GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

HABILITATION THESIS

"CONTRIBUTIONS IN THE FIELD OF FOOD QUALITY, FOOD SAFETY AND THE IMPACT OF DIET ON VARIOUS PATHOLOGIES"

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REZUMAT

Teza de abilitare cuprinde rezultatele activității mele științifice, profesionale și academice dobândite după obținerea titlului de doctor în domeniul Farmacie, precum și direcțiile viitoare de cercetare. Realizările științifice postdoctorale au fost inițiate și dezvoltate pornind de la activitatea desfășurată în cadrul studiilor doctorale finalizate cu susținerea publică a tezei de doctorat intitulată "Determinarea reziduurilor medicamentoase din mierea de albine" – coordonator științific, Prof. dr. Rodica Cuciureanu. Titlul de Doctor în Științe Medicale - Domeniul Farmacie a fost conferit prin Ordinul Ministrului Educației, Cercetării, Tineretului și Sportului nr. 3930 din 20.06.2013.

Lucrarea reflectă preocupările mele în domeniile științelor farmaceutice, calității și siguranței alimentare, nutriției și dietoterapiei, cu evidențierea rezultatelor cercetărilor efectuate în cadrul Disciplinei de Chimia Mediului și Alimentului a Universității de Medicină și Farmacie" Grigore T. Popa" din Iași.

Activitatea de cercetare s-a îmbinat perfect cu activitatea didactică (desfășurată la obiectele de studiu Chimia Mediului și Alimentului, Dietoterapie, Elemente de nutriție din cadrul Facultății de Farmacie, dar și la obiectele de studiu Chimia Alimentelor și Toxicologia Alimentară din cadrul Facultății de Medicină, specializarea Nutriție și Dietetică), cele două fiind de fapt complementare.

Principalele direcții de cercetare pe care le-am dezvoltat sunt:

- Determinarea diferitelor clase de contaminanți din produsele stupului: miere (reziduuri medicamentoase) și polen (micotoxine);
- Studiul toxicității contaminanților din miere asupra parametrilor imunologici, hematologici, biochimici la animalele de laborator;
- Validarea și aplicarea metodelor de analiză accesibile (imunochimice) pentru detecția urmelor de reziduuri din miere;
- Determinarea parametrilor de compoziție chimică pentru diverse produse alimentare de origine vegetală;
- Studii privind impactul dietei în diverse patologii cum ar fi afecțiuni gastro-intestinale, boli cardiovasculare și obezitate.

Teza de abilitare este structurată în conformitate cu criteriile recomandate și aprobate de către Consiliului Național de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU).

Teza cuprinde 3 secțiuni principale ce includ realizările științifice în perioada postdoctorală (Secțiunea A), perspectivele de dezvoltare a activității științifice, profesionale și academice (Secțiunea B) și lista referințelor bibliografice utilizate (Secțiunea C). Cele 3 secțiuni sunt precedate de o sinteză a întregii mele cariere, în care sunt prezentate cele mai importante realizări profesionale, academice și științifice.

Realizările științifice din perioada postdoctorală (**Secțiunea A**) sunt structurate în două direcții principale de cercetare.

Prima direcție de cercetare prezentată în **Capitolul I** este axată pe evaluarea calității și siguranței alimentare a produselor stupului (miere și polen), precum și studiul compoziției

chimice alimentare în vederea evidențierii claselor de nutrienți valoroși pentru alimentația umană. Studiile au fost realizate printr-o colaborare amplă cu colegii din disciplina Chimia mediului și alimentului, dar și alte colaborări extinse multidisciplinare. Rezultatele obținute au fost diseminate la diverse manifestări științifice de profil și publicate în reviste de specialitate cu vizibilitate națională și internațională, cotate ISI și BDI.

Subcapitolul 1 este o continuare a preocupărilor din timpul studiilor doctorale privind tehnicile de analiză de mare sensibilitate pentru detecția reziduurilor medicamentoase în miere în contextul asigurării calității și siguranței alimentare, dar și studii de evidențiere a efectelor toxice ale acestora asupra parametrilor hematologici, imunologici și biochimici la animalele de laborator. În plus, cercetările au fost extinse și pe alți contaminanți alimentari (micotoxine), precum și pe alte produse alimentare (polen). Astfel, am prezentat studiile efectuate pe mierea de albine privind validarea metodei de determinare simultană a patru nitrofurani și a cloramfenicolului, estimarea conținutului de chinolone, cefalosporine, amfenicoli, precum și influența reziduurilor medicamentoase (sulfonamide) din miere asupra unor parametri biochimici și hematologici la animale de laborator (șobolani).

Subcapitolul 2 face referire la cunoașterea compoziției chimice a produselor alimentare care este deosebit de importantă, atât pentru stabilirea valorii lor biologice în scopul unei alimentații corecte, echilibrate, cât și pentru înțelegerea și dirijarea proceselor care au loc în alimente în timpul cultivării, păstrării, conservării, prelucrării culinare sau industriale. Produsele alimentare de origine vegetală conțin, pe lângă componentele cu rol biologic cunoscut și indispensabile în alimentația omului (macronutrienţi și micronutrienţi) și alte substanțe bioactive (pigmenți naturali, acizi organici, enzime, uleiuri volatile etc), în concentrații foarte mici, care pot fi benefice sănătății organismului uman.

Activând în cadrul disciplinei Chimia Mediului și Alimentului, m-am dedicat domeniului alimentației și al nutriției, care este unul extrem de vast ce necesită o abordare complexă multidisciplinară prin aprofundarea aspectelor legate și de impactul dietei asupra diverselor boli umane.

Astfel, a **doua direcție** de cercetare abordată, prezentată în **Capitolul II** s-a axat pe impactul dietei/alimentației asupra unor patologii moderne, foarte răspândite în ultima vreme.

Acest capitol cuprinde 2 subcapitole care detaliază impactul dietei în patologiile din sfera gastro-intestinală, precum și în suprapondere și obezitate.

Contribuțiile personale cercetărilor incluse în Capitolul I au fost publicate în reviste de prestigiu, precum: *Farmacia*. IF:1,607, *Farmacia*. IF: 1,433, *Farmacia*. IF: 1,55, *Medicina.* IF: 2,6, *Revista de Chimie*. IF: 1,605, *Revista de Chimie*. IF: 1,412, *Revista de Chimie*. IF: 1,412, *Revista Medico-chirurgicală*.

Subcapitolul 1 al celui de al doilea capitol face referire la rezultatele unor colaborări multidisciplinare cu privire la impactul dietei asupra patologiilor din sfera gastro-intestinală (refluxul gastroesofagian, sindromul intestinului iritabil, boala celiacă), precum și implicațiile microbiomului intestinal în diverse boli (hipertensiune arterială, diabet zaharat etc).

Subcapitolul 2 din acest capitol abordează o temă de maximă actualitate și anume, obezitatea care este una dintre cele mai mari provocări de sănătate publică a secolului nostru. Metodele de prevenire și tratare a obezității includ educația nutrițională, activitatea fizică, dietele, administrarea de medicamente, terapiile psihologice sau intervențiile bariatrice. Industrializarea, urbanizarea, ultratehnologizarea, sedentarismul creează premisele extinderii acestor patologii metabolice cum ar fi obezitatea, diabetul zaharat, bolile cardiovasculare, cancerul etc. Alimentația sănătoasă presupune un echilibru între aportul alimentar și consumul energetic al organismului. Calitatea vieții este dependentă de calitatea și cantitatea hranei, iar o alimentație săracă în nutrienți va determina efecte negative asupra sănătății organismului uman.

Dietele cu restricții energetice care utilizează suplimente nutritive și alimente cu un aport proteic ușor crescut au dus la o pierdere a masei grase cu reținerea masei musculare, acestea dovedindu-se metode eficiente de slăbire aplicate în special la nivel individual.

Contribuțiile personale cercetărilor incluse în Capitolul II au fost publicate în reviste de prestigiu, precum: *Turkish Journal of Gastroenterology*. IF: 0,966, *Cells*. IF: 6, *Nutrients*. IF: 5,9, *Nutrients*. IF: 5,9, *Nutrients*. IF:5,9, *Revista de Cercetare și Intervenție socială*, IF: 1,076.

Secțiunea B are ca obiectiv, prezentarea unor strategii specifice pentru activitatea profesională, didactică și de cercetare viitoare.

Pe plan profesional și academic, îmi doresc dobândirea de noi aptitudini, competențe și cunoștințe teoretice/practice care să asigure pregătirea și perfecționarea continuă în cadrul disciplinei pe care o coordonez. Atingerea unui nivel de excelență prin dezvoltarea capacității de predare și cercetare medicală va fi posibilă doar prin integrarea în procesul educațional a celor mai noi cunoștințe și metodologii specifice domeniului studiat. În plus, voi prioritiza direcțiile de evoluție privind parteneriatul cu studenții și farmaciștii rezidenți cu scopul creșterii eficacității procesului universitar de predare și învățare.

Pe plan științific, voi rămâne și îmi voi intensifica prezența în spațiul rezervat științelor farmaceutice, pe domeniul în care activez, pentru a face ca rezultatele cercetărilor mele, desfășurate alături de colegii mei din comunitatea academică, să fie de o înaltă valoare științifică și cât mai vizibile pe plan național și internațional. În următoarea perioadă, voi continua direcțiile de cercetare actuale, voi extinde aria de cercetare pe alte clase de contaminanți (pesticide, metale grele, biostimulatori) din multiple clase de produse alimentare, dar mă voi dedica și determinărilor cantitative pe alte elemente de mediu, precum apă, aer, sol.

În acest sens, accesarea de fonduri pentru proiecte de cercetare centrate pe aceste teme va fi un alt obiectiv major urmărit prin care să-mi asigur finanțarea și suportul material, necesare realizării obiectivele științifice propuse.

Secțiunea C conține referințele bibliografice care au stat la baza realizării prezentei teze de abilitare.

Întrucât, atât activitatea profesională, didactică, cât și de cercetare se poate realiza eficient doar în echipă și cu multă implicare, muncă susținută și responsabilitate, adresez alese mulțumiri și nespusă considerație tuturor colegilor și specialiștilor cu care am colaborat interdisciplinar pentru obținerea acestor rezultate.

ABSTRACT

The habilitation thesis includes the results of my scientific, professional and academic activity acquired after obtaining the title of doctor in the field of Pharmacy, as well as future research directions. The postdoctoral scientific achievements were initiated and developed starting from the activity carried out within the doctoral studies completed through the doctoral thesis titled "Determination of medicinal residues in bee honey" – under the scientific coordination of Prof. Rodica Cuciureanu, PhD. The PhD title in Medical Sciences - Pharmacy Domain was conferred through the Order of the Minister of Education, Research, Youth and Sports no. 3930 of 20.06.2013.

The work reflects my concerns in the fields of pharmaceutical sciences, food quality and safety, nutrition and diet therapy, highlighting the research results carried out within the Environmental and Food Chemistry Discipline of the "Grigore T. Popa" University of Medicine and Pharmacy from Iasi.

The research activity was perfectly combined with the didactic activity (carried out for the study subjects Environmental and Food Chemistry, Dietotherapy, Nutritional Elements within the Faculty of Pharmacy, but also in Food Chemistry and Food Toxicology within the Faculty of Medicine, specialization Nutrition and Dietetics), the two being actually complementary.

The main research directions we have developed are:

- Determination of the classes of medicinal residues in honey;
- Study of the toxicity of honey contaminants on immunological, hematological, biochemical parameters in laboratory animals;
- Validation and application of accessible analysis methods (immunochemical) for the detection of traces of residues from honey;
- Determination of chemical composition parameters for various food products of vegetable origin;
- Studies on the impact of diet in various pathologies such as obesity, gastrointestinal disorders, cardiovascular diseases and obesity.

The habilitation thesis is structured in accordance with the criteria recommended and approved by the National Council for Attestation of University Titles, Diplomas and Certificates (CNATDCU).

The thesis includes three main sections that include the scientific achievements during the postdoctoral period (Section A), the prospects for the development of scientific, professional and academic activity (Section B) and the list of bibliographic references used (Section C). The three sections are preceded by a summary of my entire career, in which the most important professional, academic and scientific achievements are presented.

The scientific achievements of the postdoctoral period (**Section A**) are grouped into two main research directions.

The first direction of research presented in **Chapter I** is focused on the evaluation of the quality and food safety of beehive products (honey and pollen), as well as the study of the food chemical composition in order to highlight the classes of valuable nutrients for human

nutrition. The studies were carried out through extensive collaboration with colleagues from the field of Environmental and Food Chemistry, as well as other extensive multidisciplinary collaborations. The obtained results were disseminated at various scientific events and published in specialized journals with national and international visibility, ISI and BDI ratings.

Subchapter 1 is a continuation of the concerns of the doctoral studies regarding high-sensitivity analysis techniques for the detection of drug residues in honey in the context of ensuring the quality and safety of food, but also studies highlighting their toxic effects on hematological, immunological and biochemical parameters in laboratory animals. In addition, research has been extended to other food contaminants (mycotoxins) as well as other food products (pollen). Thus, we presented the studies carried out on bee honey regarding the validation of the method for the simultaneous determination of four nitrofurans and chloramphenicol, the estimation of the content of quinolones, cephalosporins, amphenicols, as well as the influence of medicinal residues (sulfonamides) in honey on some biochemical and hematological parameters in laboratory animals (rats).

Subchapter 2 refers to the knowledge of the chemical composition of food products, which is essential, both for establishing their biological value for the purpose of a correct, balanced diet, and for understanding and directing the processes that take place in food during cultivation, storage, conservation, culinary or industrial processing. Food products of vegetable origin contain, in addition to the components with a known and indispensable biological role in human nutrition (macronutrients and micronutrients), other bioactive substances (natural pigments, organic acids, enzymes, volatile oils, etc.), in low concentrations, which can be beneficial to the health of the human body.

Working in the field of Environmental and Food Chemistry, I dedicated myself to the field of food and nutrition, which is an extremely vast one that requires a complex multidisciplinary approach by deepening the aspects related to food composition, food safety and security, but also the impact of diet on various human diseases.

Thus, the **second direction** of research, presented in **Chapter II** focused on the impact of diet/nutrition on some modern pathologies, very widespread lately.

This chapter includes two sub-chapters detailing the impact of diet in gastrointestinal pathologies, as well as in overweight and obesity.

The personal research contributions included in Chapter I were published in remarkable journals, such as: *Farmacia*. IF: 1.607, *Farmacia*. IF: 1.433, *Farmacia*. IF: 1.55, *Medicina*. IF: 2.6, *Revista de Chimie*. IF: 1.605, *Revista de Chimie*. IF: 1.412, *Revista de Chimie*. IF: 1.412, *The Medical-Surgical Journal*.

Subchapter 1 of the second chapter refers to the results of multidisciplinary collaborations regarding the impact of diet on gastrointestinal pathologies (gastroesophageal reflux, irritable bowel syndrome, celiac disease), as well as the implications of the intestinal microbiome in various diseases (arterial hypertension, diabetes, etc.).

Subchapter 2 of this chapter addresses a current issue, such as obesity, which is one of the greatest public health challenges of our century. Methods to prevent and treat obesity include nutritional education, physical activity, diets, medication, psychological therapies, or bariatric interventions. Industrialization, urbanization, ultra-technology, sedentarism create the premises for the expansion of these metabolic pathologies such as obesity, diabetes, cardiovascular diseases, cancer, etc. Healthy meals involves a balance between food intake and the body's energy consumption. The quality of life is dependent on the quality and quantity of food, and a diet poor in nutrients will cause negative effects on the health of the human body.

Energy-restricted diets with nutritional supplements and foods with a slightly increased protein intake have resulted in a loss of fat while retaining muscle mass, proving to

be effective weight loss methods applied especially at the individual level.

Personal research contributions included in Chapter II were published in prestigious journals, such as: *Turkish Journal of Gastroenterology*. IF: 0.966, *Cells*. IF: 6, *Nutrients*. IF: 5.9, *Nutrients*. IF: 5.9, *Nutrients*. IF: 5.9, *Revista de Cercetare si Intervenție socială*, IF: 1.076.

Section B aims to present specific strategies for future professional, didactic and research activity.

On a professional and academic level, I want to acquire new skills, competences and theoretical/practical knowledge to ensure continuous training and improvement in the discipline I coordinate. Achieving a level of excellence through the development of teaching and medical research capacity will only be possible by integrating the latest knowledge and methodologies specific to the studied field into the educational process. In addition, I will prioritize development directions regarding the partnership with students and resident pharmacists in order to increase the effectiveness of the university teaching and learning process.

On a scientific level, I will remain and intensify my presence in the space reserved for pharmaceutical sciences, in the field in which I am active, in order to make the results of my research, carried out with my colleagues in the academic community, of high scientific value and as much as possible visible nationally and internationally. In the next period, I will continue the current research directions, I will expand the research area into other classes of contaminants (pesticides, heavy metals, biostimulants) from multiple classes of food products, but I will also devote myself to quantitative determinations on other environmental elements, such as water, air, soil.

In this sense, accessing funds for research projects centered on these themes will be another major objective pursued to secure the funding and material support necessary to achieve the proposed scientific objectives.

Section C contains the bibliographic references that were the basis for the realization of this habilitation thesis.

Since both the professional, didactic and research activity can only be carried out effectively in a team and with a lot of involvement, sustained work and responsibility, I express special thanks and immense consideration to all the colleagues and specialists with whom I have collaborated interdisciplinary to obtain these results.

LIST OF ABBREVIATIONS

95% CIs = 95% confidence intervals
a = age in years
ACE inhibitors = angiotensin converting enzyme inhibitors
AHD = aminoimidazolidine-2,4-dione
ALT = alanine aminotransferase
AMOZ = 3-amino-5-morpholinomethyl-2-oxazolidinone
AOZ = 3-amino-2-oxazolidone
ARBs = angiotensin receptor blockers
ASD = autism spectrum disorder
AST = aspartate aminotransferase
BEE = basic energy expenditure
BIA = Bioelectrical Impedance Analysis
BMI = body mass index
BRD = commonly recommended diet
bsh = bile salt hydrolase
C-plan = nutritionally balanced conventional plan
CC_{α} = decision limit
CC_{β} = detection capability
CD = celiac disease
CDA = celiac disease autoimmunity
CD-SLE = celiac disease –systemic lupus erythematosus
CEFT = ceftiofur
CR% = cross-reactivity percentage
CRP = C-reactive protein
CV% = coefficient of variation
EMA = anti-endomysial
FAP = functional abdominal pain
FAT% = body fat percentage
fCal = fecal calprotectin
FL = fecal lactoferrin
FMT = fecal microbiota transplantation
FODMAPs = oligosaccharides, disaccharides, monosaccharides and fermentable polyols
FOS = high fructose-oligosaccharide diet
GDA = general dietary advice
GERD = Gastroesophageal reflux disease
GFD = gluten-free diet
GI = gastrointestinal tract
Hct = hematocrit
HF = heart failure
HFpEF = heart failure with reduced ejection fraction
HP = high-protein plan

IBD = inflammatory bowel disease
IBS = irritable bowel syndrome
IC50 = half maximal inhibitory concentration
IgA = immunoglobulin A
IgG = immunoglobulin G
IgM = immunoglobulin M
LC-MS/MS = liquid chromatography tandem mass spectrometry
LDL-C = low-density-lipoprotein-C
LFD = low-FODMAP diet
Lmax = maximum length
LPS = lipopolysaccharides
MCHC = mean corpuscular hemoglobin concentration in a given volume of erythrocytes
MD = Mediterranean diet
mNICE = modified National Institute of Health and Clinical Excellence dietary intervention
MR = mendelian randomization
MRM = multiple reaction monitoring mode
MRPL = minimum required performance limit
ND = normal diet
NSAIDs = non-steroidal anti-inflammatory drug
NYHA = New York Heart Association
QNL = quinolone
QoL = quality of life
r = correlation coefficient
RAP = reference point for action
RBC = red blood cell count
RCTs = randomized control trials
RLU = relative light units
RSD = relative standard deviation
SCFAs = short-chain fatty acids
SD = standard deviation
SE = standard error of mean
SEM = semicarbazide
SLE = systemic lupus erythematosus
SSS = standardized complex score
T1D = type 1 diabetes
T2DM = type 2 diabetes mellitus
T3 = triiodothyronine
T4 = thyroxine
TAF = thiamphenicol
TGA = transglutaminase antibody
TMA = trimethylamine
TMAO = trimethylamine N-oxide

OVERVIEW OF ACADEMIC, PROFESSIONAL AND SCIENTIFIC ACHIEVEMENTS

Over the last 14 years I have carried out my professional activity within the Faculty of Pharmacy, Department of Pharmaceutical Sciences I, Discipline of Environmental and Food Chemistry. This discipline proved to possess a continuous attraction for me by the nature and multitude of information, knowledge passed from one generation to another, finding myself so well in the values and principles that this field promotes.

During this period, I participated in various activities of didactic, scientific, research and collaboration nature with various specialized institutions in the country and abroad. I have also developed and maintained professional and close friendships with many colleagues in this field.

The professional competence at my disposal represents the set of cognitive, affective, motivational capacities, which together with the personality traits give me the necessary qualities to carry out the didactic and scientific activity by fulfilling the proposed objectives.

My primary goal consists in continuous self-improvement in order to reach and maintain the professional standards imposed by the evolution of the discipline where I work, as well as the transmission of this information to interested persons (students, residents, colleagues).

Education and training

The education and training activity in the professional field began with the high school studies that I followed during 1986-1990 at the Sanitary High School from Iași, in the pharmacy laboratory assistants department. This field fascinated me and determined me to attend the courses of the Faculty of Pharmacy within the University of Medicine and Pharmacy "Grigore T. Popa" from Iași between 1992 and 1997.

After graduation, I was admitted to the residency training, specializing in "General Pharmacy" between 2000-2002.

In 2009, the year that also coincides with the beginning of my teaching career, I was admitted to a 4-year postgraduate PhD program (2009-2013). During the PhD training, I obtained a doctoral scholarship (2010-2013) within the POSDRU 2007-2013 project - "Doctoral scholarships for increasing competitiveness in the medical and pharmaceutical field", project ID POSDRU/88/1.5/S/63117, which included a 3-month international mobility carried out in between 01.05.2012 and 31.07.2012, at the University of Valencia, Spain.

The doctoral studies were finally completed in 2013 by obtaining the PhD degree in the fundamental field of Medical Sciences, Pharmacy Domain, Specialization in Analytical Chemistry with the presentation at the University of Medicine and Pharmacy "Grigore T. Popa" from Iași, of the PhD thesis with the title "Determination of medicinal residues in bee honey".

During 2017-2018, I followed the postgraduate course of psychopedagogical studies

for level I and II, within the Department for the training of teaching staff of the "Alexandru Ioan Cuza" University, Iași.

Professional experience

On 30.09.2019 I have obtained through competition the Associated Professor position at the discipline of Environmental and Food Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" from Iași and meanwhile I have also been the coordinator of didactic activity of the discipline.

I have obtained through competition the Lecturer position on the 29th of September 2014 which I have filled until the 29th of September 2019, at the discipline of Environmental and Food Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" from Iași as well as the coordinator of didactic activity of the discipline position.

I have filled the assistant position in between 01.10.2009 and 29.09.2014 which I had obtained through competition, at the discipline of Environmental and Food Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" from Iași, with a 3-month suspension for the international mobility in Valencia (01.05.2012-31.07.2012).

I have won the Specialist pharmacist title on the 1st of May 2002, after I have been resident pharmacist through competition in the period 01.03.2000-01.05.2002.

