi were isolated. In all tested varieties, it was determined that between 65% and 90% of the pests were harmful, and that sainfoin varieties and lines were later determined against the pest in question, Çiftçi et al. (2016) studied

the morphology of the egg of *B. scopigera*. He also stated that *Onobrychis atropatana* is a new host plant for *B. scopigera.*



Figure. 3. Pupa of *Bembecia scopigera* (Scopoli, 1763)

Later, Barış and Yücel (2021) stated that the pest lays eggs on the parts of *B. scopigera* close to the root and root collar, and the hatched larva feeds inside the root. He states that the plants dry out as a result of feding (Figure 2). They stated that in some lines and varieties tried as an alternative to the control of the sainfoin rootworm, the pest caused 65-90% damage, and there was no difference between the varieties. In addition to the damage caused by the pest on the root, it has been reported that fungal agents (*Fusarium oxysporium*, *Fusarium solani*) infect the root part and cause indirect damage as a result of the damage to the roots.

RESULTS

Studies on *B.scopigera* in Turkey started in the 1950s and have survived to the present day. It has been observed that there is limited data on both the biology and control of the pest. When the data obtained are evaluated, it is seen that the sainfoin spends the winter in the larval stage on

the roots, then it turns into a pupa by knitting a cocoon, the emerging adults live between 1-5 days, the adults lay their eggs on the plant after mating, the hatched larva moves into the root, burrowing in the root and harmful. specified. It has been stated that as a result of feeding in the root, the yield of the sainfoins began to dry over time, and the yield decreased, as well as the damage caused by direct feeding on the root, as well as indirectly damaging the root as a result of infecting the root with some fungal factors. It has been predicted that the damage to the plant can be up to 100%. As a result of the studies, it was determined that parzatiot, bacillus and fungal pathogens were found as natural enemies. In addition, chemical control studies have been carried out against the pest, but an adequate effect has not been achieved. It is thought that especially the adults of the pest live for a very short time, laying a high number of eggs in this period, making it difficult for the adults to get to the root. It is thought that feeding the pest at the root is effective in the inability to obtain resistant varieties, especially in breeding studies.

B. scopigera has been detected in Spain, most of southwestern and central Europe, the Balkans, Greece, Russia, Ukraine and in a wide geographical area, including Turkey (Spatenka et al. 1999). It has been observed that the studies carried out abroad are generally studies on the detection and morphology of the pest (Jin et al. 2008). Among the studies, it was seen that detailed data on the biology of the pest were given by Bournier and Khial (1968). Accordingly, they reported that the pest began to appear from June to July, the adult flight period lasted for about two months, the adults mate as soon as they emerge, and a female lays up to 500 eggs on the leaves, branches and buds of the plant. He stated that the larvae that hatched from the egg caused damage by entering the roots through the root collar, they started wintering as of October, the larvae formed a pupa by knitting a cocoon from the end of March, and the adults reappeared. He stated that there is no obligatory wintering of the pest, which gives only one offspring per year. It was evaluated that the findings of Bournier and Khial (1968) coincided with the findings obtained from the studies conducted in Turkey. However, it has been evaluated that some factors such as climatic conditions, host and regions may have an effect for the pest to enter diapause.

Sainfoin is one of the most important forage plants. It is especially important in terms of evaluating areas with arid or low rainfall. In addition, it

is important in other useful groups such as pollinators, parasiotes, predators, especially honey bees, because it blooms constantly, especially in arid or low rainfall areas. In studies conducted in Turkey and abroad, it has been observed that one of the most important pests of sainfoin is *Bembecia scopigera*. The potential for harm can be up to 100%. Feeding the pest at the root, the inability of its natural enemies to suppress more or less the pest, the decrease in the effectiveness of drugs due to the fact that the pest is fed inside the root complicates the struggle. Sainfoin is an important forage plant both for animal nutrition and for the evaluation of arid areas. For this reason, research is needed on both the biology and the struggle of *Bembecia scopigera*, which limits sainfoin cultivation.

REFERENCES

Açıkgöz, E(2001). Yem Bitkileri. Uludağ Üniversitesi Ziraat Fakültesi Tarla Bitkileri Bölümü, 83-94, Bursa.

Barış A., C. Yücel (2021). Determination of Reactions of Some Sainfoin Varieties and Inbreds Against the Six-belted Clearwing Moth *Bembecia scopigera* (Scopoli, 1763) (Lepidoptera: Sesiidae). 8th Plant Protection Congress with International Participation, August24-28,online. p, 164.

Bournier, A.,B.Khial (1968).*Dipsosphecia scopigera* Seop. La sesie du sainfoin. Ann. Epiphyties. 19 (2): 235-260.

Ciftci, D.,S. Seven., A. Hasbenli (2016). The morphology of egg chorion of *Bembecia scopigera* (Scopoli, 1763) with data on a new host plant and contribution to Turkish distribution (Lepidoptera: Sesiidae). Shılap-Revista De Lepidopterologia,.44(174):313-317.

Doğanlar, M. (1982). Hymenopter parasites of some lepidopterous pests in eastern Anatolia. Türkiye Bitki Koruma Dergisi, 6: 197-205.

Doğanlar, M., H. Özbek., O.Ecevit., H. Yüksel. (1981). Doğu Anadolu Bölgesinin bazı Lepidopter'leri. Bitki Koruma Bülteni, 21(3): 155-172.

Elçi, Ş(2005). Baklagil ve Buğdaygil Yem Bitkileri. T. C. Tarım ve Köy İşleri Bakanlığı, 223-257, Ankara.

Gültekin, L., Ş. Güçlü (1997). Bioecology of *Bembecia scopigera* (Scopoli) (Lepidoptera: Sesiidae) pest of sainfoin in Erzurum. Plant Protection Bull. 37 (3-4): 101-110.

Koçak, AÖ., M., Kemal (2018). A synonymous and distributional list of the species of the Lepidoptera of Turkey.Centre for Entomological Studies, ISSN:1015-8227, Ankara

Jin Q, S.X., Wang,H.H. Li (2008). Catalogue of the family Sesiidae in China (Lepidoptera: Sesiidae). SHILAP Revta. lepid., 36(144): 507-526.

Özbek, H. (1989). Tahıl, Sebze, yem ve Endüstri Bitki Zararlıları. Atatürk Üniversitesi, Ziraat Fakültesi Yayınları, Bitki Koruma Bölümü, 227, Erzurum.

Spatenka, K., O., Gorbunov, Z., Uvka, I., Tosevski, Y., Arita(1999). Sesiidae -Clearwing Moths. In: Naumann, C. (Ed.), Handbook of Palaearctic Macrolepidoptera 1. 569 pp. Wallingford, England (Gem Publishing Company)

Tamer, A.,M.,Özer(1990). Investigations on the bio-ecology and control of *Bembecia scopigera* (Scopoli) (Lepidoptera: Sesiidae) that damages sainfoin in Ankara Province. Turk. J. Agric. For. 14: 149-180.

Turkısh Sitatistical Institute. (2021). Crop production statistics. https://biruni.tuik.gov.tr/medas/?kn=92&locale=tr

Yüksel, M., S., Çalışkaner (1970). Orta Anadolu'da korungalarda zararlı olan *Dipsosphecia ichneumoniformis* ve *Sphenoptera carceli*'nin 'nin yayılış alanları, ekonomik önemleri ve pratik mücadele metotları üzerine araştırmalar. Ankara Bölge Zirai Mücadele Araştırma Enstitüsü.

CHAPTER 4

CRISPR/CAS IMMUNE SYSTEM WITH ITS MECHANISM, APPLICATIONS AND ETHICS ASSESSMENT

Ass. Dr. Özgür KEBABCI[6] & Prof. Dr. Ali Fazıl YENİDÜNYA[7]

[6] Sivas Cumhuriyet University, Science Faculty, Department of Molecular Biology and Genetics, Sivas, Turkey e-mail: ozgur.kebabci@gmail.com ORCID ID: 0000-0002-9404-747X
[7] Sivas Cumhuriyet University, Science Faculty, Department of Molecular Biology and Genetics, Sivas Cumhuriyet University, Sivas, Turkey e-mail: alifazilyenidunya@gmail.com ORCID ID: 0000-0002-9886-977X

INTRODUCTION

Life on Earth has been existing as cells for about 4 billion years. Biodiversity encountered today have emerged from two different types of cells, prokaryotic and eukaryotic, which had in turn been derived from LUCA, the last universal common ancestor. Although not cellular, viruses are a crucial part of life in general terms. The trees of life always excluded viruses. It appears that while bacteria have evolved to become more complicated, viruses have become simpler. They have achieved this by propagating only the "essential" genes.

The fiercest evolutionary battles take place in nature between invading entities and their hosts at molecular level. A substantial number of these wars happens between bacteria and bacteriophages. Bacteriophages are viruses that infect bacteria. Both sides in these wars have developed unique attack and defence systems. Likewise, Archaea are also attacked by archaeal viruses, and they have also developed specific barriers against their viral invaders. The most abundant creatures on earth are Bacteria and Archaea. Viruses (bacteriophages or phages) of these two domains pose a constant menace to prokaryotic life (Hille and Charpentier, 2016).

CRISPR/Cas (clustered regularly interspaced short palindromic repeats/ CRISPR-associated proteins), a kind of adaptive immune system, exists against phages and mobile genetic elements (MGEs) in prokaryotes. Ironically, the invaders are the ones who establish the essential components of this nucleic acid-based adaptive immune systems (Gök and Tunalı, 2016). CRISPRs are the repeating elements in the CRISPR/Cas locus and are made up of conserved short sequences, separated by relatively varying spacer DNA. MGEs are the source of these unique nucleotide sequences. Examples of MGEs are plasmids, phages, and transposons. (Hille and Charpentier, 2016). Small guide RNAs (crRNAs) play a key role in the CRISPR/Cas system. This role is at the interference process, that will be described later in detail.

An adenine-thymine-rich leader sequence precedes CRISPR. *Cas* genes flank CRISPRs and encode Cas proteins (Hille and Charpentier, 2016). Cas protein family encompasses polynucleotide binding proteins, helicases, nucleases, and polymerases. CRISPR/Cas systems are thus formed by CRISPR and Cas proteins (Horvath and Barrangou, 2010). Initially, a two-step classification approach had been adopted by the research team,

identifying every *cas* genes in all of the CRISPR/Cas loci. Then, distinctive gene architectures and signature genes were determined. Thus, the loci had been divided into types and subtypes. A recent classification has been expanded to include 2 classes, 5 types and 16 subtypes, while retaining the general structure of the previous one (Makarova et al., 2015) (Fig. 1).

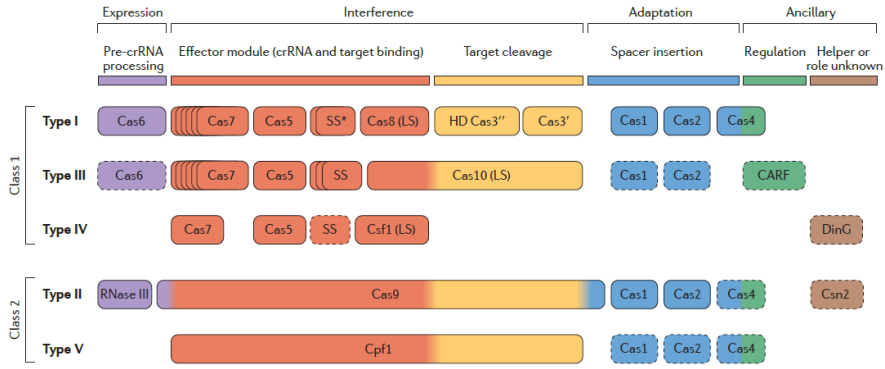


Figure 1. Cas Proteins' Functional Classification. (For detailed information, see Makarova et al., 2015 review)

This adaptive immune system in prokaryotes has become adaptable to all cells through genetic engineering. Many studies have indicated that the CRISPR/Cas system can be used in the future as an alternative to the existing methods in the fields such as agriculture, food, and medicine.

1. CRISPR/CAS HISTORY

The CRISPR/Cas system has been first identified in *Escherichia coli* where it has been shown that a 32 bp spacer sequence is inserted in the 29 bp sequence of CRISPR loci. However, the meaning of these findings has not been fully appreciated at the time (Ishino et al., 1987; Ishino et al., 2018; Javed et al., 2018). Following discoveries have been made on *Haloferax mediterranei*, a halophilic *Archaea* species. In this organism, it has been determined that CRISPR loci was formed by the repetition of a nearly perfect, roughly palindromic 30bp sequence, separated by roughly 36 bp spacers (Mojica et al., 1993). These structures that were primarily called SRSRs (short regularly spaced repeats), later were renamed as CRISPR (Jansen et al.,

2002). CRISPR elements have then been identified in many archaea and bacteria (Makarova et al., 2002). Finally, thanks to comparative genomic analysis studies, it has been comprehended that CRISPR and Cas proteins must work together as an adaptive immune system in prokaryotic cells against invading viruses and parasites and that this system has similarities with the RNA_i (RNA interference) phenomenon in eukaryotes (Makarova et al., 2006; Ishino et al., 2018).

Studies on the adaptive immune system has changed their direction when the eminence of CRISPR/Cas systems has been envisaged in the evolution of prokaryotik genome. Nowadays hundreds of studies have been focusing on the genome regulation role of CRISPR/Cas and on the devising of this entity in the diverse areas of research while keeping in mind its immense industrial implications (Figs. 2 and 3).

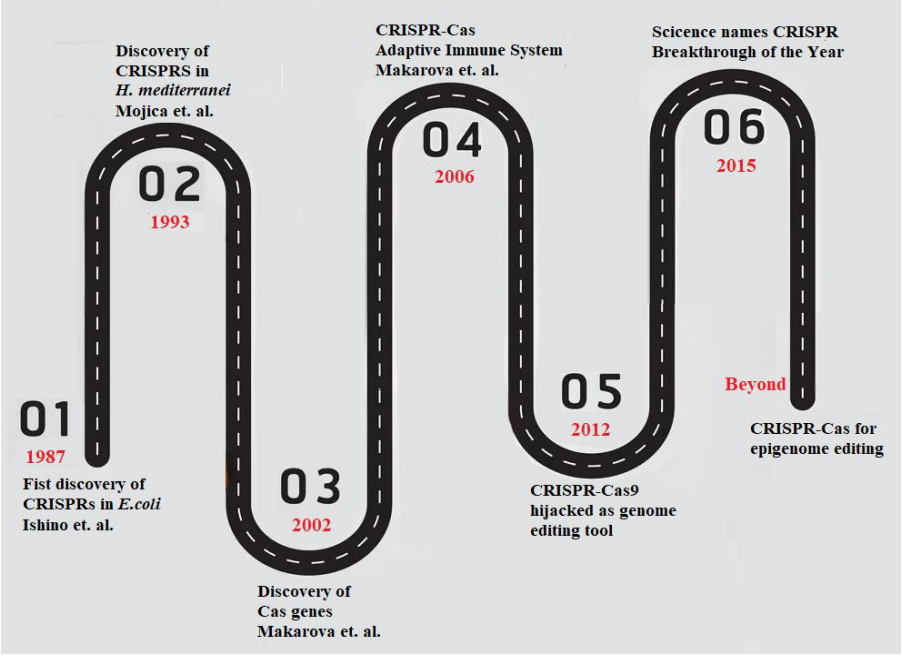


Figure 2. CRISPR/Cas Timeline

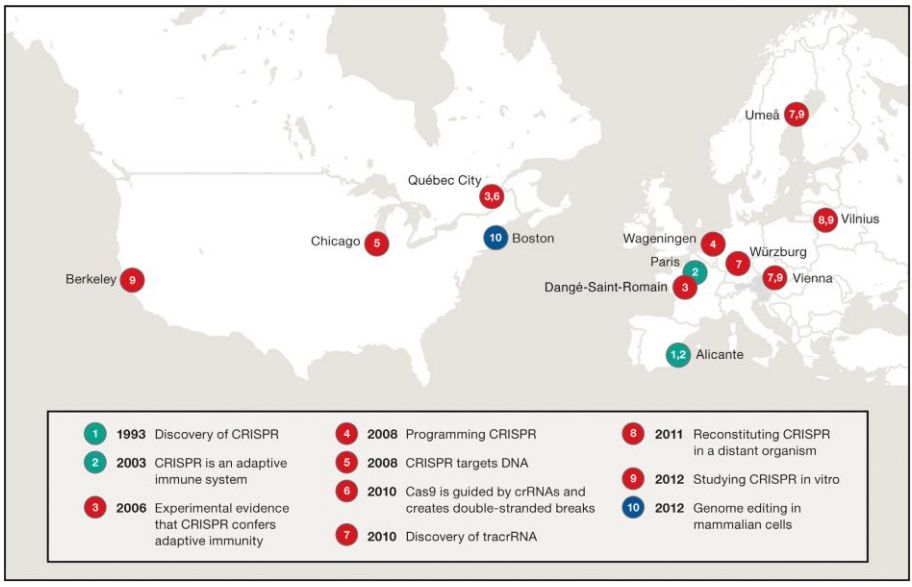


Figure 3. CRISPR/Cas History With Studies In Twelve Cities Of Nine Countries (Lander, 2016)

2. EDITING OF GENOMES

Since the emergence of genome editing technology in the 1990s, several methods have been developed for targeted gene editing. Three of these systems frequently used are TALENs, ZNFs and CRISPRs. Each of these systems has its own advantages and disadvantages. TALENs and ZFNs are essentially nucleases. The former comprises transcription activator-like effector nucleases and the latter embraces zinc finger nucleases. CRISPR approach expectedly involves Cas nucleases (Tavakoli et al., 2021).

Zinc-finger nucleases have been used to generate site-specific modifications in a number of model organisms. They are also used for various therapeutic purposes (Javed et al., 2018). Although applications of ZFNs have not been frequently encountered (Urnov et al., 2010), site-specific genome modifications have been achieved so far by TALENs in plants, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, *D. melanogaster*, *Xenopus* embryos, zebrafish, mice, humans, and somatic and pluripotent cells (Chen and Gao, 2013). Yet, due to the difficulties in the cloning and engineering of zinc finger nucleases, the use of TALENs have been significantly hindered. In this respect, CRISPR has revolutionized the

efficiency of genome editing studies as it is relatively more stable, and its use is much simpler and more flexible (Adli, 2018) (Tables 1 and 2).

Table 1: Fundamental Comparison Of Three Genome Editing Techniques (Tavakoli vd., 2021)

Feature	CRISPR-Cas	TALEN	ZFN
Cost	Low	High	Low
Ease of Design	Simple	A little complex	Moderate
Specificity	High	Intermediate	Low
Pros	Modifies multiple sites in tandem	Highly effective and specific	Highly effective and specific
Cons	PAM motif required next to target sequence	Time consuming	Time consuming
Multiplex genome editing	High-yield multiplexing	Few models	Few models

Table 2: Comparison of Genome Regulation Tools (Kozovska vd., 2021)

Features	ZNFs	TALENs	CRISPRs
DNA-binding moiety	Protein	Protein	RNA
Nuclease	*Fok*I	*Fok*I	Cas
Target recognition size	18-36 nt	30-40 nt	22 nt
Toxicity	Variable to high	Low	Low
Ease of targeting multiple targets	Low	Low	High
Complexity of design	Very complex	Complex	Simple
Off-target effects	Moderate	Low	Variable

3. CLASSIFICATION OF CRISPR/CAS SYSTEMS

The CRISPR/Cas system is essentially a complex entity. Many criteria have been employed in its classification, including Cas operons' organization, conserved Cas proteins' phylogenesis, and signature *cas* genes (Koonin ve Makarova, 2019). Phylogenetic studies have defined different versions of the CRISPR systems. Basically, two classes of CRISPR/Cas systems have been identified. Multi-subunit crRNA effector complexes are appeared in class 1 systems. In class 2 systems, a single protein such as Cas9 is responsible for all functions (Makarova et al., 2015). Class 1's subtypes are I, III and IV and class 2's subtypes are II, V and IV (Fig. 4 and 5). Effector modules' architectures are used to characterize for each type. (Koonin and Makarova, 2019). Both type IV in class 1 and class II are putatively named.

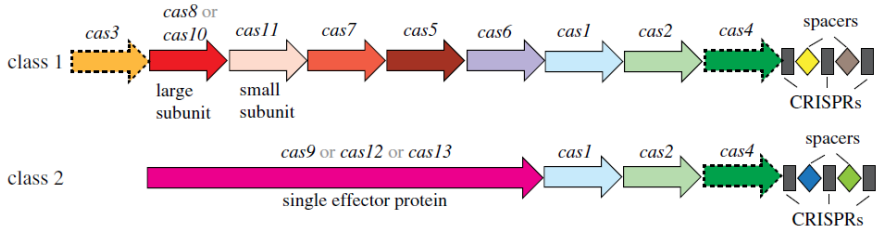


Figure 4. Class 1 And Class 2 Systems' General Architectures (Koonin and Makarova, 2019)

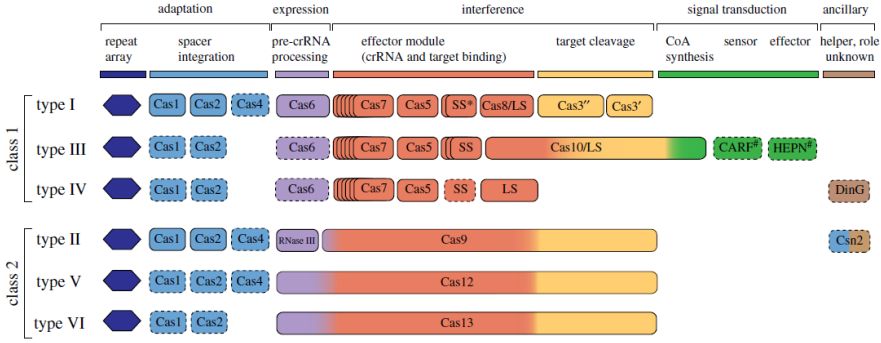


Figure 5. CRISPR/Cas Systems' Modular Organization (Koonin and Makarova, 2019)

Class 1 type I systems are divided into seven subtypes in total. Six of these subtypes are named from I-A to I-F, and another one called I-U (Fig. 6a). In systems from type I-C to type I-F, there is a single operon that encodes proteins from Cas1 to Cas3. This operon also contains genes containing subunits of the Cascade complex—CRISPR-associated complex for antiviral defence. In contrast, a different organization appears in many of the type I-A and type I-B loci. Here Cas proteins are encoded in two or more operons. Differences are also seen in type I-E and type I-F systems. For instance, these systems do not have Cas4. Cas 3 is also fused with Cas2 in the type I-F system.

In Class 1-type III systems, four subtypes are defined from type III-A to type III-D. (Fig. 6b). The marker gene *cas10* is present in all type III systems. A multi-domain protein that also contains a PALM domain is encoded by this gene. This is a variant of the RNA recognition motif (RRM), homologous to the nuclear domain of multiple nucleic acid polymerases and cyclases (Makarova et al., 2015). Cas10 is the largest subunit of crRNA-effector complexes in type III systems. There are other proteins encoded by all type III loci, such as the Cas7 protein, the Cas5 protein, and a small subunit protein. Most subtype III-B loci lack *cas1*, *cas2*, and *cas6* genes, but these genes are present in III-A loci. Subtypes III-A and III-B have been shown to be involved in co-transcriptionally targeting DNA and RNA.

Cas1 and Cas2 are absent in the class 1 type IV system. Csf1, the large subunit, can operate as a marker gene in this system. A minimal multi-subunit crRNA-effector complex is encoded by type IV system. This complex comprises of Csf1, Cas5 and Cas 7 (Fig. 6c). Two different variants have been identified in type IV systems. One of them contains DinG family helicase when other one does not. The one that lacks DinG contains a gene that encodes a small α-helix protein with a small subunit.

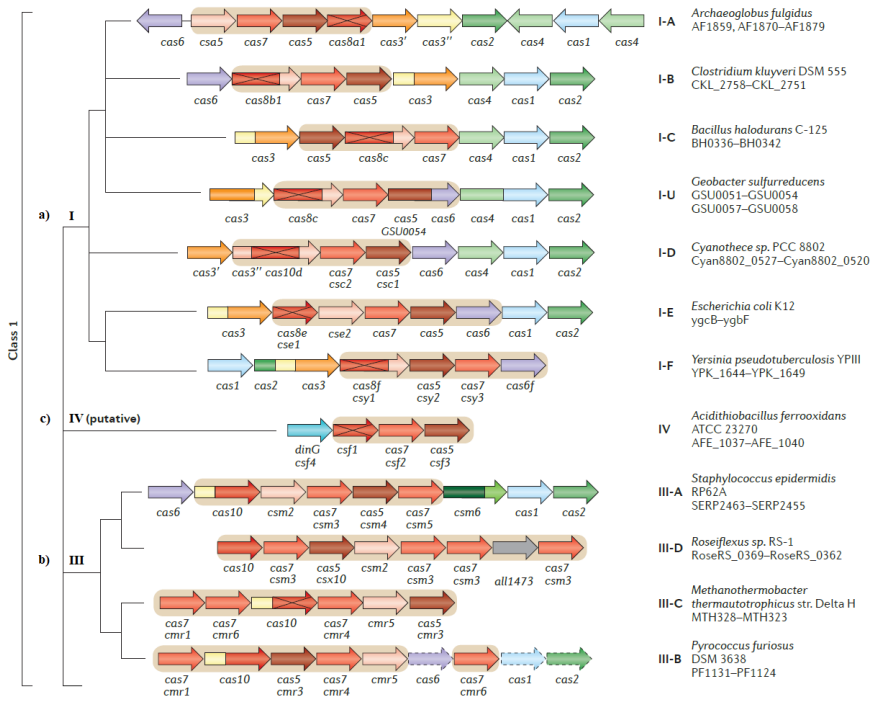


Figure 6. Type-I, Type-III And Type-IV (Putative) Systems (Makarova et al., 2015)

The class 2 type II system, which differs significantly from the type I and type III systems, is quite simpler. Its marker gene is *cas9*. Cas9 is a protein that combines the functions of the crRNA-effector complex with target DNA cleavage and also contributes to adaptation. cas1 and *cas2* are located at all type II CRISPR/Cas loci. In type II systems, 3 subtypes are identified as type II-A, II-B and II-C. An additional gene, *csn2*, is considered as a marker gene in subtype II-A systems. Subtype II-B, instead, contains *cas4*. The genes encoding the Cas1, Cas2 and Cas9 proteins are present in subtype II-C loci. This subtype is the commonest system in bacteria (Fig. 7a). *Cpf1*, *cas1*, *cas2*, and *cas4* are located in type V systems (Fig. 7b).

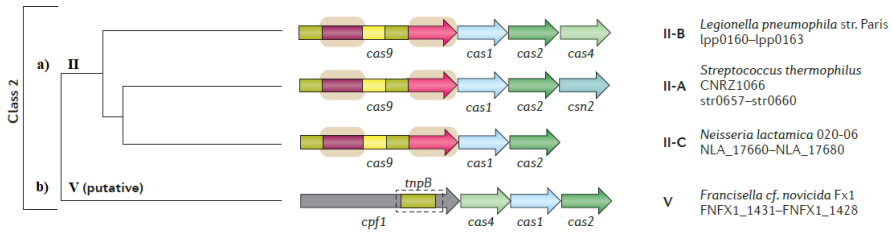


Figure 7. Type-II And Type-V (Putative) Systems (Makarova et al., 2015)

4. THE MECHANISM OF CRISPR/CAS SYSTEM

Adaptive immunity in prokaryotes progresses through three distinct stages: (1) foreign DNA acquisition (adaptation), (2) biogenesis of crRNA, and (3) interference of the target (Wilkinson and Wiedenheft, 2014).

4.1. Adaptation

Adaptation is the step in which spacer segments from external nucleic acids are inserted into the CRISPR region. In this step, the selection of protospacers takes place. The nucleotide sequence found in the phage or plasmid was first identified as a protospacer, flanked by a system-specific, highly conserved CRISPR motif. It was later renamed as PAM (protospacer adjacent motif) (Barrangou and Marraffini, 2014). PAMS are consisted of 2-5 nucleotides. Their sequence motifs were highly conserved (Gök and Tunalı, 2016; Barrangou and Marraffini, 2014). In adaptation, these PAMs are specifically recognized. The invasive DNA spacer segment is inserted into the CRISPR region along with the repetitive genes. This segment consists of the PAM sequence. Recognition of the protospacer adjacent motif by Cas9 nuclease is thought to destabilize the adjacent sequence, resulting in RNA-DNA coupling. Protospacer adjacent motifs have been associated with both immunization (sampling of invader DNA for spacer recruitment) and targeting (Barrangou and Marraffini, 2014) (Fig. 8).

The proteins involved in nearly all CRISPR/Cas systems are Cas1 and Cas2. These proteins are metal-dependent endonucleases and are involved in the process of acquiring spacers in adaptation. Cas2's catalytically active site is indispensable for the spacer acquisition (Barrangou and Marraffini, 2014; Hile and Carpentier, 2016). Types I, II and V also require Cas4.

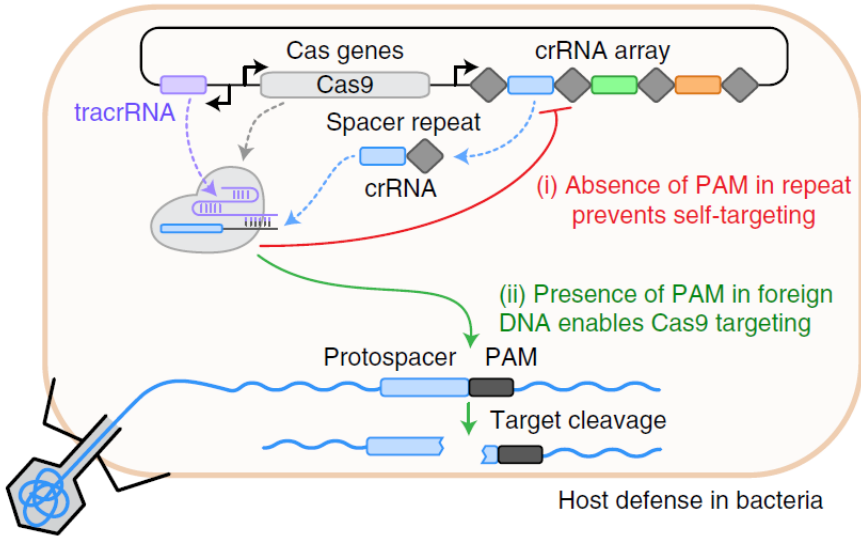


Figure 8. The Role Of PAM In Defence (Walton et al., 2021)

4.2. The Biogenesis of crRNA

In CRISPR RNA biogenesis, pre-crRNAs are created first. In other words, the region in the invasive DNA is transcribed into pre-crRNAs. This region of invasive DNA is the target sequence inserted into the CRISPR locus. The resulting pre-crRNA transcripts are cleaved into small crRNAs. They base pair with invasive DNA sequences. This process is carried out by Cas endoribonucleases (Gök and Tunalı, 2016). Members of the Cas6 family in types I and III of CRISPR/Cas systems perform the processing step. After this step, intermediate crRNA species are produced. A short 5′ tag surrounds these intermediate crRNA types. In type I-C systems an exception exists. In these systems, Cas6 proteins are not encoded. Instead, the Cas5d protein is involved. This protein processes pre-crRNA, resulting in 11 bp 5′-tags intermediate crRNAs. (Hile and Carpentier, 2016).

There are significant differences in the stage of maturation of crRNA in class 2 systems. Trans-activating crRNA (tracrRNA) is an imperative in type II systems for the processing of pro-crRNA. In this system, tracrRNA and preRNA form an RNA duplex. This RNA duplex is recognized by the host RNase III. After the processing, the intermediate crRNA is formed. Finally,

mature small guide RNA is obtained. Early crRNAs are processed by cpf1 in the type V-A system.

4.3. Target Interference

In the final stage, the target of crRNA is the invasive nucleic acid. Cas nucleases, for example Cas9, cut homologous sequences, preventing replication of viruses and plasmids (Shabbir et al., 2019). Class 1 systems contain Cascade-like complexes. These complexes perform the target interference. A single effector protein is sufficient for target interference in class 2 systems. In type I systems, the task of Cas3 nuclease is to create a nick. This nick is created on the invading DNA, initiating its fragmentation. The Cas9 effector protein has been directed by tracrRNA/crRNA duplex in type II systems. The result is a double-stranded cleavage in the target DNA. Types III-A and III-D contain the Cas10-Csm complex. In addition to this, the Cas10-Cmr complex is found in III-B and III-C systems. DNA and RNA are both can be targeted by these complexes. In Type V-A and V-B systems, crRNA is used for target localization and interference, and tracrRNA/crRNA duplex for target interference, respectively (Hile and Carpentier, 2016).

To sum up, the evolutionary immune response in bacteria against exogenous nucleic acids follows the stages of adaptation, crRNA biosynthesis, and interference (Fig. 9 and 10).

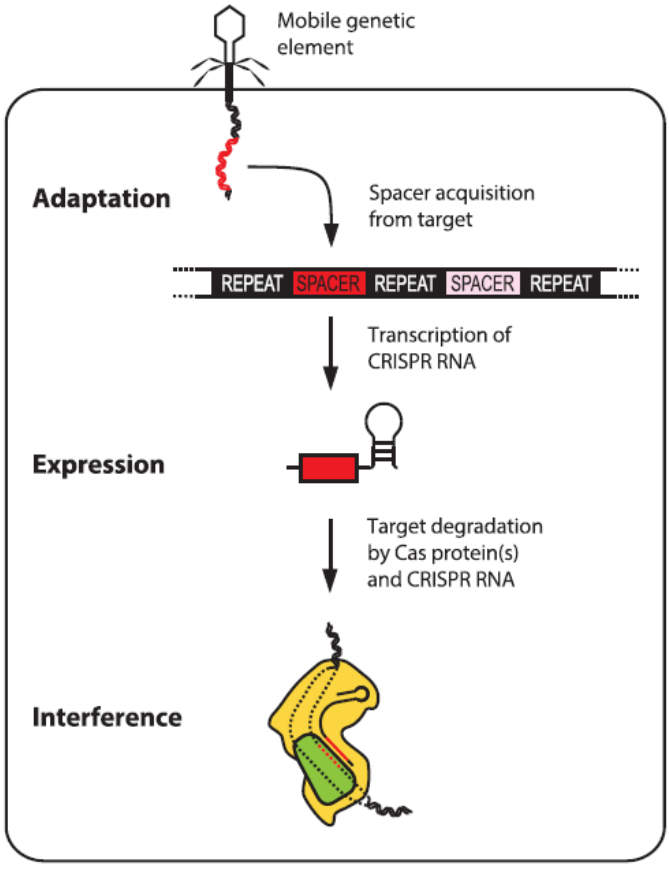


Figure 9. The Whole Procedure Of CRISPR/Cas Immunity (Rath et al., 2015)

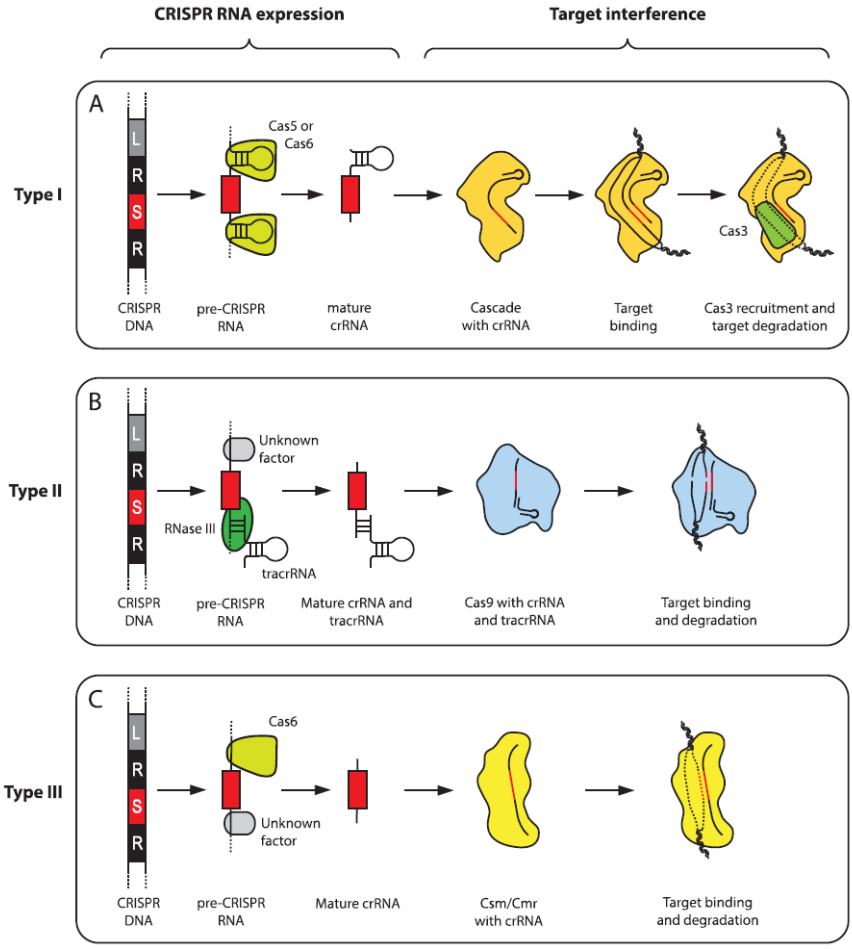


Figure 10. Representation Of crRNA Expression And Interference Patterns In Type I And III Systems (Rath et al., 2015)

5. ANTI-CRISPR MECHANISMS

Archaea and bacteria develop adaptive immunity to viruses, while viruses develop counter-strategies. The most important of these strategies is random mutations. These mutations occur either in the PAM sequence or in the protospacer. In recent years, another strategy has been noticed when *Pseudomonas aeruginosa* has been infected by Mu-like phages. These phages are able to inhibit the CRISPR/Cas system. Various proteins encoded by Mu-like phages adversely affect the activity of Type I-E and I-F systems (Rath et

al., 2015) and these are called anti-CRISPR proteins. Three types of anti-CRISPR proteins have been identified: AcrF1, AcrF2, and AcrF3. AcrF1 and AcrF2 interact with type I-F interference components, preventing binding to target DNA. AcrF3 binds Cas3 nuclease, thus preventing target interference ICP1 phages, infecting *Vibrio cholerae*. These phages have developed a different strategy to evade the bacterial immune system (Seed et al., 2013).

6. CRISPR/CAS DELIVERY TECHNIQUES

An important step in the gene editing stages in various applications is the delivery of the system to the target cell. Viral and non-viral tools have been exploited in this procedure (Karimian et al., 2019). Viral delivery includes the transmission of lentiviruses, herpes viruses, retroviruses, adenoviruses, and AAVs—adeno-associated viruses. Bacteriophages are also employed recently (Fage et al., 2020) (Table 3 and 4). Non-viral delivery include electroporation, hydrodynamic transfer, and conjugative delivery as well as the use of nanoparticles (Yeh et al., 2022).

Table 3: Delivery Strategies For CRISPR/Cas9 System (Karimian et al., 2019)

Strategies	Delivery Systems	Advantages	Disadvantages
Plasmid-based CRISPRCas9 system	▪ Electroporation ▪ Hydrodynamic injection ▪ Lipid nanoparticles ▪ AAV (Adeno-associated viruses) ▪ Lentivirus	▪ Simple ▪ Easy ▪ More stable than sgRNA and Cas9 mRNA ▪ Avoiding multiple transfections	▪ Difficulty in transferring the plasmid to the nucleus ▪ Slow effect on gene editing ▪ Undesired random integration in the host genome
Cas9 mRNA and sgRNA	▪ Electroporation ▪ Lipid nanoparticles	▪ Low cytotoxicity ▪ Temporary expression ▪ Fast effect	▪ Multiple transfection ▪ High degradability of mRNA
Cas9 protein and sgRNA	▪ Electroporation ▪ Lipid nanoparticles ▪ Gold nanoparticles	▪ The fastest effect ▪ High efficiency ▪ Avoiding undesirable integration ▪ Low antigenicity ▪ Avoiding multiple transfections	

Table 4. Delivery Of CRISPR/Cas Systems To Bacteria With Phages (Yeh et al., 2022)

Phage vector	Pathogen	
Phagemid	*Staphylococcus aureus*	In vivo
	Escherichia coli	In vivo (*Galleria mellonella* larvae)
Phage genome	*S. aureus*	In vivo (rat)
	E. coli	In vitro
	Clostridium difficile	In vitro and vivo (mice)

7. APPLICATIONS OF CRISPR/CAS

The CRISPR/Cas system is applied in many fields, for example, disease diagnosis and drug discovery (Zhan et al., 2021). CRISPR/Cas systems with RNA-targeted nuclease activity are type I and type III. These systems operate with a multimeric crRNA-Cas ribonucleoprotein complex. Therefore, the use of these systems as a molecular tool in gene editing becomes troublesome (Gök and Tunalı, 2016). Type II systems are more applicable because the cleavage of target DNA entails only one nuclease (Sapranauskas et al., 2011). The CRISPR/Cas type II system of *Streptococcus pyogenes* has been adapted in genome regulation (Jinek et al., 2012).

CRISPR/Cas9 system could be adaptable to single gene repair: For example, *cftr* gene has been targeted and repaired in the cells cultured from cystic fibrosis patients (Schwank et al., 2013); the dominant cataract disorder was ameliorated by *crygc* gene repair in mouse germ cells (Wu et al., 2013); patients with Duchenne muscular dystrophy were cured (Long et al., 2013); and some progress has been made on treatment of hereditary tyrosinemia in adult mice (Yin et al., 2014). A more detailed picture of the gene therapy will be provided later on.

7.1. Applications in Agriculture and Food Industry

Agricultural and food products are exposed to various stresses and attacks during their production, storage, and even marketing. Scientific research is aimed at increasing the productivity of agricultural products as well as preventing these stresses and attacks. CRISPR/Cas9, a recent breeding technique, has the possibility for rapidly and accurately improving many features in crops, such as aspects of abiotic stress tolerance, nutritional value, disease resistance, quality, and yield (Wan et al., 2021). It is applied in the production of new germplasm sources, as it readily enables scientists to introduce gene-directed mutations.

Application of the CRISPR/Cas system in plant gene engineering has started in 2013. First, argonaute 7 was knocked out using CRISPR/Cas9 to generate coniferous mutants (Brooks et al., 2014). Later on, studies have been carried out on the protection of plants against various stress conditions, on the improvement of plant architecture, fruit-vegetable quality, and increasing shelf life (Kulus, 2018). Agrobacterium-mediated transformation is applied in

these studies. Because of its high economic value, tomato has been chosen as a model crop to test the efficiency CRISPR/Cas9 methodology (Wan et al., 2021). The first sugarcane varieties, Cana Flex I and Cana Flex II, edited by using CRISPR have been developed in Brazil. It has been reported that the respective distinctive features of these varieties were easy cell wall digestibility and high sucrose concentration.

Many microorganisms cause various diseases in plants. Among them are bacteria. CRISPR/Cas gene editing has been applied against such bacterial plant pathogens. *Xanthomonas* is a bacterial genus that causes Citrus canker disease in grapefruit cultivars. CRISPR/Cas9 has been applied to treat this disease. *PthA4* is a dominant pathogenicity gene located in *X. citri*. In this study, the *pthA4* gene has been successfully targeted with the CRISPR/Cas9 system (Jia et al., 2015).

7.2. Applications in Microorganisms

CRISPR/Cas systems have had many applications in archaea and bacteria. For this purpose, DNA technologies based on CRISPR/Cas have been developed and diversified. Among them are genome-wide screening and silencing of key genes in archaeal and bacterial genomes (Ishino et al., 2018). A CRISPR/Cas-mediated genome editing technique have been developed and successfully applied in *E coli*. In this technique, CRISPR/Cas was combined with heterologous recombination using single-stranded (single-stranded DNA recombination [SSDR]) or double-stranded (double-stranded DNA recombination [DSDR]) template DNAs (Mougiakos et al., 2016). Gene silencing has thus been successfully performed in a number of archaeal species, including *Haloferax volcanii*, *Sulfolobus solfataricus*, and *S. islandicus* (Ishino et al., 2018).

Researchers combined several technologies with the CRISPR system to improve the performance of pathogen detection methods. An example of these technologies is nucleic acid amplification (Tian et al., 2022). There are still many hurdles and limitations in applications of detection of pathogens' nucleic acids. However, there are also promising developments. For example, there have been significant advances in the production of CRISPR/Cas-based biosensors in detecting pathogens.

Bacterial infections with multiple drug resistance (MDR) pose a global threat in human health. One of the promising approaches to combat MDR bacteria appears to be phage therapy. A CRISPR/Cas system has been applied to develop antimicrobials by effectively delivering them to target bacteria with phage-based vectors and in vivo bactericidal effects of these antimicrobials have been determined (Yeh et al., 2022).

The CRISPR/Cas has been used to identify pathogenic microorganisms, drug resistance, virulence, and knock out specific virulence genes, and to kill pathogenic bacteria (Bikard et al., 2014). Antibiotic resistance is a serious worldwide problem, and resistance genes play an important role in the pathogenicity of bacteria. By targeting antibiotic resistance genes with CRISPR/Cas system, the killing of pathogenic bacteria has been indirectly achieved (Jothi et al., 2021).

Pathogenic microorganisms do not exist alone in the host and antimicrobials agents can also be detrimental to benign, cohabiting microorganisms. The CRISPR/Cas system can be targeted more specifically to pathogens. *Staphylococcus aureus* is a kanamycin-resistant pathogen. In this bacterium, the *aph-3* gene provides resistance to kanamycin. By applying a plasmid-based CRISPR/Cas, this gene has been targeted, and thus only the bacteria sensitized to kanamycin were killed (Bikard et al., 2014). Similar studies have been performed on *S. aureus*, targeting both *sec* and *mecA* genes. The *sec* gene encodes the superantigen enterotoxin, an important virulence factor. The *mecA* gene provides resistance to penicillin derivatives. By applying phagemid-based CRISPR/Cas, the pathogen was killed by the elimination of these virulence factors of the bacteria (Jothi et al., 2021). Many researchers around the world have used CRISPR gene editing to eradicate ESBL (extended spectrum beta-lactamase) *Escherichia coli*. In parallel, they developed a CRISPR system in *E. coli*, suitable for generating double-strand breaks (DSB). In this study, β-lactamase encoding genes, $bla_{\text{NDM-1}}$ and $bla_{\text{SHV-18}}$ were targeted (Citorik et al., 2014).

There are also CRISPR/Cas applications on fungi. For instance, CRISPR/Cas13a has been applied on *Aspergillus fumigatus*, the causative agent of aspergillosis infection, to develop a reliable and rapid diagnostic tool (Li et al., 2021). Many studies have also been conducted on industrially important fungus, *Saccharomyces cerevisiae* and heat stress resistant *S.*

cerevisiae mutants have been generated (Satish et al., 2020). The CRISPR/Cas9 system has also been applied on a lactic acid-producing industrial strain of *S. cerevisiae*, in which a DNA knock-in and gene-cleavage system was successfully established (Stovicek et al., 2015). These latter two examples may suffice to exemplify the effective engineering of diverse industrial strains by the CRISPR system. Furthermore, and a multiple gene targeting study have achieved high-efficiency lipid synthesis in *Yarrowia lipolytica* (Friedlander et al., 2016).

CRISPR/Cas9 also has the potential to be used in the treatment of viral infections. It has been reported that HIV infection was significantly reduced in all founder strains produced after CRISPR TatDE transfection or lentivirus treatments (Herskovitz et al., 2021). Cas13a encoded with messenger RNA (mRNA) has been used to reduce influenza virus A and SARS-CoV-2—severe acute respiratory syndrome coronavirus 2. When given to mice upon post-infection, Cas13a effectively damaged influenza RNA in lung tissue, while reducing replication of SARS-CoV-2 and disease symptoms in hamsters (Blanchard et al., 2021). Today, with specific Cas9-based methods, HIV-1 DNA can be destroyed with high efficiency and its reinfections can be prevented (Hu et al., 2014). Guide RNAs could be designed to target Cas9 to specific viral genes (Mandal et al., 2014). Induced pluripotent stem cells (iPSCs) could be produced by using TALENs or a combination of CRISPR/Cas9 (Ye et al., 2014).

The infectious disease COVID-19, caused by SARS-CoV-2 has posed a persistent global threat. Because the spreading of SARS-CoV-2 is rapid, it has been necessary to accurately detect the agent in order to reduce its infection speed worldwide. To this end, two technologies are widely used: mNGS—clinical metagenomic next-generation sequencing and RT-qPCR—quantitative reverse transcription polymerase chain reaction (Wu et al., 2020). mNGS technology has advantages as well as disadvantages. Its advantages are unlimited usability of the culture, extensive analysis and high efficiency. The disadvantages primarily lie in the complexity of the process, long-term detection, and excessive cost. The latter technique, RT-qPCR, is rather sophisticated but it shortens the time of diagnosis. The third approach, CRISPR, has seemed to offer better means of manipulation. Diagnostic process can be speeded up by CRISPR/Cas with its ease of operation and at

relatively low cost. CRISPR/Cas9, Cas12a/b, and Cas13a-based diagnostic tools have now been made available for CoViD-19, with up to 100% sensitivity and specificity (Zhan et al., 2021) (Table 5). Beside these advantages the only problem with this approach remains to be the requirement of substantial amounts of target RNA. The false-positive rate becomes another concern.

Table 5: CRISPR/Cas Methods Used In The Diagnosis Of CoVid-19 (Zhan et al., 2021)

Platform Name	Cas protein	Time	Sensitivity	Specificity	Visualization	Target genes
contamination-free visual detection	Cas12a	40 min	100%	100%	Lateral flow	ORF1ab, N, E
SENA	Cas12a	N/A	99%	99%	Fluorescence	ORF1ab, N
opvCRISPR	Cas12a	45 min	N/A	N/A	Naked eye	S
iSCAN	Cas12a	40 min	86%	100%	Fluorescence/lateral flow	N, E
ITP-CRISPR	Cas12a	30-40 min	93.8%	100%	Fluorescence	N, E
CRISPR-FDS	Cas12a	15 min	100%	100%	Fluorescence	ORF1ab
CRISPR/Cas12a-NER	Cas12a	45 min	N/A	N/A	Fluorescence	E
STOPCovid.v1	Cas12b	50 min	N/A	N/A	Lateral flow	N
STOPCovid.v2	Cas12b	15-45 min	93.1%	98.5%	Fluorescence/lateral flow	N
DETECTR	Cas12a	45 min	95%	100%	Lateral flow	E, N
MeCas12a	Cas12a	45 min	100%	100%	Naked eye	E
ENHANCE	Cas12a	40-60 min	N/A	N/A	Fluorescence/lateral flow	N
AIOD-CRISPR	Cas12a	20 min	N/A	N/A	Naked eye	N
COVID-19 CRISPR-FDS	Cas12a	50 min	100%	71.4%	Fluorescence	ORF1ab, N
CRISPR-ABC	Cas12a	30-40 min	91.2%	99.2%	Fluorescence	ORF1ab
OR-DETECTR	Cas12a	50 min	N/A	N/A	Fluorescence	RdRp, N
CASdetec	Cas12b	60 min	N/A	N/A	Naked eye	RdRp
SHERLOCK	Cas13a	40 min	100%	100%	Fluorescence	ORF1ab, N, S
SHINE	Cas13a	50 min	90%	100%	Smartphone/lateral flow	ORF1ab
DISCoVER	Cas13a	30 min	N/A	100%	Fluorescence	N
CARMEN	Cas13a	N/A	N/A	N/A	Fluorescence	N/A
Ultralocalized Cas13a assay	Cas13a	N/A	single-molecule	single-nucleotide	Fluorescence	ORF1a, N
TL-LFA	Cas9	< 1 h	100%	97.1%	Fluorescence	ORF1ab, E

CRISPR/Cas13 is a system guided and targeted by RNA. It can be used to inhibit the replication capacity of RNA viruses and to destroy viral RNA. SARS-CoV-2 is a single-stranded RNA virus. The CRISPR/Cas13d system has been used to damage this RNA (Nguyen et al., 2020). In another study, the most likely sites for targeting viral RNA has been identified by using bioinformatics analyses (Abbott et al., 2020). As a result, 6 crRNAs were found that can target approximately 91% of SARS-CoV-2 genomes. In addition, 22 crRNAs have been developed and reported to cover all sequences of coronaviruses. Finally, it has been shown that the CRISPR/Cas13a can alleviate Covid-19 infection in laboratory animals (Blanchard et al., 2021). These findings suggest that viruses causing respiratory tract infections can be targeted with Cas13a, and their severity could be reduced. These studies show that CRISPR/Cas-based therapeutic agents offer an important option in the treatment of Covid-19.

Members of the phylum Apicomplexa are single-celled parasitic organisms. Parasites naturally need a host to live in. CRISPR/Cas applications are also involved in the treatment and diagnosis of these diseases. CRISPR/Cas9 was first studied in protists of the phylum Apicomplexa, such as shown that *Plasmodium spp*., *Cryptosporidium* spp. and *Toxoplasma gondii* (Di Cristina and Carruthers, 2018). Expression vectors harbouring donor DNA, sgRNA and cas9, have been constructed and adapted to *P. falciparum* (Ghorbal et al., 2014). They have been used to disrupt the chromosome of *P. falciparum*. With the ensuing studies it has been demonstrated that CRISPR/Cas9 could be successfully used in many other parasites, including *Toxoplasma gondii* (Shen et al., 2014) *Cryptosporidium parvum* (Vinayak et al., 2015), *Strongyloides stercoralis* (Gang et al., 2017), and *Trichomonas vaginalis* (Janssen et al., 2018) (Table 6). Another study has used CRISPR/Cas9 as a genetic tool in *Entamoeba histolytica* (Kangussu-Marcolino et al., 2020). And Cas9 has been stably expressed in *Trypanosoma brucei*, *Leishmania major*, and *L mexicana* (Beneke et al., 2017).

Table 6: CRISPR/Cas9 Applications In Protozoa (optimized from Grzybek et al., 2021).