I have started my professional career as a pharmacy laboratory assistant (19.09.1990-16.09.1992), then beginner trainee pharmacist (01.01.1998-01.04.1999), junior pharmacist 01.04.1999-01.08.1999, and then pharmacist (01.08.1999-01.03.2000).

Didactic and scientific experience

In October 2009, based on competition results, I was confirmed as an assistant at the Environmental and Food Chemistry discipline, Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" from Iași.

In this position, my didactic responsibilities consisted of leading Environmental and Food Chemistry practical laboratory activities for the IV year students from the Faculty of Pharmacy, Romanian and English departments. Also, I lead practical laboratory activities for the III year students from the Pharmacy Assistant Department of the Faculty of Pharmacy, for the same subject of study. Scientific Research Methodology represented another object of study on which I conducted the seminaries for the IV year students of the Faculty of Pharmacy.

My main objective during that period was thorough professional training, and acquiring theoretical and practical experience, in order to optimize my teaching expertise of Environmental and Food Chemistry, as well as of Pharmaceutical Scientific Research Methodology. The aim of the teaching team in our discipline was and is to systematically guide the students, so that they acquire as efficiently as possible the theoretical and practical knowledge of the studied program, as well as to use the information accumulated in their chosen profession.

Since October 2014, after confirmation in the Lecturer position and after the retirement of Prof. Rodica Cuciureanu, PhD as head of the discipline, I took over the management of all activities within the discipline, becoming the coordinator of didactic activity of the discipline. This designation includes the organization of the studies curriculum for students and residents, management of rotations and teaching contributions of tenured and associate teaching staff, supervision of examination of work procedures, facilitating career advancement for subordinate staff, scientific research, participation in scientific events, publicizing research results, etc.

My activity as didactic activity coordinator, took place in parallel to leading practical

laboratory activities and teaching courses.

I am currently teaching the Environmental and Food Chemistry course to students from the Faculty of Pharmacy,: IVth year, I and II semesters, both the Romanian and English Department and the optional course Nutrition and Diet Therapy for Vth year, second semester. My didactic activity was also extended by teaching the Food Chemistry (year I) and Food Toxicology (year II) courses to students specializing in Nutrition and Dietetics from the Faculty of Medicine.

Within our discipline, I also teach the courses and I lead the laboratory activities for two residency modules corresponding to the Pharmaceutical Laboratory specialization: Food Hygiene (year I) and Analysis of biological fluids. Water and food quality control (year II).

I led the laboratory activities for the following: Environmental and food chemistry (year IV, semester I, II, Faculty of Pharmacy, Romanian and English departments), Scientific research methodology (year IV, semester I, Faculty of Pharmacy), Food chemistry (year I, semester II, Faculty of Medicine, Nutrition and Dietetics section), and Food toxicology (Year II, semester I, Faculty of Medicine, Nutrition and Dietetics section).

Since October 2019, after confirmation on the Associated Professor position, I continued coordinating all activities within the discipline, focusing on teaching, scientific and administrative activities, in order to proceed, improve and achieve all the proposed objectives. In that position, my didactic activity was carried out for the same subjects of study presented at the lecturer position, but it was adapted to the structure corresponding to the Associated Professor position.

In the 2022-2023 academic year, within the discipline I coordinate, a new subject of study, Elements of Nutrition, was added for second-year students specializing in Medical Cosmetics and Cosmetic Product Technology of the Faculty of Pharmacy, and my teaching activity was expanded.

The objectives proposed as the coordinator of the didactic activity within the discipline of Environmental and Food Chemistry were closely followed, checked and optimized in order to ensure a high-quality didactic act for all the guided students.

The main strategies approached for the implementation of the curriculum and the achievement of the established objectives aimed at continuous improvement and progress in all fields of activity.

We have always pursued the modernization of student-centered teaching and learning methods by using state-of-the-art IT techniques. In order to increase the students' interest in achieving the curricular objectives, we approached interactive learning methods and approached the curricular objectives integratively, transdisciplinary, analyzing the basic notions of the discipline, notions that correlate the information accumulated to the previously studied objects and make the transition to the knowledge offered by the disciplines of the higher years. Also in this sense, we applied new knowledge verification and evaluation methods to raise the level of training of future graduates.

In order to train students as future researchers, we have proposed exciting research projects and themes within the discipline and department.

Thus, I realized that the results obtained depend to a large extent on the way of organizing all my didactic and scientific activities as a whole.

All my teaching activity has always had an educational side, in the spirit of correctness, honesty, punctuality, conscientiousness and professional deontology.

My interest in documentation, but also my teaching experience, were also reflected in the contribution to the development of the following teaching materials (course notes and practical laboratory works), as well as a book chapter:

1. Cuciureanu Rodica, **Morariu Ionela Daniela**, Chimia Mediului și Alimentului - Metode de Analiză, Ed. Performantica, Iași, 2009, ISBN: 978-973-730-669-2;

2. Bogdan Gabriel Șlencu, Liliana Avasilcăi, **Ionela Daniela Morariu**, Environmental Chemistry, Editura Gr. T. Popa, Iași, 2021, ISBN: 978-606-544-643-4;
3. **Ionela Daniela Morariu**, Liliana Avasilcăi, Bogdan Gabriel Șlencu, Food Chemistry, Editura Gr. T. Popa, Iași, 2021, ISBN: 978-606-544-641-0;
4. **Ionela Daniela Morariu**, Liliana Avasilcăi, Bogdan Gabriel Șlencu, Nutriție aplicată, Editura Gr. T. Popa, Iași, 2022, ISBN: 978-606-544-762-2;
5. **Ionela Daniela Morariu**, Liliana Avasilcăi, Bogdan Gabriel Șlencu, Elemente de nutriție, Editura Gr. T. Popa, Iași, 2022, ISBN: 978-606-544-754-7;
6. Cioanca Oana, **Morariu Ionela Daniela**, Hritcu Lucian. (2021) Natural Antioxidants for the Prevention and Treatment of Cancer. In: Chakraborti S. (eds) Handbook of Oxidative Stress in Cancer: Therapeutic Aspects. Springer, Singapore, pp 1-1.

The research results present scientific, didactic and applied utility. All these are revealed by the contributions made, both in the professional development on the medical level, and in the updated approach to the topics in the analytical curriculum of my discipline.

In the 01.05.2012-31.07.2012 period, my teaching activity was suspended and later resumed, due to a mobility internship carried out at the University of Valencia, Spain, from which I benefited through the POSDRU 2007-2013 project - "Doctoral scholarships for the growth competitiveness in the medical and pharmaceutical field", project POSDRU/88/1.5/S/63117.

Experience exchanges, conferences, research and specialization internships gave me the opportunity to get to know different education systems, but also special people, true professionals from numerous medical, cultural and academic centers, thus bringing my contribution to the internationalization of education in University of Medicine and Pharmacy "Grigore T. Popa" from Iasi.

My professional performances and the satisfaction of the work done were a natural consequence of the continuous improvement achieved through documentation, by consulting the specialized bibliography, but also by participating in training courses, national and international scientific events, multiple opportunities that broadened my scientific horizon and helped establish collaborative relationships with various personalities in the field.

The articles published in scientific journals and in the volumes of some scientific events prove the capacity for synthesis, the inclusion in the scientific concept of the issue and demonstrate the technical-scientific level acquired.

I published in "Gr. T. Popa" Publishing House Iasi, scientific works on various topics of interest in the field of nutrition and pharmaceuticals, as follows:

1. Determinarea profilului de citokine la șobolani în urma administrării de miere de albine îmbogățită cu sulfonamide. **Ionela-Daniela (Morariu) Popa**, Elena-Corina Schiriac, Rodica Cuciureanu 50 de ani de învățământ universitar farmaceutic la Iași, Ed. Gr T. Popa, 2011, ISBN 978-606-544-073-9, pag.143-145;
2. Vitaminele, mit și realitate. Vlad Teodor, Magdalena Bîrsan, **Ionela-Daniela Morariu** Medicina și fenomenul migrației, Ed. Gr T. Popa, 2016, ISBN 978-606-544-398-3, pag. 336-342;
3. Consumul de ouă și bolile metabolice. Vlad Ioan Teodor, Magdalena Birsan, **Ionela Daniela Morariu**, Bogdan Gabriel Slencu, Liliana Avasilcai Uniformity and diversity in teaching medical sciences today, Ed. Gr T. Popa, 2018, ISBN 978-606-544-468-3, pag. 84-89;
4. Crema cosmetică – Secret de frumusețe. Magdalena Birsan, **Ionela Daniela Morariu**, Ana-Caterina Cristofor, Nicoleta Todoran, Adriana Ciurba Uniformity and diversity in teaching medical sciences today, Ed. Gr T. Popa, 2018, ISBN 978-606-544-468-3, pag. 270-275;

5. Vitamina soarelui - rol, surse și beneficii pentru sănătatea umană. Liliana Avasilcai, Bogdan Gabriel Șlencu, Ioan Vlad Teodor, Branco-Adrian Morariu, **Ionela Daniela Morariu** Learning solutions in medical higher education-an interdisciplinary approach, Ed. Gr T. Popa, 2018, ISBN 978-606-544-596-3, pag 208-214;
6. Extractul de cafea verde – o alternativă modernă în tratamentul obezității. **Morariu Ionela Daniela,** Morariu Branco-Adrian, Avasilcăi Liliana, Cioancă Oana, Hâncianu Monica Medicamentul, tradiție și modernitate, vol I, Editura "Gr T. Popa" U.M.F.Iași, 2019, ISBN 978-673-544-624-0, pag 108-118.

The relevance and impact of my scientific results have materialized in multiple published works in scientific journals with national and international visibility, ISI and BDI ratings. The personal contributions of the research carried out were materialized in 22 papers in ISI listed journals (16 main author and 6 co-author), 7 BDI 7 papers (4 main author and 3 co-author), 3 articles published in summary in ISI indexed journals, 5 articles in summary in non-indexed supplements and over 70 papers presented in the form of oral or poster communication at national and international conferences.

Among the ISI listed journals in which I published the results of scientific research, I would mention: *Nutrients* (IF: 5.9), *Cells* (IF: 6), *Farmacia* (IF: 1.607), *Farmacia* (IF: 1.433), *Farmacia* (IF: 1.55), *Medicina* (IF: 2.6), *Revista de Chimie* (IF: 1.605), *Revista de Chimie* (IF: 1.412), *Turkish Journal of Gastroenterology* (IF: 0.966), and *Revista de Cercetare și Intervenție socială* (IF: 1.076).

The visibility of the articles that I published are reflected by the Hirsch Index 7 according to data from the Web of Science Core Collection.

In the 2016-2022 period, I have coordinated 40 bachelor theses of graduates of the Faculty of Pharmacy, University of Medicine and Pharmacy "Grigore T. Popa" from Iași, in the field of Environmental and Food Chemistry. Those bachelor theses had a strong scientific impact, thus proving the indestructible link between correct dietary behavior and drug therapy, both participating in the improvement of the patient's quality of life, as well as the desired therapeutic success.

Coordinated the bachelor theses of these graduates helped me a lot in developing practical skills in scientific writing and inspired me to pursue greater objectives, such as, guidance and coordination of PhD theses. Among the undergraduate theses completed with exceptional results, I would mention: Dietary behavior in cardiovascular diseases, Aflatoxins – contaminants in food products, Safety of using sodium monoglutamate as a food additive, Dietary behavior in kidney diseases, Prebiotics, probiotics, symbiotics in food products and supplements, Dietary behavior in gastrointestinal diseases, Interactions of antibiotics and antimicrobial chemotherapeutics with macronutrients and micronutrients in food products, Residual ecotoxicity of antibiotics, Microbial contamination of food, Safety and toxicity of synthetic food dyes, Natural antibiotics in food, Mycotoxins in food and their effects on human health, etc.

In recognition of my research studies, I have received several awards from scientific conferences, such as:

1. 3rd Award for the paper "Studiu comparativ al influenței procesului de refrigerare asupra micronutrienților din fructe", Daniela Cladcov, Liliana Avasilcăi, **Ionela-Daniela Morariu**, National Congress of Pharmaceutical Students from Romania, 27-28 April 2023;
2. 3rd Award for the paper "Eficacitatea antiinflamatoarelor nesteroidiene în formă nanoparticulată", Liliana Avasilcai, Bogdan Gabriel Șlencu, **Ionela-Daniela Morariu**

National Conference "Medicamentul de la idee la clinică", under the auspices of Zilele Medicamentului, 27th edition, Iasi, 10-12 October 2019;

3. 1st Award for "Salvestrolii-o nouă speranță în lupta cu cancerul", **Morariu Ionela Daniela**, Avasilcai Liliana, Șlencu Bogdan Gabriel, Chirilă Ioan, National Symposium "Medicamentul de la idee la clinică", under the auspices of Zilele Medicamentului, 26th edition, Iasi, 29-31 March 2018;
4. Award for novelty for the paper "Evaluarea parametrilor biochimici, imunologici și hematologici la șobolani după administrarea de miere conținând rezidurii de sulfonamide", **Ionela Daniela Morariu**, Bogdan Gabriel Șlencu, Liliana Avasilcai, Conference "Medicamentul de la idee la clinică", 25th edition, Iasi, 12-14 October 2017.

In 2019, I participated in various promotion projects of the Faculty of Pharmacy and the University of Medicine and Pharmacy Grigore T. Popa, Iași, by getting involved in various activities carried out in the high schools of our city ("Mihail Sadoveanu" National College Pașcani and Alexandru Ioan Cuza Theoretical Highschool, Iași).

At the moment I am a member of various national and international scientific societies in the medical field, such as: the College of Pharmacists from Romania, the Society of Doctors and Naturalists from Iasi, the Society of Pharmaceutical Sciences from Romania, the Romanian Society for the History of Pharmacy, the International Society for the History of Pharmacy, the Catalan Society for the History of Pharmacy.

I was part of the organizing committee of the National Congress of Pharmacy in Romania, 15th edition (2014) and of the scientific committee of various scientific events: National Congress of Lifestyle Medicine in Romania (2021), and Medical Congress of the Health and Education Association (2022, 2023).

My concern for continuous improvement was recognized by the trust that the faculty/university administration as well as colleagues had in me when they nominated/voted me in various academic activities as follows:

- Member of the Council of the Faculty of Pharmacy within the "Grigore T. Popa" University of Medicine and Pharmacy from Iași;
- Member of the contest comissions for various teaching positions in the discipline I coordinate, but also in other disciplines in the Faculty of Pharmacy;
- Member of the eligibility commissions, evaluation of files and scores during the admission contest of international candidates;
- Member of the teaching staff committee for supervising the candidates for the admission competition;
- Member of the examination committee of candidates enrolled in the exam to obtain the title of specialist biologist;
- Member of the commission for the resolution of appeals for competitions for teaching positions;
- Member of the commission for the evaluation of undergraduate theses.

During all these academic activities, I proved to possess team spirit, positive group attitude, cooperation, conscientiousness, professionalism, flexibility, and a lot of adaptability to the requests and challenges that arose.

A university career is a continuous process of thorough training and improvement, harmoniously combining teaching and research activities in order to achieve academic excellence and personal satisfaction.

SECTION A: THE MAIN RESULTS OF THE POSTDOCTORAL RESEARCH

Chapter I. FOOD QUALITY AND SAFETY MANAGEMENT

Food quality and safety is a relevant topic, and the control of drug residues in food products of animal origin is a global priority to protect the health of consumers, especially in the context of the extensive use of numerous medicinal substances in veterinary therapy. Pharmaceutical preparations administered to domestic animals, including antibiotics, hormones, anesthetics, tranquilizers, chemotherapy, etc., are retained as residues in various products. Moreover, there is a tendency not to perceive drugs as a polluting factor, although there is a high probability that they are transferred as such or as metabolites in food of animal origin.

An essential condition that food must meet is that it must not contain substances harmful to consumers. The presence of harmful substances in food has concerned and worried specialists for many years. This problem is taking on a new dimension in recent decades due to the increasing pollution of the environment, as well as through the uncontrolled use of some chemical substances in order to optimize their quality.

The adverse effects on consumers are manifested in the form of acute or chronic illnesses having negative consequences on human health. The risks associated with the consumption of food products containing drug residues are different depending on the chemical structure and the action of the drugs involved, allergic reactions, disturbances of the intestinal microbiota, carcinogenic and teratogenic effects, etc. being frequently encountered.

Ensuring the integrity of the food chain and protecting consumer health is currently a global priority. Food quality sums up aspects related to values such as nutritional, hygienic-sanitary, energetic, sensory, technological, socio-ecological, etc. To assess the quality of a food product, two characteristics are considered defining, namely, hygienic, and nutritional quality. The nutritional quality is ensured by the composition of the food product, which provides all the nutrients needed by the body. Hygienic quality (innocuity) refers to the absence or presence at acceptable levels of contaminating substances likely to make the food harmful to health or unsuitable for human consumption.

Food safety represents all the measures taken at national and international level, along the entire chain of the production process, from the raw material to the finished product. This aims to keep the food safe and reduce any risk of illness for the consumer.

With Romania's accession to the European Union, a new perspective was opened to address the issue of food quality.

Food quality and safety testing ensures that food products are not contaminated and meet national and international hygiene standards.

An essential condition that food must meet is that it does not contain substances harmful to consumers. The presence of harmful substances in food represents a problem of the

last decades due to the increasing pollution of the environment, as well as the uncontrolled use of various chemical substances to optimize their quality. The adverse effects on consumers are manifested in the form of acute or chronic illnesses with negative consequences on human health.

The control and monitoring of medicinal residues in food of animal origin is regulated by the European Union. The main objective of the legislation is to develop rules to detect the illegal use of substances in products of animal origin, the misuse of veterinary medicinal products and to ensure that appropriate actions are implemented to minimize the recurrence of all such residues in foods of animal origin.

I.1. EVALUATION OF THE QUALITY AND SAFETY OF BEE PRODUCTS: HONEY AND POLLEN

Currently, Romania is among the countries where beekeeping is quite wide-spread, a consequence of the favorable geographical position, with rich honey resources, significant flocks of bee families at our disposal, based on the amount of honey obtained, the diversification of beekeeping production and the results of scientific research and specialist training activities.

Honey and bee hive products are used not only as food, but also as medicine. The quality of beekeeping products is more important because, due to the general context of using the products offered by nature directly, they have acquired increased importance through their use as natural sources for the promotion of a healthy diet and a therapy free of chemical substances.

Honey is the main beekeeping product, appreciated both for its nutritional properties and for its therapeutic effects, being one of the most complex products from a chemical point of view. Over 200 very important substances for the human body have been discovered in its composition: water, carbohydrates, proteins, vitamins, minerals, enzymes, flavonoids, phenolic acids, volatile compounds, and many more. This food product has not only nutritional qualities, but also an effective therapeutic action, which is exercised both on digestive disorders, as well as in hepato-biliary, cardiovascular, respiratory, nervous system, urinary system, nutritional and infectious diseases, blood, and skin diseases.

In recent years, honey has borne the effects of industrialization and the evolution of agricultural production, which can result in contamination with pesticides and other harmful substances. Added to these are the occurrence of bee diseases, which involve the administration of chemical substances, which can remain in the form of residues in honey. From here, derives a problem of particular importance, both for members of the beekeeping sector and for consumers, i.e., that is the presence of residues in honey.

The honey “industry” is facing new demands, and this is due to its sensitivity to a series of “aggression factors”, such as: environmental pollution, non-compliance with the correct technologies for growing and treating bee families, production conditions, adulteration additives.

Recently, the interest of beekeepers and consumers has also extended to medicinal residues in honey. These residues were considered uncommon in honey; but with the intensification of chemical control and the improvement of analytical methods, a significant contamination with veterinary drugs (antibiotics and other antimicrobial chemotherapeutics - sulfonamides, quinolones (QNL), nitrofurans, chloramphenicol), used to treat bee diseases, was identified.

Sulfonamides are among the most widely used drug substances in veterinary medicine, due to their low price (compared to antibiotics), broad antibacterial spectrum and valuable therapeutic efficacy in some infectious diseases [1–4]. Antimicrobial chemotherapies are

effective against bee diseases, but drug residues may persist for long periods in tissues (sometimes up a month), and the consumption of honey containing such residues represents a potential risk to human health [5–9]. The intensity, the duration of action and the toxicity of the sulfonamides derive from the body's ability to metabolize those substances [10–12]. Sulfonamides are considered to have an average toxicity that includes gastrointestinal disorders (nausea, vomiting, diarrhea), hypersensitivity reactions (allergic) consisting of rash, eosinophilia and rarely anaphylactic shock. Their presence in bee honey as residues may cause allergic reactions or antibiotic resistance phenomena in humans [13–20].

Various bioassays have shown that some sulfonamides cause tumors in different locations. In addition, sulfonamides can sometimes cause drug fever, serum sickness and systemic lupus erythematosus (SLE) (type III hypersensitivity mediated by immunoglobulin G), and liver toxicity (including necrosis). Evidence of sulfonamide toxicity to the thyroid gland has also been reported [21]. Some sulfonamides inhibit lactoperoxidase and thyroid peroxidase, which are mediators in the synthesis of thyroid hormones, by competitive mechanisms, which can lead to hyperthyroidism.

The thyroid gland exerts essential regulatory influences on cell growth, on hormonal balance in general, on differentiation and metabolism, as well as on maintaining metabolic activity and affects the development of the entire skeletal system and other organs. Diseases that affect the normal functioning of the thyroid exhibit a wide range of symptoms, which can often be confused. Measurements of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) by immunological methods are the most reliable tests for assessing the presence of thyroid dysfunction.

Chloramphenicol has bacteriostatic activity, and it is effective in treating infectious diseases [22]. The mechanism of action of chloramphenicol is based on the inhibition of the transport of activated amino acids to the site of protein synthesis (ribosomes) i.e., inhibiting the synthesis of bacterial proteins. Because of the side effects that chloramphenicol has on human health, its use in veterinary medicine has been banned by the FDA in the US, by the Canadian Health Protection Branch and by the European Union in those animals whose products and by-products are used for human consumption. However, chloramphenicol has been shown to be one of the most commonly found drug residues found in honey.

Nitrofurans have been used in veterinary practice as antibacterial agents to treat infections caused by bacteria and protozoa. Although their use was banned for the first time in the EU since January 1, 1997 (Annex IV of Regulation 2377/90/EC), they are currently in use for animals not bred for consumption [23].

Nitrofurantoin, furazolidone, nitrofurazone and furaltadone are the most used nitrofurans. Studies have shown that they are rapidly converted to toxic metabolites [24], which bind proteins in high proportions and thus persist for long periods (weeks or even months) in food products. Those metabolites are 1- aminoimidazolidine-2,4-dione (AHD), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 3-amino-2-oxazolidone (AOZ) and semicarbazide (SEM) [25–27]. Although honey is considered a very healthy natural product, the incidence of honey samples contaminated with residues of nitrofurans is quite high [28].

Bee pollen has been used for its high nutritional value for many centuries and its multiple benefits have been widely praised [29–31]. The presence of mycotoxins in pollen poses a high risk to human health. Human exposure occurs mainly after ingestion of mycotoxin-contaminated products and it can lead to serious health problems, including immunosuppression and even carcinogenesis [32].

The trichothecene mycotoxins are the largest group of mycotoxins produced as secondary metabolites by some species in the fungal genera *Fusarium, Myrothecium, Trichothecium* and *Cephalosporium.* Their molecules are based on the same basic chemical structure, a 12, 13- epoxytrichothec-9-ene ring system [29,33].

European Union (EU) honey quality standards severely restrict the entry of honey into any of the European states. When we discuss about the quality of honey, we refer to compliance with quality parameters by applying a correct beekeeping technique, but also to the absence of medicinal residues.

The current consumption tendency and the need to meet all quality requirements for food products, which includes honey and other bee products, determines the implementation of quality standards and high-performance analysis methods accepted by the EU in our country.

Community regulations allow the use of several classes of veterinary drugs (antibiotics, sulfonamides, nitrofurans, QNL) to treat diseases in bees and do not allow the presence of drug residues in honey.

The development of novel, modern methods, and the implementation at national level of some international standards for the control of drug residues will allow compliance with quality parameters in accordance with EU regulations, in order to ensure the quality and safety of beekeeping products and their recognition on the market of authentic natural products.

The development and improvement of analytical methods in order to monitor the levels of residues in bee honey ensures the marketing of safe food products.

Currently, there are few methods capable of measuring the concentrations of drug residues at the limits imposed by the EU because some of them are not soluble in organic solvents, which made it difficult to extract the residues and establish their concentrations from the tissues. Other substances are not volatile enough or are too unstable at high temperatures to allow their analysis using GC or GC-MS. Thus, many of the methods for measuring drug residues are based on the HPLC method.

The research described in chapter I of section A presents the results regarding the determination of drug residues (antibiotics and other antimicrobial chemotherapeutics - sulfonamides, QNL, nitrofurans) in bee honey using a new method of determination, namely the Biochip technology.

Personal contributions regarding the quality and safety of bee products (honey and pollen) were presented in the following publications:

ISI ARTICLES

1. **Ionela Daniela Morariu**, Liliana Avasilcăi, Mădălina Vieriu, Oana Cioancă, Monica Hăncianu. Immunochemical assay of chloramfenicol in honey. *Farmacia*, 2019, 67(2): 235-239, **IF = 1.607**
   https://doi.org/10.31925/farmacia.2019.2.6.

2. **Ionela Daniela Morariu**, Liliana Avasilcăi, Mădălina Vieriu, Ionuț Iulian Lungu, Bogdan Huzum, Denisa Batir Marin, Lăcrămioara Șerban, Monica Hăncianu, Oana Cioancă. Experimental study on the influence of sulfonamide drug residues from honey on biochemical parameters in lab rats, *Farmacia*, 2020, 68(3): 470-475, **IF = 1.433**
   https://doi.org/10.31925/farmacia.2020.3.12

3. **Ionela Daniela Morariu,** Liliana Avasilcăi, Mădălina Vieriu, Ionuț Iulian Lungu, Branco Morariu, Silvia Robu, Dana Tiutunaru, Oana Cioancă, Monica Hăncianu. Estimation of quinolones, ceftiofur and thiamphenicol residues levels in honey, *Farmacia*, 2021, 69(3): 515-520, **IF = 1.550**
   https://doi.org/10.31925/farmacia.2021.3.14

4. **Ionela Daniela Morariu**, Liliana Avasilcăi, Oana Cioancă, Branco-Adrian Morariu, Mădălina Vieriu, Corneliu Tănase. The Effects of Honey Sulfonamides on

Immunological and Hematological Parameters in Wistar Rats, *Medicina*, 2022, 58(11): 1-9, **IF = 2.6**
https://doi.org/10.3390/medicina58111558

5. **Ionela Daniela Morariu**, Liliana Avasilcăi, Mădălina Vieriu, Alina Diana Panainte, Nela Bibire. Validation and Application of an Analysis Method of Four Metabolites of Nitrofurans in Honey. *Revista de Chimie*, 2018, 69(10): 2808-2812, IF = 1.605
https://doi.org/10.37358/RC.18.10.6629

6. **Ionela Daniela Morariu,** Liliana Avasilcai, Madalina Vieriu, Alina Diana Panainte, Nela Bibire. Novel Multiresidue Method for the Determination of Eight Trichothecene Mycotoxins in Pollen Samples Using QuEChERS-Based GC-MS/MS, *Revista de Chimie*, 2017, 68(2): 304-306, IF = 1.412
https://doi.org/10.37358/RC.17.2.5441.

I.1.1. Chloramphenicol Residue Determination

I.1.1.1. Aim of the Study

The current study aimed to validate a quantitative approach for chloramphenicol determination utilizing Biochip technology and to validate the new method for the determination of chloramphenicol residues in Romanian honey samples.

I.1.1.2. Materials and Methods

The validation parameters evaluated were linearity, sensitivity (half maximal inhibitory concentration - IC50), specificity and selectivity, precision (intermediate and reproducibility), accuracy, detection limit and recovery [13,34,35].

The validation method and the honey analysis procedure were performed in accordance with 2002/657/EC Decision, FDA approved validation guidelines and validation guidelines for screening methods for veterinary drug residues.

The linearity of the method was evaluated by performing a 9-point calibration using the calibrators included in the Anti-Microbial Array III kit. Considering the complex honey composition as a sample matrix, the linearity of the method was also checked by spiking blank honey samples to obtain nine concentration levels: 0, 0.001, 0.01, 0.05, 0.1, 1, 4, 10 and 50 µg chloramphenicol/kg. The analysis software used a specific calibration equation [36,37]:

$$y = D + [(A-D)/1 + (x/C)B]$$

where:
x = analyte concentration (µg/kg);
y = the intensity of the chemiluminescent signal expressed as relative light units (RLU);
A, B, C, D = parameters of the competitive method, predefined in the analyzer software as A = the intensity of the chemiluminescent signal of the blank, B = slope factor, C = the inflection point of the calibration curve and D = the intensity of the luminous response signal at an infinite theoretical concentration of the analyte [34].

The following stages were included in the process used to obtain the calibration curves: adding 50 µL of each calibrator to the surface of each Biochip followed by the addition of 150 µL of reaction buffer (AM III DIL ASY); incubating the Biochips at 25°C while stirring at 370 rpm for 30 minutes; adding 100 µL of enzyme conjugate solution to each Biochip; incubating for 30 minutes the Biochips at 25°C while stirring at 370 rpm; removing the reaction mixture by washing the reaction surface 6 times, in order to remove the components that did not bind to the polyclonal antibodies present on the solid substrate on surface of the Biochip; after

complete removal of the reaction mixture, 250 μL of reagent (luminol: peroxide 1:1 v/v) had been added; the Biochips were left to rest in the dark for the development of the reaction and exactly 2 minutes after, they were placed in the image capture chamber for processing and interpretation of the signal.

The sensitivity of the method expressed as IC50 was calculated based on 50% of the value of the RLU signal generated by the zero-concentration calibrator and extrapolating the RLU value thus obtained on the X-axis of the calibration curve where the concentration units were expressed as μg/kg. The concentration thus obtained corresponded to IC50 [35].

The specificity and selectivity of the method were analyzed by adding the analyte separately in known concentrations of (10 and 100 μg/kg) to the zero-concentration calibrator in serial dilutions. To assign the cross-reactivity percentage (CR%), three replicates were assessed for each analyte level in the serial dilution and CR% was calculated using the formula:

$$CR\%=[IC50_{analyte}/IC50_{cross\text{-}reactant}]\times 100.$$

Mean concentration, standard deviation (SD), and coefficient of variation (CV%) were also calculated.

The precision in the same analytical series was determined by analyzing 20 replicates of negative honey samples, spiked to obtain 3 different concentration levels: 0.5, 1 and 1.5 μg/kg.

The precision in different analytical series was determined by analyzing two replicates of blank honey samples spiked with chloramphenicol at three different concentration levels (0.5, 1 and 1.5 μg/kg) in 10 different days of analysis.

Precision and accuracy are acceptable if the CV% in the concentration of the control samples measured does not exceed ±15% for determinations on the same day or on different days or analytical series.

In order to determine the decision limit (CC_α) and the detection capability (CC_β) 20 blank honey samples were selected and spiked at the target concentration for screening - 0.5 μg/kg. CC_α was calculated as the arithmetic mean of the concentration in 20 spiked samples. The concentration level of each analyte was minimum required performance limit (MRPL) plus 1.64 × SD of repeatability at a = 5%. CC_β was calculated as the arithmetic mean of the concentration analyte at CC_α plus 1.64 × SD of repeatability at a = 5%.

The recovery percentage was calculated for three chloramphenicol concentration levels 0.5, 1, 1.5 μg/kg in spiked honey samples. Recovery percentages were calculated by plotting the ratio of the analyte concentration in the sample against the theoretical concentration of the analyte in the standard solution. According to the validation guidelines, the requirement for the recovery percentage for the determination of drug residues in honey must be higher than 70%.

The examination of samples of Romanian honey obtained from supermarkets or independent producers was performed using the validated technique. The samples had been kept in the dark and at room temperature.

Derivatization and extraction from honey samples were part of sample processing. The procedure included the following steps: 1g of honey sample was mixed with 4 mL distilled water, incubated at 37°C for 30 minutes, and stirred 10 minutes until dissolved; 0.5 mL of 1M HCl and 100 μL of 10 mM 4-nitrobenzaldehyde solution were added to the sample solution; the mixture was stirred for 10 minutes, and then incubated for 16-24 hours at 37°C; after incubation, 5 mL dipotassium phosphate 0.1M solution was added to each sample, and the pH was adjusted to 7.4 with 1M NaOH solution; 15 mL ethyl acetate were added to 5 mL of derivatized honey sample, which was stirred on a Vortex shaker for 12 minutes; the sample was centrifuged for 10 minutes using 4500 relative centrifugal force; 6 mL of supernatant from each sample was transferred into microtubes, which were then evaporated at 60°C and 15 psi; the residue was mixed with 375 μL of sample diluent provided in the kit (AM III DIL SPE) and

stirred for 2 minutes.

A validated LC-MS/MS method using an Agilent 1100 LC (Agilent Technologies, USA) coupled with a 4000 Q TRAP mass spectrometer (Applied Biosystems, USA) was used to verify the findings obtained using the Biochip approach for the tested honey samples.

I.1.1.3. Results and Discussions

Following the analyses, we discovered a correlation coefficient (r) of 0.991 for the linearity range of 0–5 g/kg, which is an excellent result, considering the acceptance criterion of the calibration curve (r>0.949). The calibration curve produced by this method is represented in Figure 1.

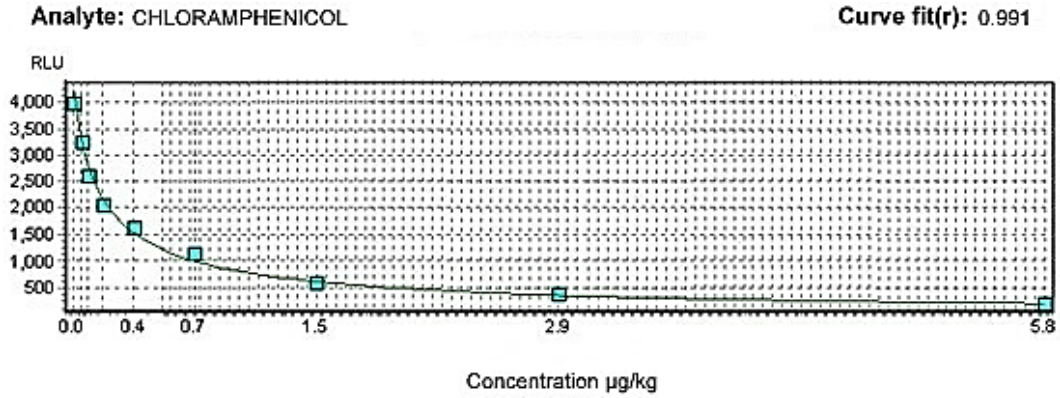

Figure 1. Calibration curve for chloramphenicol

The sensitivity of the method expressed as IC50 for the simultaneous quantitative determination of chloramphenicol was 0.74 µg/kg.

While studying specificity and selectivity, the cross-reactivity (%) for chloramphenicol was 100 and for cross-reactant - chloramphenicol glucuronide, it was 17. According to the validation guides, the CR% for the determination of drug residues in honey must not exceed 25% for the analyte concentration at the minimum limit of quantification.

In Table I and Table II, the assayed validation parameters are presented.

Using the validated Biochip method, 16 samples of honey from different Romanian locations were analyzed. Using the LC-MS/MS method, the results were verified.

The Biochip approach had excellent performance, with values that were comparable to those of both positive and negative sample results (Table III).

Table I. Precision of the method

Series	Level	Concentration (µg/kg)	CV%
Identical	1	0.32	9.06
	2	0.75	6.97
	3	1.06	7.34
Different	1	0.44	8.92
	2	0.75	7.26
	3	1.06	9.84

For the majority of the samples, the outcomes of the Biochip method were comparable to those of the LC-MS/MS method.

There were also some samples that generated different results, most likely as a result of the honey samples varied composition, viscosity, and sugar content.

However, the Biochip method proved to be an effective monitoring tool for screening purposes.

Table II. Recovery, decision limit and detection capability

	Level 1	Level 2	Level 3
Concentration (µg/kg)	0.32	0.75	1.06
Recovery (%)	64	75	71
Average concentration (µg/kg)	0.32		
SD	0.03		
1.64×SD	0.05		
CC_α (µg/kg)	0.37		
CC_β (µg/kg)	0.42		

Table III. Comparison of the results determined through Biochip and LC-MS/MS methods

Sample No	Biochip (µg/kg)	LC-MS/MS (µg/kg)
1	0.79	0.88
2	0.84	0.33
3	3.07	2.34
4	0.23	0.07
5	0.76	0.61
6	0.49	0.29
7	0.77	0.43
8	0.44	0.94
9	0.11	0.32
10	0.11	0.32
11	0.11	0.32
12	0.85	0.12
13	0.44	0.14
14	0.11	0.02
15	0.11	0.53
16	0.11	0.26

Our data is in accordance to other published studies that sustain the use of Biochip assay for the fast screening of banned antibiotic residues in different food samples.

In terms of literature similar studies, there is more data that confirms the capacity and sensitivity of the Biochip method for identification of nitrofuran antibiotics in food [38,39]. The limit of detection for the investigated Biochip technique was below 0.9 µg/kg for all metabolites, where the threshold limit was generally set at 1 µg/kg.

Moreover, the detection costs, speediness and reliability of this method represent more arguments for its use in food safety domain.

I.1.1.4. Conclusions

Chloramphenicol residues in honey could be quantified at levels below the minimum necessary performance limits due using biochip technology. The novel quantitative approach for determining chloramphenicol has a high sensitivity and it was evaluated as IC50 (0.74 g/kg). For concentration levels of 0.5, 1 and 1.5 g/kg, the approach demonstrated extremely excellent accuracy both within the same analytical series and in distinct analytical series with typical values lower than 15%.

Chloramphenicol's decision limit (CC_α) was 0.37 µg/kg, while its detection capacity (CC_β) was 0.42 µg/kg.

The study of 16 samples of honey from different geographic areas in Romania was conducted using the validated Biochip technology, and the outcomes were verified using the LC-MS/MS method.

Although honey is recognized as a natural, healthy product, samples of honey were often contaminated with chloramphenicol residues.

I.1.2. Quinolones, Cephalosporins, Amphenicols Residue Determination

I.1.2.1. Aim of the Study

The study included a method validation for the simultaneous quantitative determination of several QNL, cephalosporins (ceftiofur), and amphenicols (thiamphenicol) using biochip technology, as well as an analysis of QNL, ceftiofur, and thiamphenicol in various honey samples bought from the Romanian market.

I.1.2.2. Materials and Methods

Antimicrobial II panel was used to determine 3 types of antibiotics, namely, ceftiofur (CEFT), thiamphenicol (TAF) and generic QNL.

The method of simultaneous quantitative determination using the biochip technology was validated following a protocol that simultaneously met the requirements of decision 2002/657/EC and the possibilities of any laboratory processing a large number of test samples [40]. The validation parameters evaluated were linearity, sensitivity, specificity, selectivity, accuracy, intermediate accuracy and reproducibility, the limit of detection and recovery.

A standard multi-analyte solution was prepared. It contained 10 μg/kg of each analyte dissolved in methanol. Calibration curves were obtained by spiking the negative honey samples at 9 levels of concentration of the analytes: 0, 0.001 μg/kg, 0.01 μg/kg, 0.05 μg/kg, 1 μg/kg, 1 μg/kg, 4 μg/kg, 10 μg/kg and 50 μg/kg.

To evaluate the sensitivity of the method, IC50 was calculated for each analyte. IC50 represents 50% of the RLU value corresponding to the zero concentration standards and extrapolating that RLU value from the X-axis of the calibration curve on which the units of concentration were expressed as μg/kg.

That concentration corresponded to the concentration, which produced 50% inhibition To determine the specificity and selectivity, known concentrations of each analyte were used in serial dilutions. Three replicates were analyzed for each drug level in serial dilution to assign the percentage of cross-reactivity.

According to the validation guides, the percentage of cross-reactivity for drug residue determination in honey should not exceed 25% for concentrating the analytes at the minimum limit of quantification [41]. According to the current legislation and validation guides for the methods of determining the drug residues in honey, the accuracy and precision are evaluated for concentrations representing 50 %, 100% and 150 % respectively of MRPL required for antibiotic residues in honeybees: MRPL = 1 μg/kg [13,42]. The accuracy within the same analytical series was determined by analyzing 20 replicates of negative samples of honey that were spiked to achieve those 3 concentration levels of antibiotics.

The accuracy of different analytical series was determined by analyzing 2 replicates of negative samples of honey that were spiked to achieve 3 concentration levels of antibiotics that were analyzed during 10 working days. Accuracy and precision were acceptable if CV% of the concentration in the control samples did not exceed ±15% for all determinations executed during the same day.

To determine the decision limit (CC_α) and the detection capacity (CC_β), 20 negative honey samples (blank samples) were selected. Aliquots of those samples were spiked with drugs at the target-screening concentration of 0.5 μg/kg for CEFT and TAF, and 1 μg/kg for QNL. The blank and spiked samples were tested. CC_α was calculated as the average of the analyte concentration in the 20 samples spiked with the analyte concentration at MRPL level plus 1.64×SD of repeatability at $\alpha = 5\%$. CC_β was calculated as the arithmetic average of the analyte concentration at CC_α + 1.64×SD of repeatability at $\alpha = 5\%$.

To estimate the recovery percentage, negative honey samples were used, spiked with

the analytes at 3 different levels of concentration.

For the purpose of identifying antibiotic residues, 43 samples of honey from various assortments were examined with the validated biochip method. The validation technique utilized honey samples that had already performed LC-MS/MS analysis and had been shown to be drug-free as negative samples. Using the LC-MS/MS technique, the results of the biochip method on the samples analyzed of honey were verified.

The determinations were conducted using the Agilent 1100 LC system (Agilent Technologies, USA) coupled with the 4000 Q TRAP mass spectrometer (Applied Biosystems, USA).

I.1.2.3. Results and Discussions

Nine of the 54 biochips were utilized for the calibration curve, while the other biochips were used to evaluate the precision and accuracy of the same analytical series.

The calibration intervals were 0-7 µg/kg for QNL, 0-11.5 µg/kg for CEFT and 0-5 µg/kg TAF. The r obtained for the drugs were in the range 0.982-0.998, and the lowest coefficient was obtained for TAF.

The calculated IC50 were for each analyte 0.52 µg/kg for QNL, 0.25 µg/kg for CEFT and 0.5 µg/kg TAF. Specificity and selectivity were studies based on CR% that was determined against the parent compound and the corresponding chain of related compounds (Table IV).

Table IV. Cross-reactivity study results

Cross-Reactant	CR%			Cross-Reactant	CR%		
	QNL	CEFT	TAF		QNL	CEFT	TAF
Amoxicillin		< 1		Levofloxacin	13		
Ampicillin		< 1		Marbofloxacin	16		
Cefadroxil		< 1		Nadifloxacin	14		
Cefazolin		< 1		Nafcillin		< 1	
CEFT	< 1	100	< 1	Nalidixic acid	< 1		
Chlortetracycline		< 1	< 1	Norfloxacin	100	< 1	< 1
Cinoxacin	< 1			Ofloxacin	21		
Ciprofloxacin	19			Orbifloxacin	11		
Cloxacillin		< 1		Oxacillin		< 1	
Danofloxacin	10			Oxolinic acid	12		
Dicloxacillin		< 1		Pazufloxacin	3		
Difloxacin	3			Pefloxacin	24		
Enoxacin	5			Penicillin G		< 1	
Enrofloxacin	8			Pipemidic acid	9		
Fleroxacin	12			Sarafloxacin	6		
Florfenicol			23	Streptomycin		< 1	< 1
Florfenicol amine			< 1	TAF	< 1	< 1	100
Flumequine	< 1			Ticarcillin		< 1	
Gatifloxacin	< 1			Tylosin		< 1	< 1

The approach demonstrated good accuracy both within and throughout analytical series (Table V), with typical values less than 15% for the determined concentrations.

In Table VI, the values of decision limit and detection capacity are presented. The decision limit obtained for the classes of antibiotics determined was between 1.25 µg/kg and 5.5 µg/kg. Additionally, the detection capacity acquired for the same drugs was between 2.05 µg/kg and 8.81 µg/kg.

Table V. Precision and accuracy data

Validation parameters	CEFT	QNL	TAF
Average concentration (µg/kg)	0.49	0.93	0.38
SD	2.44	1.52	0.53
1.64×SD	4.01	2.50	0.87
CC_α (µg/kg)	4.50	3.43	1.25
CC_β (µg/kg)	8.51	5.93	2.05

Table VI. Decision limit and detection capacity

Concentration	Concentration (µg/kg)			CV%		
	CEFT	QNL | TAF		CEFT	QNL	TAF
The same series						
0.5 µg/kg	1.25	0.63	0.31	6.8	4.30	3.40
1 µg/kg	2.50	1.25	0.63	5.80	6.60	4.50
2 µg/kg	5.00	2.50	1.25	5.00	6.80	6.80
Different series						
0.5 µg/kg	0.49	0.93	0.38	6.80	4.30	3.40
1 µg/kg	1.05	1.80	0.59	5.80	6.60	4.50
2 µg/kg	1.97	3.29	1.17	5.00	6.80	6.80

The recovery percentage was calculated using the formula:

$$\text{Recovery (\%)} = ((A-B)/C) \times 100$$

where: A was the average concentration determined for the analyte, B was the average analyte concentration in the sample and C was the analyte concentration in the spiked sample.

Table VII shows the recovery rates for samples of honey. A recovery percentage > 70% required for each analyte was achieved with values in the range of 77-125%.

Only QNL residues were measured in 6 samples of honey after 43 honey samples were tested. The LC-MS/MS method verified samples that were both positive and negative.

Table VII. Recovery data

Concentration	Analyzed Concentration (µg/kg)			Recovery (%)		
	CEFT	QNL	TAF	CEFT	QNL	TAF
0.5 µg/kg	0.56	0.62	0.60	112	123	119
1 µg/kg	0.87	1.00	1.00	87	100	100
2 µg/kg	1.54	1.74	1.98	77	87	99

The findings shown in Table VIII demonstrate that the concentration levels obtained using the biochip approach and the LC-MS/MS method are comparable.

The samples with drug concentrations greater than 1 µg/kg were verified to be positive after the use of the LC-MS/MS method.

The results of the 6 positive samples identified by the biochip method and confirmed by the LC-MS/MS method revealed the same QNL at concentration levels above MRPL, namely: ciprofloxacin and norfloxacin. Private beekeepers from different geographical regions in Romania provided the 6 QNL-positive samples of honey. The results obtained in the study were comparable to those previously published by other research teams in the scientific literature [43,44].