Organism	**Purpose**	**Strategy (Vectors)**	**Repair mechanism**	**References**
Plasmodium falciparum	Gene knockout	2	HDR	Ghorbal et al., 2014
Toxoplasma gondii	Gene knockout/in	1	NHEJ, HDR	Shen et al., 2014
Cryptosporidium parvum	Gene knockout/in	1	HDR	Vinayak et al., 2015
Trichomonas vaginalis	Gene knockout/in	1 - 2	NHEJ, HDR	Jannsen et al., 2018
Strongyloides stercoralis	Gene knockout	2	HDR	Gang et. al., 2017
Trypanosoma brucei	Gene knockin	1	MMEJ	Beneke et. al., 2017
Leishmania mexicana	Gene knockin	1	MMEJ	
Leishmania major	Replacement of a gene tandem	2	HDR	
Entamoeba histolytica				Kangussu-Marcolino et. al., 2020

7.3. Applications in Cancer Diagnosis and Treatment

Cancer is a global, high-mortality disease, involving various genetic and epigenetic changes within and on the chromatin (Sung et al., 2021). The CRISPR/Cas9 has been the most suitable system in cancer studies. It appeared to have a great potential in the interference of gene expression, altering oncogenes' activity, and in introducing deletions (Zhang et al., 2021). Knockdown of CD133 with CRISPR/Cas9 has been reported to significantly reduce cell proliferation and colony formation in colon cancer cells. In addition, significant inhibitory effects were observed on cell invasion and migration (Li et al., 2019). In a study with pancreatic cancer cells, CRISPR/Cas9 has inactivated microRNA 3064 (miR-3064), and thus reduced the rate of invasion and proliferation (Yan et al., 2019). It has also been applied experimentally in the treatment of TNBC—triple-negative breast cancers. The E3 ubiquitin ligase UBR5 was deleted in these experiments, and in this way it has been shown that in vivo metastasis and tumour growth could be inhibited in mice (Liao et al., 2017). Another important feature in the initiation and progression of cancer is the inactivation of tumour suppressor genes. In one study, these genes (*p53*, *nf1*, *pten* and *ptch1*) have been deleted by CRISPR/Cas9 (Zuckermann et al., 2015). A number of studies have shown that disrupted tumour suppressor genes can also be repaired using CRISPR/Cas9 technology (Zhang et al., 2021).

Cancer cells can develop resistance to cancer drugs. Chemoresistance occurs due to the chemoresistance genes. It has been demonstrated that these genes could be eliminated by CRISPR/Cas9-mediated treatments (Fig. 11).

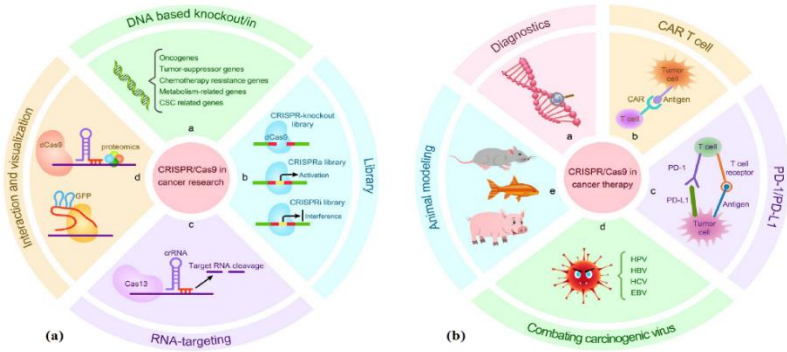


Figure 11. CRISPR/Cas9 Applications In Cancer Research And Therapy (Zhang et al., 2021)

In the diagnosis of cancer, liquid biopsy is also used in addition to traditional tissue biopsy. Liquid biopsy is an alternative approach to conventional tissue biopsy. It facilitates early detection of cancer. Moreover, better evaluation of mutations and intra-tumour heterogeneity in metastasizing cancer types is possible with this biopsy. With liquid biopsy, the drug resistance profile of cancer can be determined before and during treatment. Orthogonal Cas13 enzymes have been used in one of the studies on liquid biopsy, where multiple mutations associated with cancer could be detected at once (Palaz et al., 2021). Early detection of cancer has a great impact on survival. Research on cancer diagnosis and treatment by CRISPR/Cas systems appears to continue to increase.

8. CRISPR AND ETHICS

Although the CRISPR/Cas can be as an efficient system in genome editing and in its other applications, the use of this system in humans has brought some concerns. The CRISPR/Cas system can be applied in human gene therapy. This possibility bears many ethical factors with it. Spreading of CRISPR/Cas9's in human genome could be the first concern with its most fearsome consequences (Jothi et al., 2021). Four problems appear to be prominent in editing human embryos with CRISPR/Cas9. First, there is a potential risk factor for genome rearrangement. Another is the validity of the medical implications. Third is the criteria set for the selection of individuals. Finally, the concerns on consequent casualties which could likely occur during the establishment of relevant technologies (Lander, 2015; Jothi et al., 2021).

Genetically modified organisms, GMOs, constitute another form of concern in human societies in general, as they are already at the centre of ethical discussions. CRISPR/Cas systems have also contributed to these concerns as they bring much more powerful manipulative means in this realm (Table 7). The accelerated development of the CRISPR/Cas system and the rapid increase in its application areas have already created a worldwide CRISPR/Cas market, amounting to US$1.5 billion. And it is expected to grow by 20% until 2026.

Tablo 7. The Risk Factors And Ethical Issues Involving CRISPR/Cas9 (Takavoli et al., 2021).

Organisms	Risks	Bioethical Issues
Bacteria	Gene mutations/drifts	Disruption of ecological balance
Plants	Gene mutations/drifts	Disruption of ecological balance
Animals/ Chimeric Animals	Gene mutations	Disruption of ecological balance
Humans	Gene mutations Side effects Cost Genetic mosaicism	Eugenics Informed consent Enhancement Accessibility Patenting Safety Incomplete or over legislation

CONCLUSION

CRISPR/Cas systems have been rapidly developing into powerful DNA manipulation tools since their discovery in 1987. Although not included in the references, hundreds and even thousands of forthcoming studies apply the CRISPR/Cas system in medicine in order to treat a diverse cohort of diseases, to increase the yield and nutritive quality of agriculture products and of food industry. Despite the valid ethical concerns, the future success of this technology seems to be inevitable.

REFERENCES

Abbott, T. R., Dhamdhere, G., Liu, Y., Lin, X., Goudy, L., Zeng, L., Chemparathy, A., Chmura, S., Heaton, N. S., Debs, R., Pande, T., Endy, D., La Russa, M. F., Lewis, D. B., and Qi, L. S. (2020). Development of CRISPR as an Antiviral Strategy to Combat SARS-CoV-2 and Influenza, Cell, Vol. 181, pp 865–876. Doi: 10.1016/j.cell.2020.04.020.

Adli, M. (2018). The CRISPR tool kit for genome editing and beyond, Nature Communications, Vol. 9:1911, pp 1–13. Doi: 10.1038/s41467-018-04252-2.

Barrangou, R., Marraffini, L. A. (2014). CRISPR-Cas Systems: Prokaryotes Upgrade to Adaptive Immunity, Molecular Cell, Vol. 54, No. 2, pp 234-44. Doi: 10.1016/j.molcel.2014.03.011.

Beneke, T., Madden, R., Makin, L., Valli, J., Sunter, J., and Gluenz, E. (2017). A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids, Royal Society Open Sciences, Vol. 4, No. 5, 170095. Doi: 10.1098/rsos.170095.

Bikard, D., Euler, C.W., Jiang, W., Nussenzweig, P.M., Goldberg, G.W., Duportet, X., Fischetti, V.A., and Marraffini, L.A. (2014). Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials, Nature Biotechnology, Vol. 32, No. 11, pp 1146–1150. Doi: 10.1038/nbt.3043.

Blanchard, E. L., Vanover, D., Bawage, S. S., Tiwari, P. M., Rotolo, L., Beyersdorf, J., Peck, H. E., Bruno, N. C., Hincapie, R., Michel, F., Murray, J., Sadhwani, H., Vanderheyden, B., Finn, M. G., Brinton, M. A., Lafontaine, E. R., Hogan, R. J., Zurla, C., and Santangelo, P. J. (2021). Treatment of influenza and SARS-CoV-2 infections via mRNA-encoded Cas13a in rodents, Nature Biotechnology, Vol. 39, pp 717-726. Doi: 10.1038/s41587-021-00822-w.

Brooks, C., Nekrasov, V., Lippman, Z. B., and Van Eck J. (2014). Efficient Gene Editing in Tomato in the First Generation Using the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated9 System, Plant Physiology, Vol. 116, pp 1292-1297. Doi: doi/10.1104/pp.114.247577.

Citorik, R. J., Mimee, M., Lu, T. K. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases, Nature Biotechnology, Vol. 32, No. 11, pp 1141–1145. Doi: 10.1038/nbt.3011

Chen, K., and Gao, C. (2013). TALENs: customizable molecular DNA scissors for genome engineering of plants, Journal of Genetics and Genomics, Vol. 40, No. 6, pp 271–279. Doi: 10.1016/j.jgg. 2013.03.009.

Di Cristina, M., and Carruthers, V. B. (2018). New and emerging uses of CRISPR/Cas9 to genetically manipulate apicomplexan parasites, Parasitology, Vol. 145, pp 1119–1126. Doi: 10.1017/S003118201800001X.

Fage, C., Lemire, N., Moineau, S. (2020). Delivery of CRISPR-Cas systems using phage-based vectors, Current Opinion in Biotechnology, Vol. 68, pp 174–180. Doi: 10.1016/j.copbio.2020.11.012.

Friedlander, J., Tsakraklides, V., Kamineni, A., Greenhagen, E. H., Consiglio, A. L., MacEwen, K., Crabtree, D. V., Afshar, J., Nugent, R. L., Hamilton, M. A., Joe Shaw, A., South, C. R., Stephanopoulos, G., Brevnova, E. E. (2016). Engineering of a high lipid producing *Yarrowia lipolytica* strain. Biotechnology for Biofuels, Vol. 9, No. 1(77). Doi: 10.1186/s13068-016-0492-3.

Gang, S. S., Castelletto, M. L., Bryant, A. S., Yang, E., Mancuso, N., Lopez, J. B., Pellegrini, M., Hallem, E. A. (2017). Targeted mutagenesis in a human-parasitic nematode. PLOS Pathogens, Vol. 13, No. 10(e1006675). Doi: 10.1371/journal.ppat.1006675.

Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., Scherf, A., and Lopez-Rubio1, J-J. (2014). Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRIScrisPR-Cas9 system, Nature Biotechnology, Vol. 32, No. 8, pp 819-21. Doi: 10.1038/nbt.2925.

Gök, Z.G., and Tunalı, B. Ç. (2016). CRISPR-Cas İmmün Sisteminin Biyolojisi, Mekanizması ve Kullanım Alanları (Biology, Mechanism and Applications of CRISPR-Cas Immune System), International Journal of Engineering Research and Development, Vol. 8, No.2, pp 11-23.

Grzybek, M., Golonko, A., Górska, A., Szczepaniak, K., Strachecka, A., Lass, A., Lisowski, P. (2018). The CRISPR/Cas9 system sheds new lights on the biology of protozoan parasites, Applied Microbiology and Biotechnology, Vol. 102, No. 11, pp 4629–4640. Doi: 10.1007/s00253-018-8927-3.

Herskovitz, J., Hasan, M., Patel, M., Blomberg, W. R., Cohen, J. D., Machhi, J., Shahjin, F., Mosley, R. L., McMillan, J. E., Kevadiya, B. D., Gendelman, H. E. (2021). CRISPR-Cas9 Mediated Exonic Disruption for HIV-1 Elimination, EbioMedicine, Vol. 73(103678). Doi: 10.1016/j.ebiom.2021.103678.

Hille, F., and Charpentier, E. (2016). CRISPR-Cas: biology, mechanisms and relevance, Philosophical Transactions of the Royal Society B, Vol. 371(20150496). Doi: 10.1098/rstb.2015.0496.

Horvath, P., and Barrangou, R. (2010). CRISPR/Cas, the Immune System of Bacteria and Archaea, Science, Vol. 327, pp 167-170. Doi: 10.1126/science.1179555.

Hu, W., Kaminskia, R., Yanga, F., Zhang, Y., Cosentino, L., Lia, F., Luob, B., Alvarez-Carbonellc, D., Garcia-Mesac, Y., Karn, J., Mo, X., Khalili, K. (2014). RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection, Proceedings of the National Academy of Sciences, Vol. 111, No. 31, pp 11461-6. Doi: 10.1073/pnas.1405186111.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., and Nakata, A. (1987). Nucleotide Sequence of the iap Gene, Responsible for Alkaline Phosphatase Isozyme Conversion in *Escherichia coli*, and Identification of the Gene Product, Journal of Bacteriology, Vol. 169, No. 12, pp 5429-5433. Doi: 10.1128/jb.169.12.5429-5433.1987.

Ishino, Y., Krupovic, M., Forterrea, P. (2018). History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology, Journal of Bacteriology, Vol. 200, No. 7(e00580-17). Doi: 10.1128/JB.00580-17.

Jansen, R., Embden, J. D., Gaastra, W., and Schouls L. W. (2002). Identification of genes that are associated with DNA repeats in prokaryotes. Molecular Microbiology, Vol. 43, No. 6, pp 1565–1575. Doi: 10.1046/j.1365-2958.2002.02839.x.

Janssen, B. D., Chen, Y-P., Molgora, B. M., Wang, S. E., Simoes-Barbosa, A. and Johnson, P. J. (2018). CRISPR/Cas9-mediated gene modification and gene knock out in the human-infective parasite *Trichomonas vaginalis*. Scientific Reports, Vol. 10, No. 8(1) pp 270. Doi: 10.1038/s41598-017-18442-3.

Javed, M. R., Sadaf, M., Ahmed, T., Jamil, A., Nawaz, M., Abbas, H., Ijaz, A. (2018). CRISPR-Cas System: History and Prospects as a Genome Editing Tool in Microorganisms, Current Microbiology, Vol. 75, pp 1675–1683. Doi: 10.1007/s00284-018-1547-4.

Jia, H., Orbovic, V., Jones, J. B., and Wang, N. (2015). Modification of the PthA4 effector binding elements in Type I CsLOB1 promoter using Cas9/sgRNA to produce transgenic Duncan grapefruit alleviating XccDpthA4:dCsLOB1.3 infection, Plant Biotechnology Journal, Vol. 14, No. 5, pp 1291-301. Doi: 10.1111/pbi.12495.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science, Vol. 337(6096), pp 816-21. Doi: 10.1126/science.1225829.

Jothi, R., Karthika, C., Kamaladevi, A., Satish, L., Pandian, S. K., and Gowrishankar, S., (2021). CRISPR based bacterial genome editing and removal of pathogens, Progress in Molecular Biology and Translational Science, Vol. 179, pp 77-92. Doi: 10.1016/bs.pmbts.2020.12.013.

Kangussu-Marcolino, M. M., Morgado, P., Manna, D., Yee, H., and Singh, U. (2020). Development of a CRISPR/Cas9 system in *Entamoeba histolytica*: proof of concept, International Journal for Parasitology, Vol. 51, No. (2–3), pp 193-200. Doi: 10.1016/j.ijpara.2020.09.005.

Karimian, A., Azizian, K., Parsian, H., Rafieian, S., Shafiei-Irannejad, V., Kheyrollah, M., Yousefi, M., Majidinia, M., and Yousefi, B. (2019). CRISPR/Cas9 technology as a potent molecular tool for gene therapy. J. Cell Physiol., Vol. 234, No. 8, pp 12267-12277. Doi: 10.1002/jcp.27972.

Koonin, E. V., and Makarova, K. S. (2019). Origins and evolution of CRISPR-Cas systems, Philosophical Transactions of the Royal Society B., Vol. 374(20180087). Doi: 10.1098/rstb.2018.0087.

Kozovska, Z., Rajcaniova S., Munteanu P., Dzacovska S., Demkova L. (2021). CRISPR: History and perspectives to the future, Biomedicine & Pharmacotherapy, Vol. 141(111917). Doi: 10.1016/j.biopha.2021. 111917.

Kulus, D. (2018). Genetic resources and selected conservation methods of tomato, Journal of Applied Botany and Food Quality, Vol. 91, pp 135–144. Doi:10.5073/JABFQ.2018.091.019.

Lander, E. S. (2015). Brave new genome, New England Journal of Medicine, Vol. 373, No. 1, pp 5-8. Doi: 10.1056/NEJMp1506446.

Lander, E. S. (2016). The Heroes of CRISPR, Cell, Vol. 164, No. 1-2, pp 18-28. Doi: 10.1016/j.cell.2015.12.041.

Li, W., Cho, M. Y., Lee, S., Jang, M., Park, J., Park, R. (2019). CRISPR-Cas9 mediated CD133 knockout inhibits colon cancer invasion through reduced epithelial-mesenchymal transition. PLoS One, Vol. 14, No. 8(e0220860). Doi: 10.1371/journal.pone.0220860.

Li , Z., Wang, M., Xu, T., Zhan Y., Chen, F., Lin, Y., Li, S., Cheng, J., Ye, F. (2021). Development and clinical implications of a novel CRISPR-based diagnostic test for pulmonary Aspergillus fumigatus infection, Journal of Microbiology, Immunology and Infection, In Press. Doi: 10.1016/j.jmii.2021.11.008.

Liao, L., Song, M., Li, X., Tang, L., Zhang, T., Zhang, L., Pan, Y., Chouchane, L., and Ma, X. (2017). E3 ubiquitin ligase UBR5 drives the growth and metastasis of triple-negative breast cancer. Cancer Research, Vol. 77, No. 8, pp 2090-2101. Doi: 10.1158/0008-5472.CAN-16-2409.

Long, C., McAnally, J. R., Shelton, J. M., Mireault, A. A., Bassel-Duby, R., Olson, E. N. (2013). Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA, Science, Vol. 345(6201), pp 1184-1188. Doi: 10.1126/science.1254445.

Makarova, K. S., Aravind, L., Grishin, N. V., Rogozin, I. B., Koonin, E. V. (2002). A DNA repair system specific for thermophilic *Archaea* and *Bacteria* predicted by genomic context analysis. Nucleic Acids Research, Vol. 30, No. 2, pp 482–496. Doi: 10.1093/nar/30.2.482.

Makarova K. S., Grishin, N. V., Shabalina, S. A., Wolf, Y. I., Koonin E .V. (2006). A putative RNA-interference-based immune system in

prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biology Direct, Vol. 1, No. 7. Doi: 10.1186/1745-6150-1-7.

Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J. J., Charpentier, E., Haft, D. H., Horvath, P., Moineau, S., Mojica, F. J. M., Terns, R. M., Terns, M. P., White, M. F., Yakunin, A. F., Garrett, R. A., van der Oost, J., Backofen, R., and Koonin, E. V. (2015). An updated evolutionary classification of CRISPR–Cas systems, Nature Reviews Microbiology, Vol. 13, pp 1–16. Doi:10.1038/nrmicro3569.

Mandal, P. K., Ferreira, L. M., Collins, R., Meissner, T. B., Boutwell, C. L., Friesen, M., Vrbanac, V., Garrison, B. S., Stortchevoi, A., Bryder, D., Musunuru, K., Brand, H., Tager, A. M., Allen, T. M., Talkowski, M. E., Rossi, D. J., Cowan, C. A. (2014). Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9, Cell Stem Cell, Vol. 15, No. 5, pp 643–52. Doi: 10.1016/j.stem. 2014.10.004.

Mojica, F. J. M., Juez, G., and Rodrı´guez-Valera, F. (1993). Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. Molecular Microbiology. Vol. 9, pp 613–621. Doi: 10.1111/j.1365-2958.1993.tb01721.x.

Mougiakos, I., Bosma, E. F., de Vos, W. M., van Kranenburg, R., and van der Oost, J., (2016). Next generation prokaryotic engineering: the CRISPR-Cas toolkit, Trends In Biotechnology, Vol. 34, No. 7, pp 575–587. Doi: 10.1016/j.tibtech.2016.02.004.

Nguyen, T. M., Zhang, Y., Pandolfi, P. P. (2020). Virus against virus: a potential treatment for 2019-nCov (SARS-CoV-2) and other RNA viruses, Cell Research, Vol. 30, pp 189–190. Doi: 10.1038/s41422-020-0290-0.

Palaz, F., Kalkan, A. K., Can, Ö., Demir, A. N., Tozluyurt, A., Özcan, A., and Ozsoz, M. (2021). CRISPR-Cas13 System as a Promising and Versatile Tool for Cancer Diagnosis, Therapy, and Research, ACS Synthetic Biology, Vol. 10, No. 6, pp 1245–1267. Doi: 10.1021/acssynbio. 1c00107.

Rath, D., Amlinger, L., Rath, A., and Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications, Biochimie, Vol. 117, pp 119–128. Doi: 10.1016/j.biochi.2015.03.025.

Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., Siksnys, V. (2011). The Streptococcus thermophilus CRISPR/Cas system provides immunity in *Escherichia coli*, Nucleic Acids Research, Vol. 39, No. 21, pp 9275–9282. Doi: 10.1093/nar/gkr606.

Satish, L., Shamili, S., Muthubharathi, B. C., Ceasar, S. A., Kushmaro, A., Singh, V., and Sitrit, Y. (2020). CRISPR-Cas9 system for fungi genome engineering toward industrial applications, Singh, V., and Dhar, P. K. (ed.). Academic Press, pp 69–81.

Schwank, G., Koo, B-K., Sasselli, V., Dekkers, J. F., Heo, I., Demircan, T., Sasaki, N., Boymans, S., Cuppen, E., van der Ent, C. K., Nieuwenhuis, E. E. S., Beekman, J. M., and Clevers, H. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients, Cell Stem Cell, Vol. 13, No. 6, pp 653–8. Doi: 10.1016/j.stem.2013.11.002.

Seed, K. D., Lazinski, D. W., Calderwood, S. B., and Camilli A. (2013). A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity, Nature, Vol. 494(7438), pp 489–491. Doi:10.1038/nature11927.

Shabbir, M. A. B., Shabbir, M. Z., Wu, Q., Mahmood, S., Sajid, A., Maan, M. K., Ahmed, S., Naveed, U., Hao, H., and Yuan, Z. (2019). CRISPR‐cas system: biological function in microbes and its use to treat antimicrobial resistant pathogens, Annals of Clinical Microbiology and Antimicrobials, Vol. 18, No. 21, pp 1-9. Doi: 10.1186/s12941-019-0317-x.

Shen, B., Brown, K. M., Lee, T. D., Sibley, L. D. (2014) Efficient gene disruption in diverse strains of Toxoplasma gondii using CRISPR/CAS9. mBio, Vol. 5, No. 3(e01114-14). Doi: 10.1128/mBio. 01114-14.

Stovicek, V., Borodina, I., Forster, J. (2015). CRISPReCas system enables fast and simple genome editing of industrial Saccharomyces cerevisiae strains. Metabolic Engineering Communications, Vol. 20, No. 2, pp 13–22. Doi: 10.1016/j.meteno.2015.03.001.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal. A., and Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians, Vol. 71, No. 3, pp 209–49. Doi: 10.3322/caac.21660.

Tavakoli, K., Pour-Aboughadareh, A., Kianersi, F., Poczai, P., Etminan A., and Shooshtari L., (2021). Applications of CRISPR-Cas9 as an Advanced Genome Editing System in Life Sciences, BioTech, Vol. 10, No. 14, pp 1-17. Doi: 10.3390/biotech10030014.

Tian, Y., Liu, T., Liu, C., Xu, Q., Liu, Q. (2022). Pathogen detection strategy based on CRISPR, Microchemical Journal, Vol. 174(107036). Doi: 10.1016/j.microc.2021.107036.

Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases, Nature Reviews Genetics, Vol. 11, No. 9, pp 636–646. Doi: 10.1038/nrg28 42.

Vinayak, S., Pawlowic, M. C., Sateriale, A., Brooks, C. F., Studstill, C. J., Bar-Peled, Y., Cipriano, M. J., and Striepen, B. (2015). Genetic modification of the diarrhoeal pathogen Cryptosporidium parvum. Nature, Vol. 523, pp 477–480. Doi: 10.1038/nature14651.

Walton, R. T., Hsu, J. Y., Joung, J. K., and Kleinstiver, B. P. (2021). Scalable characterization of the PAM requirements of CRISPR–Cas enzymes using HT-PAMDA, Nature Protocols, Vol. 16, pp 1511–1547. Doi: 10.1038/s41596-020-00465-2.

Wan, L., Wang, Z., Tang, M., Hong, D., Sun Y., Ren, J., Zhang N., and Zeng H. (2021). CRISPR-Cas9 Gene Editing for Fruit and Vegetable Crops: Strategies and Prospects, Horticulturae, Vol. 7, No. 193, pp 1-19. Doi: 10.3390/horticulturae7070193.

Wilkinson, R., and Wiedenheft B. (2014). A CRISPR method for genome engineering, F1000Prime Reports, Vol. 6, No. 3. Doi:10.12703/P6-3.

Wu, Y., Liang, D., Wang, Y., Bai, M., Tang, W., Bao, S., Yan, Z., Li, D., and Li, J. (2013). Correction of a Genetic Disease in Mouse via Use of CRISPR-Cas9, Cell Stem Cell, Vol. 13, No. 6, pp 659-62. Doi: 10.1016/j.stem.2013.10.016.

Wu, F., Zhao, S., Yu, B., Chen, Y-M., Wang, W., Song, Z-G., Hu, Y., Tao, Z-W., Tian, J-H., Pei, Y-Y., Yuan, M-L., Zhang, Y-L., Dai, F-H., Liu, Y.,

Wang, Q-M., Zheng, J-J., Xu, L., Holmes, E. C., & Zhang, Y-Z. (2020). A new coronavirus associated with human respiratory disease in China, Nature, Vol. 579, pp 265-269. Doi: 10.1038/s41586-020-2008-3.

Yan, J., Jia, Y., Chen, H., Chen, W., Zhou, X. (2019). Long non-coding RNA PXN-AS1 suppresses pancreatic cancer progression by acting as a competing endogenous RNA of miR-3064 to upregulate PIP4K2B expression, Journal of Experimental & Clinical Cancer Research, Vol. 38, No. 1(390). Doi: 10.1186/s13046-019-1379-5.

Ye, L., Wang, J., Beyer, A. I., Teque, F., Cradick, T. J., Qi, Z., Chang, J. C., Bao, G., Muench, M. O., Yu, J., Levy, J. A., Kan, Y. W. (2014). Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Δ32 mutation confers resistance to HIV infection, Proceedings of the National Academy of Sciences, Vol. 111, No. 26, pp 9591-6. Doi: 10.1073/pnas.1407473111.

Yeh, T-K., Jean, S-S., Lee, Y-L., Lu, M-C., Ko, W-C., Lin, H-J., Liu, P-Y., Hsueh, P-R. (2022). Bacteriophages and phage-delivered CRISPR-Cas system as antibacterial therapy, International Journal of Antimicrobial Agents, Vol. 59, No. 1(106475). Doi: 10.1016/j.ijantimicag. 2021.106475.

Yin, H., Xue, W., Chen, S., Bogorad, R. L., Benedetti, E., Grompe, M., Koteliansky, V., Sharp, P. A., Jacks, T., Anderson, D. G. (2014). Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype, Nature Biotechnology, Vol. 32, No. 6, pp 551-3. Doi: 10.1038/nbt.2884.