As a result of the current study, we were able to confirm that the biochip method was suitable for the proposed purpose. The performances of the biochip method were adequate, and the results obtained were similar to the concentration values determined using the confirmatory method, for both positive and negative samples.

Table VIII. Comparison of the results obtained using the biochip method versus the LC-MS/MS method

Sample No.	Method	QNL (µg/kg)	CEFT (µg/kg)	TAF (µg/kg)
1	biochip	10.4	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 5 µg/kg Norfloxacin 7 µg/kg	< LOD	< LOD
2	biochip	54.9	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 41 µg/kg Norfloxacin 10 µg/kg	< LOD	< LOD
3	biochip	12.7	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 4 µg/kg Norfloxacin 2 µg/kg	< LOD	< LOD
4	biochip	11.4	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 7 µg/kg Norfloxacin 3 µg/kg	< LOD	< LOD
5	biochip	9.4	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 6 µg/kg Norfloxacin 4 µg/kg	< LOD	< LOD
6	biochip	24.6	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 3 µg/kg Norfloxacin 15 µg/kg	< LOD	< LOD

I.1.2.4. Conclusions

The method validation criteria (specificity, accuracy, linearity, limits of detection and quantification) complied with the recommendations of European Commission Decision 2002/657/EC and proved that the method can detect and quantify drug residues, without necessarily having to be tested by mass spectrometry or derivatization for fluorescence analysis of analytes.

The biochip method presented excellent accuracy both within the same analytical series and in different analytical series, with typical values less than 15% for concentrations of 0.5, 1 and 1.50 µg/kg. The decision limit was between 1.25 µg/kg and 4.5 µg/kg. The detection capacity recorded values in the range of 2.05-8.51 µg/kg. The recovery coefficient obtained was in the range of 77-125% compared to the initial concentration.

The immunological approach suggested in our study had the advantage of not requiring the extraction of antibiotics from the biological matrix with organic solvents. Another advantage was that it produced a lot of results extremely fast.

Instrumental methods, like LC/MS are sensitive and specific, excellent for confirmation, but would be too laborious for screening a large number of samples.

This study demonstrated that the biochip method is suitable for the proposed purpose, namely, the determination of the concentration of each analyte during European food safety monitoring programs.

I.1.3. Four Metabolites of Nitrofurans Residue Determination

I.1.3.1. Aim of study

The study's objective was to provide an immunochemical method as a alternative for detecting nitrofuran residues in honey.

A novel technique has been applied for the determination of four metabolites of the nitrofurans most frequently found in honey using biochip technology. The contaminants in samples of honey from various sources have also been quantified using this method.

I.1.3.2. Materials and Methods

The honey analysis procedure and the validation method were carried out in accordance with the FDA approved validation guidelines, the guideline for the implementation of Commission Decision 2002/657/EC, the drug residue validation guides, and the validation guidelines for screening methods for residues of veterinary drugs.

Validation parameters included: linearity, sensitivity (IC50), specificity and selectivity, precision (intermediate and reproducibility), accuracy, detection limit and recovery [16,45].

The Anti-Microbial Array III kit was assessed to determine the method's linearity, producing a calibration curve with nine points for each of the four nitrofurans. Considering the complex honey composition as a sample matrix, the linearity of the method was also checked by enriching blank honey samples in order to obtain 9 concentration levels 0, 0.001, 0.01, 0.05, 0.1, 1, 4, 10, 50 μg/kg for AHD, AOZ and AMOZ. For SEM, concentrations of 0, 0.001, 0.01, 0.05, 2, 20, 40, 100 and 500 μg/kg were used [12,46].

The analyzer software used to calibrate a particular equation for the competitive immune-enzymatic detection technique:

$$y = D + [(A-D)/1+(x/C)^B]$$

where:

x = analyte concentration (μg/kg),

y = the intensity of the chemiluminescent signal expressed as RLU,

A, B, C, D = parameters of the competitive method, predefined in the analyzer software as A = the intensity of the chemiluminescent signal when the concentration of the analyte is 0, B = slope factor, C = the inflection point of the calibration curve and D = the intensity of the luminous response signal at an infinite theoretical concentration of the analyte [36,39].

An initial estimate was made for each parameter, then it was optimized by minimizing the sum of the squares of the residuals using the Microsoft Excel Solver.

The calibration curve initially assumed adding of 50 μL of each calibrator frame on the surface of each biochip followed by the addition of 150 μL of reaction buffer (AM III DIL ASY). The biochips were incubating at 25°C while stirring at 370 rpm for 30 min. After to each biochip was adding 100 μL of enzyme conjugate solution, and all biochips was incubating at 25°C while stirring at 370 rpm for 30 min. The next step consisted in removing the reaction mixture by washing the reaction surface, repeated 6 times. After complete removal of the reaction mixture, was adding 250 μL of working signal reagent (obtained by separate mixing luminol and peroxide in equal volumes). The biochips was placed in the support in the dark for the development of the reaction. Within about 2 min of its input, the image capture camera processed and interpreted the signal.

The specificity and selectivity of the method was analyzed by adding each analyte separately in known concentrations of (10 and 100 μg/kg) to the zero concentration calibrator in serial dilutions. To assign the cross reactivity percentage, three replicates were assessed for

each analyte level in the serial dilution.

Cross-reactivity was calculated as CR% = [IC50 (analyte)/IC50(cross reactant)]×100.

Because the residues of nitrofurans have a 1 μg/kg reference point for action (RAP), that was the reason the target concentration for screening was set at 0.5 μg/kg (50% of RAP).

According to current legislation and validation guidelines for methods of drug determination in honey, precision and accuracy must be assessed for the MRPL which was 1 μg/kg, and 50 and 150% MRPL [47,48].

The precision in the same analytical series was determined by analyzing 20 replicates of negative honey samples, enriched at 3 different concentration levels: 0.5, 1.0 and 1.5 μg/kg.

The accuracy in different analytical series was determined by analyzing 2 replicates of blank honey samples enriched with nitrofurans at three different concentration levels (0.5, 1.0 and 1.5 μg/kg) in 10 different rounds of work. Accuracy and accuracy are acceptable if CV% in the concentration of the control samples measured does not exceed ±15% for determinations on the same day or on different days or analytical series.

In order to determine the decision limit (CC_α) and the detection capability (CC_β) 20 blank honey samples were selected and spiked at the target concentration for screening - 0.5 μg/kg. CC_α was calculated as the arithmetic mean of the concentration in 20 spiked samples. The concentration level of each analyte was MRPL plus 1.64×SD of repeatability at a = 5%. CC_β was calculated as the arithmetic mean of the concentration analyte at CC_α+1.64×SD of repeatability at α = 5%.

Five samples of honey obtained from private producers and eleven samples purchased from supermarkets in various parts of Romania were both determined using the validated technique. At room temperature and in the dark, the samples had been kept.

Derivatization and nitrofuran extraction from honey samples were part of the sample processing, which was done as follows:

- 1g of honey sample was mixed with 4 mL distilled water, incubated at 37°C for 30 min, and stirred 10 min until dissolved;
- 0.5 mL of 1M HCl and 100 μL of 10 mM 4-nitrobenzaldehyde solution were added to the sample solution; the mixture was stirred for 10 min, and then incubated for 16-24 h at 37°C;
- after incubation, 5 mi dipotassium phosphate 0.1M solution was added to each sample, and the *p*H was adjusted to 7.4 with 1M NaOH solution;
-15 mL ethyl acetate were added to 5 mL of derivatized honey sample, which was stirred on a Vortex shaker for 2 min and then homogenized for another 10 min; the sample was centrifuged for 10 min at 4500 relative centrifugal force;
- 6 mL of supernatant was transferred from each sample into microtubes, which were then evaporated for 30 minutes at 60°C and 15 psi;
- the residue was mixed with 375 μL of sample diluent provided in the kit (AM III DIL SPE) and stirred for 2 min.

In Table IX, the method's performance measures are shown. Confirmation of the results obtained using the biochip method for the analyzed honey samples was performed by using a LC-MS/MS method using an Agilent 1100 LC (Agilent Technologies, USA) coupled with a 4000 Q TRAP mass spectrometer (Applied Biosystems, USA).

To confirm the results, the following parameters were followed: signal/noise ratio >3±2.5% differentiation of analyte retention time and corresponding standard, and ±20% deviation of the relative abundance of the analyte and ±50% deviation of the corresponding standard.

Table IX. Performance parameters of the LC-MS/MS method

Analyte	Mean concentration±SD (µg/kg)	CC_α (µg/kg)	CC_β (µg/kg)	Precision (%)	Accuracy (%)
AOZ	0.4±0.17	0.69	0.96	6.26	11.70
AMOZ	0.6±0.20	0.93	1.26	5.11	9.73
AHD	0.5±0.14	0.73	0.96	4.41	16.23
SEM	0.5±0.2S	0.96	1.42	4.90	12.60

I.1.3.3. Results and Discussions

The calibration curves for all four metabolites of nitrofurans are presented in Figure 2. Following the determinations, r values greater than 0.98 were obtained. The lowest value for r was obtained for SEM (r = 0.982). The obtained results prove that the admittance criteria for the calibration curve has been met as the r must be higher than 0.949.

The sensitivity of the method expressed as IC50 for the simultaneous quantitative determination of the 4 nitrofurans had values equal or even lower than 2.32 µg/kg (Table X).

Table X. Linearity and sensitivity

Parameter	Analyte			
	AOZ	AMOZ	AHD	SEM
Calibration range (µg/kg)	0-10	0-20	0-10	0-10
r	0.987	0.996	0.989	0.982
IC50 (µg/kg)	0.09	0.44	0.40	2.32

The values obtained for the cross-reactivity of the analytes analyzed are shown in Table XI.

Table XI. Specificity and selectivity

Cross-reactivity (%)								
Nitrofuran metabolite	4-NP-AOZ	100	4-NP-AMOZ	100	4-NP-AHD	100	4-NP-SEM	100
Cross-reactant	furazolidone	4	furaltadone	18	nitrofurantoin	16	4-nitro-2-furaldehyde semicarbazone	24

The recovery percentage was calculated for 3 concentration levels representing 50, 100 and 150% of the MRPL for nitrofurans residues in honey (MRPL = 1 µg/kg). Honey samples were spiked with nitrofurans at the following concentrations levels 0.5, 1, 1.5 µg/kg. Recovery percentages were calculated by plotting the ratio of the analyte concentration in the sample against the theoretical concentration of the analyte in the standard solution.

According to the validation guidelines, the requirement for the recovery percentage for the determination of drug residues in honey must be higher than 70%. The recovery rate obtained was in the range of 64-192% relative to the initial concentration, as shown in Table XII.

The decision limit (CC_α) for the determined nitrofurans ranged between 0.70 µg/kg and 1.05 µg/kg. The detection capability (CC_β) obtained for nitrofurans ranged between 78 µg/kg and 1.14 µg/kg (Table XII).

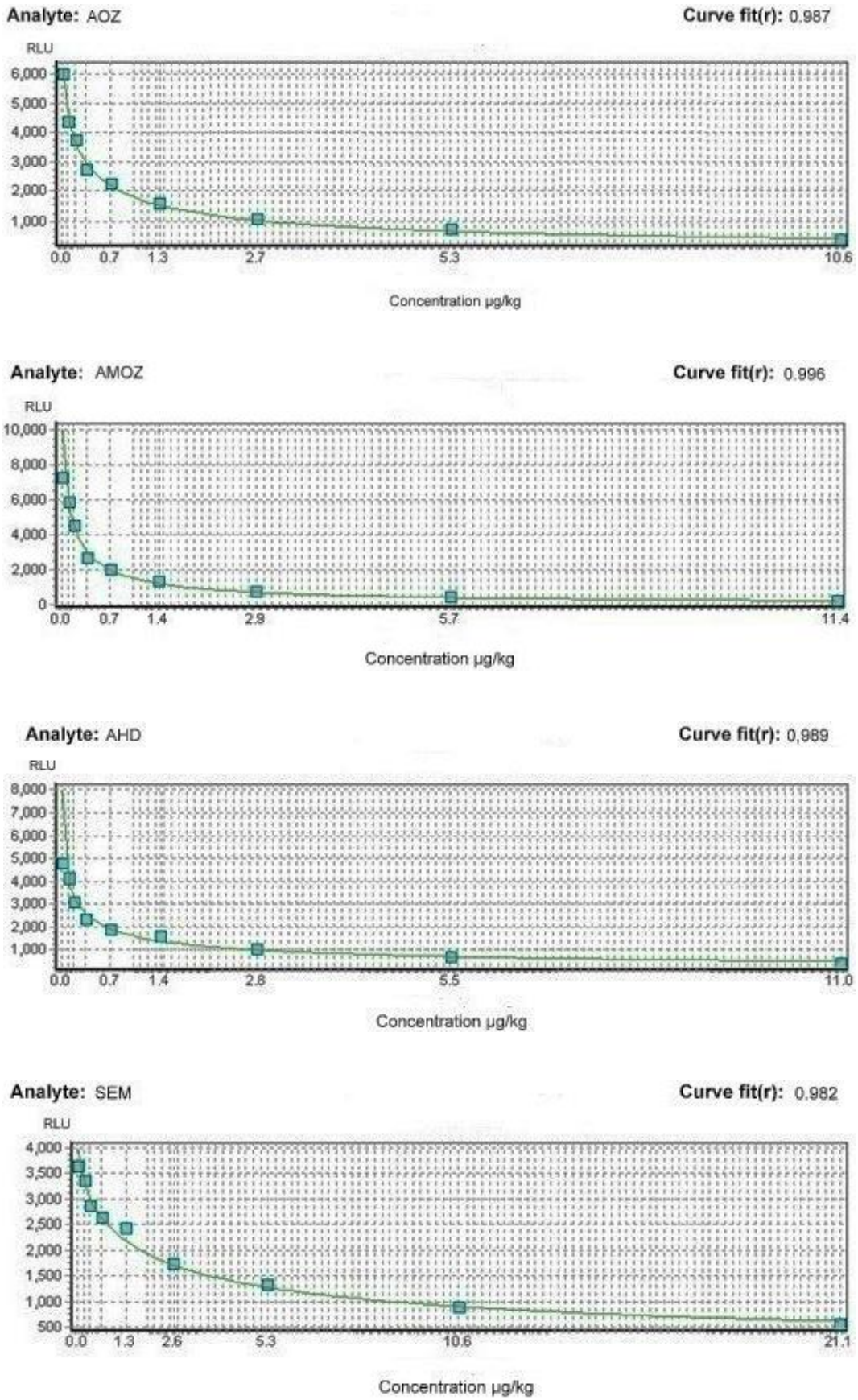

Figure 2. Calibration curves for AOZ, AMOZ, AHD and SEM

Table XII. Recovery, decision limit and detection capability

Parameter (n = 20)		Analyte				
		AOZ	AMOZ	AHD	SEM	CAP
1	Concentration (µg/kg)	0.62	0.62	0.68	0.96	0.32
	Recovery (%)	124	124	136	192	64
2	Concentration (µg/kg)	1.06	1.11	1.19	1.25	0.75
	Recovery (%)	106	111	119	125	75
3	Concentration (µg/kg)	1.82	1.73	1.81	2.25	1.06
	Recovery (%)	123	115	121	150	71
Mean concentration (µg/kg)		0.62	0.62	0.68	1.96	0.32
Standard deviation		0.05	0.05	0.05	1.06	0.03
1.64 × Standard deviation		0.08	0.08	0.08	0.09	0.05
CC_α (µg/kg)		0.70	0.70	0.76	1.05	0.37
CC_β (µg/kg)		0.78	0.78	0.84	1.14	0.42

The precision of the method (Table XIII) was very good, both within the same analytical series and in different analytical series, with typical values lower than 15% for concentrations of 0.5, 1 and 1.50 µg/kg. Within the different analytical series, the coefficients of variation reached higher values than those for samples from the same analytical series, but those values were within the acceptability limit.

Table XIII. Precision and accuracy

Concentration level		AOZ	AMOZ	AHD	SEM
Same series (n = 20)					
1	Concentration (µg/kg)	0.62	0.62	0.68	0.96
	CV(%)	8.05	7.87	7.32	5.86
2	Concentration (µg/kg)	1.06	1.11	1.19	1.25
	CV(%)	7.32	8.10	5.72	5.59
3	Concentration (µg/kg)	1.64	1.82	1.81	2.08
	CV(%)	7.36	6.88	6.89	3.93
Different series (n = 20)					
1	Concentration (µg/kg)	0.68	0.64	0:61	0.96
	CV(%)	10.15	7.87	8.32	5.86
2	Concentration (µg/kg)	1.16	1.14	1.07	1.25
	CV(%)	8.02	8.10	5.72	7.46
3	Concentration (µg/kg)	1.84	1.73	1.81	2.25
	CV(%)	8.33	11.78	8.71	12.93

The validated biochip method was applied to the analysis of 16 samples of honey from various geographic regions in Romania. One of the analyzed samples was found positive with values higher than 1 µg/kg for AOZ and SEM.

All results were confirmed by the LC-MS/MS method. The performance of the biochip method was very good, the values obtained were comparable to the results obtained for both positive samples and negative samples (Table XIV).

Table XIV. Comparison of results determined through biochip and LC-MS/MS methods

Simple Nº	Method	Analyte (µg/kg)			
		AOZ	AMOZ	AHD	SEM
1	biochip	0.50	0.74	0.46	0.75
	LC-MS/MS	0.17	0.12	0.32	0.19
2	biochip	0.76	0.75	0.78	0.66
	LC-MS/MS	0.41	0.42	0.13	0.28
3	biochip	1.24	2.88	4.16	7.21

Simple Nº	Method	Analyte (μg/kg)			
		AOZ	AMOZ	AHD	SEM
	LC-MS/MS	1.11	1.46	2.34	6.54
4	biochip	0.68	0.53	0.55	0.54
	LC-MS/MS	0.08	0.34	0.11	0.38
5	biochip	0.35	0.65	0.73	0.44
	LC-MS/MS	0.12	0.18	0.65	0.22
6	biochip	0.44	0.88	0.67	0.57
	LC-MS/MS	0.43	0.65	0.60	0.52
7	biochip	0.41	0.54	0.65	0.72
	LC-MS/MS	0.09	0.16	0.45	0.43
3	biochip	0.28	0.78	0.89	0.34
	LC-MS/MS	0.22	0.98	0.99	0.21
9	biochip	0.27	0.99	0.54	0.88
	LC-MS/MS	0.07	0.76	0.76	0.72
10	biochip	1.07	0.99	1.14	0.88
	LC-MS/MS	0.72	0.77	0.76	0.72
11	biochip	0.43	0.43	0.54	0.88
	LC-MS/MS	0.44	0.76	0.76	0.72
12	biochip	0.76	0.65	0.42	0.63
	LC-MS/MS	0.42	0.48	0.53	0.26
13	biochip	0.43	0.74	0.89	0.34
	LC-MS/MS	0.87	0.81	0.19	0.21
14	biochip	0.89	0.89	0.54	0.88
	LC-MS/MS	0.80	0.16	0.26	0.42
15	biochip	0.89	0.79	0.54	0.88
	LC-MS/MS	0.80	0.76	0.46	0.62
16	biochip	0.89	0.89	0.54	0.88
	LC-MS/MS	0.48	0.32	0.32	0.21

I.1.3.4. Conclusions

The use of biochip technology made it possible to detect nitrofuran residues in honey simultaneously and selectively at concentrations significantly lower than the minimally necessary performance limits. All of the IC50 values for the simultaneous quantitative detection of 4 nitrofurans were lower than 2.32 g/kg, indicating the method's sensitivity and specificity for each target analyte.

For concentration levels of 0.5, 1 and 1.5 g/kg, the approach demonstrated extremely excellent accuracy both within the same analytical series and in distinct analytical series with typical values lower than 15%. The range of the decision limit for the identification of 4 nitrofuran metabolites was between 0.37 and 1.05 μg/kg. The detection capacity was measured in the range of 0.42 to 1.14 μg/kg.

The range of the recovery coefficient for the initial concentration was between 64 and 192%. The study of 16 samples of honey from different geographic locations in Romania was conducted using the validated biochip technology, and the outcomes were verified using the LC-MS/MS technique.

I.1.4. Influence of Sulfonamide Drug Residues on Biochemical Parameters

I.1.4.1. Aim of study

The objectives of the research were to study the influence of some sulfonamides residues in honey on Wistar rats, by evaluating various biochemical parameters. Farmers often use the selected sulfonamides to treat various bee infectious diseases; thus, remains of theses antibiotics are usually found as traces in bee products.

I.1.4.2. Materials and Methods

Wistar females weighing 180–220 g were utilized for this research, and the batches were set up as follows: group l - the control group, received by gastric gavage standard feed and 2 mL bee honey, group 2 of rats received 2 mL of honey spiked with 100 μg/kg mixture of sulfonamide by gastric gavage for 5 days and group 3 received 2 mL of honey spiked with 100 μg/kg mixture of sulfonamide by gastric gavage, for 14 consecutive days.

The mixture of sulfonamides contained 20 μg of each substance (sulfadiazine, sulfamethazine, sulfathiazole and sulfamethizole, sulfadimethoxine).

Blood samples were collected in vacutainers containing anticoagulant (1 part 1% EDTA-Na_2 anticoagulant to 9 parts of whole blood), and they were used immediately to determine the biochemical parameters.

The study was conducted in accordance with the approval issued by the Research Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy from Iași, Romania.

Utilizing the Rx Imola automatic analyzer manufactured by Randox Laboratories, UK, the biochemical parameters (urea, creatinine, uric acid, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined quantitatively.

The quantitative determination of urea was performed based on the UV enzymatic kinetic method with urease. Briefly, urea was hydrolyzed by urease to ammonium ion and CO2. The ammonia produced was combined with a-ketoglutarate and NADH in the presence of glutamate dehydrogenase, when glutamate and NAD^+ were obtained. The change in absorbance due to the formation of NAD^+ instead of the consumed NADH was proportional to urea concentration [49].

The quantitative determination of creatinine was based on its reaction with picric acid in alkaline medium.

The concentration of the newly formed compound was directly proportional to creatinine concentration. For the quantitative determination, the uric acid was converted by uricase to allantoin and hydrogen peroxide. The latter finally formed quinonimine with 4-aminophenazone, whose color was directly proportional to uric acid concentration.

Quantitative determination of total bilirubin was performed based on the Jendrassik Grog colorimetric method [50]. Total bilirubin was released from the albumin molecule in the presence of caffeine and reacts with diazotized sulfanilic acid to form a colored compound whose color intensity was directly proportional to total bilirubin. Determinations on hemolyzed samples were avoided because it interfered with the analysis.

The determination of direct (conjugated) bilirubin was based on the ability of direct bilirubin to react with sulfanilic acid to form an intermediate reaction compound to which the diazo reagent was added. A red compound was formed, and color intensity was directly proportional to serum concentration of direct bilirubin.

Quantitative determinations of ALT and AST respectively, were performed using the standardized UV method, according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The decrease in absorbance measured at 340 nm, due to NADH consumption and NAD^+ formation, was inversely proportional to the activity of the enzyme.

The statistical description of the samples was done in order to obtain the descriptors of interest (mean, median, standard error of mean (SE), SD, amplitude, Skewness coefficient, Kurtosis coefficient). Also, the Kolmogorov-Smirnov test was applied to assess the normality of the data distribution. The values were considered as following: $p < 0.05$ - significant, $p < 0.01$ - distinctly significant, $p < 0.001$ - highly significant.

I.1.4.3. Results and Discussions

The findings are shown in Table XV and Table XVI as a consequence of processing the data collected in determining the biochemical parameters and performing descriptive statistical tests and significance.

Table XV. Experimental data for the group of rats that received a 5-day treatment with sulfonamides spiked honey

Statistical parameter	Creatinine (mg/dL)	Urea (mg/dL)	Uric Acid (mg/dL)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)	AST (IU/L)	ALT (IU/L)
Mean±SE	0.45±0.02	17.04±1.70	1.71±0.13	0.62±0.02	0.08±0.01	60.71±3.87	72.75±1.85
Median	0.47	17.00	1.64	0.63	0.07	61.20	74.64
Standard deviation	0.05	3.80	0.29	0.04	0.02	8.66	4.14
Variance	0.002	14.47	0.08	0.001	0.0003	75.00	17.14
Skewness coefficient	-0.53	0.98	0.68	-0.58	-0.32	-0.10	-0.53
Kurtosis coefficient	-3.04	1.43	0.98	-1.22	-0.74	-1.67	-3.01
Amplitude	0.1	10.10	0.94	0.10	0.04	21.31	8.64
Minimum value	0.39	12.90	1.36	0.56	0.05	49.82	67.79
Maximum value	0.49	23	2.14	0.66	0.10	71.13	76.43
Confidence level (95%)	0.06	4.72	0.39	0.05	0.02	10.75	5.14
Confidence interval (95%)	0.39-0.50	12.32-21.76	1.35-2.07	0.57-0.67	0.05-0.10	49.95-71.46	67.61-77.89

Table XVI. Experimental data for the group of rats that received a 14-day treatment with sulfonamides spiked honey

Statistical parameter	Creatinine (mg/dL)	Urea (mg/dL)	Uric Acid (mg/dL)	Total bilirubin (mg/dL)	Direct bilirubin (mg/dL)	AST (IU/L)	ALT (IU/L)
Mean±SE	0.72±0.02	18.36±0.39	2.40±0.19	1.27±0.04	0.32±0.03	109.57±3.29	128.23±2.68
Median	0.74	18.60	2.28	1.22	0.35	110.18	130.61
Standard deviation	0.03	0.87	0.42	0.09	0.07	7.36	5.99
Variance	0.001	0.76	0.18	0.008	0.005	54.21	35.90
Skewness coefficient	-0.59	-1.09	0.32	1.09	-1.02	0.45	-0.99
Kurtosis coefficient	-2.98	0.53	-3.04	0.06	-0.30	-0.33	0.66
Amplitude	0.07	2.15	0.87	0.23	0.17	18.19	15.51
Minimum value	0.68	17.00	1.98	1.18	0.21	101.17	119.00
Maximum value	0.75	19.15	2.85	1.41	0.38	120.08	134.51
Confidence level (95%)	0.04	1.08	0.52	0.12	0.09	9.14	5.00
Confidence interval (95%)	0.68-0.76	17.27-19.44	1.87-2.92	1.15-1.38	0.23-0.40	110.43-118.71	120.79-135.67

As predicted, two weeks of consuming infected honey resulted in noticeably higher levels of ALT and AST compared to a five-day therapy (Figure 3).

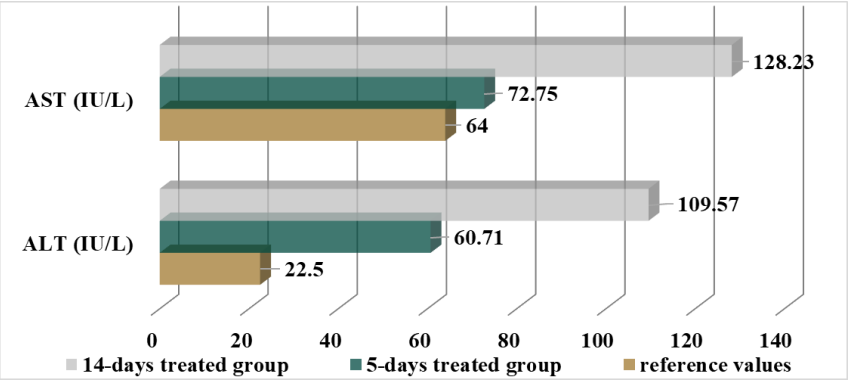

Figure 3. Changes of transaminases levels after 5 days and 14 days of treatment with spiked honey

Serum concentrations of AST and ALT increased following administration of honey with sulfonamides, compared with the values determined in the control group. Those results were in agreement with data from the literature, according to which the toxic effect of sulfonamides, even administered in very small quantities, manifested by modifying the liver parameters in laboratory animals [51]. Thus, ALT and AST activity recorded statistically significant increases ($p < 0.0001$) in rats that received honey spiked with sulfonamides, compared to the control group, thus proving the change in hepatocyte membrane permeability and the degree of liver dysfunction in rats.

However, more significant changes were observed on the biliary pathways (Figure 4). Similar effects were observed on zebrafish metabolomic model from sulfonamides contaminated water [52].

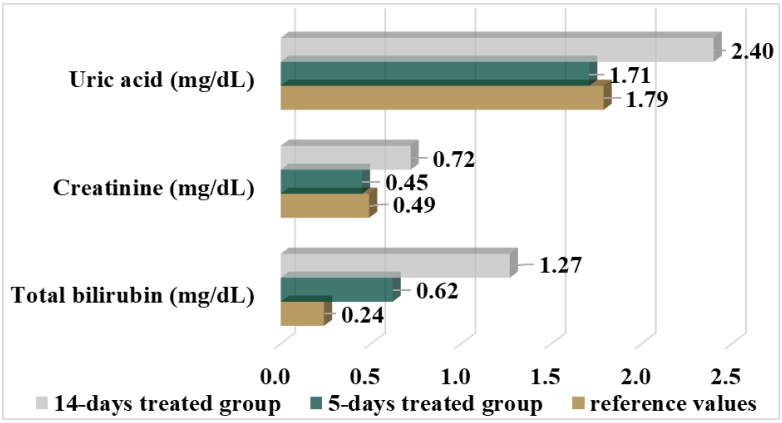

Figure 4. Comparison between the experimental data and the reference values for female Wistar rats

As the groups were small ($n \leq 15$), the Kolmogorov-Smirnov test was applied to assess the normality of the data distribution, as well as for the other parameters analyzed in that part of the study. The application of the statistical test highlights the existence of a significant difference. According to the evaluated parameters, for both treated groups we established that the samples had both normal and similar distribution, so they were comparable. Moreover, two tailed t-Student indicated that the obtained results were very significant ($p < 0.001$, $T = 2.92$, $r = 0.9871$, $R^2 = 0.7068$, $t = 1.86$) for all tested parameters except for urea and uric acid, when the significance was distinctive with $p < 0.015$ and $p < 0.01$ respectively. Therefore, all the

obtained values were statistically significant in terms of changes from the control values. Noteworthy is that Romanian bee honey samples are not the only contaminated products, residues of veterinary medicines (chloramphenicol, sulfathiazole and tetracycline) were found in Spanish, Belgian, Turkish and Chinese market samples [1,2,4,10–12,48,53,54]. Therefore, precise methods are important for proper detection of contaminants [9,10,45,55–58]. However, the true impact of the consumption of contaminated honey was not assessed on animal models thus far. To date, only one study has tested the relevance of sulfonamides metabolites found in soil and water on zebrafish [52]. Our research showed a statistically insignificant increase in uremia (18.36±0.87 mg/dL) for the sulfonamide-treated groups, compared with the reference group for which the average urea concentration was 17.04±3.80 mg/dL.

It was helpful to interpret the findings for that parameter by connecting it with the results of other tests such as creatinine and uric acid since serum concentrations of urea rely on three factors: protein catabolism, diuresis, and renal functional capacity.

The effect of the administration of honey with sulfonamide in rats on renal function resulted in a significant increase in the values of uric acid and creatinine concentrations ($p < 0.05$) in the test groups compared with the control group.

Evaluation of liver activity by analyzing total bilirubin and direct bilirubin revealed a significant increase in total bilirubin concentration ($p < 0.0001$) following administration of bee honey spiked with sulfonamide. The average concentration obtained on the tested group was 1.27 mg/dL (14 days treatment), compared to the shorter treatment group (5 days) with an average concentration of 0.62 mg/dL. The values of the direct bilirubin concentration varied similarly to those of total bilirubin. In the group that consumed honey spiked with sulfonamides for 14 days, the values of the direct bilirubin were 0.32±0.07 mg/dL compared to 0.08±017 mg/dL recorded for the control group. Similarly, the Korean researchers concluded that xenobiotics and its metabolites influence the hepatobiliary metabolic pathways [52]

Sulfonamides, particularly nitrogen-containing sulfonamides and their metabolites, have been discovered by other research groups to have the ability to trigger allergies in T-cells [5–7]. Our results allow us to make the conclusion that the presence of sulfonamides in honey and other bee products negatively affects the health of the liver and kidneys. Legislation in Romania should thus adhere to international standards set by nations like Germany, Switzerland, and Japan, which prohibit using certain medicines to treat bees [1,2,6,10].

I.1.4.4. Conclusions

Administration of bee honey spiked with sulfonamides to Wistar rats led to significant alterations in biochemical parameters. The observed changes in the activities of ALT and AST indicated noteworthy increases, pointing towards the permeabilization of hepatocyte membranes and subsequent migration of those enzymes into intercellular spaces. This permeabilization is hypothesized to stem from damage incurred due to the interaction between sulfonamides or their metabolites and membrane proteins.

The increased plasma levels of total bilirubin and direct bilirubin further validated the substantial disruption in hepato-biliary function. That disruption was pronounced after 14 days of administering through gastric gavage honey that contained sulfonamide.

Furthermore, the investigation into renal function parameters highlighted elevated concentrations in the experimental groups. That might be attributed to the toxicity due to the consumption of honey containing sulfonamides. Consequently, those findings provide concrete evidence of the toxic potential of sulfonamides, even if present in small doses or as traces within food products.

Considering these outcomes, it is imperative to caution both beekeepers and consumers against the consumption of such products and to highlight the importance of avoiding products containing sulfonamides, emphasizing the potential risks they pose.

I.1.5. Influence of Sulfonamide Drug Residues on Immunological and Hematological Parameters

I.1.5.1. Aim of the Study

The objective of the study was to evaluate the influence of sulfonamide residues in bee honey on Wistar rats by quantifying immunological parameters, such as T3 and T4, and hematological parameters, such as hematocrit (Hct), hemoglobin, red blood cell count (RBC), and mean corpuscular hemoglobin concentration in a given volume of erythrocytes (MCHC) after administration of sulfonamide-containing honey.

I.1.5.2. Materials and Methods

For the quantitative determination of the immunological parameters, the kit for the determination of T3 cod EH-500 and the kit for the determination of T4 cod EH-501 (produced by ClinPro International Co., LLC, Union City, CA, USA) were used. These contained 1 mL of calibrator solutions for the following concentrations 0, 0.75,1.5,3.0, 6.0,10.0 ng/mL for T3 and 0, 2,5,10,15, 25 μg/dL for T4, respectively.

Reagents used for the determination of eosinophils (hemoleukogram) were dedicated reagents for the automatic hematology analyzer Sysmex XT 1800i: Cellpack (diluent for hematological analyzers), the Stromatolyser FB (diluent for hematological analyzers), the Stromatolyser 4DL (diluent for hematological analyzers), the Stromatolyser 4DS (used for staining leukocytes from previously diluted and lysed samples), and Cellclean (strong alkaline detergent used to remove lysate residues, cell residues and proteins from the hydraulic system of the analyzer).

Wistar females weighing 180–220 g were utilized for this research, and the rats were divided into two groups of 10 rats each: a control group that were fed 2 mL of sulfonamide free honey by gastric gavage and a sample group fed by gastric gavage for 5 days with honey spiked with 100 μg mixture per kilogram of body weight (μg/kgBW). The 100 μg mixture contained 20 μg of each of the five sulfonamides (i.e., sulfadiazine, sulfamethazine, sulfathiazole, sulfamethizole and sulfadimethoxine). All rats were individually housed under specific microclimate conditions and fed a standard plant-based diet with free water and food access.

EU Regulation no. 37/2010 of the Commission has established a total maximum residue level of 100 μg/kg for all substances belonging to the sulfonamide group. This level applies to lean and fatty meat, liver, and kidney from all animals raised for consumption; it also applies to milk and honey [59]. The study was conducted in accordance with the 2010/63/EU directive and followed the recommendations of the National Institutes of Health (NIH) Guide for the Care and the Use of Laboratory Animals. Prior to the beginning of the study, the protocol received ethical approval from the University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Ethics Committee.

Blood samples were collected in vacutainers containing anticoagulants (1% EDTA-Na_2 - 1 part to 9 parts of whole blood) and were analyzed immediately to determine the hematological parameters (complete blood count). Blood samples collected in vacutainers without anticoagulant were immediately centrifuged and serum was sampled to determine the immunological parameters. Serum samples were kept frozen at -25 °C until testing.

The quantitative determinations of the immunological parameters (T3, T4) were performed on the Stat Fax 303 Plus device (Awareness Technology, Ramsey Minnesota, MI, USA), and of hematological parameters were performed on the automatic hematology analyzer, model Sysmex XT 1800i (Sysmex Europe Gmbh, Norderstedt, Germany).

Three duplicates of each experiment were carried out. The mean and SD are used to express all data. To remove biological variability and calculation mistakes, the data were

statistically analyzed. In order to do this, the samples were statistically described using the descriptors of interest (mean, median, SE, SD, variance, skewness coefficient, and kurtosis coefficient).

I.1.5.3. Results and Discussions

A comparative analysis of the T4 results from the two groups showed a highly statistically significant p value that highlighted the presence of a marked decrease in T4 values in the sample group compared to the control group (Table XVII). A comparative analysis showed that the T3 values of the sample group were significantly lower than those of the control group indicated by a statistically significant p value (Table XVIII).

Table XVII. Data processing of thyroxine values (T4 as µg/dL)

Parameter	Control Group		Sample Group	
Mean±SD	4.50±0.30		3.32±0.21	
Mean±SE	4.50 ±0.13		3.32±0.09	
Median	4.33		3.34	
Standard deviation	0.30		0.21	
Variance	0.04		0.09	
Skewness coefficient	0.81		0.55	
Kurtosis coefficient	-1.54		-0.41	
Amplitude	0.70		0.52	
Minimum value	4.22		3.10	
Maximum value	4.92		3.62	
Number of replicates (n)	5		5	
Confidence level (95%)	0.37		0.26	
Confidence interval (95%)	4.12-4.87		3.06-3.58	
Kolmogorov-Smirnov Test	p		0.98	0.63
Student's t-test Control vs. Sample Group	t (t stat)	t (t critic)	p (T < t)	p (F-test)
	-7.21	1.86	$4.58\text{-}10^{-5}$ <0.001 *	0.49
(*) p < 0.05 significant, p < 0.01 distinctly significant, p < 0.001 very significant.				

Table XVIII. Data processing of triiodothyronine values (T3 as ng/dL)

Parameter	Control Group		Sample Group	
Mean±SD	0.70±0.14		0.34±0.03	
Mean±SE	0.70±0.06		0.34 ±0.02	
Median	0.69		0.35	
Standard deviation	0.14		0.03	
Variance	0.001		0.02	
Skewness coefficient	-0.15		-0.84	
Kurtosis coefficient	-1.70		0.70	
Amplitude	0.34		0.09	
Minimum value	0.52		0.29	
Maximum value	0.86		0.38	
Number of replicates (n)	5		5	
Confidence level (95%)	0.17		0.04	
Confidence interval (95%)	0.53-0.88		0.30-0.38	
Kolmogorov-Smirnov Test	p		0.98	0.97
Student's t-test Control vs. Sample Group	t (t stat)	t (t critic)	p (T < t)	p (F-test)
	-5.57	2.13	0.003 *	0.018
(*) p < 0.05 significant, p ≤ 0.01 distinctly significant, p ≤0.001 very significant				

The results obtained for the hematological parameters following the administration of honey containing sulfonamide residues in Wistar rats are presented in Table XIX.

Analyzing the results, it was found that the values for Hct in the sample group were

higher compared to the control group (Table XX).

Table XIX. Hematological parameters after administration of honey with sulfonamide residues to rats

Parameter (Mean±SD)	Control Group	Sample Group
Hct (%)	47.80±3.69	52.70±6.64
RBC (million/mm^3)	9.72±1.14	5.14±0.38
Hb (g/dL)	16.10±0.92	14.50±1.16
MCHC (g/dL)	38.60±2.79	34.50±2.20

Table XX. The data processing of hematocrit values

Parameter	Control Group		Sample Group	
Mean±SE	47.80±1.65		52.70±2.97	
Median	47.36		50.16	
Standard deviation	3.69		6.64	
Variance	13.61		44.03	
Skewness coefficient	-0.30		0.71	
Kurtosis coefficient	1.12		-0.98	
Amplitude	10.15		16.40	
Minimum value	42.47		45.76	
Maximum value	52.62		62.16	
Number of replicates (n)	5		5	
Confidence level (95%)	4.58		8.24	
Confidence interval (95%)	43.22-52.38		44.46-60.94	
Kolmogorov-Smirnov Test	p		0.94	0.86
Student's t-test	t (t stat)	t (t critic)	p (T < t)	p (F-test)
Control vs. Sample Group	-1.44	1.86	0.09 *	0.28
(*) p <0.05 significant, p ≤ 0.01 distinctly significant, p ≤ 0.001 very significant.				

The description and statistical analysis of the two groups for RBC showed a significant decrease in the values obtained in the sample group compared to the control group (Table XXI).

Table XXI. Data processing of red blood cell count values (RBC as million/mm^3)

Parameter	Control Group		Sample Group	
Mean±SE	9.72±0.51		5.14±0.17	
Median	9.86		4.93	
Standard deviation	1.14		0.38	
Variance	1.31		0.15	
Skewness coefficient	-0.13		1.02	
Kurtosis coefficient	0.53		-0.76	
Amplitude	3.10		0.88	
Minimum value	8.14		4.83	
Maximum value	11.24		5.71	
Number of replicates (n)	5		5	
Confidence level (95%)	1.42		0.48	
Confidence interval (95%)	8.30-11.14		4.66-5.62	
Kolmogorov-Smirnov Test	p		0.99	0.64
Student's t-test	t (t stat)	t (t critic)	p (T < t)	p (F-test)
Control vs. Sample Group	8.49	1.86	$1.42 \cdot 10^{-5}$*	0.057
(*) p < 0.05 significant, p ≤ 0.01 distinctly significant, p ≤ 0.001 very significant.				

For Hb, the data analysis highlighted changes of statistical significance. The values for the sample group were significantly lower than those for the control group (Table XXII).

Table XXII. Data processing of hemoglobin values (Hb as g/dL)

Parameter	Control Group		Sample Group	
Mean±SE	16.10±0.41		14.50±0.52	
Median	16.47		14.86	
Standard deviation	0.92		1.16	
Variance	0.85		1.34	
Skewness coefficient	-1.34		-0.41	
Kurtosis coefficient	1.19		-0.14	
Amplitude	2.19		3.05	
Minimum value	14.62		12.87	
Maximum value	16.81		15.92	
Number of replicates (n)	5		5	
Confidence level (95%)	1.15		1.44	
Confidence interval (95%)	14.96-17.25		13.06-15.94	
Kolmogorov-Smirnov Test	p		0.85	0.94
Student's t-test	t (t stat)	t (t critic)	p (T < t)	p (F-test)
Control vs. Sample Group	2.42	1.86	0.02*	0.67
(*) p <0.05 significant, p < 0.01 distinctly significant, p < 0.001 very significant.				

In terms of the MCHC values, it was found that there were statistically significant differences between the two groups. For the sample group, the values obtained for MCHC were lower than those of the control group (Table XXIII).

Table XXIII. Mean corpuscular hemoglobin concentration in a given volume of erythrocytes (MCHC as g/dL)

Parameter	Control Group		Sample Group	
Mean±SE	38.60±1.25		34.50±0.98	
Median	37.07		34.58	
Standard deviation	2.79		2.20	
Variance	7.77		4.82	
Skewness coefficient	0.65		-0.71	
Kurtosis coefficient	-2.72		-0.03	
Amplitude	5.94		5.56	
Minimum value	36.24		31.25	
Maximum value	42.18		36.81	
Number of replicates (n)	5		5	
Confidence level (95%)	3.46		2.73	
Confidence interval (95%)	35.14-42.06		31.78-37.23	
Kolmogorov-Smirnov Test	p		0.64	0.99
Student's t-test	t (t stat)	t (t critic)	p (T < t)	p (F-test)
Control vs. Sample Group	2.58	1.86	0.02 *	0.66
(*) p <0.05 significant, p < 0.01 distinctly significant, p < 0.001 very significant.				

Normal hematological values for Wistar rats are listed in Table XXIV. There are limited data in the literature on changes to hematological parameters resulting from toxicity produced by administration of honey containing sulfonamide residues.

Table XXIV. Normal values of hematological parameters for Wistar rats [60]

Hematological Parameter	Unit of Measurement	Range of Variation
RBC	millions/mm^3	5.5-10
Hct	%	53-60
Hb	g/dL	14-16
MCHC	g/dL	31-33
MCHC	pg/cell	17-20

The results of this study showed that the observed variations in hematological parameters in laboratory rats fed with sulfonamide-spiked honey showed decreased values for Hct, Hb, and MCHC. Among these, reduced values for erythrocyte count, the amount of hemoglobin and the value of Hct are the main parameters for the diagnosis of anemia.

The scientific data indicates that 15% of the reported clinical cases of sulfonamide use have been associated with hematological changes (anemia, thrombocytopenia, neutropenia, etc.) in humans. These results concern adverse reactions to medication. No food-related sulfonamide contamination has so far been reported, contaminated batches have been disposed of prior to being consumed [61].

The T3 values results confirmed the hypothesis that sulfonamides interfere with thyroid hormone production. For the group of animals given sulfonamide-spiked honey, the mean T3 concentration decreased from 0.70±0.14 ng/dL to 0.34±0.03 ng/dL. The intervention of sulfonamides in the metabolism of thyroid hormones resulted in changes in T3 concentrations which were uncorrelated with the quantity of sulfonamides administered with bee honey by gastric gavage in laboratory rats.

In the same time, animals fed with contaminated honey with sulfonamide had considerably lower plasma T4 levels than the control group. These results are consistent with those published by Cribb et al. when evaluating the adverse effects of sulfonamides and sulfonamides/trimethoprim on rats [62].

In rats, administration of high doses of various sulfonamides has been associated with decreased T3 and T4 serum levels as well as increased TSH (thyroid stimulating hormone) levels [63]. These results are consistent with hypothyroidism reported in the literature in human studies as part of hypersensitivity reactions following treatment with one or more sulfonamides [64]. According to the same article, when administered to human subjects, the sulfonamides were converted by thyroid peroxidase into reactive metabolites that caused localized destruction of the thyroid gland. The thyroid lesions were found to be reversible after discontinuation of the drug.

Thyroid adenoma is the main health issue associated with sulfonamide consumption. Studies on animals that received chronic feeding for 18 to 24 months revealed dose-dependent cancerous consequences. After obtaining these findings, the United States imposed an interdiction on the use of sulfonamides in cows above 20 months of age as such substances can transfer to milk as residues. More than 73% of the examined milk samples revealed sulfonamides, according to a market analysis [61]. From a biological point of view, the toxicity of substances is not the same for all species of living organisms. This is a result of the different rates and ways of eliminating the toxic metabolite, as well as of the different sensitivities of the species to that metabolite. Observed differences between species are primarily due to metabolic factors, which, in turn, depend on the enzymes that control the biotransformation of the toxic substance. These variations reflect the kinetic parameters of transport, distribution, storage, redistribution, and blood/tissue distribution that change among various species, as well as the methods and rates of bio-inactivation or elimination of the toxic substance.