Zhan, Y., Li X-P., and Yin J-Y. (2021). COVID-19 one year later: a retrospect of CRISPR-Cas system in combating COVID-19, International Journal of Biological Sciences, Vol. 17, No. 8, pp 2080-2088. Doi: 10.7150/ijbs.60655.

Zhang, H., Qin, C., An, C., Zheng, X., Wen, S., Chen, W., Liu, X., Lv, Z. Yang, P., Xu, W., Gao, W., and Wu, Y. (2021). Application of the CRISPR/Cas9-based gene editing technique in basic research, diagnosis, and therapy of cancer, Molecular Cancer, Vol. 20, No. 1, pp 126. Doi: 10.1186/s12943-021-01431-6.

Zuckermann, M., Hovestadt, V., Knobbe-Thomsen, C. B., Zapatka, M., Northcott, P. A., Schramm, K., Belic, J., Jones, D. T. W., Tschida, B., Moriarity, B., Largaespada, D., Roussel, M. F., Korshunov, A., Reifenberger, G., Pfister, S. M., Lichter, P., Kawauchi, D. and Gronych, J. (2015). Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. Nature Communications, Vol. 6(7391). Doi: 10.1038/ncomms8391.

CHAPTER 5

POSTBIOTICS AND PARAPROBIOTICS: A NEW TREND FOR FUNCTIONAL FOODS

Res. Asst. Dr. Betül AYDIN[1]

[1] Gazi University, Faculty of Science, Department of Biology, Ankara, Turkey. barslan@gazi.edu.tr ORCID ID: 0000-0002-9092-1350

INTRODUCTION

Social, economic, and technological factors in human life cause many habits to change. One of the habits affected by these factors is the daily diet of people. Access to information more quickly with developing technology triggers people to review their eating habits for a healthier life. In response to the demands made on this subject, the term functional food has entered our lives. Functional foods are defined as foods that improve health status as well as meet basic nutritional needs (Cencic & Chingwaru, 2010; Shahidi, 2004). Studies show that functional foods have positive effects on health conditions such as cardiovascular diseases (Chiu et al., 2018), diabetes (Alkhatib et al., 2017), obesity (Sunkara & Verghese, 2014), neurodegenerative diseases (John et al., 2020), healthy aging (Ferrari, 2004) and cancer (Islam & Siddiqua, 2020). Fermented products containing probiotic microorganisms constitute an important group among functional foods that attract attention with their many health-beneficial features. However, the use of postbiotics and paraprobiotics, which are a discovery in functional food ingredients, has increased the interest in this subject (Cuevas-González et al., 2020).

1. PROBIOTICS

Originating from a Greek word meaning "for life", the term probiotic is defined in a 2001 report by "the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO)" as "live microorganisms that, when taken in adequate amounts, benefit the health of the host" (FAO/WHO, 2001). When people started to consume fermented milk and meat products, probiotic cultures met the human body; however, their existence was discovered centuries later. Studies conducted for many years have shown the prophylactic and therapeutic effects of probiotic bacteria on the gastrointestinal system, immune system, cardiovascular system, and many types of cancer. It is thought that these beneficial effects of

probiotic bacteria are realized with three different approaches (Figure 1).

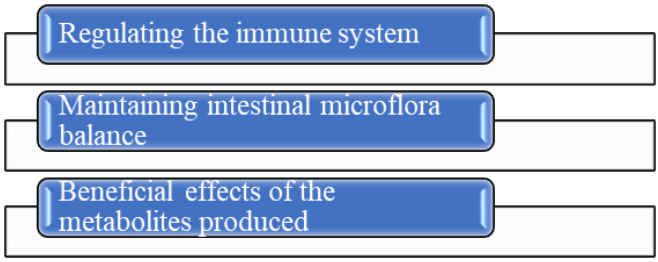


Figure 1. Three Different Approaches To The Health Benefits Of Probiotic Bacteria

In the first approach, probiotic bacteria regulate the innate or acquired immune system of the host. This emphasizes the importance of it in the management or prevention of infectious diseases, as well as the reduction of digestive tract inflammationProbiotic bacteria are advantageous in maintaining the microbial balance in the gastrointestinal system, according to the second approach, by altering the commensal or pathogenic microorganisms that live in the intestines. In the third approach, probiotic bacteria generate metabolites that have detoxification-like properties (Oelschlaeger, 2010). Although bacteria are generally included in probiotics, there are also molds and yeasts with probiotic properties, and lactic acid bacteria are the most common probiotic bacteria group due to their generally recognized as safe (GRAS) (Aguilar-Toalá et al., 2018; Singh et al., 2011). The most commonly used lactic acid bacteria species in probiotic formulations are listed in Table 1.

Table 1. Lactic Acid Bacteria Species Most Commonly Used In Probiotic Preparations (Parvez et al., 2006)

Lactobacillus sp.	*Bifidobacterium* sp.	*Enterococcus* sp.	*Streptococcus* sp.
L. acidophilus	*B. bifidum*	*E. faecalis*	*S. cremoris*
L. casei	*B. adolescentis*	*E. faecium*	*S. salivarius*
L. delbrueckii ssp. *(bulgaricus)*	*B. animalis*		*S. diacetylactis*
L. cellobiosus	*B. infantis*		*S. intermedius*
L. curvatus	*B. thermophilum*		
L. fermentum	*B. longum*		
L. lactis			
L. plantarum			
L. reuteri			
L. brevis			

Especially *Lactobacillus* and *Bifidobacterium* species are preferred as starter cultures in fermented food products, but *Enterococcus* species are also used in foods due to their technological advantages (such as acid and salt resistance) (Hanchi et al., 2018) and some beneficial effects on health (Nascimento et al., 2019). When deciding whether to use any microbial culture in food applications, the strain must have met many competencies. A decision tree form was created for a safety assessment process for microbial cultures (Pariza et al., 2015). Even though probiotic bacteria to be used in foods have passed all these safety levels, some factors prevent these bacteria from showing their beneficial properties. Probiotic bacteria taken into the body through food must be able to survive based on stomach and intestinal conditions, to be able to adhere to the intestinal epithelium after coming to the intestine and colonize and reach a sufficient number there. Only after that, bacteria can show beneficial effects on health with the metabolites they produce. It is not always possible for all these conditions to occur in every individual. For this reason, the concept of probiotics has been reshaped today with the definitions of "paraprobiotic" and "postbiotic".

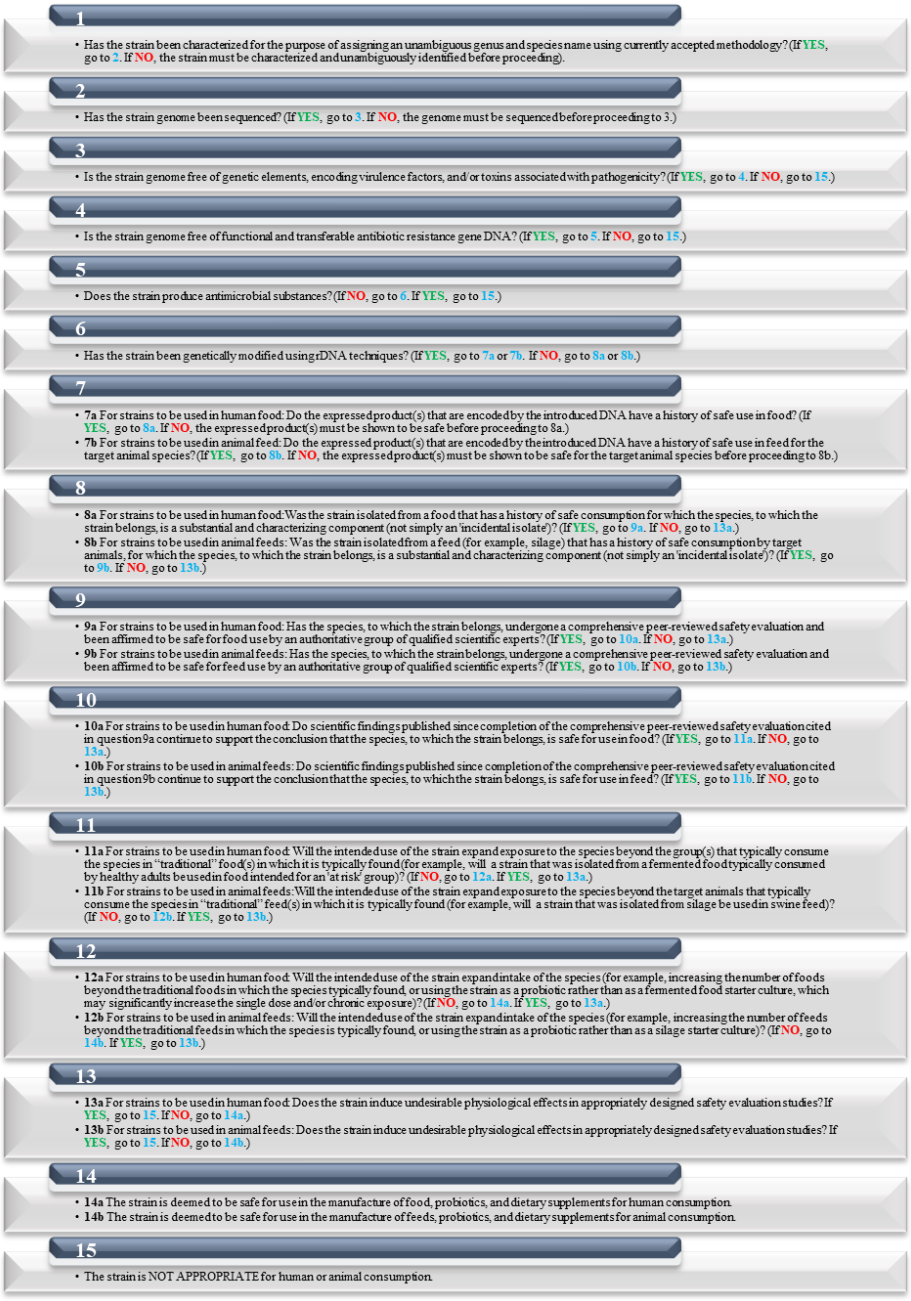


Figure 2. A Decision Tree For Evaluating The Safety Of Microbial Cultures ForHuman Or Animal Consumption (Pariza et al., 2015)

2. PARAPROBIOTICS

Some factors such as gastrointestinal system conditions and antibiotic use may adversely affect the viability of probiotic microorganisms. For this reason, the use of only living microorganisms for the treatment of some diseases may not be sufficient. Paraprobiotics, also known as ghost probiotics, non-viable probiotics, or inactivated probiotics, are defined as "inactivated microbial cells that, when taken in adequate amounts, provide benefits to the consumer" (Nataraj et al., 2020). To obtain paraprobiotics, probiotic microorganisms are inactivated by using certain chemical or physical methods indicated in Figure 3 (Akter et al., 2020; Barros et al., 2021; de Almada et al., 2016; Kim et al., 2020; Nakamura & Mitsunaga, 2018).

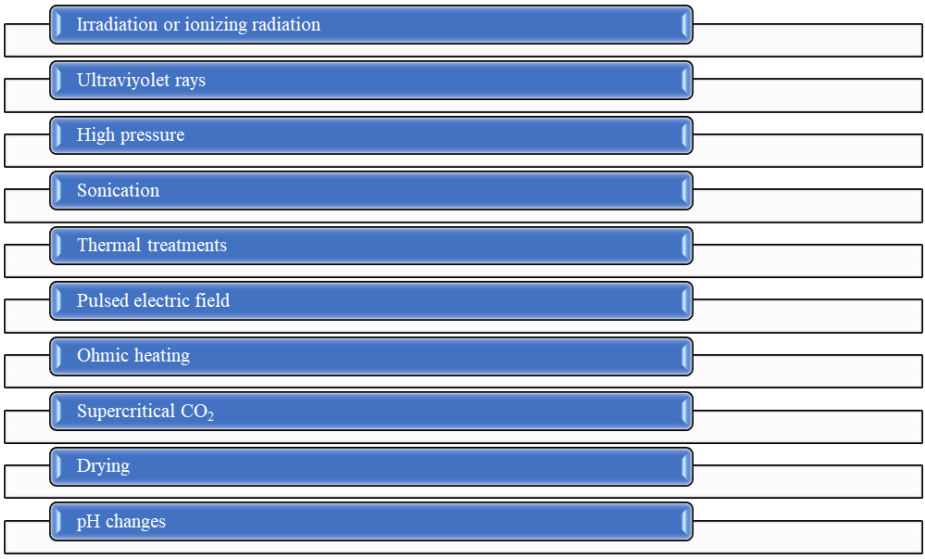


Figure 3. Inactivation Methods Of Microorganisms Used To Obtain Paraprobiotics (Akter et al., 2020; de Almada et al., 2016)

Paraprobiotics have been reported in several studies to provide a wide range of health benefits (Figure 4) (de Almada et al., 2016; Öner et al., 2021). Some of these properties are antioxidant activity (Aydin et

al., 2021), antibacterial activity (Chen et al., 2020), antiobesity (Lim et al., 2022), antiproliferative activity (Maghsood et al., 2020).



Figure 4. Health Benefits Of Paraprobiotics (de Almada et al., 2016; Siciliano et al., 2021)

It is thought that non-living bacteria exert their beneficial effects on health through the cell membrane, cell wall, and cytoplasmic components they contain (Abd El-Ghany, 2020; Teame et al., 2020).

3. POSTBIOTICS

The word postbiotic, also known as metabiotic, biogenic, or metabolite/cell-free supernatant, refers to substances produced by living bacteria or released following bacterial lysis. These products include short-chain fatty acids (SCFA), peptides, organic acids, enzymes, teichoic acid, exopolysaccharides, muropeptides, cell surface proteins, vitamins (Figure 5) (Aguilar-Toalá et al., 2018; Kerry et al.,

2018). Disadvantages such as the inability of living microorganisms to keep the cell viability at the desired value due to many factors affecting their viability in the gastrointestinal tract, their potential to transfer antibiotic resistance genes to pathogenic bacteria, the risks of increasing acute inflammation and mortality rate lead to limitations in the use of live probiotic bacteria. However, postbiotics draw attention to their advantages such as being more easily produced on a pure and industrial scale and being easily stored (Erginkaya & Konuray-Altun, 2022).

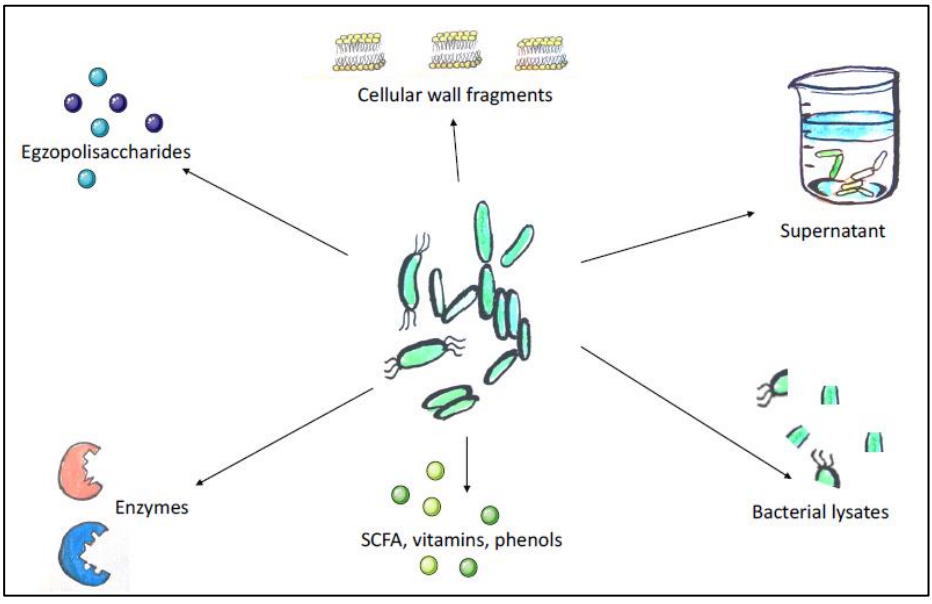


Figure 5. Types Of Postbiotics (Żółkiewicz et al., 2020)

In addition to having local effects on health such as antimicrobial (Indira et al., 2018), anti-inflammatory (Maghsood et al., 2018), and immune regulation, postbiotics also have systemic effects such as antioxidant (Liang et al., 2016), anticancer, antihypertensive, and hypocholesterolemic (Bhat & Bajaj, 2018) effects (Figure 6) (Aguilar-Toalá et al., 2018).

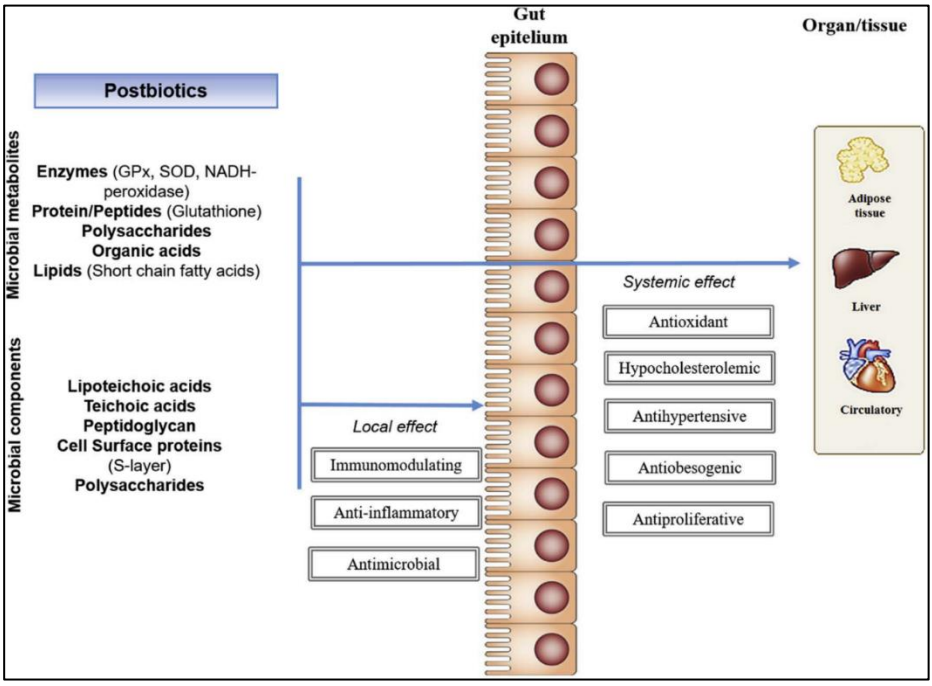


Figure 6. Potential Local And Systemic Beneficial Effects Of Postbiotics (Aguilar-Toalá et al., 2018)

Postbiotics exert their therapeutic effects through a variety of methods, including improved epithelial barrier function, beneficial microbiota regulation, immune response modulation, systemic metabolism modulation, and nervous system signaling (Figure 7) (Salminen et al., 2021).

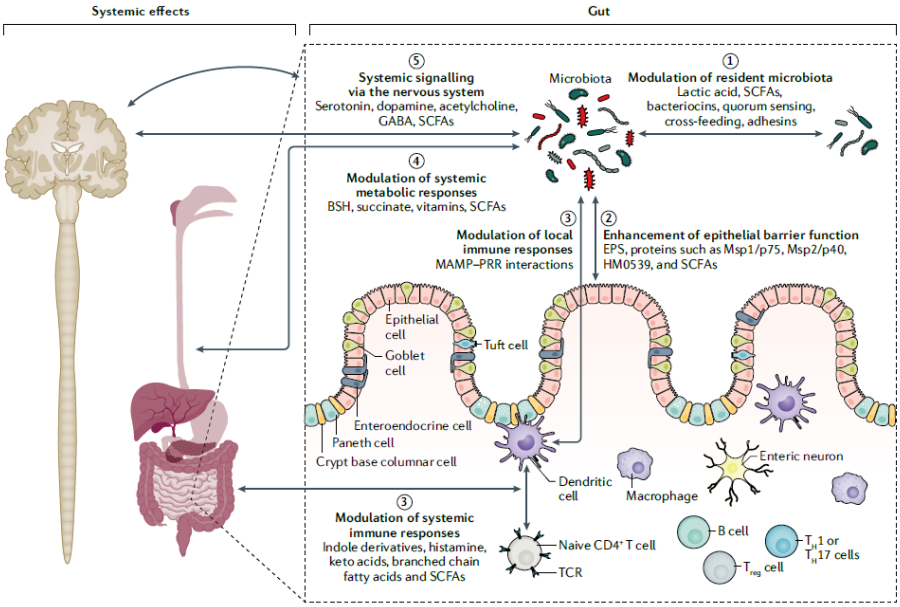


Figure 7. Mechanisms Of Action Of Postbiotics (Salminen et al., 2021)

CONCLUSION

Due to the strong relationship between nutrition and health, people pay more attention to using functional foods in their diets to lead a healthier life and to help treat their existing diseases. The beneficial effects of probiotic bacteria found in fermented foods that people often consume in their daily diet have been known for a long time. The discovery of many beneficial effects of postbiotics and paraprobiotics, which have just entered the probiotic world, has opened a new door for the development of new functional foods.

REFERENCES

Abd El-Ghany, W. A. (2020). Paraprobiotics and postbiotics: Contemporary and promising natural antibiotics alternatives and their applications in the poultry field. Open Veterinary Journal, 10(3), 323–330.

Aguilar-Toalá, J., Garcia-Varela, R., Garcia, H., Mata-Haro, V., González-Córdova, A., Vallejo-Cordoba, B., & Hernández-Mendoza, A. (2018). Postbiotics: An evolving term within the functional foods field. Trends in Food Science & Technology, 75, 105-114.

Akter, S., Park, J.-H., & Jung, H. K. (2020). Potential Health-Promoting Benefits of Paraprobiotics, Inactivated Probiotic Cells. Journal of Microbiology and Biotechnology, 30(4), 477-481.

Alkhatib, A., Tsang, C., Tiss, A., Bahorun, T., Arefanian, H., Barake, R., Khadir, A., & Tuomilehto, J. (2017). Functional foods and lifestyle approaches for diabetes prevention and management. Nutrients, 9(12), 1310.

Aydin, B., Çiydem, T., Esra, K., & Leyla, A. (2021). Evaluation of the Antioxidant Effects of Postbiotics and Paraprobiotics in Lactic Acid Bacteria Isolated from Traditional Fermented Sausages. Avrupa Bilim ve Teknoloji Dergisi, (28), 849-852.

Barros, C. P., Pires, R. P., Guimarães, J. T., Abud, Y. K., Almada, C. N., Pimentel, T. C., Sant'Anna, C., De-Melo, L. D. B., Duarte, M. C. K., & Silva, M. C. (2021). Ohmic heating as a method of obtaining paraprobiotics: Impacts on cell structure and viability by flow cytometry. Food Research International, 140, 110061.

Bhat, B., & Bajaj, B. K. (2018). Hypocholesterolemic and bioactive potential of exopolysaccharide from a probiotic *Enterococcus faecium* K1 isolated from kalarei. Bioresource Technology, 254, 264-267.

Cencic, A., & Chingwaru, W. (2010). The role of functional foods, nutraceuticals, and food supplements in intestinal health. Nutrients, 2(6), 611-625.

Chen, Y. T., Hsieh, P. S., Ho, H. H., Hsieh, S. H., Kuo, Y. W., Yang, S. F., & Lin, C. W. (2020). Antibacterial activity of viable and heat- killed probiotic strains against oral pathogens. Letters in Applied Microbiology, 70(4), 310-317.

Chiu, H.-F., Shen, Y.-C., Venkatakrishnan, K., & Wang, C.-K. (2018). Popular functional foods and nutraceuticals with lipid lowering activity and in relation to cardiovascular disease, dyslipidemia, and related complications: an overview. Journal of Food Bioactives, 2, 16–27-16–27.

Cuevas-González, P., Liceaga, A., & Aguilar-Toalá, J. (2020). Postbiotics and paraprobiotics: From concepts to applications. Food Research International, 136, 109502.

de Almada, C. N., Almada, C. N., Martinez, R. C., & Sant'Ana, A. S. (2016). Paraprobiotics: Evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. Trends in Food Science & Technology, 58, 96-114.

Erginkaya, Z., & Konuray-Altun, G. (2022). Potential biotherapeutic properties of lactic acid bacteria in foods. Food Bioscience, 101544.

FAO/WHO. (2001). Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria.

Ferrari, C. K. (2004). Functional foods, herbs and nutraceuticals: towards biochemical mechanisms of healthy aging. Biogerontology, 5(5), 275-289.

Hanchi, H., Mottawea, W., Sebei, K., & Hammami, R. (2018). The genus *Enterococcus*: Between probiotic potential and safety concerns—An update. Frontiers in microbiology, 9.

Indira, M., Venkateswarulu, T., Prabhakar, K. V., Peele, K. A., & Krupanidhi, S. (2018). Isolation and characterization of bacteriocin producing *Enterococcus casseliflavus* and its antagonistic effect on *Pseudomonas aeruginosa*. Karbala International Journal of Modern Science, 4(4), 361-368.

Islam, S. R., & Siddiqua, T. J. (2020). Functional foods in cancer prevention and therapy: Recent epidemiological findings. Functional Foods in Cancer Prevention and Therapy, 405-433.

John, T., Samuel, B., Abolaji, O., Folashade, O., Oyetooke, A., & Oluwatosin, F. (2020). Functional foods and bioactive compounds: Roles in the prevention, treatment and management of neurodegenerative diseases. GSC Biological and Pharmaceutical Sciences, 11(2), 297-313.

Kerry, R. G., Patra, J. K., Gouda, S., Park, Y., Shin, H.-S., & Das, G. (2018). Benefaction of probiotics for human health: A review. Journal of Food and Drug Analysis, 26(3), 927-939.

Kim, H., Jeon, B., Kim, W. J., & Chung, D.-K. (2020). Effect of paraprobiotic prepared from Kimchi-derived *Lactobacillus plantarum* K8 on skin moisturizing activity in human keratinocyte. Journal of Functional Foods, 75, 104244.

Liang, T.-W., Tseng, S.-C., & Wang, S.-L. (2016). Production and characterization of antioxidant properties of exopolysaccharide (s) from *Peanibacillus mucilaginosus* TKU032. Marine drugs, 14(2), 40.

Lim, J.-J., Jung, A.-H., Suh, H. J., Choi, H.-S., & Kim, H. (2022). *Lactiplantibacillus plantarum* K8-based paraprobiotics prevents obesity and obesity-induced

inflammatory responses in high fat diet-fed mice. Food Research International, 111066.

Maghsood, F., Johari, B., Rohani, M., Madanchi, H., Saltanatpour, Z., & Kadivar, M. (2020). Anti-proliferative and anti-metastatic potential of high molecular weight secretory molecules from *probiotic Lactobacillus reuteri* cell-free supernatant against human colon cancer stem-like cells (HT29-ShE). International Journal of Peptide Research and Therapeutics, 26(4), 2619-2631.