I.1.5.4. Conclusions

Our study demonstrates the potential for the quantification and individual identification of other antimicrobial substances, as well as other pollutants (pesticides, toxic metals, biostimulators, mycotoxins), used in the treatment of diseases in bees and which are found as residues in honey. It also highlights the importance of the evaluation of changes in immunological and hematological parameters and oxidative stress when investigating the effects of metabolite residues resulting from the administration of bee honey and other bee products (e.g., pollen, royal jelly, propolis) as well as other food products.

By following the suggested time interval between the administration of chemotherapy

and the harvest of the bee product, as well as by lowering the frequency of application of veterinary treatments, it is possible to reduce the amount of sulfonamide residues in honey and other bee products.

I.1.6. Determination of Mycotoxins in Pollen

I.1.6.1. Aim of the Study

An innovative multiresidue approach for the assessment of eight trichothecene mycotoxins (nivalenol, fusarenon-x, diacetoxyscirpenol, 3-acetyl-deoxynivalenol, neosolaniol, deoxynivalenol, T-2 and HT-2 toxins) has been performed using gas chromatography coupled with mass spectrometry. The mycotoxins had been extracted from pollen samples using a QuEChERS-based extraction procedure.

I.1.6.2. Materials and Methods

Quick, Easy, Cheap, Effective, Rugged, and Safe, the QuEChERS ("catchers") method is a fast, simple, and effective alternative to conventional sample prep for multiresidue analysis of various types of substances [65–73].

Sample preparation involved successive stages of extraction and derivatization.

Mycotoxins were extracted from spiked pollen samples using a QuEChERS-based extraction procedure without applying any further clean-up step. 10mL of distilled water were added to 5g of each homogenized sample and the mixture was sonicated for 15 minutes. The main extraction involved the addition of 7.5mL acetonitrile, 4g $MgSO_4$ and 1g NaCl. To induce phase separation and mycotoxins partitioning, the test tube was shaken on a vortex for 30 s and then it was centrifuged for 10 min at 4000 rpm. Then the upper layer was submitted to a dispersive Solid-Phase Extraction clean up with a mixture of 900mg $MgSO_4$, 300mg octadecyl silica-bonded sorbent and 300 mg primary secondary amine sorbent. The mixture was vortexed for 30 s and centrifuged for 10 min at 4000 rpm. After purification, the extract was transferred into a vial and evaporated under a nitrogen flow to dryness.

The dry extract was mixed with 50p, L of N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and N-trimethylsilyimidazole (3:2:3) mixture and the sample was kept for 30 min at room temperature. The derivatized sample was diluted with hexane to 250pL. The hexane solution was washed with 1mL of phosphate buffer (60mM, pH 7.2). In the end, the hexane layer was filtered and transferred into an autosampler vial to be analyzed through GC-MS/MS.

Chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler. The mass spectrometer was operated in electron impact ionization (70 eV). The transfer line and source temperatures were 280 and 230^{o}C, respectively. Nitrogen gas was used as collision gas for MS/MS, and helium was used as quenching gas. Data was acquired and processed using the Agilent Masshunter version B.04.00 software.

Analytes were separated on a HP-5MS 30m×0.25mm×0.25μm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode at 250^{o}C in programmable temperature vaporization inlet employing helium as carrier gas at 20.3psi pressure. The oven temperature program was initially 80^{o}C, and then the temperature was increased to 245^{o}C at a rate of 60^{o}C·min^{-1}. After 3 min spent at 245^{o}C, the temperature was increased to 260^{o}C at a rate of 3^{o}Cmin^{-1} and finally to 270^{o}C at a rate of 10^{o}C·min^{-1} and then the temperature was maintained constant for 10 more min.

I.1.6.3. Results and Discussions

Two transitions per compound were used, fulfilling the European Council Directives regarding mass spectrometric detection [28,40,74]. For each compound, the most abundant multiple reaction monitoring mode (MRM) transition was used for quantification while the other transition was used for confirmation (Table XXV).

Figure 5 and Figure 7 present the chromatographic peak of neosolaniol and its mass spectrum and the characteristic transitions viewed in MRM mode are presented, while Figure 6 illustrates the MS/MS spectrum of a spiked pollen sample obtained in full scan mode in which neosolaniol has a mass/charge ratio of 252.0.

Linearity and matrix effects were studied during the validation of the method using standard solutions and matrix matched calibrations (Table XXVI). Matrix matched calibration curves were built by spiking blank samples with selected mycotoxins before extraction. The limit of quantification was defined as the concentration with a signal-to-noise ratio 10:1. That parameter was determined by analysis decreasing concentrations of mycotoxins in spiked pollen samples. To assess matrix, effect the slope of pollen matrix matched (A) and the slope of external calibration (B) were calculated. Thus, the ratio (A/B×100) is defined as the matrix effect (%). A value of 100% indicated that there was no matrix effect. There was signal enhancement if the value was higher than 100% and signal suppression if the value was lower than 100%.

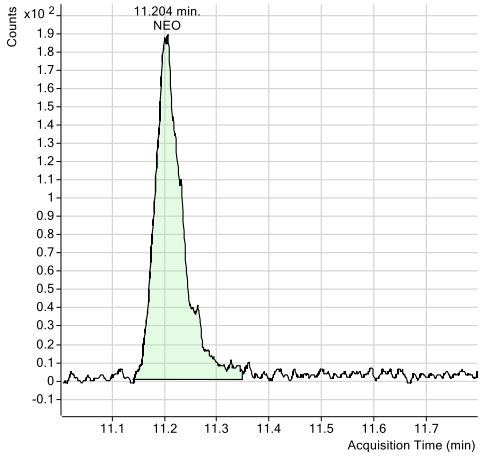

Figure 5. GC chromatogram peak of neosolaniol

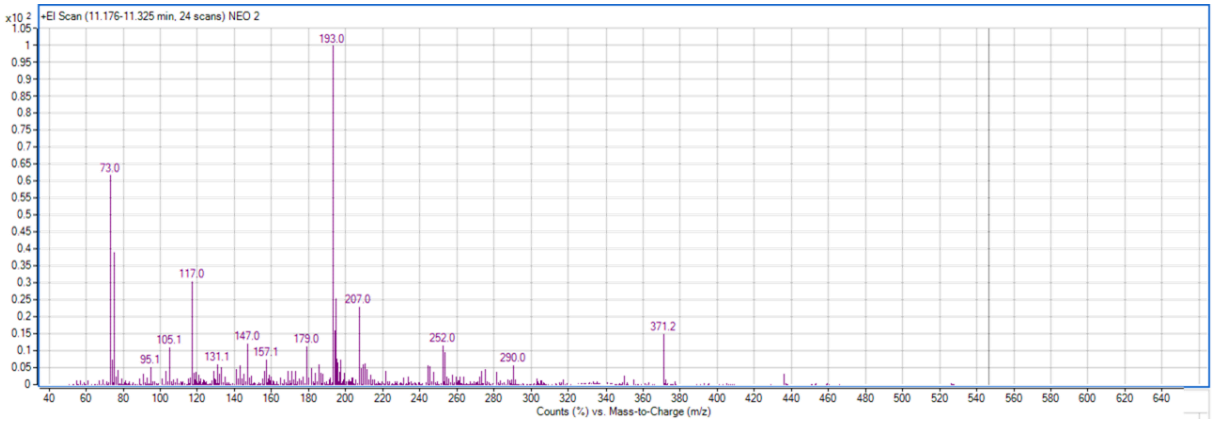

Figure 6. MS/MS spectrum of a spiked pollen sample

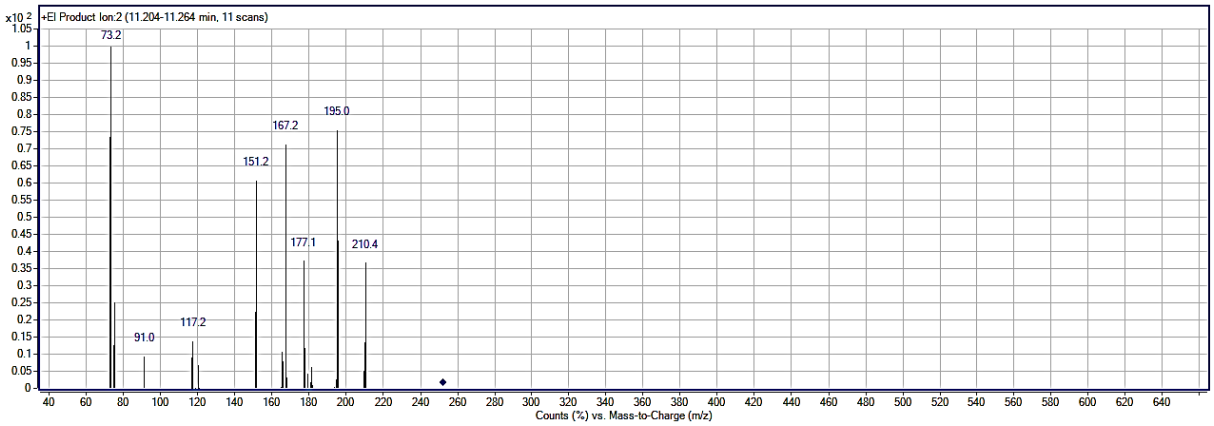

Figure 7. The mass spectrum of neosolaniol

Table XXV. Mass spectrometry parameters for the optimized GC-MS/MS method

Compound	Retention time (min)	MRM transitions (m/z)		Ratio Q/q±%RSD
deoxynivalenol	8.38	392>259	407>197	41.6 ± 3.2
3-acetyl-deoxynivalenol	9.42	392>287	467>147	47.5 ± 12.3
fusarenon X	9.48	450>260	450>245	11.9 ± 7.0
diacetoxyscirpenol	9.53	350>229	378>124	56.9 ± 10.3
nivalenol	9.89	289>73	379>73	29.6 ± 2.7
neosolaniol	11.24	252>195	252>167	40.6 ± 4.3
HT-2	14.66	347>157	347>185	86.7 ± 7.8
T-2	14.71	350>259	350>229	81.9 ± 5.8

All limits of quantification for the analytes were in between 1 and 4qg-Kg^{-1}. In terms of the matrix effect the optimized method demonstrated a good linearity for all mycotoxins except for neosolaniol, HT-2 and T-2 which showed a slight enhancement in the analytical response.

The method's recovery and precision were calculated by analyzing spiked pollen samples at three concentration levels: 80µg·Kg^{-1}, 250µg·Kg^{-1} and 1000µg·Kg^{-1}, repeatedly.

In general, recoveries were satisfactory, with values in between 91 and 104% with RSDs > 11% for the analytes except for deoxynivalenol and nivalenol.

The obtained results from Table XXVI showed a good linearity for all analytes within the tested range.

Table XXVI. Analytical performance of the proposed method

Analyte	LOQ (µg·Kg^{-1})	Recovery (%)			Precision (%RSDs)	Linearity r^2	Matrix effect (%)
		80µg·Kg^{-1}	250µg·Kg^{-1}	1000µg·Kg^{-1}			
deoxynivalenol	1	86.2	119.5	82.9	17.5	0.9307	68
3-acetyl-deoxynivalenol	1	88.1	118.9	88	5.7	0.9447	70
fusarenon X	4	83.4	119.6	87.4	10.6	0.9317	63
diacetoxyscirpenol	4	85.2	119.4	83.2	3.1	0.9265	86
nivalenol	1	77.5	119.4	83.5	26.3	0.9111	30
neosolaniol	2	76.8	118.7	90.1	1.0	0.9230	141
HT-2	1	72.8	141.6	86.9	6.4	0.9110	115
T-2	4	77.9	138.7	85.8	2.8	0.9001	122

I.1.6.4. Conclusions

The new nation parameters in terms of linearity, precision and LOQ. For the purpose of assessing the health risks to humans, knowledge of the mycotoxins present in bee pollen can be crucial.

A QuEChERS-based extraction technique was employed to extract a total of eight mycotoxins from pollen samples. Remarkably, this procedure eliminated the necessity for an additional purification step. The extraction, chromatographic, and detection parameters were meticulously fine-tuned with the dual aim of enhancing both the efficiency of sample processing and the sensitivity of the analysis.

This methodology not only enables a higher throughput of samples but also ensures the accurate quantification of the targeted mycotoxins. The validation of this approach yielded favorable results across various important parameters, including linearity, precision, and limit of quantification.

I.2. CHEMICAL COMPOUNDS WITH NUTRITIONAL AND THERAPEUTIC VALUE IN VEGETABLE PRODUCTS

Food represents one of the physiological factors indispensable to human health. All the body's vital functions depend, to a great extent, on food. It ensures cellular and tissue recovery, the basic energy requirement and the relationship with the environment. Rational nutrition ensures the health of the population and increases the body's resistance to harmful factors.

Therefore, to ensure the nutritional needs of the body, the quality of the nutritional principles ingested through food intake is defining.

Knowing the chemical composition of food products is absolutely essential, both for establishing their biological value for the purpose of a correct, balanced diet, and for understanding and directing the processes that happen on food during storage, preservation, culinary or industrial processing [75].

The main groups of chemical compounds that enter the chemical composition of all food products are: water, mineral substances, organic substances (proteins, carbohydrates, lipids, vitamins, organic acids, enzymes, dyes). Another general classification divides food principles into 3 large categories: macronutrients (carbohydrates, lipids, proteins), micronutrients (mineral substances, vitamins) and other nutrients (water, dietary fibers, bioactive substances).

In order to achieve the nutritional requires of the body, it is necessary to take into account not only the nutritional value of the ingested food, respectively their content in nutritional principles, but also how much of it is absorbed and used by the body and the transformations that the nutrients go through along the digestive tract.

After ingestion, the nutritional principles present in food are subjected, in a first phase, to biodegradation (catabolic) processes, after which small molecules (metabolites) are produced; in the next stage, within the biosynthesis (anabolic) processes, the compounds formed in the previous stage are transformed into the body's own compounds - "bioconstituents", which will ensure growth, development, differentiation and maintenance of vital functions.

Inadequate intake of food, respectively incomplete absorption of nutritional principles progressively leads to states of imbalance materialized by symptoms of deficiency or abuse. Exploring the nutritional status of the body is necessary to promptly correct the phenomena of deficiency or excessive supply of nutritional principles.

Knowing and appreciating food from a nutritional point of view, but also from a biological point of view, allows us to select them according to the satisfaction of the body's requirements.

Food analysis is a requirement for verifying product quality, implementing regulatory regulations, and verifying compliance with national and international food standards, manufacturer specifications, and nutrition labeling requirements.

This is a diverse and interdisciplinary field of research with a significant impact on health, but also from a social and economic point of view. The chemical composition, quality, traceability, sensory perception, nutritional value of food products are strong points that characterize the food from a hygienic-sanitary point of view. The composition of food is often very complex and depends on a variety of factors, including genetic and geographic origin, environmental and climatic conditions, type of agriculture, cultivation and processing practices, and the presence of contaminants, etc.

Foods of vegetable origin are rich in carbohydrates but contain less protein and lipids.

Just like animal-based foods, plant-based foods should not be missing from a balanced diet, because they provide a multitude of macro and micronutrients, along with extremely valuable bioactive substances.

Legumes, such as beans, peas, lentils, soy, contain proteins and carbohydrates, essential fatty acids, minerals (calcium, potassium, phosphorus, iron, magnesium) and vitamins (B1, B2, C, E, PP).

Phaseolus genus includes a variety of species, among which *Phaseolus vulgaris* (common beans) are the most widely cultivated in the world, and *Phaseolus coccineus* (runner beans) are cultivated predominantly in America and Europe, for seeds and ornamental purposes [76].

Although less cultivated and studied in Romania, *Phaseolus coccineus L.* are of particular interest on the international level [77]. Some of their aspects that were relatively recently studied were: chemical composition, organoleptic properties, physico-chemical characteristics [76], the emulsifying and foaming properties of proteins [78], the effect of oligosaccharides on protein foaming properties [79], oligosaccharides degradation capacity of a-galoxidase [80], the anti-proliferative and anti-oxidative effect of dimeric lectin [81], the antineoplastic potential and the specific antifungal action of sialic-lectin [82], the influence of hydrothermal treatment on digestibility [83], the influence of methyl jasmonate on the response to oxidative stress in copper-treated *P. coccineus L.* [84]. Those studies have been conducted on dry seeds of *P. coccineus*, as those were the most researched vegetal material compared to the pods or the green seeds of the plant [85–87].

Food products of vegetable origin contain, in addition to the components with a known and indispensable biological role in human nutrition (macronutrients and micronutrients) and other substances (natural pigments, organic acids, enzymes, volatile oils, etc.), in low concentrations, which can fulfill, in the food or in the body, specific roles.

Currently, the following categories of components are included in the group of bioactive substances: organic acids, alkaloids, phytoncides, enzymes, hormones, glycosides, natural pigments, tannins, and essential oils.

Plant pigments are organic substances with a varied structure that are found in flowers, leaves, fruits, and plant tissues, giving the aroma, taste and color of plant products. Ingested together with the foods that contain them, they participate in numerous biochemical processes in the body: they participate in redox reactions, influence vascular permeability, participate in the transformations the ascorbic acid goes through in the human body. The main groups of plant pigments are chlorophylls, carotenes, flavonoid pigments, anthocyanins and betaines.

Flavonoids are low molecular weight polyphenolic phytochemicals, derived from the secondary metabolism of plants and play an important role in different biological processes. The intake of flavonoid-containing foods may vary depending on the culinary habits of different countries.

Depending on their structure, the large group of flavonoids can be divided into six categories: flavones, flavanones, flavonols, flavanol, isoflavones and anthocyanidins. Given the immense number of known flavonoid derivatives, their diversity has a direct implication on the molecular targets that have been found to represent important hubs for therapeutics. This is exactly why flavonoids still represent key compounds for therapy and research [88–90]. Flavonoids have several health benefits such as being powerful antioxidants, assisting in reducing oxidative stress and protect cells against damage caused by free radicals. They can also have anti-inflammatory, antiviral, antibacterial and anti-cancerous effects.

Personal contributions related to chemical compounds with nutritional and therapeutic value in plant products are presented below:

ISI ARTICLES

1. Liliana Avasilcai, Gabriel Teliban, **Daniela Ionela Morariu**, Vasile Stoleru, Nela Bibire, Mădălina Vieriu, Alina Diana Panainte, Neculai Munteanu. Parameters of Chemical Composition of Phaseolus coccineus L. Pods Grown in Protected Areas, *Revista de Chimie*, 2017, 68(12): 2955-2958, **IF = 1.412** https://doi.org/10.37358/RC.17.12.6015

BDI ARTICLES

1. I.I. Lungu, B. Huzum, Ioana Alexandra Humulescu, Oana Cioancă, **Daniela Morariu**, Ionela-Lăcrămioara Șerban, Monica Hăncianu. Flavonoids as promising therapeutic and dietary agents. *The Medical-Surgical Journal*, 2020, 124(1):151-156 https://www.revmedchir.ro/index.php/revmedchir/article/view/2062/1600.

I.2.1. Determination of Chemical Composition of Food

I.2.1.1. Aim of the Study

Our study focused on figuring out crop-specific characteristics in protected areas of four varieties of *P. coccineus L.* homologated in the UK (Lady Di, Desiree, Polestar and White Apollo) and also on the determination of chemical composition parameters of their pods, in order to highlight the particularities that could determine the expansion of their cultivation in our country.

I.2.1.2. Materials and Methods

The biological material consisted of four varieties of bean originated from the UK: Lady Di, Polestar, Desiree, and White Apollo as there are no homologated varieties in Romania. The experimental protocol consisted of organizing the area in subdivided parcels with three replicates, each replicate comprising of six hills with two seeds each.

The research was initiated at the Teaching Reservation of the Ion Ionescu de la Brad University of Agricultural Sciences and Veterinary Medicine of Iasi by preparing a protected area, covered with a polyethylene film. The soil conditions were cambic chernozem (black earth) with medium fertility, 3% organic substance and pH = 6.5. The temperature during the vegetation period was 18°C and the relative humidity of the air was 64% (AgroExpert 2014).

The determination of the chemical composition parameters was conducted at the Faculty of Pharmacy of Grigore T. Popa University of Medicine and Pharmacy of Iasi on samples of two types of pods: pods of maximum length (Lmax) and of % of maximum length. Each parameter was determined three times on three replicates.

The determination of moisture, ash, organic nitrogen, proteins, glucose, vitamin C, calcium, magnesium, iron and copper were performed according to AOAC International methods [91–99].

Evaluation of the global mineral content in foodstuffs is conducted gravimetrically after the calcinations of the sample. The residue obtained after dry ashing contains the total amount of mineral substances such as salts or oxides.

The Kjeldahl method was used to determine the presence of proteins.

Organic matter is oxidized with boiling sulfuric acid, in the presence of a catalyzer (HgO and K_2SO_4, for the increase of the boiling point of the mixture); proteic nitrogen is fixed as ammonium sulfate; by using vapors as carriers, in alkaline medium, ammonia is released and fixed in a solution of sulfuric acid of known titer.

The determination of calcium and magnesium was conducted by the titrimetric method.

Calcium is titrated at pH 12-13 (NaOH) with complexon III in the presence of murexide, with a change of color from pink red to intense violet.

The sum calcium + magnesium is titrated with complexon III at alkaline pH (ammonia buffer) in the presence of eriochrome black T, with a change of color from red to blue.

The quantitative determination of ascorbic acid can be conducted using titrimetric or spectrophotometric methods.

The determination of copper was carried out by a spectrophotometric method and is based on the following principle: Cu+2 ion forms a yellow complex with sodium dithiocarbamate at pH 9-10, extractable in organic solvents, with a maximum absorption at 436 nm.

The determination of glucose was conducted by a titrimetric method using Feeling reagent in excess, and the excess reagent was iodometrically titrated with sodium thiosulphate. The volume of sodium thiosulphate used for titration is proportional with the content of reducing sugar in the sample.

The determination of iron was carried out by a spectrophotometric method through the reaction with ortho-phenanthroline, after the reduction of trivalent iron, to bivalent iron with hydroxyl amine hydrochloride, when a red colored compound results.

I.2.1.3. Results and Discussions

Runner beans Lady Di produces red flowers that form 30cm long tender pods. The pods are completely without strings, delicious and especially succulent, due to the very slow development of purple seeds with black markings.

Desiree produces a very abundant crop of large beans that grows well in normal and drought conditions. That variety produces white flowers and wide and stringless pods up to 30cm long with few white seeds. The pods are glossy, aromatic and savory.

Polestar is a British variety created for high production and reliability, which led it to become the favorite of the growers. The succulent, aromatic, stringless pods grow in bunches and reach up to 25cm in length. It is precocious in producing red flowers and yields high throughout the season.

White Apollo produces straight, smooth and stringless pods up to 37cm long, with an excellent flavor. This variety produces prolific crops over a long harvest period, with high yields throughout the summer.

All of these features (Figure 8) were based on the observations recorded during the experiment and they were consistent to literature data [76].

The humidity of fresh pods was above 90% in all investigated samples, and it varied for the ¾ Lmax pods in between 91.13% (White Apollo variety) and 93.68% (Polestar variety), and for the Lmax pods in between 91.31% (Desiree variety) and 93.00% (Polestar variety). In the literature there is data on the moisture content only for seeds of Nata and Karo varieties of *P. coccineus L.*, for which the recorded values were 11.1±0.0, and 12.0±0.0% respectively [83].

The determination of mineral substances as ash obtained through calcination provided the following results: in the case of the ¾ Lmax pods the value was 0.60% for the Lady Di and Desiree while for the variety White Apollo 0.76%, 0.63% for Polestar and 0.73% for White Apollo, the pods at maximum length. Other authors obtained values of 5.24±0.24% and 4.29±0.26% for the fresh seeds of *P. coccineus L.* of the Nata and Karo varieties [83].

Determination of organic nitrogen using the Kjeldahl method led to a range of values for ¾ Lmax pods in between 1.03% for Lady Di and 2.43% for Desiree and for the Lmax pods in between 1.59% for White Apollo and 3.01% for Desiree.

Lady Di Desiree

Polestar White Apollo

Figure 8. The aspect of the four varieties of *P. coccineus L.*

The percentage of proteins in the composition of the pods was calculated based on organic nitrogen concentrations by multiplying it by 6.25 [83]. The values obtained were between 6.43% (Lady Di variety) and 15.18% (Desiree variety) for fresh % Lmax green beans, and between 9.93% (White Apollo variety) and 18.81% (Desiree variety) for pods at maximum length.

The glucose concentration in the samples harvested at % of the maximum length was 2.79 g% for Lady Di, 1.51 g% for Desiree, 4.01 g% for Polestar and 2.13 g% for White Apollo variety. The values of 1.92 g%, 2.59 g%, 2.15 g% and 2.08 g% respectively were determined for the pods harvested at their maximum length.

Calcium concentration in fresh pods samples harvested at ¾ of the maximum length varied between 11.90mg% for Desiree and 45.10mg% for Lady Di. For the fresh pods harvested at maximum length, the variation range was between 33.76mg% for Lady Di and 58.58mg% for White Apollo.

Magnesium concentration ranged between 27.09mg% (Lady Di) and 44.02mg% (Desiree) in freshly harvested samples of % of the maximum length, respectively between 28.31mg% (White Apollo) and 52.48mg% (Desiree) on samples of fresh pods harvested at maximum length.

The iron concentration ranged from 0.23 mg% (Desiree) to 0.54 mg% (Lady Di) for fresh pods harvested at % of the maximum length and from 0.43 mg% (Lady Di) to 0.51 mg% (Polestar) when the samples had maximum length. The spectrophotometric method applied for the determination of iron, the regression equation was $A = 0.0136C + 0.0048$ and $R^2 = 0.9946$.

Copper concentration varied between 0.1mg% in White Apollo and 0.31 mg% in Lady Di for pods at % Lmax, while for Lmax pods it varied between 0.18 mg% for Polestar and 0.54 mg% for Desiree. The equation of the regression line of the spectrophotometric determination of copper was $A = 0.0331C + 0.0023$ with $R^2 = 0.9988$.

The concentration of vitamin C in frozen pods samples was determined using a spectrophotometric method within a 49 days period after freezing, because that method of conservation is equally relevant to both consumers and processors of vegetables [76]. The calibration line and the equation of the regression line are shown in Figure 9.

Table XXVII provides the results obtained, comparatively, for samples of fresh green beans harvested at % of maximum length and at maximum length (L).

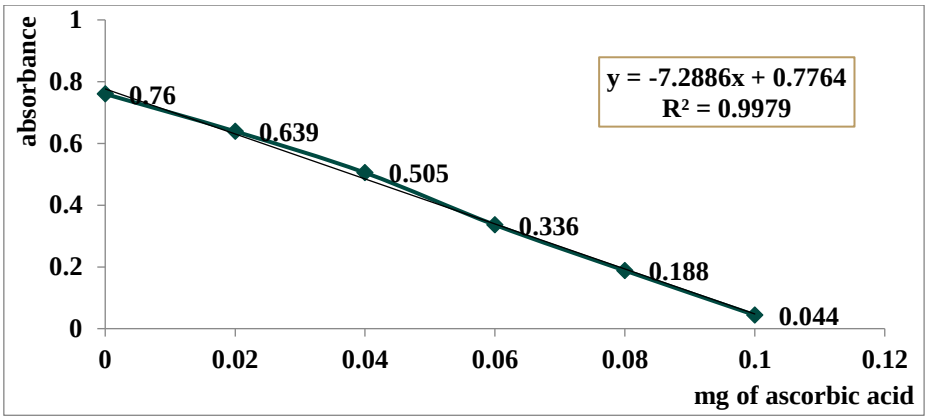

Figure 9. Calibration curve for the determination of ascorbic acid

Table XXVII. Macro- and micronutrient content of fresh pods

Variety	Lady Di		Desiree		Polestar		White Apollo	
Vegetation stage	¾ L_{max}	L_{max}	¾ L_{max}	L_{max}	¾ L_{max}	L_{max}	¾ L_{max}	L_{max}
Moisture (g%)	92.90	92.96	92.97	91.31	93.68	93.00	91.13	91.39
Ash (g%)	0.60	0.65	0.60	0.72	0.66	0.63	0.76	0.73
Organic nitrogen (g%)	1.03	1.81	2.43	3.01	1.78	2.07	1.73	1.59
Proteins (g%)	6.43	11.31	15.18	18.81	11.12	12.93	10.81	9.93
Glucose (g%)	2.79	1.92	1.51	2.59	4.01	2.15	2.13	2.08
Ca (mg%)	45.10	33.76	11.90	46.56	24.22	33.99	40.20	58.58
Mg (mg%)	27.09	40.51	44.02	52.48	39.97	33.98	33.33	28.31
Fe (mg%)	0.54	0.43	0.23	0.46	0.45	0.51	0.45	0.49
Cu (mg%)	0.31	0.26	0.19	0.54	0.25	0.20	0.17	0.18

The first determination was performed 7 days after freezing, and then the next two determinations were done 28 and 49 days after freezing. Evolution of ascorbic acid concentration during storage at -18°C of pods is shown in Figure 10 and the percentage decrease of vitamin C concentration for each *P. coccineus L.* variety correlated with the notations in Figure 10 is represented in Table XXVIII.

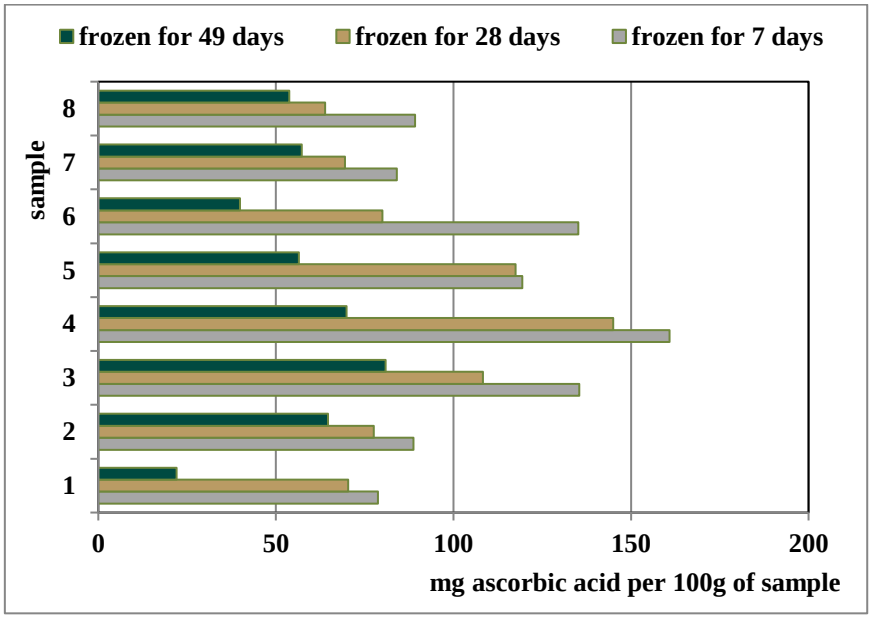

Figure 10. Evolution of ascorbic acid concentration in the *P. coccineus L.* samples

Table XXVIII. Percentage decrease of ascorbic acid content after freezing

Sample	Variety	The length of the harvested pods	Percentage decrease of baseline (%)
1	Lady Di	¾ L_{max}	72.00
2		L_{max}	27.08
3	Desiree	¾ L_{max}	40.29
4		L_{max}	56.55
5	Polestar	¾ L_{max}	39.15
6		L_{max}	70.49
7	White Apollo	¾ L_{max}	31.81
8		L_{max}	39.71

There is no data in the literature on the production of *P. coccineus L.* and no chemical compositional determinations; the vast majority of the data in the literature refer to the physico-chemical and sensory properties of *P. coccineus L.* seeds, most of the time compared to *Phaseolus vulgaris*. From this point of view, our study can be regarded as a starting point in extending the study of the runner beans.

In order to achieve a good correlation between our results and those published by other authors, we also performed the same determinations of chemical composition parameters for dehydrated pods, the results of which are shown in Table XXIX.

Table XXIX. Macro- and micronutrients content of dehydrated pods

Variety	Lady Di		Desiree		Polestar		White Apollo	
Vegetation stage	**¾ L_{max}**	**L_{max}**	**¾ L_{max}**	**L_{max}**	**¾ L_{max}**	**L_{max}**	**¾ L_{max}**	**L_{max}**
Moisture (g%)	8.21	8.21	7.39	8.38	8.60	8.00	8.13	7.69
Ash (g%)	7.73	7.56	8.59	9.06	8.80	8.15	8.71	8.96
Organic nitrogen (g%)	2.21	2.36	2.91	3.24	2.83	2.61	2.77	3.35
Proteins (g%)	13.81	14.75	18.81	20.25	17.68	16.31	17.31	20.93
Ca (mg%)	351.95	403.83	261.90	327.92	234.47	294.14	284.50	414.76
Mg (mg%)	370.92	365.93	415.11	400.90	333.67	394.70	347.43	370.25
Fe (mg%)	3.80	1.61	1.50	3.36	1.52	4.45	3.46	6.70
Cu (mg%)	0.97	0.63	0.78	0.85	0.64	0.55	0.70	0.68

The values obtained for protein concentration in dehydrated pods were comparable to those reported by other authors for *P. coccineus L.* seeds: 24.94±0.05% and 27.56±0.07% respectively [83]. From a nutritional point of view the intake of 100g of *P. coccineus L.* pods provides 4.60% Ca, 4.68% Mg and 3.97% Fe, out of the recommended daily intake for those minerals [75,100].

Regarding the vitamin C intake, known for its antioxidant properties, it is obvious that fresh pods offer the maximum amount of ascorbic acid compared to the frozen or dry pods. The dynamic determination of ascorbic acid revealed the inverse proportionality between the duration of freezing and the amount of vitamin C preserved through that conservation method. There was a decrease of over 70% of the initial amount of vitamin C in the early pods with % Lmax of Lady Di and for and Lmax pods for Polestar, while in Desiree and White Apollo varieties, the decrease in concentration of ascorbic acid was 40.29% (% Lmax Desiree), 56.55% (Lmax Desiree), 31.81% (White Apollo-Lmax) and 39.71% (White Apollo-Lmax) respectively.

These data support *P. coccineus* to be recommended as an important source of antioxidants among other vegetables [101] that should be taken into account as part of the medical nutrition therapy in conditions associated with high oxidative stress status [102].

I.2.1.4. Conclusions

The biological material, either seedling or seeds, used to set up the crop had a major influence on harvest time as the highest production values were recorded for crops obtained

from seedlings as far as early production and total production.

The results obtained for the chemical composition parameters varied within narrow range in-between varieties and were consistent with the literature data when comparing the dehydrated pods with the fresh seeds of *P. coccineus L.* Also, the results obtained for each parameter were consistent for the two considered vegetation stages (maximum length and % Lmax) and were comparable to the results reported in the literature.

I.2.2. The Importance of Bioactive Compounds in Nutrition and Therapeutics

I.2.2.1. Aim of the Study

The purpose of this review is to present the benefits of flavones used as food and therapeutic agents. This analysis includes recent relevant reports from the databases accessed in order to develop scientific knowledge about this subject.

I.2.2.2. Materials and Methods

Pharmacokinetic properties and therapeutic potential are described based on data from the literature using the following keywords in the search: flavonoids, oxidative stress, therapy, pharmacokinetics.

I.2.2.3. Results and Discussions

Pharmacokinetics

The absorption of flavonoids depends on the processing of the vegetal matrix and the chemical structure of each compound. Flavonoids are highly metabolized in the intestine especially by P-glucosidases (acts on monoglucosides) lactase-phlorizin hydrolase (active on glucose and galactose derivatives) and, as a result, their metabolites are transported to the liver. Metabolites formed in the liver can be transported to the target cells, reenter the enterohepatic circulation through bile excretion, be hydrolyzed or excreted through urine or feces. Catechins are usually absorbed rapidly from the intestine, but dimerization leads to a decrease in their absorption. Also, trimers can be metabolized, but larger oligomers can only be transformed by the gut microbiota in the larger intestine [88,103,104].

Numerous research has concentrated on improving the bioavailability of flavonoids using various strategies, such as inhibiting the enzymes that restrict their bioavailability, changing the composition of food matrices, or increasing the rate of dissolution. Moreover, the addition of alcohol (as wine) or proteins (milk) did not influence the absorption of flavonols and catechins [89,105]. For example, hesperidin and its aglycone (hesperidin) are two flavonoids from citrus species that have different biological properties. Hesperidin is transformed into hesperidin by the colonic microflora, and it can be directly absorbed by enterocytes, is a cytochrome P450 inhibitor that can affect the metabolism of other drugs such as diltiazem, verapamil and felodipine [106].

Biologic and therapeutic potential

Flavones have antioxidant, antiproliferative, antitumor, antimicrobial, estrogenic, anti-inflammatory activity and are also used in cancer, cardiovascular disease, neurodegenerative disorders [90,104,107–109]. Also, flavonoids affect several mammalian enzymes such as protein kinases that regulate multiple cells signaling pathways and modifications of multiple cell signaling pathways. Apigenin and luteolin are commonly found in a variety of foods such as parsley, celery, artichoke, peppers, carrots, thyme, rosemary, oregano, olive oil, peppermint, salad, pomegranate, cucumber, lemon, beet, cabbage, cauliflower, spinach, and green tea. It is well documented that luteolin acts as an antioxidant, anti-inflammatory, anticancer and inhibitor of prooxidant enzymes, both *in vitro* and *in vivo* [108,110–113].

Antioxidant potential

High concentrations of free radicals can oxidize biomolecules in biological systems, causing tissue damage, cell death, or a number of illnesses like cancer, cardiovascular disease, arteriosclerosis, neurological problems, skin irritation, and inflammation [110,111,114]. Free radicals are highly reactive and therefore can attack membrane lipids, generating carbon radicals and producing radicals that cause lipid peroxidation. Therefore, a single radical can damage many molecules by initiating lipid peroxidation reactions. To counteract the vicious effect of free radicals, the body has several mechanisms of defense of antioxidants in the form of enzymes such as superoxide dismutase and catalase, copper and iron transport proteins, as well as water-soluble and lipid-soluble antioxidants. It has been studied that the imbalance between free radicals and the antioxidant defense mechanism is associated with several human diseases [103,107].

Antioxidants can stop oxidation from starting or they can break the chain by acting as antioxidants. Preventing the initiation of oxidation occurs by inhibiting the production of superoxide anions, degradation of hydrogen peroxide and chelation or reduction of metal ions, while chain-breaking antioxidants act by eliminating radicals, largely hydroxyl radicals, thus inhibiting the chain of events. Oxidative agents lead to damage of lipid membranes, proteins and DNA. Moreover, oxidative species and free radicals are involved in the pathophysiology of many diseases, such as neurodegenerative disorders, cardiovascular, cerebrovascular, autoimmune disorders such as diabetes, rheumatoid arthritis, psoriasis, etc. Therefore, different natural and synthetic antioxidants are used to eliminate free radicals [103,104,112].

Flavones have been discovered to have several actions including a well-known antioxidant activity. Therefore, flavones are significantly used in the pharmaceutical and food industry. Flavones, such as luteolin and apigenin, which contain two or three free hydroxyl groups in A / B rings, exhibit antioxidant properties at low concentrations [112,113].

According to certain research that used a QSAR model for various flavones to assess the relationship between the structure and the strength of the antioxidant activity, the activity gets better as the number of hydroxyl groups increases. Moreover, two neighboring hydroxyl groups have better effects. Different flavone conjugates, such as complexes C_{60}-flavone, have been evaluated for the antioxidant potential in which the flavone phenolic fragment reacts with peroxyl radicals, while the C_{60} part of the molecule acts synergistically by trapping alkyl radicals under reduced O**2** partial pressure. Also, a natural flavone extracted from the tropical plant *Coronopus didymus* was tested for their ability to inhibit lipid peroxidation induced by y radiation, Fe (III) and Fe (II) radiation and showed a better protective effect [107].

The 5-hydroxyl group in combination with the 4-keto and catechuic hydroxyl groups chelates catalytic active metal ions involved in redox reactions, which may prevent the formation of oxidizing species. The most harmful of the reactive oxygen species generated in the Fenton reaction is the hydroxyl radical, which can induce lipid peroxidation (Figure 11).

$$Fe^{2+} \longrightarrow H_2O_2 \longrightarrow Fe^{3+} + H\dot{O} + OH^-$$

$$O^{2-} + H_2O_2 \longrightarrow O_2 + OH^- + H\dot{O}$$

Figure 11. Fenton reaction

The hydroxyl groups on ring B donate the hydrogen and one electron to the hydroxyl, peroxyl- and peroxynitrite radicals, stabilizing them, thus forming a relatively stable flavone radical (Figure 12) [107].

Other investigations have demonstrated the connection between inflammation brought on by various processes and cognitive impairment and the effectiveness of luteolin/apigenin therapy in reducing inflammation in the brain [111,115,116]. Some studies have also reported

that the neuroprotective effect of luteolin is related to its antioxidant activity. Improvements in cognitive function due to luteolin treatment have been linked to its antioxidant activity in a streptozotocin-induced diabetes model. For example, neuronal injury and cognitive dysfunction are found in streptozotocin-induced diabetes, and chronic luteolin exposure can reduce neuronal injury and improve cognitive performance. This effect of luteolin is related to its ability to increase antioxidant activity [117,118].

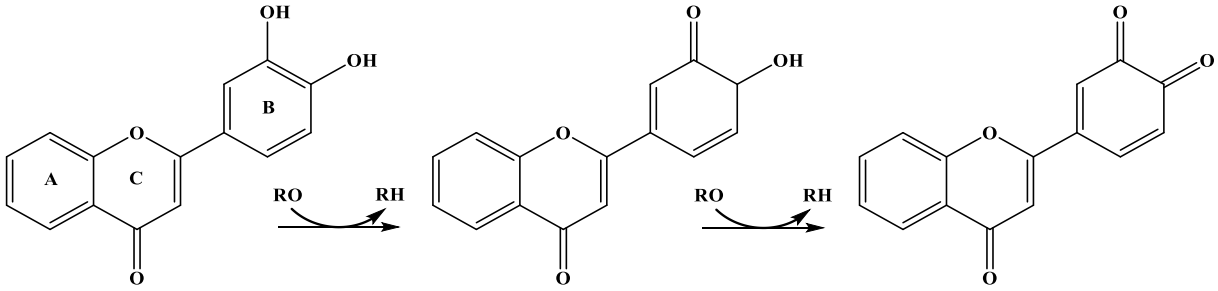

Figure 12. Changes in the structure of 3,4'-dihydroxyflavone (RO-free oxidized radicals; RH-reduced radicals)

Lipid peroxidation is increased, while antioxidant enzyme activities in the cortex and hippocampus are decreased in diabetes. However, chronic luteolin treatment may alleviate such changes induced by diabetes. In addition, apigenin rich extracts have been shown to reduce scopolamine-induced neuroinflammation, 1-p interleukin level, acetylcholinesterase, butyryl cholinesterase, lactate dehydrogenase and malondialdehyde activity. Moreover, the same doses (25 mg/kg and 75 mg/kg) restored the normal level of all antioxidant enzymes and the expression of the brain-derived neurotrophic factor [111,115].

According to this research, the anti-inflammatory properties of apigenin and luteolin, as well as other biological properties including antioxidant activity, could make them attractive for the prevention and treatment of neurodegenerative illnesses, such as Alzheimer's [88,103,111,115,116].

Antidiabetic potential

The apathetic lifestyle and energy-rich diet are now aggressively contributing to an astonishing increase in obesity, leading to insulin resistance, to type 2 diabetes along with retinopathy and nephropathy. Various enzymes such as a-glucosidase, aldose reductase, protein tyrosine phosphatases, protein kinase C are associated. The a-glucosidase enzyme is an intracellular enzyme involved in the breakdown of complex carbohydrates, which aids in the absorption of ingested carbohydrates, increases post-prandial blood sugar. Aldose reductase is a key enzyme in the pathway of polyol in which glucose is changed to sorbitol and its accumulation is linked to diabetic complications. It also involves the formation of advanced glycation end products (AGEs). Diabetic complications such as retinopathy, nephropathy, neuropathy is caused by an increase in the advanced formation of glycation end products and activation of protein kinase C isomers. Among them, AGEs are known to cause ageing, as well as diabetic complications [103,104,118].

Various flavone derivatives, both natural and synthetic, have been reported by numerous scientists to be effective in treating diabetes and associated consequences. In one study, various flavone 6- and 7-hydroxy flavone hybrids with aminopropanol were synthesized and evaluated as novel antidiabetic agents. Of all the synthesized hybrids only two were found to be the strongest. These active compounds possess a voluminous lipophilic substitution on ring B of flavone and smaller substituents on the nitrogen atom. Hybrids with tert-butyl and isopropylamine functionality at the N atom are of higher activity [103,104,107,118,119].

On the other hand, luteolin has inhibited the a-glucosidase enzyme up to 36% at a

concentration of 0.5 mg/mL and has the potential to suppress postprandial hyperglycemia in non-insulin-dependent diabetes mellitus. 6-amino-5,7-dihydroxyflavone is a unique flavone having an amino moiety together with the usual 5,7-dihydroxyl substituents, which have been shown to be active against rat intestinal a-glucosidase. Initial studies suggested that 6-amino and 7-hydroxyl groups were essential for activity. The 5-hydroxyl substitution was favorable, while the 8-amino group was unfavorable for activity [120,121].

Cardiovascular protective activity

It is commonly established that memory and cognition are positively impacted by a healthy cardiovascular system. Furthermore, most of the risk factors are determinant both for cardiovascular and brain dysfunction. Observations in regard to flavonoid-rich food intake indicated that the best benefit is in at-risk populations. Various cocoa products were administered to a wide range of people and the results indicated a decrease in blood pressure of older generation, especially in those with impaired vascular function. Nevertheless, there are still conflicting studies in which tea and other foods associated with high fat load meals could or couldn't influence blood pressure. Therefore, further investigations are necessary to elucidate the mechanisms and the real potential of such compounds [89,103,104].

Numerous isoflavones, including daidzein, genistein, and equal, which are known to generate hormone-like action, have been detected in human studies and were recognized to have a beneficial effect on cardiovascular diseases and osteoporosis. Such flavonoids have shown a positive influence on various parameters associated with cardiovascular complications (atherosclerosis, platelet aggregation, lipoprotein peroxidation). A significant improvement was identified for selective extracts rich in anthocyanins. In this case, several randomized trials indicated no secondary effects and the restoration of the HDL/LDL-cholesterol balance to normal parameters [89,104,112].

I.2.2.4. Conclusions

Metabolic imbalances, cardiovascular issues, and neuronal degeneration stand as significant lifestyle hurdles in contemporary society. In light of this, proactive measures are of utmost importance, with dietary decisions playing a pivotal role in addressing these challenges. Optimal nutritional and functional attributes should guide food choices.