Maghsood, F., Mirshafiey, A., Farahani, M. M., Modarressi, M. H., Jafari, P., & Motevaseli, E. (2018). Dual effects of cell free supernatants from *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* GG in regulation of MMP-9 by up-regulating TIMP-1 and down-regulating CD147 in PMA-differentiated THP-1 cells. Cell Journal (Yakhteh), 19(4), 559.

Nakamura, S., & Mitsunaga, F. (2018). Anti-Allergic Effect of para-probiotics from non-viable acetic acid bacteria in ovalbumin-sensitized mice. Food and Nutrition Sciences, 9(12), 1376-1385.

Nascimento, L. C. S., Casarotti, S. N., Todorov, S. D., & Penna, A. L. B. (2019). Probiotic potential and safety of enterococci strains. Annals of Microbiology, 69(3), 241-252.

Nataraj, B. H., Ali, S. A., Behare, P. V., & Yadav, H. (2020). Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. Microbial cell factories, 19(1), 1-22.

Oelschlaeger, T. A. (2010). Mechanisms of probiotic actions–a review. International Journal of Medical Microbiology, 300(1), 57-62.

Öner, Z., Uğur, E., Bektas, A., & Ulusoy, M. (2021). Paraprobiotics, postbiotics and their effects on health. GIDA-Journal of Food, 46(2), 428-442.

Pariza, M. W., Gillies, K. O., Kraak-Ripple, S. F., Leyer, G., & Smith, A. B. (2015). Determining the safety of microbial cultures for consumption by humans and animals. Regulatory Toxicology and Pharmacology, 73(1), 164-171.

Parvez, S., Malik, K. A., Ah Kang, S., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. Journal of Applied Microbiology, 100(6), 1171-1185.

Salminen, S., Collado, M. C., Endo, A., Hill, C., Lebeer, S., Quigley, E. M., Sanders, M. E., Shamir, R., Swann, J. R., & Szajewska, H. (2021). The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nature Reviews Gastroenterology & Hepatology, 18(9), 649-667.

Shahidi, F. (2004). Functional foods: Their role in health promotion and disease prevention. Journal of Food Science, 69(5), R146-R149.

Siciliano, R. A., Reale, A., Mazzeo, M. F., Morandi, S., Silvetti, T., & Brasca, M. (2021). Paraprobiotics: A new perspective for functional foods and nutraceuticals. Nutrients, 13(4), 1225.

Singh, K., Kallali, B., Kumar, A., & Thaker, V. (2011). Probiotics: A review. Asian Pacific Journal of Tropical Biomedicine, 1(2), S287-S290.

Sunkara, R., & Verghese, M. (2014). Functional foods for obesity management. Food and Nutrition Sciences, 2014.

Teame, T., Wang, A., Xie, M., Zhang, Z., Yang, Y., Ding, Q., Gao, C., Olsen, R. E., Ran, C., & Zhou, Z. (2020). Paraprobiotics and postbiotics of probiotic Lactobacilli, their positive effects on the host and action mechanisms: A review. Frontiers in Nutrition, 191.

Żółkiewicz, J., Marzec, A., Ruszczyński, M., & Feleszko, W. (2020). Postbiotics—a step beyond pre-and probiotics. Nutrients, 12(8), 2189.

CHAPTER 6

BIOLOGY OF NATURAL AND SYNTHETIC LANTIBIOTICS AND THEIR CURRENT APPLICATION STATUS

Prof. Dr. Ali Fazıl YENİDÜNYA[1] & Dr. Serap ÇETİNKAYA[2]

[1] Sivas Cumhuriyet University, Science Faculty, Department of Molecular Biology and Genetics, Sivas, Turkey
e-mail: alifazilyenidunya@gmail.com, ORCID ID: 0000-0002-9886-977X
[2] Sivas Cumhuriyet University, Science Faculty, Department of Molecular Biology and Genetics, Sivas, Turkey
e-mail: serapcetinkaya2012@gmail.com, ORCID ID: 0000-0001-7372-1704

INTRODUCTION

Lantibiotics are the posttranslationally modified antimicrobial peptides. They assume their functional forms through a proteolytic processing, and are extruded by the release machinery to the extracellular milieu. Repeating peptidoglycan units of the target cell wall or integral lipids of the membrane, such as phosphatidylethanolamine and phosphatidylserine, appear to serve as the specific binding targets of these antibacterial agents. A cohort of natural lantibiotics from diverse species of bacteria now exist along with their modified and engineered forms. Insights have been gained into the practical aspects of this inventory covering areas from bacterial pathogens to deadly viruses and cancer types. This chapter attempted to reflect the current status of the lantibiotics biology and of their functional magnitude.

OVERVIEW

Antimicrobial peptides (AMPs) are one of the "narrow range" biological arsenals of Bacteria to fight for survival in adverse situations, since AMPs of two major groups of Bacteria, Gram (+) and Gram (-), are only effective from within, in other words, they are ineffective on the other group. Producing organism is expectedly immune to its own AMPs (Smits et al., 2020).

Lantibiotics are the posttranslationally modified AMPs, bacteriocins, and two prominent types (I, and II) are featured by their two unique amino acid residues, lanthionine (Lan) and 3-methyllanthionine (MeLan). Two dehydrated amino acids, 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) are also frequently encountered. Besides, few lantibiotics harbour S-aminovinyl-D-cysteine, S-aminovinyl-D-methylcysteine, lysinoalanine, or erythro-3-hydroxyaspartic acid. Genes encoding these peptides are clustered together and the genes responsible for their modifications, proteolytic cleavage, and transport are also grouped together in *lan* cluster. Lantibiotics assume their functional forms through a proteolytic processing, and are finally extruded by the release machinery to the extracellular milieu. Cationic lantibiotics form holes on the membrane of target organisms by anchoring onto the specific membrane lipids, such as lipid II or phosphoethanolamine (Guder et al., 2000).

Molecular weight of lantibiotics differ in the two major groups of bacteria. The ones produced by the members of Gram (-) bacteria appear to be much larger, within 30–70 kDa range, whereas the other major group of bacteria, Gram (+), synthesizes small peptides between 2 and 5 kDa. Typical examples for the former lantibiotics include the unmodified colicin lantibiotics (Table 1). Gram (-) bacteria also produce modified AMPs, microcins, which are less than 10 kDa (Guder et al., 2000).

Table 1: Names and structures of identified lantibiotic peptides (Guder et al., 2000).

Lantibiotic	Molecular Mass (Da)	Producer Species	Modified Residues				Ref.
			Lan	MeLan	Dha	Dhb	
Nisin Group							
Nisin A	3353	*Lactococcus lactis*	1	4	2	1	4,5
Nisin Z	3330	*Lactococcus lactis*	1	4	2	1	4,5
Subtilin	3317	*Bacillus subtilis*	1	4	2	1	4,5
Epidermin group							
Epidermin	2164	*Staphylococcus epidermidis*	2	1	0	1	4,5
[Val1-Leu6]-epidermin[a]	2151	*Staphylococcus epidermidis*	2	1	0	1	4,5
Gallidermin	2164	*Staphylococcus epidermidis*	2	1	0	1	4,5
Mutacin B-Ny266[a]	2270	*Streptococcus mutans*	2	1	1	1	4,5
Mutacin 1140[a]	2263	*Streptococcus mutans*	2	1	1	1	84
Mutacin III[a]	2266	*Streptococcus mutans*	2	1	1	1	85
Pep5 group							
Pep5	3488	*Staphylococcus epidermidis*	2	1	0	2	4,5
Epilancin K7	3032	*Staphylococcus epidermidis*	2	1	2	2	4,5
Epicidin 280[a]	3133	*Staphylococcus epidermidis*	1	2	0	1	4,5
Lacticin 481 group							
Lacticin 481	2901	*Lactococcus lactis*	2	1	0	1	4,5
Streptococcin A-FF22[a]	2795	*Streptococcus pyogenes*	1	2	0	1	4,5
Butyrivibriocin OR79A[a]	?	*Butyrivibrio fibrisolvens*	1	2	0	1	86
Salivaricin A[a]	2315	*Streptococcus salivarius*	1	2	0	0	4,5
[Lys2, Phe7]-salivaricin A[a]	2321	*Streptococcus salivarius*	1	2	0	0	4,5
Variacin[a]	2658	*Micrococcus varians*	2	1	0	1	4,5
Lactocin S	3764	*Lactobacillus sake*	2	0	0	1	4,5
Cypemycin	2094	*Streptomyces* ssp	0	0	0	4	4,5
Plantaricin C	2880	*Lactobacillus plantarum*	1	3	1	0	87
Mersacidin group							
Mersacidin	1825	*Bacillus* ssp.	0	3	1	0	4,5
Actagardine	1890	*Actinoplanes liguriae*	1	2	0	0	4,5
Ala(0)-actagardine[a]	1961	*Actinoplanes liguriae*	1	2	0	0	88
Cinnamycin group							
Cinnamycin	2042	*Streptomyces cinnamoneus*	1	2	0	0	4,5
Duramycin	2014	*Streptomyces cinnamoneus*	1	2	0	0	4,5
Duramycin B	1951	*Streptoverticillium* sp.	1	2	0	0	4,5
Duramycin C	2008	*Streptomyces griseoluteus*	1	2	0	0	4,5
Ancovenin	1959	*Streptomyces* ssp.	1	2	1	0	4,5
Structures incomplete							
Sublancin 168	3877	*Bacillus subtilis*					89
Mutacin II	3245	*Streptococcus mutans*					90
Carnocin UI 49	4635	*Carnobacterium piscicola*					4,5
Nukacin ISK-1	2960	*Staphylococcus* ssp.					[b]
Structures incomplete/two-component lantibiotics							
Cytolysin A1	4164	*Enterococcus faecalis*					13
Cytolysin A2	2631						
Staphylococcin C55α	3339	*Staphylococcus aureus* C55					14
Staphylococcin C55β	2993						
Lacticin 3147 A	3322	*Lactococcus lactis* DPC3147					15
Lacticin 3147 B	2847						

[a] Bridging patterns are not proven but assumed to be identical to those of the related peptides in the respective group. References 4 and 5 contain detailed literature on structures identified before 1995 or 1998.

[b] Sonomoto, K. pers. communication.

Earlier attempts have tried to classify lantibiotics into two types, I and II, on the basis of their primary structures. Linear lantibiotics, like nicin, are included into type I. These are cationic, pore forming, and relatively more flexible peptides. The second type includes globular molecules, such as ancovenin, cinnamycin, and duramycin, as their heads and tails are cross-linked (Fig. 1). This latter group differs greatly in its antimicrobial activity (Kogler et al., 1991; Zimmermann et al., 1995; Guder et al., 2000).

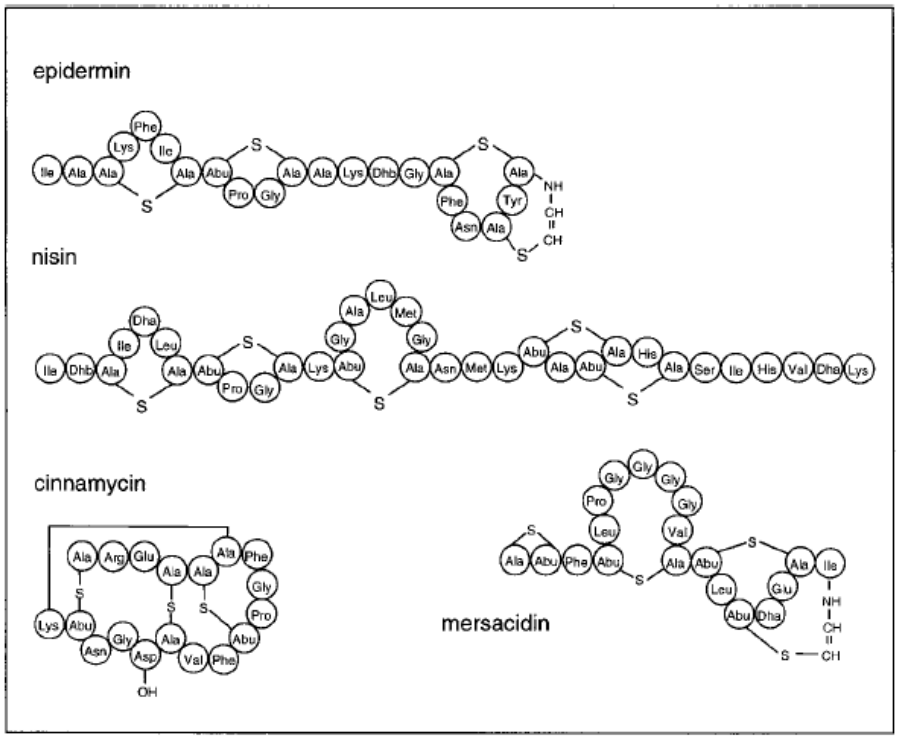


Figure 1: Characteristic structures of lantibiotics. Dha: 2,3-didehydroalanine; Dhb: 2,3-didehydrobutyrine; Abu: a-aminobutyric acid; Ala-S-Ala: lanthionine; Abu-S-Ala: 3-methyllanthionine (Guder et al., 2000).

N-terminal of lantibiotics contains a leader peptide. C-terminal includes the amino acids to be modified and thus it is named prepeptide. Four genes responsible for the modification reactions are named *lan*B, *lan*C, *lan*M, and *lan*D. A proteolytic cleavage gene (*lan*P), a transport gene (*lan*T), immunity genes (*lan*I, *lan*EFG), and regulatory genes (*lan*K, *lan*Q, and *lan*R) constitute the remaining of the cluster. The cluster can either be located on the chromosome or can be extrachromosomal. Each of the two type lantibiotics

have their own modifying, biosynthetic, genes. Type I lantibiotics are modified by *lan*B and *lan*C (Kuipers et al., 1993) while the members of type II are modified by a single gene product LanM (Fig. 2) (Altena et al., 2000; Guder et al., 2000). One or two genes, responsible for the self-immunity, are found within the gene clusters of types I and II. These encode a lipoprotein, LanI, and LanFEG proteins. LanI is anchored on the outer membrane surface through its fatty acid moiety (Smits et al., 2020).

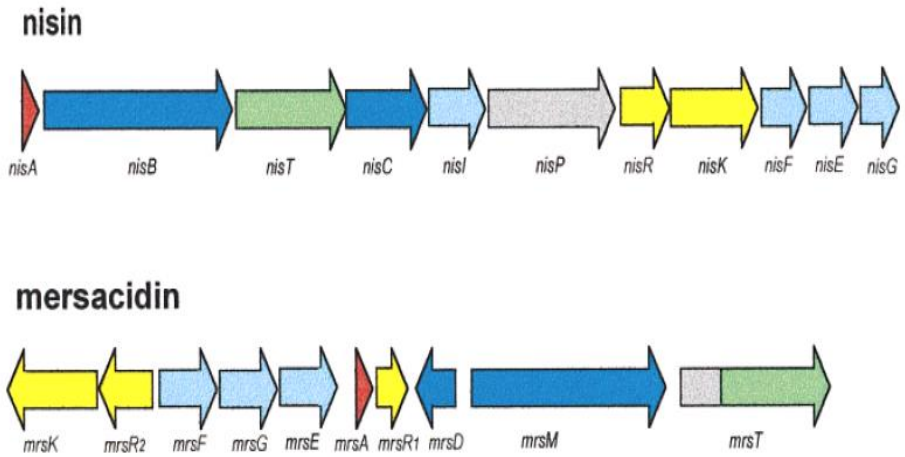


Figure 2: Nisin and mersacidin gene clusters. Structural genes (red), genes modification genes (dark blue) necessary, export genes (green), regulating genes (yellow), self-immunity genes (light blue), and protease genes (grey) (Guder et al., 2000).

In dehydration reactions Ser and Thr residues serve as the substrate, yielding Dha (2,3-didehydroalanine) and Dhb (2,3- didehydrobutyrine), respectively. When cysteine is present in the vicinity, these amino acids are further modified by Michael addition (Fig. 3). LanD, the oxidoreductase, oxidizes cysteine at C-terminus. This cysteine is the substrate for decarboxylation with the ultimate product being the enethiol anion. It has been envisaged that this enethiol reacts with an internal 2,3-didehydroalanine (Weil et al., 1990; Guder et al., 2000).

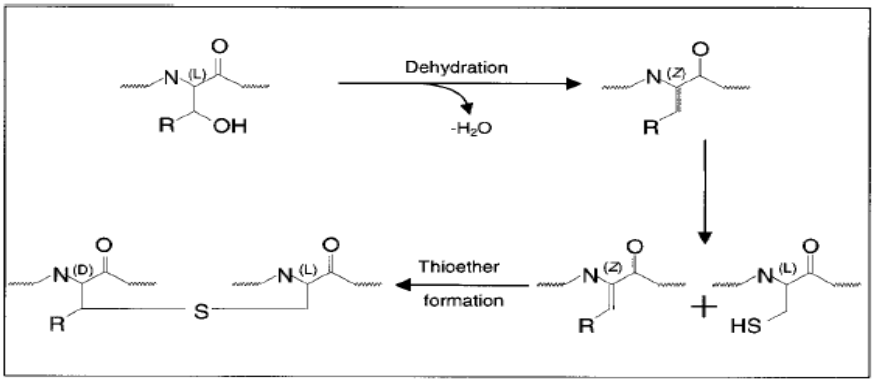


Figure 3: Chemistry of Lan and MeLan formation. Ser (R: H) 3 Dha 3 Lan; Thr (R: CH3) 3 Dhb 3 MeLan (Guder et al., 2000).

Overall, bacteriocin biosynthesis involves a two-component regulatory system, comprising a histidine kinase, LanK, on the cell membrane and a response regulator, LanR. Upon induction by an external signal, the kinase phosphorylates itself at its cytoplasmic histidine and this phosphate group is finally transferred onto LanR at an Asp residue. This phosphorylation induces a conformational change in LanR and leads it to bind to a DNA element, the operator sequence of a defined gene.

After modification, the leader sequence of the lantibiotic prepeptide (LanA) was excised by LanP. This protease has similarities to subtilisin and is specific for type I lantibiotics. For this protease to cleave, the leader amino acid sequence should harbour the FNLD consensus sequence as well as a proline at position 22.

The transport protein LanT belongs to the ABC (ATP-binding-cassette) superfamily. It has transmembrane N-terminal- and cytoplasmic ATP binding C-terminal domains. Hence, the lantibiotic extrusion is an energy driven process (Guder et al., 2000).

Some of the lantibiotics do not fall into either of the two types. These include lantibiotics having two polypeptide components. Each of the components, by itself, seems to display insignificant or no antibacterial activity. Synergistically however they are capable of behaving as robust lantibiotics. This group is as yet made up of relatively few members which can be closely- or distantly related. Structurally related members include lacticin 3147, plantaricin W, and staphylococcin C55A, and the distant one is

cytolysin. A distinct subgroup has been created for these lantibiotics (Asaduzzaman and Sonomoto, 2009).

Not all lantibiotics are characterised by Lan and MeLan residues. Best known examples of these are labyrinthopeptins. These peptides contain labionin (Lab), a variant of Lan which is made up of two Dha (2,3-didehydroalanine) and one cysteine. Lab is in essence a compound in which cysteine is bonded with Dha through a methylene bridge. In addition, they have a disulphide bridge between two cysteine residues. Labyrinthopeptins form the third type of lantibiotics and are relatively shorter peptides, ranging from 18 to 21 amino acids. One bacterium could synthesize more than one type labyrinthopeptin. So is the case with *Actinomadura namibiensis* DSM 6313, which produces three types of labyrinthopeptins (LabA1-3). The differences can arise from the addition of one single amino acid (Fig. 4e). LabA1 appears to be an effective antiviral agent against human immunodeficiency- and herpes simplex viruses. This peptide interferes with the entry of these viruses into the cell and with the invasion of the uninfected cells. Thus LabA1 could be an alternative to a number antiviral drugs in the treatment of drug-resistant viral strains (Gomes et al., 2017).

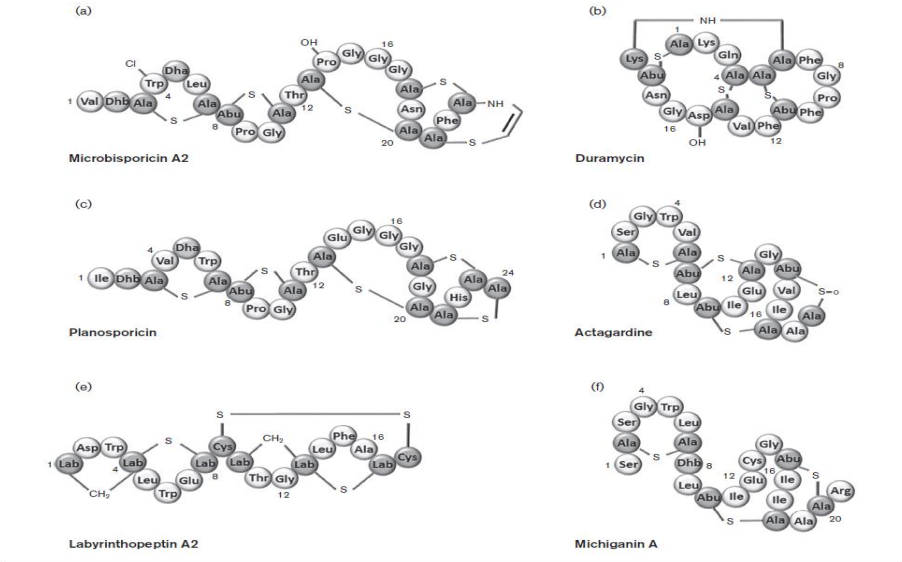


Figure 4: Typical examples of actinobacterial lantibiotics.

Lantibiotic production requires the immunity of the producing organism. Immunity appears to be rendered by small peptides of specific bacteriocin antagonists. Lantibiotic producing organisms also possess transport proteins belonging to the group B of ABC superfamily. These transport proteins send the bacteriocins bound to the cell membrane, back to the extracellular environment.

So far, it has been evidenced that lantibiotics often disrupt the integrity of cytoplasmic membrane as well as interfering with the *de novo* synthesis of Gram (+) cell wall. Such destructive activities have been shown on *Bacillus* members and erythrocytes. It appears that outer membrane of Gram (-) bacteria is an impasse to the attacks of lantibiotic peptides.

In vitro studies have revealed that lantibiotics, in general, form tight complexes with the essential component of cell wall, undecaprenyl-diphosphoryl-N-acetylmuramic acid-[pentapeptide]-N-acetylglucosamine, and that they can inhibit transglycosylation (Fig. 5) (Gomes et al., 2017).

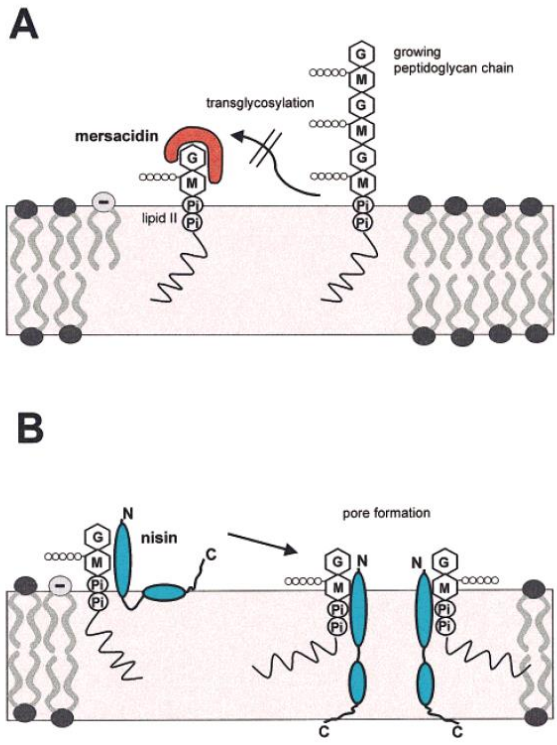


Figure 5: Interference of mersacidin (A) and nisin (B) with cell wall synthesis over lipid II, and with pore formation, respectively (Guder et al., 2000).

The kinetics of type A lantibiotics seems to be much faster and they exert a detergent effect on the target cell membrane and cell lysis ensues immediately. This has been evidenced on Clostridia cells treated with nisin. Hydrophilic interactions between the positively charged residues of the lantibiotic peptide and the lipid's negatively charged phosphate groups seem to govern this destructive process. Kinetics of nisin- binding on purified lipid targets has been demonstrated to be two- or three fold faster than on the whole cells.

For some of the type II lantibiotics specific target lipids have been identified in the cell membrane. Phosphatidylethanolamine, for example, can bind cinnamycin or duramycin, and such interactions interfere with the activity of phospholipase II.

Nisin is a commercialised type I lantibiotic and has been effectively used in food industry. In canned food products, combined with pasteurization, nisin is a proteinaceous protective agent against *Clostridium botulinum*. Because of its stability in acidic pH, it can also be used against *Helicobacter pylori* (Delves-Broughton et al., 1996). Decades of studies with nisin has prompted the notion that lantibiotics could replace some of the currently used antibiotics, offering a partial solution to the problem of antibiotic resistance. An antibiotic, vancomycin, for example, is also capable of targeting membrane lipids, lipid II, through sugar-phosphate parts. Nisin, on the other hand, has been shown to bind both to lipid I and to lipid II. Peptide "antibiotics" are more versatile molecules in that their action mechanisms can be elucidated through introducing predetermined changes in their primary structure (Gomes et al., 2017).

IDENTIFICATION OF UNKNOWN LANTIBIOTICS FROM UNCULTIVABLE MICROORGANISMS

Most of the prokaryotes are currently uncultivable and they have been thought to have an immense potential for the discovery of "novel" genes of interest. Whole genomic sequences have been used in respective studies and such efforts are called genome mining. Existing sequence data on the genes of interest are employed as the driver to identify similar but unknown genes in the databases. For example, the genes *hal*A1 and *hal*A2 for a two-component bacteriocin, haloduracin, has been mapped from *Bacillus halodurans* by using

the nucleotide sequence information of mersacidin. The production of the two polypeptides, HalA1 and HalA2, have been successfully established in *E. coli* by means of recombinant manipulations (Sandiford, 2017).

Two bioinformatics tools, BAGEL (BActeriocin GEnome mining tool) (van Heel et al., 2013) and antiSMASH (antibiotics and Secondary Metabolite Analysis SHell) (Medema et al., 2011) and their versions have been employed in the identification of unknown lantibiotic gene family members. New versions have included additional algorithms, enabling the prediction of the lantibiotic fine structure and functional amino acid motifs and mapping the biochemically active sites. Recent identification studies could be performed without relying on the sequence markers, defining the open reading frames (Sandiford, 2017).

THE TRANSPORT OF LANTIBIOTICS TO THE EXTRACELLULAR ENVIRONMENT

The release of lantibiotics into the exterior of the producing organism serves dual functions, to protect the self and to combat others. ATP-Binding Cassette (ABC) system serves in the transport (Figs. 6 and 7). Members of ABC transporters' superfamily are common in the three domains of life with multiple functions. They export signalling- and toxic molecules, import cellular food, and play a crucial role in rendering the host to be a multidrug resistant cell. As the name suggests, these processes are all driven at the expense of cellular ATP. ABC system comprises two domains: twelve helix-transmembrane domain (TMD) enables the translocation of the export molecules, and a dimeric nucleotide binding domain (NBD) binds and hydrolyses ATP (Smits et al., 2020).

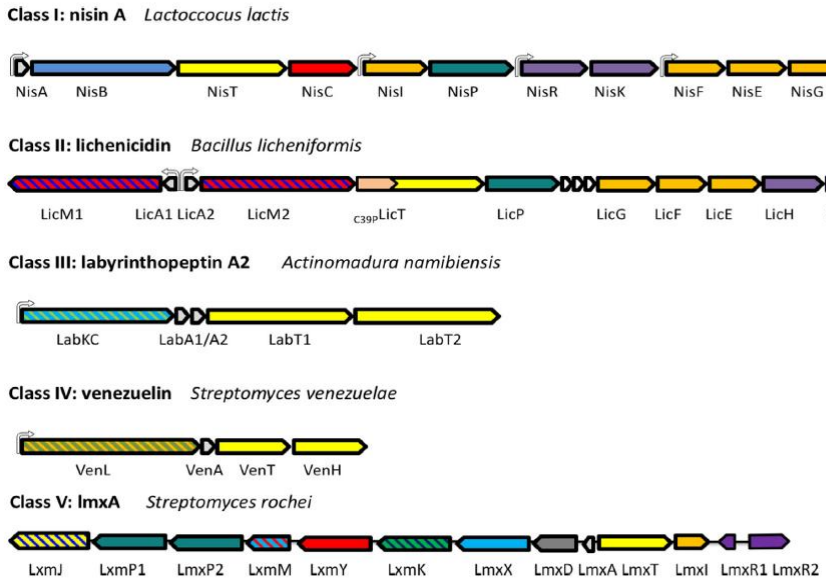


Figure 6: Genes of class I to class V lanthipeptides. Grey precursor peptide LanA;, class I LanB (blue) and LanC (red); class II: bifunctional LanM (blue, red striped); class III: trifunctional LanKC (blue, grey striped); class IV trifunctional LanL (orange, green striped); exporter (yellow); bifunctional exporter/protease (light orange/yellow); immunity proteins (orange); two-component system (violet); class V trifunctional synthetases LanK, LanX and LanY (green striped, blue and red, respectively). Known promoters have been indicated as white arrows (Smits et al., 2020).

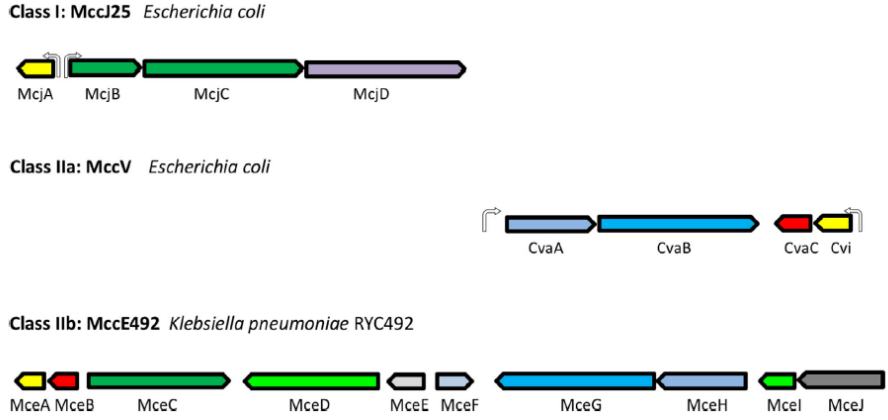


Figure 7: Class I and IIa/b microcins genes. Precursor peptide (yellow); posttranslational modification enzymes (green); self-immunity by transporters (blue and purple); self-immunity proteins (excluding export) (red); unknown function (grey); promoters (white arrows) (Smits et al., 2020).

A LanT type ABC system enables the export of type I lantibiotics and this involves NisT, SpaT or PepT transporters (Fig. 8; Table 2). Before transportation, the leader peptide is removed from the peptide to be exported. As mentioned above, this cleavage is exerted by a specific protease, LanP, which can be membrane-bound (PepP, EciP or ElxP) or free in the cytoplasm (EpiP or NisP). The secretion of type III lantibiotics also involves LanT-type transporters (as in class I) and leader peptide is suggested to be removed during the final maturation phase by POPs (prolyl oligopeptidases). In the secretion of type II lanthipeptides, final peptide maturation- and transport processes are combined and the final maturation stage requires a second peptidase activity (Fig. 6). Here, SunT family members, for example BovT, LctT, LicT, MrsT or NukT, furbish the cell with both secretion and proteolytic activities (Smits et al., 2020).

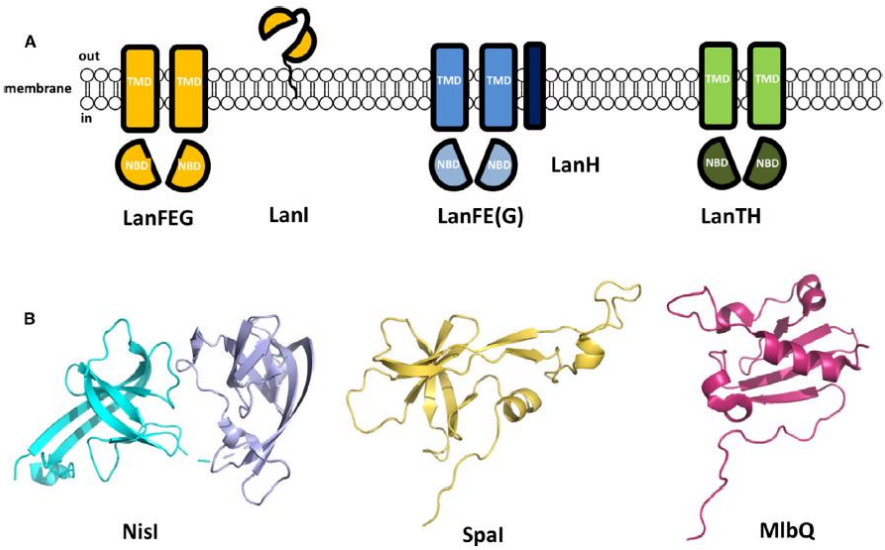


Figure 8: Proteins of self-immunity. (A) LanFEG ABC transporter LanI (yellow), LanFE(G) ABC transporter and LanH (blue). LanTH ABC transporter (green). (B) LanI family: NisI of *Lactococcus lactis* (left). SpaI of *Bacillus subtilis* (middle panel) and MlbQ of *Microbispora* ATCC PTA-5024 (right panel). NisI (turquoise and blue), SpaI (yellow), and MlbQ (purple) (Smits et al., 2020).

Table 2: Physical and biochemical features of ABC transporters in immunity or resistance (Smits et al., 2020).

Lanthipeptide ABC transporters	*LanT*	*SunT (fused NBD and TMD) + C39*	*LanFEG*	*BceAB*
Sequence length (amino acids)	602	710	225 + 242 + 214	253 + 648
Predicted transmembrane helices	6	5 or 6	LanE: 6 LanG: 6	BceB: 10
Stoichiometry	Homodimer	Homodimer	Heterodimer LanFEG: 2 : 1 : 1	BceAB: unknown (dimeric BceA)
Special sequence motif	–	N-terminal C39 peptidase domain	LanF: E-loop	BceA: Q-loop BceB: 210-amino acid-long extracellular domain between helices 7–8
Inactive transport mutant	H-loop	H-loop	H-loop	H-loop
Observed mechanism	Secretion of produced and or processing of lantibiotic	Leader processing and secretion	Expelling AMP	Expelling AMP/ shielding of membrane target
Substrate specificity	AMP (recognised by leader sequence)	AMP (recognised by leader sequence)	Immunity against produced AMP Nisin: recognition of C terminus	Resistance against AMP and bacitracin Nisin: recognition of N terminus

Type II lantibiotics duramycin and cinnamycin bind the PE head at equal stoichiometric ratios and with high propensity by virtue of their conserved hydrophilic pocket, spanning from phenilalanine-7 and cysteine-14 (Fig. 9). An ionic bond, between the ammonium head and asparagine15 of the peptide, stabilizes the binding. Thus the peptide pocket and the ionic bond both account for the high affinity binding. A third factor might also come into play in the specificity of binding. This is the length of the hydrophobic lipid chain. Longer chains, containing more than eight carbons, appear to favour the peptide-lipid interaction. These features have formed the basis of developing lantibiotic probes in order to target PE within the closely related membrane lipids in order to understand membrane dynamics of healthy and diseased cells, and the course of membrane division during mitosis (Zhao, 2011).

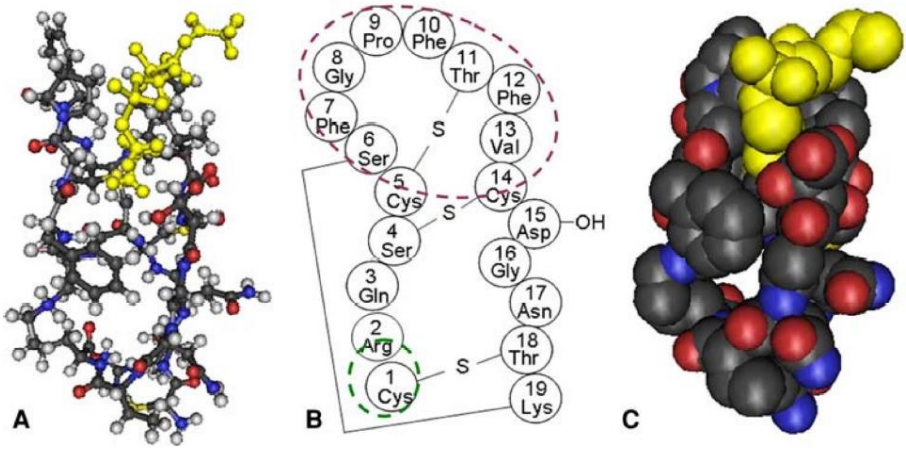


Figure 9: The interaction between of cinnamycin and phosphatidylethanolamine (yellow): PE-binding site (purple circle) (Zhao, 2011).

Owing to their robust structures lantibiotics are naturally suitable proteins in the design and production of fruitful probes, as the transversal intramolecular bridges further stabilize their closed ring structure. N-terminal primary amines located further away from the PE-binding region, are also readily available for labelling efforts, using biotin esters or radiolabels. Two of the radiolabels used are technetium (Tc) and gadolinium (Gd). Duramycin is also attached onto the fluorescent liposomes. Cinnamycin at 5 μM could lyse 50% of human erythrocytes within a few minutes. This disadvantage seems to be completely eliminated, when it is complexed with avidin. The use of radiolabelled lantibiotics involves chelation chemistry (Choung et al., 1988; Zhao, 2011).

Phosphatidylethanolamine, PE, is an anticoagulant and normally found in the inner membrane leaflet of mammalian cells. During mitosis or in apoptotic cells it can move to the outer leaflet. This also appears to be the case in budding *Saccharomyces cerevisiae*. A duramycin probe conjugated with avidin has identified a glycoprotein (Ros3p) was responsible for the translocation of PE, in other words, its movement to the outer membrane leaflet. PE deficiency interferes with cytokinesis in bacteria and mammalians (Kato et al., 2002; Zhao, 2011).

One of the main characteristics of apoptotic cells is the disruption of asymmetric organisation of their membrane lipids. In apoptotic cells, besides

PE, phosphatidylserine (PS) translocates to the outer membrane leaflet. Hence, these two lipids serve apoptotic markers and they can be localised with labelled lantibiotics. This approach could also differentiate live cells among the dead ones (Zhao, 2011).

ENGINEERED LANTIBIOTICS

Lantibiotics are promising antibiotic biomolecules. Their genes can be cloned from uncultivable microorganisms and recombinantly expressed in order to enrich lantibiotic inventory. Along these lines, in vitro systems have been employed to translate lanthipeptide mRNAs and these systems have produced more than a thousand engineered variants of a single lanthipeptide. Heterologous production systems have also been utilised for the translation and screening of lantibiotic gene libraries. A workhorse of these heterologous expression efforts has been *Lactococcus lactis* for heterologous protein expression (Pipiya et al., 2020).

The Nisin- controlled expression, NICE, comprising an autoinduced system, has been established within the nisin biosynthetic cluster of the *L. lactis*. Upon the extrusion of nisin into the extracellular milieu, a membrane-bound kinase, NisK, phosphorylates itself and transfers the phosphate group onto NisR, an intracellular regulatory protein. Phosphorylated NisR specifically binds to the promoter of NisA gene and induces the expression of following genes. This whole system serves the well-ordered production of interested heterologous lantibiotic peptide. Many findings have revealed distinguishing modular amino acid motifs and shapes in lantibiotic peptides. These modules have been copied in the generation of synthetic peptide libraries. In the libraries, the modules of 12 different lantibiotics have been recombined in *L. lactis*. Screening of these libraries have identified, for example, some chimeric peptides, comprising gallidermin and nisin modules, that were significantly more effective on a Gram (+) pathogen, *Streptococcus pneumonia*, than their native- state counterparts (Pipiya et al., 2020).

Another bacterium that has been notoriously exploited in the recombinant protein production is obviously *Escherichia coli*. Prochlorosins have been produced in much higher yields in this bacterium than in the engineered strain MIT 9313 of native *Prochlorococcus marinus*.

It has been realised that the leader peptide of lantibiotics interfered with the antibiotic activity of the engineered versions. Thus engineered lantibiotics had to be treated with suitable proteases to remove the leader amino acid sequence. This step which appears to be the most cumbersome of the whole production scheme, have been overcome by producing LicP, the protease within the cell and then directing it to the periplasm. This stage was controlled in a temperature-sensitive manner.

As mentioned above, antimicrobial activity of lantibiotics is normally limited to Gram (+) bacteria. Chimeric lanthipeptides have been attempted to challenge this bottleneck and go over into the kingdom of Gram (-) bacteria. One of the solutions has been the gallidermin and siderophore conjugates. Here siderophore has been expected to bind its receptor on the membrane and internalize with it the lantibiotic. These efforts have been fruitless because Gram (-) bacteria appeared to possess an inherent immunity to lantibiotics. Following studies have used short peptides with known activity, and nisin combinations. These attempts yielded some encouraging results.

Macrocyclic structures in lantibiotics have been prompted the notion that the macrocycles could interrupt the interaction of other proteins as they resemble natural ligands of proteins. Gene libraries could also provide a significant diversity of these cycles. This approach bore some fruit in the inhibition of the interaction between an HIV protein, p6, and human TSG101. This finding had positive implications because with this approach the infection of a deadly virus could be disrupted (Pipiya et al., 2020).

IN VIVO EFFORTS TO INCREASE YIELD

Many lantibiotics are naturally produced at low yields and this impedes especially their medical use. Protein engineering via amino acid substitutions have been seen a means of overcoming this problem. These efforts have not improved significantly the activity of lantibiotic of interest but it increased its yield (Escano and Smith, 2015).

Overproduction of nisin and its posttranslational enzymes have long been made possible by the use of inducible promoters. Considerable efforts have been made in order to express type II lantibiotics heterologously in *E. coli*. A successful outcome of the latter studies was the production of lichenicidin, which is a two-peptide lantibiotic of *Bacillus licheniformis*,

showing antimicrobial activity against *Listeria monocytogenes*, and resistant strains of *Staphylococcus aureus* and enterococci (Escano and Smith, 2015).

Chemical production of natural lantibiotics omits the necessity of the coexpression of modification enzymes and paves the way for the design and development of analogous lantibiotics. Here, generally toxic metabolite antibiotics can also be included in the production schemes. First and only successful production case was for nisin. Advancements in SPPS (solid phase peptide synthesis) has also enabled a number of chemical lantibiotics other than nisin. Its first fruitful product has been lactocin S of *Lactobacillus Sake* L45. Following efforts has incorporated norleucine, a non-proteinogenic amino acid, into this synthetic lantibiotic. This substitution has attempted to eliminate an oxidation problem caused by methionine. The bottleneck of the SPPS approach has been again the requirement of posttranslational modification on the nascent product. Lacticin 3147, a two-component lantibiotic, has also been produced by the same technology. This synthetic lantibiotic appeared to be compatible with its native counterpart both in activity and structure, because it has been demonstrated that the peptide components were interchangeable. This method also had its weakness in its low-yield capacity. And this defect has opened the door to the production of lantibiotic mimics (Escano and Smith, 2015).

One of the inherent problems of natural lantibiotics is their vulnerability to oxidation within the rings. Except one single natural case, oxidation generally inactivates lantibiotics. Target moieties, such as sulphur, has been replaced by ring-closing alkenes or alkanes, thus ring variants have been produced (Figure 3C). This product, however, could not bind to the target lipid on the cell membrane. Similar results have argued for the same problem and indicated that ring motifs were indispensable for the lantibiotics' structural activity (Escano and Smith, 2015).

IN VITRO MUTASYNTHESIS

A recent approach has been the *mutasynthesis* of lantibiotics in vitro. This scheme has been reported to have used the chemical synthesis of the nascent polypeptide together with modification (PTM) enzymes. Yet the low-yield problem appeared again in this method (Escano and Smith, 2015).

LEADING THERAPEUTIC CAPABILITIES OF LANTIBIOTICS

1. Antiviral Capability

T-cell Ig mucin domain (TIM) protein TIM1, like lantibiotics, specifically binds to membrane phosphatidylethanolamine. TIM1 permits the infection of T-cells by enveloped viruses. This has prompted the studies on the antiviral activity of lantibiotics over blocking TIM1. A few lantibiotics have been tried. Of these, duramycin has exhibited significant activity on the inhibition of virus entry. As expected this inhibition required the presence of phosphatidylethanolamine, and duramycin had no antiviral activity after infection. Another lantibiotic, labyrinthopeptin A1 has been demonstrated to have significant antiviral capacity against strains of HIV (human immunodeficiency virus) and HSV (herpes simplex virus) using the same mechanism. The viral range of this lantibiotic appeared to be rather expansive. Labyrinthopeptin A1 has employed target cellular proteins other than TIM1, such as TSG101 (tumour susceptibility gene 101). The latter protein seems to be involved in the budding of HIV from the host cell, and labyrinthopeptin A1 exerts its antiviral activity by impeding the interaction of TSG101with p6 protein of HIV (van Staden et al., 2021).

2. Anticancer Activity

In each type of biological activity of lantibiotics, nearly a similar set of them has been encountered. This could arise from the richness of lantibiotics in varieties, as in the case of nisin. Two types of nisins, A and Z, have been reported to possess significant anti-cancer effects on head and neck squamous cell carcinoma (HNSCC) on which they also induce apoptosis. The target molecule is this time another membrane lipid, phosphatidylcholine, which has been found in increasing numbers in the cancerous cells. For nisin to exert its apoptotic activity, first it has to initiate the calcium entry into the cell. The ion influx in turn activates calpain-1, evading caspase 3 pathway. Then it disrupts the cell membrane via the discharge of LDH (lactate dehydrogenase) which is one of the signs of cells going through apoptosis (Dreyer et al., 2019; van Staden et al., 2021).

Another prominent lantibiotic, duramycin could more selectively start apoptosis of cancer cells through its usual target, phosphatidylethanolamine.

General cytotoxicity of this lantibiotic has been lessened by fusing it to an IgG. This fusion also increased its capacity in targeting cancer cells. The end result of this treatment was the increase in phagocytosis.

In a library, several lantibiotics has been identified that bind to a serine protease, known as urokinase plasminogen activator (uPA). These findings might have strong implications for the anti-cancer potential of such peptide libraries (van Staden et al., 2021).

3. Immunomodulatory Activity

Cationic antimicrobial proteins (ctAMPs) are involved in the modulation during infection and in wounds. As the name suggests these agents are positively-charged small peptides and include hydrophobic amino acids. These features remind of lantibiotics, which also exert some immunomodulatory functions. Nisin, gallidermin, and Pep5 are among such lantibiotics that could trigger the discharge of chemokines and neutrophils as efficiently as LL-37, a member of ctAMP family. These chemokines are involved in the protection against bacterial and fungal infections (van Staden et al., 2021).

Lantibiotics could also be involved in the modulation of enzymes which hydrolyse membrane lipids. One of these enzymes is the phospholipase A2 which hydrolyse phospholipids and produce arachidonic acid. This enzyme is important in the production of responses to inflammations. The arachidonic product is the basis of strong mediators of the immune system such as leukotrienes and prostaglandins. Some cinnamycin-like lantibiotics can disable phospholipase A2 over its substrate phosphatidylethanolamine and behave as indirect mediators of ancovenin, another cinnamycin-like lantibiotic which inhibits angiotensin-converting enzyme (ACE) (Kido et al., 1983). ACE is involved in fibrosis, diabetic inflammation, and hypertension. A lantibiotic streptocollin, with a similar structure to cinnamycin-like lantibiotics could partially inhibit protein tyrosine phosphatase 1B (PTP1B) which is involved in insulin and immune cell signalling. These findings, together, argue that through lantibiotics some of the routes of immune system, especially those which involve ctAMPs, can be modulated (Través et ali., 2014; van Staden et al., 2021).

CONCLUSION

Natural lantibiotics are synthesized upon stress and they exert their antimicrobial activities on closely related genera. Their target structures on the host bacteria are the integral constituents of cell wall, undecaprenyl-diphosphoryl-N-acetylmuramic acid-[pentapeptide]-N-acetyl-glucosamine, and membrane lipids, lipid II, phosphatidylethanolamine, and phosphatidylserine.

A commercialised type I lantibiotic, nisin, arouses a detergent effect on the target cell membrane and lyse the cell instantly. This rapid destruction is catered through ionic interactions with the membrane lipids. The detergent effect could be enhanced dramatically with the use of purified lantibiotics.

Robust and motif bearing structures of lantibiotics make them suitable for the development of efficient and diverse probes. They can be affinity-directed to target molecules with avidin-biotin technology, conjugated to immunoglobulins, or readily radiolabelled.

Bioinformatics tools have been created to fish out lantibiotic genes from uncultured microorganisms. Lanthipeptide mRNAs have been translated in vitro systems and these efforts resulted in a lantibiotic inventory comprising more than a thousand variants of a single lanthipeptide. Heterologous production systems have also been utilised for the translation and screening of lantibiotic gene libraries. Two workhorses of these heterologous expression studies have been *Escherichia coli* and *Lactococcus lactis* for heterologous protein expression.

Chemical production of natural lantibiotics has offered much better means as this approach eliminated the coexpression of modifying proteins and open the way to deliberately incorporate some “foreign” constituents with known cytotoxicity.

Lantibiotics appears to mimic a surface protein of T-cell, TIM, which permits the infection with HIV. This protein also specifically binds to the phosphatidylethanolamine. These features have enabled the researches to inhibit virus entry to T-cells using duramycin. A similar inhibitory activity has also been demonstrated for labyrinthopeptin A1, which blocked the interaction of TSG101with p6 protein of HIV.

Nisin, gallidermin, Pep5 could mimic cationic antimicrobial proteins (ctAMPs), which elicit the discharge of chemokines and neutrophils upon infection or in wounds.

Two types of nisin, A and Z, through their interactions with phosphatidylcholine could trigger apoptosis in the case of head and neck squamous cell carcinoma (HNSCC).

To sum up, lantibiotics in natural, modified- or in chemically synthesized forms, seem to have a great therapeutic potential in diverse areas of medicine. They mostly act through their specific interactions with a few membrane lipids. It also becomes clear that they can mimic key eukaryotic proteins involved in microbial and viral infections.

REFERENCES

Altena, K., Guder, A., Cramer, C., & Bierbaum, G. (2000). Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster, *Appl. Environ. Microbiol.*, 66, 2565–2571.

Asaduzzaman, S. M., & Sonomoto, K. (2009). Lantibiotics: diverse activities and unique modes of action, *J Biosci Bioeng.*, 107, 475–487.

Choung, S. Y., Kobayashi, T., Inoue, J., Takemoto, K., Ishitsuka, H., & Inoue, K. (1988). Hemolytic activity of a cyclic peptide Ro09-0198 isolated from Streptoverticillium, *Biochim Biophys Acta*, 940, 171–179.

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

Dreyer, L, Smith, C, Deane, S. M, Dicks, L. M. T., & van Staden, A. D. (2019). Migration of bacteriocins across gastrointestinal epithelial and vascular endothelial cells, as determined using in vitro simulations, *Sci Rep*., 9:11481.

Escano, J., & Smith, L. (2015). Multipronged approach for engineering novel peptide analogues of existing lantibiotics. Expert Opin, *Drug Discovery*, 10(8), 857−870

Gomes, K. M., Duarte, R. S., & de Freire Bastos, M. D. (2017). Lantibiotics produced by Actinobacteria and their potential applications (a review), *Microbiology*, 163, 109–21

Guder, A., Wiedemann, I., & Sahl, H.G. (2000) Posttrans-lationally modified bacteriocins – the lantibiotics. *Biopolymers*, 55, 62–73

Kato, U., Emoto, K., Fredriksson, C., Nakamura, H., Ohta, A., Kobayashi, T., Murakami-Murofushi, K., Kobayashi, T., & Umeda, M. (2002). A novel membrane protein, Ros3p, is required for phospholipid translocation across the plasma membrane in Saccharomyces cerevisiae, *J Biol Chem*., 277, 37855–37862.

Kido, Y., Hamakado, T., Yoshida, T., Anno, M., Motoki, Y., Wakamiya, T., & Shiba, T. (1983). Isolation and characterization of ancovenin, a new inhibitor of angiotensin I converting enzyme, produced by actinomycetes. J Antibiot 36:1295–1299.

Kogler, H., Bauch, M., Fehlhaber, H.W., Griesinger, C., Schubert, W., & Teetz, V. (1991). In Nisin and Novel Lantibiotics; Jung, G., Sahl, H.G., Eds.; Leiden: Escom, 159–170.

Kuipers, O. P., Beerthuyzen, M., Siezen, R. J., & de Vos, W. M. (1993). Characterization of the nisin gene cluster nisABTCIPR of Lactococcus lactis: Requirement of expression of the nisA and nisI genes for development of immunity, *European Journal of Biochemistry,* 216, 281–291.

Medema, M. H., Blin, K., Cimermancic, P., de Jager, V., Zakrzewski, P., Fischbach, M. A., Weber, T., & Breitling, R. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences, *Nucleic Acids Research*, 39(2), 339-346.

Pipiya, S. O., Terekhov, S. S., Mokrushina, Yu. A., Knorre, V. D., Smirnov, I. V., & Gabibov, A. G. (2020). Engineering Artificial Biodiversity of Lantibiotics to Expand Chemical Space of DNA-Encoded Antibiotics, *Biochemistry (Moscow)*, 85(11), 319-1334.

Sandiford, S. K. (2017). Genome database mining for the discovery of novel lantibiotics, *Expert Opin. Drug Discov.*, 12, 489–495.

Smits, S.H., Schmitt, L., & Beis, K. (2020). Self-immunity to antibacterial peptides by ABC transporters, *FEBS Lett.*, 594, 3920–3942.

van Heel, A. J., de Jong, A., Montalbán-López, M., Kok, J., & Kuipers, O. P. (2013). BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides, *Nucleic Acids Research*, 41, 448-453.

Través, P. G., Pardo, V., Pimentel-Santillana, M., González-Rodríguez, Á., Mojena, M., & Rico, D., Montenegro, Y., Calés, C., Martín-Sanz, P., Valverde, A. M., Boscá, L. (2014). Pivotal role of protein tyrosine phosphatase 1B (PTP1B) in the macrophage response to pro-inflammatory and anti-inflammatory challenge, *Cell Death Dis.*, 5:e1125.

van Staden, A. D. P., van Zyl, W. F., Trindade, M., Dicks, L. M. T., & Smith, C. (2021). Therapeutic application of lantibiotics and other lanthipeptides: old and new findings, *Appl. Environ. Microbiol.*, 87:e0018621.

Weil, H.P., Beck-Sickinger, A. G., Metzger, J. W., Stefanovic, S., Jung, G., Josten, M., & Sahl, H. G. (1990). Biosynthesis of the lantibiotic Pep5: isolation and characterization of a prepeptide containing dehydroamino acids, Eur J Biochem., 194, 217–223.

Zhao, M. (2011). Lantibiotics as probes for phosphatidylethanolamine, *Amino Acids*, 41(5), 1071−1079.

Zimmermann, N., Metzger, J.W., & Jung, G. (1995). The tetracycline lantibiotic actagardine: 1H-NMR and 13C-NMR assignments and revised primary structure, *Eur. J. Biochem.*, 228, 786–797.

CHAPTER 7

BACTERIA SHOWING HALOVERSATILE PROPERTIES IN HYPERSALINE ENVIRONMENTS

Associate Professor Pinar CAGLAYAN[1]

[1] Marmara University, Faculty of Arts and Sciences, Department of Biology, Istanbul, Turkey, pinar.caglayan@marmara.edu.tr, ORCID ID: 0000-0002-0238-1754

INTRODUCTION

Hypersaline regions such as saline lakes, salt lakes, salt marshes, salt pans, coastal lagoons, evaporation ponds inhabit halotolerant and halophilic organisms (Ventosa et al., 1998a; Margesin and Schinner, 2001; Litchfeld and Gillevet, 2002). The term halophile is used for salt loving organisms (Ventosa et al., 1998a). The halophilic organisms require salt for growth and they are classified according to their salt requirements (Ventosa, 2006). First definition of microorganisms according to their salt tolerance by Kushner and Kamekura (1988) includes non-halophilic (require less than 1% sodium chloride), halotolerant (do not require salt, but can tolerate high salt concentrations), slightly halophilic (grow optimally at 1% to 3% sodium chloride), moderate halophilic (grow best at 3% to 15% sodium chloride), extreme halophilic (grow best at 15% to 30% sodium chloride). The definition of microorganisms according to their salt tolerance in recent years was presented in Table 1 (Caviccihioli and Thomas, 2004).

Table 1. Definition of Microorganisms According to Their Salt Tolerance (Caviccihioli and Thomas, 2004)

	Salt concentration (M)	
Category	Range	Optimum
Nonhalophilic	0-1.0	<0.2
Slightly halophilic	0.2-2.0	0.2-0.5
Moderate halophilic	0.4-3.5	0.5-2.0
Borderline extreme halophilic	1.4-4.0	2.0-3.0
Extreme halophilic	2.0-5.2	>3.0
Halotolerant	0-1.0	<0.2
Haloversatile	0-3.0	0.2-0.5

1. HALOPHILES IN HYPERSALINE ENVIRONMENTS

They are well adapted to saline environments (Caviccihioli and Thomas, 2004). The halophiles are found in all kingdoms: *Bacteria* (the phyla *Spirochaetes, Bacteroidetes, Proteobacteria, Cyanobacteria, Actinobacteria, Firmicutes*), *Archaea* (the phylum *Euryarchaeota*) and *Eukarya* (the genus *Dunaliella*) (Oren, 2008). It was previously reported that hypersaline environments harbor prokaryotic and eukaryotic organisms such as

halotolerant bacteria (Arahal and Ventosa, 2002; Logan and De Vos, 2015; Yilmaz and Birbir, 2019), moderately halophilic bacteria (Ventosa et al., 1998b; Arahal and Ventosa, 2002), extremely halophilic archaea (Rodríguez-Valera et al., 1985; Benlloch et al., 2002; Elevi et al., 2004; Birbir et al., 2007), haloversatile bacteria (Ellis-Evans 1985; James et al., 1990; Vreeland and Huval 1991; Ashour et al., 2011; Caglayan, 2019a; Caglayan2019b), fungi (Gunde-Cimerman et al., 2009) and algae (Gunde-Cimerman et al., 2009). Other eukaryotic organisms are also sometimes present (Rodríguez-Valera et al., 1985). Halophilic microorganisms isolated from hypersaline environments in some of the investigations are shown in Table 2.

Table 2. Some of the Halophilic Microorganisms Isolated from Hypersaline Environments

Microorganisms	Isolation source	Reference
halophilic bacteria	marine salt	Lloyd, 1929
halotolerant, slightly, moderately halophilic bacteria and extremely halophilic archaea	brine cured hides	Kallenberger, 1985; Bailey and Birbir, 1993; Bailey and Birbir, 1996; Vreeland et al., 1998
haloversatile bacteria	seawater (Antarctica)	Ellis-Evans, 1985
moderately halophilic bacteria	Great Salt Lake	Oren, 1986
haloversatile bacteria	Antarctic saline lakes	James et al., 1990
extremely halophilic archaeon	Dead Sea	Oren et al., 1995
extremely halophilic bacteria	water samples (Santa Pola)	Antón et al., 2000
moderately halophilic bacteria	Saltern (Spain)	Sánchez-Porro et al., 2003
moderately halophilic bacteria and extremely halophilic archaea, haloversatile bacteria	soil, brine, salt samples (Çamaltı Saltern)	Yasa et al., 2008; Guven et al., 2010; Mutlu and Guven, 2015; Caglayan, 2019a; 2019b
halophilic bacteria	hypersaline lake (Iran)	Rohban et al., 2009
bacterial and archaeal populations	curing salt samples, salted hides, soaked hides, soaking liquors	Berber and Birbir, 2010; Berber et al., 2010
haloversatile bacteria	Safaga coast (Egypt) Mangrove area (Red Sea, Egypt)	Ashour et al., 2011; Shatla et al., 2021
moderately halophilic bacteria	Indian saline desert	Khunt et al., 2011
halotolerant bacteria,	salted hides	Birbir, 1997; Aslan and

slightly halophilic bacteria, Gram positive and Gram negative bacteria, moderately halophilic bacteria, extremely halophilic archaea	salted skins	Birbir, 2011; Aslan and Birbir, 2012; Ulusoy and Birbir, 2015; Sánchez-Porro et al., 2011; De La Haba et al., 2011; Akpolat et al., 2015; Caglayan et al., 2015; Bilgi et al., 2015
halophilic bacteria and archaea extremely halophilic archaea	salt samples (Kaldirim and Kayacik Salterns; Tuzkoy Salt Mine; Sereflikochisar Salt Lake; Tuz Lake, Turkey)	Birbir et al., 2002; Birbir et al., 2004; Birbir and Eryilmaz, 2007; Birbir et al., 2015
halotolerant bacteria	salt samples used in leather industry	Yilmaz and Birbir, 2019
moderately halophilic bacterium	sediment of solar pond (Korea)	Lee et al., 2016
halophilic bacteria and archaea	hypersaline lake (Tunisia)	Karray et al., 2018
halophilic bacteria	Lake Qarun (Egypt)	Ibrahim et al., 2020
halophilic archaea	sediment sample (solar saltern, China)	Ding et al., 2020
moderately halophilic bacterium	hypersaline lake (China)	Lu et al., 2020

2. ADAPTATION TO HYPERSALINE ENVIRONMENTS

Microorganisms inhabit in hypersaline environments have different biochemical adaptations to cope with osmotic and water stress, variations in ion concentrations, changes in pH, temperature and salinity (Javor, 1989). Halophilic microorganisms produce biopolymers, compatible solutes, pigments, exopolysaccharides to protect themselves from harsh conditions of hypersaline environments (Ventosa et al., 2005; More et al., 2014; Ibrahim et al., 2020). β-caroten and glycerol produced from halophilic *Dunaliella*, which is a green alga thrive in hypersaline environments, are used in biotechnological applications as antioxidant and food coloring agent (Javor, 1989; Oren, 2010). Bacteriorhodopsin pigments produced by halophiles are used in artificial retina, optical computing, spatial light modulator (Margesin and Schinner, 2001). To combat with high salinity, halophilic organisms occumulate or synthesize compatible solutes (ectoine, proline, trehalose, betaine, glycine) inside their cells (Ventosa et al., 1998a). These compounds

are used as osmoprotective agents in biotechnological processes due to their protection of membranes, enzymes, proteins against osmotic pressure, inactivation, and denaturation (Ventosa et al., 1998a). They have potential use in different industries such as agriculture, food, pharmaceutical, medicine (Pastor et al., 2010). Compatible solutes are used as stress-protective and stabilizer agents and biopolymers are applied to enhance oil recovery (Margesin and Schinner, 2001). Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by microorganisms (Bonete and Martínez-Espinosa, 2011). It was reported that PHAs of halophilic archaea are environmentally friendly source for bioplastics (Legat et al., 2010; Bonete and Martínez-Espinosa, 2011). Moreover, halophilic organisms are capable of degrading various organic compounds such as benzoate, phenol, benzoic acid, ferulic acid, salicylic acid (Rosenberg, 1983; Oren, 1988; García et al., 2004; Ventosa et al., 2005). These organic compounds in saline environments may be degraded by halophilic microorganisms that are able to use these pollutants. Among biopolymers, biosurfactants are utilized in the oil bioremediation of water and soil (Margesin and Schinner, 2001).

3. PRODUCTS OF HALOPHILIC BACTERIA

Due to the low nutrient requirements of halophilic bacteria and their ability to live in saline conditions, recent researchs are focused on these microorganisms.The enzymes, compatible solutes, biofertilizers, pharmaceutically active compounds (PhACs), biopolymers produced by halophilic bacteria are used in diverse biotechnological and industrial applications (Galinski and Tindall, 1992; Mohammadipanah et al., 2015). Halophiles are source of various enzymes such as amylase, phospholipase, lipase, chitinase, DNase, nuclease, pullulanase, pectinase, protease, cellulase, inulinase, xylanase. These enyzmes are referred to as halozymes which are salt tolerant or salt dependent catalytic activity (Rohban et al., 2009; Patel and Saraf, 2015; Gupta et al., 2016). Microbial enzymes from bacteria and archaea, which are catalytically active at harsh conditions, play an important role in biological populations, industrial and biotechnological processes (Table 3).

Table 3. The Industrial Applications of Microbial Enzymes

Microbial enzyme	Industries	The use of enzymes	References
Amylase	Brewing Food Distilling Detergent Textile	Saccharification and hydrolysis of starch Saccharification of marine algae Removal of starch from fabrics Syrup production Reduction of turbidity of beverage	Ratnakar, 2013; Ammar et al., 2002; Kikani et al., 2010;
β-galactosidase	Food Dairy products	Lactose removal from milk Production of galactosylated products Digestibility of dairy products Improving sweetness and flavor	Heyman, 2006; Neri et al., 2008; Grosova et al., 2008; Husain, 2010
Caseinase	Food	Casein digestion in milk	Johnson and Case, 2010
Cellulase	Agriculture Textile Detergent	Biofuel production from cellulosic material Softening and polishing of clothes Agricultural biomass conversation into valuable products	Aygan and Arikan, 2008; Wang et al., 2009
DNase	Food	Flavor agents (acid 5'-guanilic, acid 5'-inosinic) Breaking the phosphodiester linkages of DNA Reducing biofilms	Delgado-García et al., 2015; Brown et al., 2015; Nahar et al., 2018
Esterase	Textile Food	Synthesis of new biopolymeric materials	De Miguel Bouzas et al., 2006
Lipase	Food Detergent Paper	Amynolysis Trans-esterification Esterification Enantioselective biocatalyst for production of fine chemicals Detergent additives	Babu et al., 2008; Jaeger and Holliger, 2010

Table 3. The Industrial Applications of Microbial Enzymes (Continued)

Urease	Soft drink	Removal of urea from wine	Liu et al., 2012
Protease	Animal feed Biomedical Baking Brewing Cheese Laundry Food Detergent Tanning Leather Pharmaceutical Chemistry	Laundry treatments Detergent formulations Peptide sythesis Production of fish sauce Dehairing of animal skins and hides Solubilize proteins in wastes Tenderization of meat	Chand and Mishra, 2003; Mitra and Chakrabartty, 2005; Samad et al., 2017; Kumar and Takagi, 1999; Singh et al., 2014
Pullulanase	Food	Glucose production Biocatalysis agents Starch saccharification Debranching starch	Hii et al., 2012; Delgado-García et al., 2015
Phospholipase	Food Dairy	Flavour improvement of cheese Producing lipolyzed milk fat	Law, 2009; Raveendran et al., 2018
Xylanase	Baking Food Pulp Paper	Bleaching Flour, feed, coffee production Lignin removal from pulp Increasing loaf volume Production of biofuel Starch production Improving wheat flour quality Food additives to poultry Extraction of plant oils and starch	Ratnakar, 2013; Khandeparker and Numan, 2008; Bedford and Classen, 1992; Maat et al., 1992; Wong and Saddler, 1992; Beg et al., 2001
Lysozyme	Food Meat Medical	Increasing shelf life of food	Thallinger et al., 2013; Nahar et al., 2018
Nuclease	Molecular biology	Cleaving phosphodiester bonds of nucleic acids Programmed cell death	Kamekura and Onishi, 1976; Onishi et al., 1983 Parrish and Xue, 2006

Table 3. The Industrial Applications of Microbial Enzymes (Continued)

Microbial enzyme	Industries	The use of enzymes	References
Chitinase	Agriculture	Biocontrol agent against mold diseases	Hatori et al., 2006 Essghaier et al., 2009
Pectinase	Food Textile Paper Pulp	Clarification of fruit juice Increasing fabric whiteness Bleaching of pulp	Chaudhri and Suneetha, 2012; Ahlawat et al., 2009; Kaur et al., 2010
Inulinase	Food	Hydrolysing inulin-containing materials into glucose, fructose	Mohan et al., 2018
Catalase	Food Dairy Bioremediation	Preservation of food Hydrogen peroxide removal from milk in cheese production	Kaushal et al., 2018; Dana and Bauder, 2011
Peroxidase	Food	Improving food quality	Regaldo et al., 2004; Raveendran et al., 2018

4. HALOVERSATILE BACTERIA

Haloversatile bacteria (James et al., 1990) or euryhaline bacteria (Vreeland, 1987) are capable of growing over a wide range of salt concentrations (0- >3.0 M NaCl, optimally 0.2-0.5 M NaCl), but also able to grow in the absence of salt (Kristjansson et al., 2000). These bacteria found in saline environments have been examined in a few studies. The characterization of haloversatile bacteria in these environments and examination of their enzymatic activities may provide important information about their industrial potentials. These microorganisms can cope with osmotic stress due to their ability to produce and accumulate organic compatible solutes. Due to their osmotic adaptation abilities, haloversatile bacteria may live in different salt concentrations found in seawater and saline environments (Kushner, 1978; Grant et al., 1998).

Research on haloversatile bacteria dates back to 1985. These bacteria were isolated from saline environments in a few experiments (Ellis-Evans, 1985; James et al., 1990; Vreeland and Huval, 1991; Ashour et al., 2011). It was reported that psychrophilic and haloversatile species such as

Flectobacillus glomeratus, *Halobacterium lacusprofundi*, *Carnobacterium alterfunditum*, *Methanococcoides burtonii*, *Carnobacterium funditum*, *Flavobacterium gondwanense*, *Halomonas subglaciescola*, *Halomonas meridiana*, *Flavobacterium salegens*, *Vesiculatum antarcticum* were isolated from seawater samples in Antarctica (Ellis-Evans, 1985). A haloversatile bacterium phylogenetically similar to *Halomonas meridiana* was isolated from Antarctic saline lakes (James et al., 1990). In addition, different haloversatile isolates obtained from saline water samples were able to grow at 0-17% NaCl (Vreeland and Huval, 1991). In another study, haloversatile, moderately halophilic and halotolerant bacteria were isolated from the Safaga coast in Egypt (Ashour et al., 2011). Those bacterial isolates belong to the genus *Bacillus*, *Streptococcus* and *Micrococcus* (Ashour et al., 2011). The researchers reported that the haloversatile bacterium isolated from a mangrove ecosystem was able to grow at 0-15% NaCl, pH 5.0-8.5, and temperature range 30-50°C (Ashour et al., 2011). In another study conducted with twenty salt samples collected from Çamaltı Saltern, sixty-nine bacterial strains showing haloversatile properties were isolated and characterized (Caglayan, 2019a). In that study, forty haloversatile bacterial species [*Bacillus subtilis* subsp. *stercoris* (8 isolates), *Kocuria sediminis* (5 isolates), *Paracoccus marcusii* (4 isolates), *Kocuria polaris* (3 isolates), *Micrococcus aloeverae* (3 isolates), *Bacillus haynesii* (3 isolates), *Microbacterium maritypicum* (2 isolates), *Brevibacterium frigoritolerans* (2 isolates), *Paracoccus hibiscisoli* (2 isolates), *Bacillus velezensis* (2 isolates), *Bacillus pumilus* (2 isolates), *Bacillus safensis* (2 isolates), *Staphylococcus petrasii* subsp. *jettensis* (2 isolates), *Staphylococcus hominis* subsp. *novobiosepticus* (2 isolates), *Staphylococcus lentus* (2 isolates), *Bacillus thioparans* (1 isolate), *Staphylococcus epidermidis* (1 isolate), *Acinetobacter radioresistens* (1 isolate), *Exiguobacterium sibiricum* (1 isolate), *Gordonia alkanivorans* (1 isolate), *Microbacterium aurantiacum* (1 isolate), *Staphylococcus pasteuri* (1 isolate), *Bacillus paraflexus* (1 isolate), *Microbacterium saccharophilum* (1 isolate), *Kocuria rosea* (1 isolate), *Staphylococcus saprophyticus* subsp. *saprophyticus* (1 isolate), *Micrococcus yunnanensis* (1 isolate), *Pseudomonas songnenensis* (1 isolate), *Bacillus nealsonii* (1 isolate), *Staphylococcus equorum* subsp. *equorum* (1 isolate), *Agrococcus lahaulensis* (1 isolate), *Sanguibacter inulinus* (1 isolate), *Virgibacillus salarius* (1 isolate),

Staphylococcus cohnii subsp. *urealyticus* (1 isolate), *Bacillus altitudinis* (1 isolate), *Exiguobacterium artemiae* (1 isolate), *Bacillus siamensis* (1 isolate), *Exiguobacterium indicum* (1 isolate), *Bacillus oryzaecorticis* (1 isolate), *Staphylococcus warneri* (1 isolate)] were identified (Caglayan, 2019a). All species could grow at 0-3 M NaCl (optimally 0.2-0.3 M NaCl), pH 6-11 and 20-40°C. While all isolates produced catalase enzyme, none of them produced esterase and xylanase enzymes. **Amylase** (*Brevibacterium frigoritolerans*, *Virgibacillus salarius*, *Pseudomonas songnenensis*, *Bacillus siamensis*); **cellulase** (*Staphylococcus hominis* subsp. *novobiosepticus*, *Staphylococcus pasteuri*, *Bacillus velezensis*, *Brevibacterium frigoritolerans*, *Kocuria rosea*, *Micrococcus yunnanensis*, *Bacillus subtilis* subsp. *stercoris*, *Pseudomonas songnenensis*, *Staphylococcus petrasii* subsp. *jettensis*, *Bacillus altitudinis*, *Bacillus oryzaecorticis*, *Staphylococcus warneri*); **pullulanase** (*Staphylococcus epidermidis*, *Gordonia alkanivorans*, *Brevibacterium frigoritolerans*, *Sanguibacter inulinus*, *Bacillus paraflexus*, *Exiguobacterium indicum*); **lecithinase** (*Staphylococcus hominis* subsp. *novobiosepticus*, *Staphylococcus epidermidis*, *Exiguobacterium sibiricum*, *Bacillus haynesii*, *Microbacterium maritypicum*, *Gordonia alkanivorans*, *Microbacterium aurantiacum*, *Kocuria polaris*, *Paracoccus marcusii*, *Micrococcus aloeverae*, *Bacillus velezensis*, *Bacillus thioparans*, *Microbacterium saccharophilum*, *Kocuria rosea*, *Micrococcus yunnanensis*, *Bacillus nealsonii*, *Staphylococcus equorum* subsp. *equorum*, *Agrococcus lahaulensis*, *Bacillus paraflexus*, *Bacillus altitudinis*, *Exiguobacterium artemiae*, *Exiguobacterium indicum*, *Staphylococcus warneri*); **lipase** (*Staphylococcus hominis* subsp. *novobiosepticus*, *Staphylococcus epidermidis*, *Staphylococcus pasteuri*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Bacillus pumilus*, *Acinetobacter radioresistens*, *Kocuria sediminis*, *Staphylococcus cohnii* subsp. *urealyticus*, *Bacillus altitudinis*, *Staphylococcus warneri*); **urease** (*Staphylococcus saprophyticus* subsp. *saprophyticus*, *Staphylococcus equorum* subsp. *equorum*, *Staphylococcus cohnii* subsp. *urealyticus*); **protease** (*Exiguobacterium sibiricum*, *Bacillus haynesii*, *Microbacterium maritypicum*, *Staphylococcus pasteuri*, *Micrococcus aloeverae*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Bacillus velezensis*, *Bacillus pumilus*, *Brevibacterium frigoritolerans*, *Microbacterium saccharophilum*, *Micrococcus yunnanensis*, *Bacillus safensis*, *Bacillus*

nealsonii, *Staphylococcus equorum* subsp. *equorum*, *Agrococcus lahaulensis*, *Sanguibacter inulinus*, *Virgibacillus salarius*, *Bacillus paraflexus*, *Pseudomonas songnenensis*, *Staphylococcus petrasii* subsp. *jettensis*, *Bacillus altitudinis*, *Exiguobacterium artemiae*, *Bacillus siamensis*, *Exiguobacterium indicum*, *Bacillus oryzaecorticis*, *Staphylococcus warneri*); **caseinase** (*Bacillus pumilus*, *Bacillus siamensis*); **deoxyribonuclease** (*Microbacterium maritypicum*, *Gordonia alkanivorans*, *Microbacterium aurantiacum*, *Bacillus thioparans*, *Staphylococcus lentus*, *Sanguibacter inulinus*, *Exiguobacterium artemiae*, *Bacillus siamensis*, *Exiguobacterium indicum*, *Bacillus oryzaecorticis*); **oxidase** (*Staphylococcus hominis* subsp. *novobiosepticus*, *Staphylococcus epidermidis*, *Exiguobacterium sibiricum*, *Bacillus haynesii*, *Staphylococcus pasteuri*, *Kocuria polaris*, *Paracoccus marcusii*, *Staphylococcus saprophyticus* subsp. *saprophyticus*, *Bacillus velezensis*, *Bacillus pumilus*, *Kocuria sediminis*, *Brevibacterium frigoritolerans*, *Bacillus thioparans*, *Paracoccus hibiscisoli*, *Kocuria rosea*, *Staphylococcus lentus*, *Bacillus safensis*, *Bacillus nealsonii*, *Staphylococcus equorum* subsp. *equorum*, *Virgibacillus salarius*, *Staphylococcus cohnii* subsp. *urealyticus*, *Bacillus paraflexus*, *Bacillus subtilis* subsp. *stercoris*, *Staphylococcus petrasii* subsp. *jettensis*, *Bacillus altitudinis*, *Exiguobacterium artemiae*, *Exiguobacterium indicum*, *Bacillus oryzaecorticis*, *Staphylococcus warneri*) were produced by haloversatile isolates (Caglayan, 2019a).

In the study of Caglayan (2019b), it was reported that six bacterial species showing haloversatile properties (*Arthrobacter ginsengisoli* (2 isolates), *Arthrobacter psychrochitiniphilus* (1 isolate), *Pseudarthrobacter polychromogenes* (1 isolate), *Glutamicibacter arilaitensis* (1 isolate) and *Arthrobacter agilis* (1 isolate) were isolated from a raw salt collected from Çamaltı Saltern. Those isolates could grow at 0-3 M sodium chloride (optimally 0.2 M sodium chloride), 20-45°C (optimally 32°C), pH 6-8 (optimally pH 7). All species produced catalase enzyme (Caglayan, 2019b).

In conclusion, products of halophilic bacteria find potential applications in different industries such as leather, textile, dairy, food, medical, detergent, pharmaceutical, beverage, pulp, paper, molecular biology, agriculture, wood, and chemical. These products are environmentally friendly, stable, produced effectively in high yield with low cost, modified and optimized easily through gene manipulation. Haloversatile bacteria may also have considerable

potential for industrial and biotechnological applications due to their important metabolic products and their tolerance towards extreme pH, temperature, salt concentrations. These microorganisms are unique in their ability to live without salt and with salt and play a key role in the global biological and biogeochemical cycles of carbon and nitrogen.

REFERENCES

Ahlawat, S., Dhiman, S. S., Battan, B., Mandhan, R. P., Sharma, J. (2009). Pectinase Production by *Bacillus subtilis* and its Potential Application in Biopreparation of Cotton and Micropoly Fabric, Process Biochemistry, 44(5), pp. 521-526.

Akpolat, C., Ventosa, A., Birbir, M., Sánchez-Porro, C., Caglayan, P. (2015). Molecular Identification of Moderately Halophilic Bacteria and Extremely Halophilic Archaea Isolated from Salted Sheep Skins Containing Red and Yellow Discoloratios, The Journal of the American Leather Chemists Association, 110, pp. 211-220.

Ammar, Y. B., Matsubara, T., Ito, K., Lizuka, M., Limpaseni, T., Pongsawasdi, P., Minamiura, N. (2002). Characterization of a Thermostable Levansucrase from *Bacillus* sp. TH4-2 Capable of Producing High Molecular Weight Levan at High Temperature, Journal of Biotechnology, 99, pp. 111-119.

Antón, J., Rosselló-Mora, R., Rodríguez-Valera, F., Amann, R. (2000). Extremely Halophilic Bacteria in Crystallizer Ponds from Solar Salterns, Applied and Environmental Microbiology, 66(7), pp. 3052-3057.

Arahal, D. R., Ventosa, A. (2002), Moderately Halophilic and Halotolerant Species of *Bacillus* and Related Genera, Berkeley, R. C. W., Heyndrickx, M., Logan, N., DeVos, P., In: Applications and Systematics of Bacillus and Relatives (pp. 83-99), Oxford, Blackwell.

Ashour, S. M., Mostafa, S. A., Ahmed, S. A. (2011). Halophilic and Halotolerant Bacteria in Mangrove Ecosystem South Safaga on the Red Sea, Egypt, African Journal of Biological Sciences, **7**, pp. 61-68.

Aslan, E., Birbir, M. (2012). Examination of Gram-Negative Bacteria on Salt-Pack Cured Hides, The Journal of the American Leather Chemists Association, 4(107), pp. 106-115.

Aslan, E., Birbir, M. (2011). Examination of Gram-Positive Bacteria on Salt-Pack Cured Hides, The Journal of the American Leather Chemists Association, 12(106), pp. 372-380.

Aygan, A., Arikan, B. (2008). A New Haloalkaliphilic, Thermostable Endoglucanase from Moderately Halophilic *Bacillus sp*. C14 isolated

from Van Soda Lake, International Journal of Agriculture and Biology, 10, pp. 369-374.

Babu, J., Pramod, W.R., George, T. (2008). Cold Active Microbial Lipases: Some Hot Issues and Recent Developments, Biotechnology Advances, 26, pp. 457-470.

Bailey, D. G., Birbir, M. (1993). A Study of the Extremely Halophilic Microorganisms Found on Commercially Brine-Cured Cattle Hides, The Journal of the American Leather Chemists Association, 88, pp. 285-293.

Bailey, D.G., Birbir, M. (1996). The Impact of Halophilic Organisms on the Grain Quality of Brine Cured Hides, The Journal of the American Leather Chemists Association, 91, pp. 47-51.

Bedford, M. R., Classen, H. L. (1992). The Influence of Dietary Xylanase on Intestinal Viscosity and Molecular Weight Distribution of Carbohydrates in Rye-fed Broiler Chick, Visser, J., Beldman, G., VanSomeren, M. A. K., Voragen, A. G. J., In: Xylans and Xylanases (pp. 361-370), Elsevier, Amsterdam.

Beg, Q. K., Kapoor, M., Mahajan, L., Hoondal, G. S. (2001). Microbial Xylanases and Their İndustrial Applications: A Review, Applied Microbiology and Biotechnology, 56, pp. 326-338.

Benlloch, S., López-López, A., Casamayor, E. O., Øvreås, L., Goddard, V., Daae, F. L., Smerdon, G., Massana, R., Joint, I., Thingstad, F., Pedrós-Alió, C., Rodríguez-Valera, F. (2002). Prokaryotic Genetic Diversity Throughout the Salinity Gradient of a Coastal Solar Saltern, Environmental Microbiology, 4(6), pp. 349-360.

Berber, D., Birbir, M. (2010). Examination of Bacterial Populations in Salt, Salted Hides, Soaked Hides and Soak Liquors, The Journal of the American Leather Chemists Association, 105, pp. 320-326.

Berber, D., Birbir, M., Mertoğlu, B. (2010). Examination of Bacterial and Archaeal Populations in Salt, Salted and Soaked Hide and Soak Liquors via Fluorescent In Situ Hybridization, Journal of the Society of Leather Technologists and Chemists, 6(94), pp. 259-261.

Bilgi, S. T., Yapici-Mericli, B., Karaboz, I. (2015). Determination of Hydrolytic Enzyme Capabilities of Halophilic Archaea Isolated from Hides and Skins and Their Phenotypic and Phylogenetic

Identification, The Journal of the American Leather Chemists Association, 110(2), pp. 33-42.

Birbir, M. (1997). Tuzla Muhafaza Edilmiş Fransız ve Rus Derilerinin Halofilik Bakteriler Yönünden İncelenmesi, Journal of Turkish Microbiology Society, 27, pp. 68-73.

Birbir, M., Kalli, N., Johansson, C. (2002). The Examination of Salt Quality of Şereflikoçhisar Lake Used in Turkish Leather Industry, Journal of the Society of Leather Technologists and Chemists, 86(3), pp. 112-117.

Birbir, M., Ogan, A., Calli, B., Mertoğlu, B. (2004). Enzymatic Characteristics of Extremely Halophilic Archaeal Community in Tuzkoy Salt Mine, Turkey, World Journal of Microbiology and Biotechnology, 20, pp. 613-621.

Birbir, M., Eryilmaz, S. (2007). Inhibiting of Lipolytic Haloarchaeal Damage on Brine Cured Hides with Halocin Producer Strains, Journal of the Society of Leather Technologists and Chemists, 91, pp. 69-72.

Birbir, M., Calli, B., Mertoglu, B., Elevi-Bardavid, R., Oren, A., Ogmen, M. N., Ogan, A. (2007). Extremely Halophilic Archaea from Tuz Lake, Turkey, and the Adjacent Kaldirim and Kayacik Salterns, World Journal of Microbiology and Biotechnology, 23, pp. 309-316.

Birbir, Y., Anik, S., Birbir, M., Caglayan, P. (2015). Annihilation of Extremely Halophilic Archaea in Hide Preservation Salt Using Alternating Electric Current, *Johnson Matthey Technology Review*, 59, pp. 109.

Bonete, M. J., Martínez-Espinosa, R. M. (2011). Enzymes from Halophilic Archaea: Open Questions, Ventosa, A., Oren, A., Ma, Y., In: Halophiles and Hypersaline Environments, Current Research and Future Trends (pp. 359-371), Chapter 19, Springer.

Brown, H. L., Hanman, K., Reuter, M., Betts, R. P., Van Vliet, A. H. (2015). *Campylobacter jejuni* Biofilms Contain Extracellular DNA and are Sensitive to DNase I Treatment. Frontiers in Microbiology, 6, pp. 699.

Caglayan, P., Birbir, M., Ventosa, A., Sánchez-Porro, C. (2015). Characterization of Moderately Halophilic Bacteria from the Salt-

pack Cured Hides, Journal of the Society of Leather Technologists and Chemists, 5, pp. 250-254.

Caglayan, P. (2019a). Enzymatic Reactions and Phylogenetic Analysis of Haloversatile Bacteria Isolated from Çamaltı Saltern Salt Samples used in Leather Industry, The Journal of the American Leather Chemists Association, 115, pp. 450-461.

Caglayan, P. (2019b). Isolation and 16S rRNA Sequence Analysis of Six Environmental Haloversatile Bacteria from Çamaltı Saltern, International Biodiversity and Ecology Symposium (Istanbul, Turkey, September 26- September 28, 2019).

Caglayan, P., Birbir, M., Sánchez-Porro, C., Ventosa, A., Birbir, Y. (2018). Investigation of Moderately Halophilic Bacteria Causing Deterioration of the Salted Sheep and Goat Skins and Their Extermination via Electric Current Applications, The Journal of the American Leather Chemists Association, 113, pp. 41-52.

Caviccihioli, R., Thomas, T. (2004). Extremophiles, Schaechter, M., Lederberg, J., In: The Desk Encyclopedia of Microbiology, Chapter 37, Elsevier Academic Press.

Chand, S., Mishra, P. (2003). Research and Application of Microbial Enzymes, India's Contribution, *Advences in Biochemical Engineering/Biotechnology*, 85, pp. 95-124.

Chaudhri, A., Suneetha, V. (2012). Microbially Derived Pectinases: A Review, Journal of Pharmacy and Biological Sciences, 2(2), pp. 1-5.

Dana, L. D., Bauder, J. W. (2011). A General Essay on Bioremediation of Contaminated Soil, Montana State University, Bozeman, Mont, USA.

De La Haba, R., Yilmaz, P., Birbir, M., Sánchez-Porro, C., Ventosa, A. (2011). *Salimicrobium salexigens* sp. nov., A Moderately Halophilic Bacterium from Salted Hides, Systematic and Applied Microbiology, 34, pp. 435-439.

Delgado-García, M., Nicolaus, B., Poli, A., Aguilar, C. N., Rodríguez-Herrera, R. (2015). Isolation and Screening of Halophilic Bacteria for Production of Hydrolytic Enzymes, Maheshwari, D., Saraf, M., In: Halophiles, Sustainable Development and Biodiversity (pp. 396), Springer.

De Miguel Bouzas, T., Barros-Velázquez, J., González Villa, T. (2006). Industrial Applications of Hyperthermophilic Enzymes: A Review, Protein & Peptide Letters, 13, pp. 645-651.

Ding, Y., Han, D., Cui, H. L. (2020). *Halorussus halophilus* sp. nov., A Novel Halophilic Archaeon Isolated from a Marine Solar Saltern, Current Microbiology, pp. 1-7.

Elevi, R., Assa, P., Birbir, M., Ogan, A., Oren, A. (2004). Characterization of Extremely Halophilic Archaea isolated from the Ayvalik Saltern, Turkey, World Journal of Microbiology and Biotechnology, 20, pp. 719-725.

Ellis-Evans, J. C. (1985). Microbial Ecology in Antarctica, *Biologist*, 32, pp. 171-176.

Essghaier, B., Fardeau, M. L., Cayol, J. L., Hajlaoui, M. R., Boudabous, A., Jijakli, H., Sadfi-Zouaoui, N. (2009). Biological Control of Grey Mould in Strawberry Fruits by Halophilic Bacteria. Journal of Applied Microbiology, 106, pp. 83-846.

Galinski, E.A., Tindall, B.J. (1992). Biotechnological Prospects for Halophiles and Halotolerant Micro-organisms, Herbert, R. H., Sharp, R. J., In: Molecular Biology and Biotechnology of Extremophiles (pp. 76–114), Blackie, Glasgow.

García, M. T., Mellado, E., Ostos, J. C., Ventosa, A. (2004). *Halomonas organivorans* sp. nov., a Moderate Halophile able to Degrade Aromatic Compounds, International Journal of Systematic and Evolutionary Microbiology, 54, pp. 1723-1728.

Grant, W. D., Gemmell, R. T., Mcgenity, T. J. (1998). Extremophiles, Horikoshi, K., Grant, W.D., In: Microbial Life in Extreme Environment (pp. 93), Wiley-Liss, New York.

Grosova, Z., Rosenberg, M., Rebros, M. (2008). Perspectives and Applications of Immobilized β-galactosidase in Food Industry: A Review, Czech Journal of Food Sciences, 26, pp. 1-14.

Gunde-Cimerman, N., Ramos, J., Plemenitas, A. (2009). Halotolerant and Halophilic Fungi, Mycological Research, 113, pp. 1231-1241.

Gupta, S., Sharma, P. Dev, K., Sourirajan, A. (2016). Halophilic Bacteria of Lunsu Produce an Array of Industrially Important Enzymes with Salt

Tolerant Activity, Hindawi Publishing Corporation Biochemistry Research, 2016, pp. 1-10.

Guven, K., Demirci, A., Mutlu, M. B., Korcan, S. E. (2010). Phenotypic Characterization of Halophilic Bacteria Isolated from Çamaltı Saltern in Turkey. Electronic Journal of Biotechnology, 1, 11-21.

Hatori, Y., Sato, M., Orishimo, K., Yatsunami, R., Endo, K., Fukui, T., Nakamura, S. (2006). Characterization of Recombinant Family 18 Chitinase from Extremely Halophilic Archaeon *Halobacterium salinarum* Strain NRC-1, Chitin Chitosan Research, 12, pp. 201.

Heyman, M. B. (2006). Committee on Nutrition, Lactose Intolerance in Infants, Children, and Adolescents, Pediatrics, 118, pp. 1279-1286.

Hii, S. L., Tan, J. S., Ling, T. C., Ariff, A. B. (2012). Pullulanase: Role in Starch Hydrolysis and Potential Industrial Applications, Hindawi Publishing Corporation Enzyme Research, 2012, pp. 1-14.

Husain, Q. (2010). B-Galactosidases and Their Potential Applications: A Review, Critical Reviews in Biotechnology, 30(1), pp. 41-62.

Ibrahim, I. M., Konnova, S. A., Sigida, E. N., Lyubun, E. V., Muratova, A. Y., Fedonenko, Y. P., Elbanna, K. (2020). Bioremediation Potential of a Halophilic *Halobacillus* sp. Strain, EG1HP4QL: Exopolysaccharide Production, Crude Oil Degradation, and Heavy Metal Tolerance, Extremophiles, 24, pp.157-166.

Jaeger, K. E., Holliger, P. (2010). Chemical Biotechnology a Marriage of Convenience and Necessity, *Current Opinion in Biotechnology*, 21, pp. 711-712.

James, S. R., Dobson, S. J., Franzmann, P.D., McMeekin, T. A. (1990). *Halomonas meidiana*, a New Species of Extremely Halotolerant Bacteria isolates from Antarctic Saline Lakes. Systematic and Applied Microbiology, 13, pp. 270-278.

Javor, B. (1989). Hypersaline Environments, Microbiology and Biogeochemistry, Springer.

Johnson, T. R., Case, C. L. (2010). Laboratory Experiments in Microbiology, Pearson Education, San Francisco.

Kallenberger, W.E. (1985). Halophilic Bacteria in Hide Curing, *PhD Thesis*, Division of Graduate Studies and Research of the University of

Cincinati, Department of Basic Science Tanning Research of the Collage of Arts and Science.

Kamekura, M., Onishi, H. (1976). Effect of Magnesium and Some Nutrients on the Growth and Nuclease Formation of a Moderately Halophile *Micrococcus varians* var. *halophilus*, Canadian Journal of Microbiology, 22, pp. 1567-1576.

Karray, F., Ben Abdallah, M., Kallel, N., Hamza, M., Fakhfakh, M., Sayadi, S. (2018). Extracellular Hydrolytic Enzymes Produced by Halophilic Bacteria and Archaea Isolated from Hypersaline Lake. Molecular Biology Reports, 45, pp. 1297-1309.

Kaushal, J., Mehandia, S., Singh, G., Raina, A., Kumar Arya, S. (2018). Catalase Enzyme: Application in Bioremediation and Food Industry, Biocatalysis and Agricultural Biotechnology, 16, pp. 192-199.

Kaur, A., Mahajan, R., Singh, A., Garg, G., Sharma, J. (2010). Application of Cellulase-free Xylano-pectinolytic Enzymes from the Same Bacterial Isolate in Biobleaching of Kraft Pulp, Bioresource Technology, 101(3), pp. 9150-9155.

Khandeparker, R., Numan, M. T. (2008). Bifunctional Xylanases and Their Potential Use in Biotechnology, Journal of Industrial Microbiology and Biotechnology, 35(7), pp. 635-644.

Khunt, M., Pandhi, N., Rana, A. (2011). Amylase from Moderate Halophiles Isolated from Wild Ass Excreta, International Journal of Pharmacy and Biological Sciences, 1, pp. 586-592.

Kikani, B. A., Shukla, R. J., Singh, S. P. (2010). Biocatalytic Potential of Thermophilic Bacteria and Actinomycetes, Mendez-Vilas, A., In: Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (pp. 1000-1007), Formatex Research Center, Badajoz.

Kristjansson, J. K., Hreggvidsson, G. O., Grant, W. D. (2000). Taxonomy of Extremophiles, Priest, F. G., Goodfellow, M., In: Applied Microbial Systematics (pp. 251), Springer-Science, Kluwer Academic Publisher.

Kumar, C. G., Takagi, H. (1999). Microbial Alkaline Proteases: From a Bioindustrial Viewpoint, Biotechnology Advances, 17, pp. 561-594.

Kushner, D. J. (1978). Life in High Salt and Solute Concentrations: Halophilic Bacteria, Microbial Life In Extreme Environments, Academic Press, pp. 317-368.

Kushner, D. J., Kamekura, M. (1988). Physiology of Halophilic Eubacteria, Rodríguez-Varela, F., In: *Halophilic Bacteria* (pp. 109-138), CRC Press: Boca Raton, FL, USA.

Law, B. A. (2009). Enzymes in Dairy Product Manufacture, Van Oort, M., Whitehurst, R. J., In: *Enzymes in Food Technology* (pp. 88-102), Oxford, UK, Wiley-Blackwell.

Lee, J. C., Kim, S. J., Whang, K. S. (2016). *Halomonas sediminicola* sp. nov., a Moderately Halophilic Bacterium Isolated from a Solar Saltern Sediment, International Journal of Systematic and Evolutionary Microbiology, 66, pp. 3865-3872.

Legat, A., Gruber, C., Zangger, K., Wanner, G., Stan-Lotter, H. (2010). Identification of Polyhydroxyalkanoates in *Halococcus* and Other Haloarchaeal Species. Applied Microbiology and Biotechnology, 87, pp. 1119-1127.

Liu, J., Xu, Y., Nie, Y., Zhao, G.A. (2012). Optimization Production of Acid Urease by *Enterobacter* sp. in an Approach to Reduce Urea in Chinese Rice Wine, Bioprocess and Biosystems Engineering, 35, pp. 651-657.

Litchfeld, C.D., Gillevet, M.P. (2002). Microbial Diversity and Complexity in Hypersaline Environments: A Preliminary Assessment, Journal of Industrial Microbiology and Biotechnology, 28, pp. 48-55.

Lloyd, D.J. (1929). Red Heat in Salted Hides, Journal of the Society of Leather Technologists and Chemists 13, pp. 538- 569.

Logan, N. A., De Vos, P. (2015). *Bacillus*, In: Bergey's Manual of Systematics of Archaea and Bacteria (pp. 1-163), John Wiley&Sons, Inc., In Association with Bergey's Manual Trust.

Lu, H., Xing, P., Zhai, L., Li., H., Wu, Q. (2020). *Halomonas montanilacus* sp. nov., isolated from hypersaline Lake Pengyanco on the Tibetan Plateau, International Journal of Systematic and Evolutionary Microbiology, pp. 1-8.

Maat, J., Roza, M., Verbakel, J., Stam, H., daSilra M. J. S., Egmond, M. R., Hagemans, M. L. D., vanGarcom, R. F. M., Hessing, J. G. M.,

vanDerhondel, C. A. M. J. J., vanRotterdam, C. (1992). Xylanases and Their Application in Bakery, Visser, J., Beldman, G., vanSomeren, M. A. K., Voragen, A.G.J., In: Xylans and Xylanases (pp. 349-360), Elsevier, Amsterdam.

Margesin, R., Schinner, F. (2001). Biodegradation and Bioremediation of Hydrocarbons in Extreme Environments. Applied Microbiology and Biotechnology, 56(5-6), 650-663.

Mohammadipanah, F., Hamedi, J., Dehhaghi, M. (2015). Halophilic Bacteria: Potentials and Applications in Biotechnology, Maheshwari, D., Saraf, M., In: Halophiles, Sustainable Development and Biodiversity, Volume 6, Springer, Cham.

More, T. T., Yadav, J. S. S., Yan, S., Tyagi, R. D., Surampalli, R. Y. (2014). Extracellular Polymeric Substances of Bacteria and Their Potential Environmental Applications, Journal of Environmental Management, 144, pp. 1-25.

Mitra, P., Chakrabartty, P. K. (2005). An Extracellular Protease with Depilation Activity from *Streptomyces nogalator*, Journal of Scientific and Industrial Research, 64(12), pp. 978-983.

Mohan, A., Flora, B., Girdhar, M. (2018). Inulinase: An Important Microbial Enzyme in Food Industry, Singh, J., Sharma, D., Kumar, G., Sharma, N., In: Microbial Bioprospecting for Sustainable Development (pp. 237-248), Springer, Singapore.

Mutlu, M. B., Guven, K. (2015). Bacterial Diversity in Çamaltı Saltern, Turkey. Polish Journal of Microbiology, 64, pp. 37-45.

Nahar, S., Mizan, F. R., Ha, A. J. W., Ha, S. D. (2018). Advances and Future Prospects of Enzyme-Based Biofilm Prevention Approaches in the Food Industry, Comprehensive Review in Food Science and Food Safety, 17, pp. 1484-1502.

Neri, D. F. M., Balcao, V. M., Carneiro-da-Cunha, M. G., Carvalho, J. L. B., Teixeira, J.A. (2008). Immobilization of β galactosidase from *Kluyveromyces lactis* onto a Polysiloxane-polyvinyl Alcohol Magnetic (mPOS-PVA) Composite for Lactose Hydrolysis, Catalysis Communications, 4, pp. 234-239.

Onishi, H., Mori, T., Takeuchi, S., Tani, L., Kobayashi, T., Kamekura, M. (1983). Halophilic Nuclease of a Moderately Halophilic *Bacillus* sp.:

Production, Purification and Characterization, Applied and Environmental Microbiology, 45, 24-30.

Oren, A. (1986). Intracellular Salt Concentrations of the Anaerobic Halophilic Eubacteria *Haloanaerobium praevalens* and *Halobacteroides halobius*, Canadian Journal of Microbiology, 32(1), pp. 4-9.

Oren, A. (1988). Anaerobic Degradation of Organic Compounds at High Salt Concentrations. *Antonie van Leeuwenhoek*, 54, pp. 267-277.

Oren, A., Gurevich, P., Gemmell, R. T., Teske, A. (1995). *Halobaculum gomorrense* gen. nov., sp. nov., a Novel Extremely Halophilic Archaeon from the Dead Sea, International Journal of Systematic Bacteriology, 45(4), pp. 747-754.

Oren, A. (2008). Microbial Life at High Salt Concentrations: Phylogenetic and Metabolic Diversity, Saline Systems, 4(2), pp. 1-13.

Oren, A. (2010). Industrial and Environmental Applications of Halophilic Microorganisms. Environmental Technology, 31, pp. 825-834.

Parrish, J. Z., Xue, D. (2006). Cuts Can Kill: The Roles of Apoptotic Nucleases in Cell Death and Animal Development, Chromosoma, 115(2), pp. 89-97.

Pastor, J. M., Salvador, M., Argandona, M., Bernal, V., Reina-Bueno, M., Csonka, L. N., Iborra, J. L., Vargas, C., Nieto, J. J., Cánovas, M. (2010). Ectoines in Cell Stress Protection: Uses and Biotechnological Production. Biotechnology Advances, 28, pp.782-801.

Patel S., Saraf M. (2015). Perspectives and Application of Halophilic Enzymes, Maheshwari, D., Saraf, M., In: Halophiles. Sustainable Development and Biodiversity (pp. 403-419), Volume 6, Springer, Cham.

Ratnakar, D. (2013). Use of Halophile Physiology and Adaptations in Various Industrial Applications, Research Journal of Biotechnology, 8(2), pp. 1-3.

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., Pandey, A. (2018). Applications of Microbial Enzymes in Food Industry, 56(1), Food Technology and Biotechnology, pp. 16-30.

Regaldo, C., García-Almendárez, B. E., Duarte-Vázquez, M.A. (2004). Biotechnological Applications of Peroxidises. Phytochemistry Reviews, 3(1-2), 243-256.

Rodríguez-Valera, F., Ventosa, A., Juez, G., Imhoff, J.F. (1985). Variation of Environmental Features and Microbial Populations with Salt Concentrations in a Multi-pond Saltern, Microbial Ecology, 11, pp. 107-115.

Rohban, R., Amoozegar, M. A., Ventosa, A. (2009). Screening and Isolation of Halophilic Bacteria Producing Extracellular Hydrolyses from Howz Soltan Lake, Iran, Journal of Industrial Microbiology and Biotechnology, 36()3, pp. 333-340.

Rosenberg, A. (1983). *Pseudomonas halodurans* sp. nov., a Halotolerant Bacterium. Archives of Microbiology, 136, pp. 117-123.

Samad, N. S. A., Amid, A., Jimat, D. N., Shukor, N. A. A. (2017). Isolation and Identification of Halophilic Bacteria Producing Halotolerant Protease, Science Heritage Journal/Galeri Warisan Sains (GWS), 1(1), pp. 7-9.

Sánchez-Porro, C., Martín, S., Mellado, E., Ventosa, A. (2003). Diversity of Moderately Halophilic Bacteria Producing Extracellular Hydrolytic Enzymes, Journal of Applied Microbiology, 94, pp. 295-300.

Sánchez-Porro, C., Yilmaz, P., De La Haba, R. R., Birbir, M., Ventosa, A. (2011). *Thalassobacillus pellis* sp. nov., a Moderately Halophilic, Gram-positive Bacterium Isolated from Salted Hides, International Journal of Systematic and Evolutionary Microbiology, 5, pp. 1206-1210.

Shatla, S.H., El-Kafrawy, S.B., Ahmed, H.A., El-Mokadem, M.T. (2021). An Integrating Geospatial Technology with Microbiology in Isolating and Characterizing Selenite-Reducing Bacteria from Two Mangrove Areas Along the Red Sea, Egypt, Egyptian Journal of Aquatic Biology & Fisheries, 25(1), 389-405.

Singh, T. A., Devi, K. R., Ahmed, G., Jeyaram, K. (2014). Microbial and Endogenous Origin of Fibrinolytic Activity in Traditional Fermented Foods of Northeast India, Food Research International, 55, pp. 356-362

Thallinger, B., Prasetyo, E. N., Nyanhongo, G. S., Guebitz, G. M. (2013). Antimicrobial Enzymes: An Emerging Strategy to Fight Microbes and Microbial Biofilms, Biotechnology Journal, 8(1), 97-109.

Ulusoy, K., Birbir, M. (2015). Identification and Metabolic Activities of Bacterial Species Belonging to the *Enterobacteriaceae* on Salted Cattle Hides and Sheep Skins, The Journal of the American Leather Chemists Association, 110, pp. 86-199.

Ventosa, A., Nieto, J. J., Oren, A. (1998a). Biology of Moderately Halophilic Aerobic Bacteria, Microbiology and Molecular Biology Reviews, 62, pp. 504-544.

Ventosa, A., Márquez, M.C., Garabito, M.J., Arahal, D.R. (1998b). Moderately Halophilic Gram-positive Bacterial Diversity in Hypersaline Environments, Extremophiles, 2(3), pp. 297-304.

Ventosa, A., Sánchez-Porro, C., Martín, S., Mellado, E. (2005). Halophilic Archaea and Bacteria as a Source of Extracellular Hydrolytic Enzymes, Gunde-Cimerman, N., Oren, A., Plemenitaš, A., In: Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya. Cellular Origin, Life in Extreme Habitats and Astrobiology, Volume 9, Springer, Dordrecht.

Ventosa, A. (2006). Unusual Microorganisms from Unusual Habitats: Hypersaline Environments, Logan, N. A., Lppin-Scott, H. M., Oyston, P. C. F., In: Prokaryotic Diversity-Mechanism and Significance (pp. 223-253), Cambridge University Press, Cambridge, UK.

Vreeland, R. H. (1987). Mechanisms of Halotolerance in Microorganisms, CRC Critical Reviews in Microbiology, 14, pp. 311-356.

Vreeland, R. H., Huval, J. H. (1991). Phenotypic Characterization of Halophilic Bacteria from Ground Water Sources in the United States, Rodríguez-Valera, F., In: General and Applied Aspects of Microorganisms (pp. 53-60), Plenum Press, New York.

Vreeland, R. H., Angelini, S., Bailey, D. G. (1998). Anatomy of Halophile Induced Damage to Brine Cured Cattle Hides, The Journal of the American Leather Chemists Association, 93, pp. 121-131.

Wang, C. Y., Hsieh, Y. R., Ng, C. C., Chan, H., Lin, H. T., Tzeng, W. S., Shyu, Y. T. (2009). Purification and Characterization of a Novel

Halostable Cellulase from *Salinivibrio sp*. Strain NTU05, Enzyme and Microbial Technology, 44, pp. 373-379.

Wong, K. K. Y., Saddler, J.N. (1992). *Trichoderma* Xylanases, Their Properties and Purification, Critical Reviews in Biotechnology, 12, pp. 413-435.

Yasa, I., Kahraman, O., Tekin, E., Kocyigit, A. (2008). Isolation and Molecular Identification of Extreme Halophilic Archaea from Çamaltı Saltern, Ege Journal of Fisheries and Aquatic Sciences, 25, pp. 117-121.

Yilmaz, E., Birbir, M. (2019). Characterization of Halotolerant *Bacillus* Species Isolated from Salt Samples Collected from Leather Factories in Turkey, The Journal of the American Leather Chemists Association, 114, pp. 118-130.



İKSAD
Publishing House

ISBN: 978-625-8405-83-5