Plant-derived products provide a wide array of bioactive compounds with potential pharmacological benefits. These substances show potential in addressing age-related deficiencies through a range of mechanisms. An ongoing focus revolves around the exploration of flavonoids, which may open doors to novel therapeutic approaches with substantial potential for improving human health and well-being.

Chapter II. THE IMPACT OF DIET IN VARIOUS PATHOLOGIES

Food plays a significant role in promoting and maintaining health throughout life, and a non-sanogenic diet is involved in the determinism of many chronic diseases with an alarmingly increasing incidence and prevalence in today's civilization, such as obesity, type 2 diabetes, cardiovascular diseases, gastrointestinal diseases, autoimmune diseases, cancer, etc.

An unhealthy diet is one of the four modifiable behavioral risk factors (along with smoking, alcohol consumption, and physical inactivity) that contribute to most chronic major diseases.

A common feature of these pathologies, mainly metabolic, however different their production mechanism may be in some cases, is that each of them can be prevented through various lifestyle improvement measures, by practicing healthy eating habits. Also, for each pathology there are food recommendations that can reduce the risk of developing that disease, as well as warnings regarding some food components that can aggravate it.

Furthermore, there is now a causal relationship between the gut microbiome and predisposition to certain human diseases, and the interactions between diet, gut flora and human health are highly complex and multidirectional.

An in-depth understanding of the molecular mechanisms by which the microbiome influences the diet-host relationship would allow the optimization of health strategies through diet, depending on the gut microbial profile.

For precision nutrition, those individual characteristics that can predict the effect on health following adherence to a particular diet must be assessed.

A correctly applied diet combined with a healthy lifestyle can significantly reduce the risk of various current diseases.

II.1. GASTROINTESTINAL PATHOLOGIES

Due to the modern lifestyle, gastrointestinal pathology has been increasing nowadays and is characterized by the appearance of disorders of the digestive tract and/or the ancillary organs.

Increasing consumption of highly processed foods, very rich in saturated fats, concentrated sugars with fast absorption intensifies gastrointestinal symptoms.

The impact of the diet on various pathologies in the gastrointestinal sphere is major because the digestive system is complex and sensitive to the foods that pass through it.

Gastroesophageal reflux involves irritation of the esophagus by gastric acid, which refluxes from the stomach and sometimes the bile. These acid secretions cause burning sensations and pain in the esophagus, difficulty in swallowing food and feelings of nausea or even vomiting.

Gastroesophageal reflux is increasingly prevalent worldwide, particularly in the Western world, with a prevalence of up to 40% in population studies. Eating habits can be a trigger for dyspeptic symptoms. Frequent consumption of non-vegetarian and fried foods, carbonated beverages, coffee and black tea has been associated with gastroesophageal reflux disease.

Irritable bowel syndrome (IBS) is a frequent functional disorder of the gastrointestinal tract (GI) designated by the Rome IV diagnostic criteria, considering the increase in the number of daily cases, as there are currently more than 3.9 million female patients and more than 3.0 million male patients who present this pathology worldwide [122]. IBS causes changes in bowel habits in terms of diarrhea and/or constipation, abdominal pain, bloating, and flatulence in adults and children [123,124]. At the same time, it causes a decrease in quality of life (QoL) [125], labor productivity, and higher care costs [126].

Even if the pathophysiological mechanism remains incompletely revealed, altered GI motility, visceral hypersensitivity, intestinal microbiota imbalance [127], altered brain-gut axis [128], inflammation of the digestive tract, and psychological factors appear to determine the occurrence and development of IBS [129].

Irritable bowel syndrome is a typical gastrointestinal disease that causes bloating, flatulence, abdominal pain, diarrhea, constipation in adults and children. A diet low in oligosaccharides, disaccharides, monosaccharides and fermentable polyols (FODMAPs) is one of the potential treatment strategies to reduce abdominal symptoms and increase QoL.

The associated gut microbiota is an important component of human physiology and metabolic homeostasis and can affect health or disease. Many methods are available to modulate the dysbiotic gut microbiota, such as dietary interventions (pre-, pro- and postbiotic), but treatment outcomes vary depending on individual characteristics, genetic background, microbiome diversity, etc.

Celiac disease (CD) is a controllable pathology, found in approximately 1% of the entire population as a reaction to environmental stimuli affecting individuals with genetic susceptibility, causing gluten intolerance, gastrointestinal and extradigestive symptoms, culminating in severe malabsorption. CD, also called gluten-sensitive enteropathy, is characterized by a disturbance of the internal environment associated with histological changes in the small intestine, the most important of which is the subtotal atrophy of the villi with hyperplasia of the crypts. Clinically, it is manifested by a wide spectrum of symptoms, from gastrointestinal disorders (diarrhea, bloating, weight loss, and abdominal pain) to extra-intestinal symptoms (iron deficiency anemia, delayed puberty, and oral ulcers), centered on variable degrees of malabsorption [130,131]. With an estimated prevalence of 1:100, serological screening is performed by titrating anti-tissue transglutaminase antibodies (TGAs) considered positive at values over two times the normal limit, doubled by genetic testing for human leukocyte antigen HLA-DQ2 or HLA-DQ8 and total immunoglobulin A dosing.

The celiac disease –systemic lupus erythematosus (CD-SLE) correlation represents a crossroads in the study of autoimmune diseases, both in terms of their onset as well as their development, overlap, and treatment.

My contribution to the management of gastrointestinal disorders resulted in the publication of the following papers:

ISI ARTICLES

1. Ioan Chirila, **Ionela Daniela Morariu**, Oana Bogdana Barboi, Vasile Liviu Drug. The role of diet in the overlap between gastroesophageal reflux disease and functional dyspepsia, *Turkish Journal of Gastroenterology*, 2016, 27(1): 73-80, **IF = 0.966** https://www.turkjgastroenterol.org/content/files/sayilar/290/buyuk/73-80Y.pdf

2. **Ionela-Daniela Morariu**, Liliana Avasilcai, Mădălina Vieriu, Vasile Valeriu Lupu, Branco-Adrian Morariu, Ancuta Lupu, Paula-Cristina Morariu, Oana-Lelia Pop, Iuliana Magdalena Starcea, Laura Trandafir. Effects of a Low-FODMAP Diet on

Irritable Bowel Syndrome in both children and adults – a narrative review, *Nutrients*, 2023, 15(10), 2295:1-21, **IF = 5.9**
https://doi.org/10.3390/nu15102295

3. Vasile Valeriu Lupu, Elena Jechel, Cristina Maria Mihai, Elena Cristina Mitrofan, Ancuta Lupu, Iuliana Magdalena Starcea, Silvia Fotea, Adriana Mocanu, Dragos Catalin Ghica, Costica Mitrofan, Dragos Munteanu, Delia Lidia Salaru, **Ionela Daniela Morariu**, Ileana Ioniuc. Connection between celiac disease and systemic lupus erythematosus in children – a development model of autoimmune diseases starting from what we inherit to what we eat, *Nutrients*, 2023, 15(11), 2535: 1-19, **IF = 5.9**
https://doi.org/10.3390/nu15112535

4. Vasile Valeriu Lupu, Anca Adam Raileanu, Cristina Maria Mihai, **Ionela Daniela Morariu**, Ancuta Lupu, Iuliana Magdalena Starcea, Otilia Elena Frasinariu, Adriana Mocanu, Felicia Dragan, Silvia Fotea. The implication of the gut Microbiome in heart failure, *Cells*, 2023, 12(8), 1158:1-24, **IF = 6**
https://doi.org/10.3390/cells12081158

5. Vasile Valeriu Lupu, Laura Mihaela Trandafir, Anca Adam Raileanu, Cristina Maria Mihai, **Ionela-Daniela Morariu**, Iuliana Magdalena Starcea, Adriana Mocanu, Lacramioara Ionela Butnariu, Gabriela Stoleriu, Delia Lidia Salaru, Tatiana Chisnoiu, Dragos Munteanu, Costica Mitrofan, Ancuta Lupu. Advances in understanding the human gut microbiota and its implication in pediatric celiac disease – a narrative review, *Nutrients*, 2023, 15(11), 2499, IF = 5.9
https://doi.org/10.3390/nu15112499

II.1.1. Gastroesophageal Reflux Disease and Functional Dyspepsia

II.1.1.1. Aim of the Study

The aim of our study was to update the prevalence data for functional dyspepsia and gastroesophageal reflux disease (GERD) and for the overlap of these diseases and to evaluate the type of diet associated with them.

II.1.1.2. Materials and Methods

The study was conducted in a medical center which serves 18,000 people in an area of the city of Iasi, in North East Romania. The sample size and demographic characteristics were calculated to be representative using Epi Info™ 3.5.2 software. We selected 250 subjects from family doctor's patient lists using a randomization function in Microsoft Excel™ software. The family doctors invited the selected subjects by telephone for an interview in their offices. The inclusion criteria were 18-79 years of age and a resident in this urban area. There were no exclusion criteria.

All participants completed an interview-based questionnaire at the family doctor's office to assess eating patterns and frequency of food consumption and to identify gastrointestinal diseases. Using the Rome III and Montreal criteria, general practitioners were trained to identify functional dyspepsia and GERD [132–134].

Socio-demographic factors and general medical history were also included in the interview together with an objective evaluation of obesity (the general practitioners measured the height and weight of subjects).

Health and health-related behaviors were investigated, such as smoking, physical activity, self-perceived stress, and general well-being.

Subjects were considered overweight or obese if the body mass index (BMI) was between 25 kg/m^2 and 29.9 and >30.0 kg/m^2, respectively.

To characterize the frequency of food consumption in the population studied, we used the median as the cutoff point, and the group was divided into two categories of consumers (less than median frequency and equal to or more than median frequency). An initial Spearman's correlation test and cross-tabulation analysis were performed.

These analyses examined whether there was any association between referral patterns, personal history of illness, eating habits, food consumption frequency, and other associated conditions.

Moreover, we used multivariate analysis for risk factors that were significant in univariate analysis, and we calculated odd ratios and 95% confidence intervals (95% CIs) for significant predictors of functional dyspepsia and GERD derived from the initial analysis. A value of $p<0.05$ in both analyses was considered to be relevant for our statistics.

The University of Medicine and Pharmacy's Ethics Committee gave permission to the study, and all participants gave their informed permission.

II.1.1.3. Results and Discussions

Prevalence of functional dyspepsia and GERD

During a period of 4 months, 184 subjects (106 women and 78 men, mean age 49.4 years) participated in the study. The participation rate was 73.6%. Functional dyspepsia was present in 7.6% (3.8% for women and 12.8 % for men, $p<0.05$) of patients, and GERD was present in 31.0% (33.0% in women and 28.2% in men, $p>0.05$) of patients (Figure 13). In total, 25.9% of GERD subjects were diagnosed with functional dyspepsia. Also, 92.9% of functional dyspepsia subjects were diagnosed with GERD. The overlap of the two diseases was 22.4% among subjects with upper gastrointestinal disorders, and in our sample, 7.1% of participants received both diagnoses.

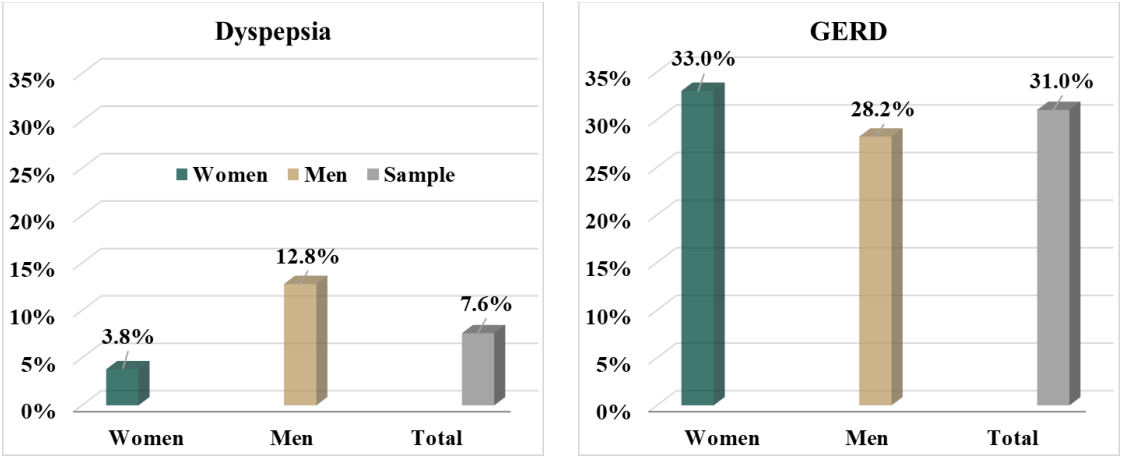

Figure 13. Prevalence of functional dyspepsia (a) and GERD (b) according to gender

The age distribution (Table XXX) indicated an increased prevalence of functional dyspepsia for subjects above the mean age of the sample (11.7% vs. 2.5%, $p<0.05$). The prevalence of GERD also increased with age ($r=0.938$, $p<0.05$).

Table XXX. Prevalence of functional dyspepsia and GERD in different age groups

Age (years)	GERD	Prevalence Dyspepsia
20-29	20.8%	0.0%
30-39	26.5%	2.9%
40-49	25.0%	4.2%
50-59	35.4%	14.6%
60-69	36.8%	13.2%
70-79	37.5%	0.0%

The educational level of the subjects significantly influenced the prevalence of functional dyspepsia in the general population ($p<0.05$). The prevalence of functional dyspepsia was 2.6% among high, 9.5% among medium, and 16.6% among low levels of education. The prevalence of GERD was 27.3% among high, 32.9% among medium, and 40.0% among low levels of education ($p>0.05$) (Figure 14).

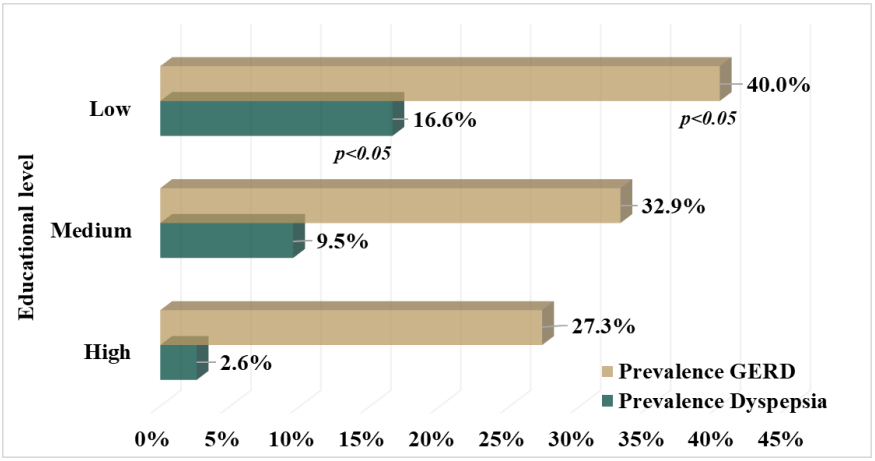

Figure 14. Educational level and prevalence of functional dyspepsia and GERD

Functional dyspepsia, GERD, and health-related behaviors/conditions

Smoking was not associated with functional dyspepsia or GERD ($p>0.05$): 35.7% of dyspeptic patients were smokers vs. 26.5% of non-dyspeptic subjects ($p>0.05$); a similar situation 0%, in dyspeptic patients) and was more frequent in non-GERD was observed in the case of GERD (22.8% vs. 29.1%, $p>0.05$).

The majority of subjects were physically inactive: 71.4% of patients with functional dyspepsia, 63.8% of non-dyspeptic subjects ($p>0.05$), 73.7% of GERD patients, and 60% of non-GERD subjects ($p>0.05$).

Functional dyspepsia and stress perception did not significantly correlate. High and medium levels of stress were perceived in 21.4% and 78.6% of functional dyspepsia patients, respectively, whereas high and medium levels of stress were perceived in 13.6% and 75.1% of non-dyspeptic subjects, respectively ($p>0.05$). However, stress was associated with GERD. High and medium levels of stress were perceived in 12.3% and 86% of GERD patients, respectively, whereas high and medium levels of stress were perceived in 15.6% and 70.6% of non-GERD subjects, respectively ($p=0.025$). A very good general well-being was perceived only in non-dyspeptic subjects (15.4% vs. 0%, in dyspeptic patients) and was more frequent in non-GERD subjects than in GERD patients (18.1% vs. 6.4%, $p=0.06$).

In the investigated group, 48.4% were overweight and 21.2% were obese. The presence of overweight and obese subjects was not significantly different in dyspeptic (85.7%) and non-dyspeptic subjects (68.2%) ($p>0.05$). Nevertheless, GERD was more frequently present in overweight subjects (35.9%) than in subjects with normal weight (19.6%) ($p<0.05$).

Food consumption frequency and eating habits

Figure 15 shows the median frequency of food consumption in the population under investigation. Using the median as a cutoff point, we analyzed the frequency of food consumption among subjects with or without functional dyspepsia and GERD (Table XXXI).

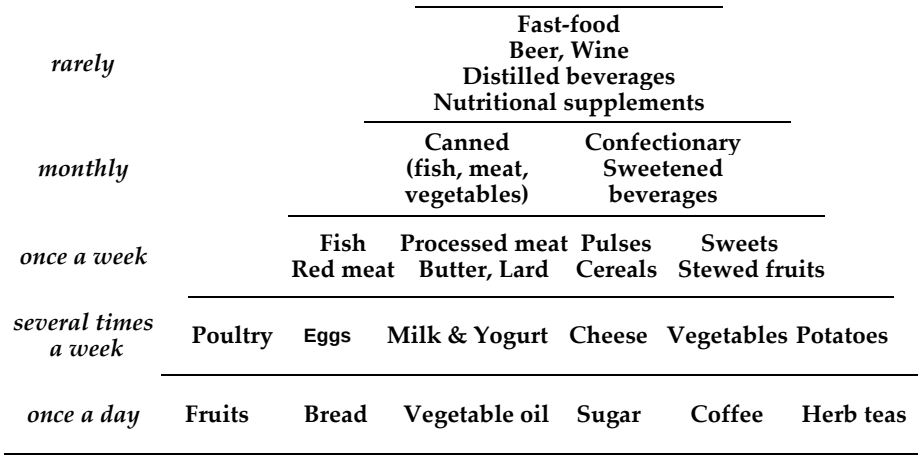

Figure 15. Median frequency of food consumption

Table XXXI. Median frequency of food consumption among subjects with or without functional dyspepsia and GERD

Food frequency consumption		Non-dyspepsia n =170		Dyspepsia n=14		p*	Non-GERD n=127		GERD n=57		p*
		No	%	No	%		No	%	No	%	
Pork	Less than once a week	79	46.5%	4	28.6%	0.196	59	46.5%	24	42.1%	0.583
	At least once a week	91	53.5%	10	71.4%		68	53.5%	33	57.9%	
Beef	Less than once a week	135	79.4%	8	57.1%	0.054	103	81.1%	40	70.2%	0.100
	At least once a week	35	20.6%	6	42.9%		24	18.9%	17	29.8%	
Poultry	Once a week or less	26	15.3%	3	21.4%	0.545	25	19.7%	4	7.0%	0.029
	At least several times a week	144	84.7%	11	78.6%		102	80.3%	53	93.0%	
Processed meat	Once a week or less	73	42.9%	3	21.4%	0.116	61	48.0%	15	26.3%	0.006
	At least several times a week	97	57.1%	11	78.6%		66	52.0%	42	73.7%	
Fish (fresh)	Less than once a week	61	35.9%	3	21.4%	0.275	51	40.2%	13	22.8%	0.022
	At least once a week	109	64.1%	11	78.6%		76	59.8%	44	77.2%	
Fish (canned)	Rarely	77	45.3%	0	0.0%	0.001	73	57.5%	4	7.0%	<0.001
	At least monthly	93	54.7%	14	100.0%		54	42.5%	53	93.0%	
Canned mixed (meat/fish +vegetables)	Rarely	84	49.4%	2	14.3%	0.011	78	61.4%	8	14.0%	<0.001
	At least monthly	86	50.6%	12	85.7%		49	38.6%	49	86.0%	
Canned vegetables	Rarely	73	42.9%	0	0.0%	0.002	70	55.1%	3	5.3%	<0.001
	At least monthly	97	57.1%	14	100.0%		57	44.9%	54	94.7%	
Canned food	Rarely	58	34.1%	0	0.0%	0.008	55	43.3%	3	5.3%	<0.001
	At least monthly	112	65.9%	14	100.0%		72	56.7%	54	94.7%	
Eggs	Once a week or less	43	25.3%	3	21.4%	0.748	32	25.2%	14	24.6%	0.927
	At least several times a week	127	74.7%	11	78.6%		95	74.8%	43	75.4%	
Milk	Once a week or less	34	20.0%	1	7.1%	0.239	33	26.0%	2	3.5%	<0.001
	At least several times a week	136	80.0%	13	92.9%		94	74.0%	55	96.5%	
Cheese	Once a week or less	21	12.4%	2	14.3%	0.837	20	15.7%	3	5.3%	0.047
	At least several times a week	149	87.6%	12	85.7%		107	84.3%	54	94.7%	

Food frequency consumption		Non-dyspepsia n =170		Dyspepsia n=14		p*	Non-GERD n=127		GERD n=57		p*
		No	%	No	%		No	%	No	%	
Butter, lard	Less than once a week	67	39.4%	3	21.4%	0.183	61	48.0%	9	15.8%	<0.001
	At least once a week	103	60.6%	11	78.6%		66	52.0%	48	84.2%	
Vegetable oil	Less than once a day	32	18.8%	3	21.4%	0.811	24	18.9%	11	19.3%	0.949
	At least once a day	138	81.2%	11	78.6%		103	81.1%	46	80.7%	
Potatoes	Once a week or less	48	28.2%	3	21.4%	0.584	40	31.5%	11	19.3%	0.087
	At least several times a week	122	71.8%	11	78.6%		87	68.5%	46	80.7%	
Vegetables with 5% carbohydrates (lettuce, spinach, tomatoes, peppers)	Once a week or less	49	28.8%	2	14.3%	0.243	44	34.6%	7	12.3%	0.002
	At least several times a week	121	71.2%	12	85.7%		83	65.4%	50	87.7%	
Vegetables with 10% carbohydrate (carrots, onions, beets)	Once a week or less	25	14.7%	2	14.3%	0.966	21	16.5%	6	10.5%	0.287
	At least several times a week	145	85.3%	12	85.7%		106	83.5%	51	89.5%	
Pulses (beans, peas, lentils, soybeans)	Less than once a week	51	30.0%	2	14.3%	0.212	46	36.2%	7	12.3%	0.001
	At least once a week	119	70.0%	12	85.7%		81	63.8%	50	87.7%	
Fruits	Less than once a day	32	18.8%	4	28.6%	0.377	28	22.0%	8	14.0%	0.205
	At least once a day	138	81.2%	10	71.4%		99	78.0%	49	86.0%	
White bread	Less than once a day	17	10.0%	1	7.1%	0.729	10	7.9%	8	14.0%	0.193
	At least once a day	153	90.0%	13	92.9%		117	92.1%	49	86.0%	
Grain bread/pasta	Once a week or less	47	27.6%	2	14.3%	0.277	45	35.4%	4	7.0%	<0.001
	At least several times a week	123	72.4%	12	85.7%		82	64.6%	53	93.0%	
Corn flour***	Less than once a week	23	13.5%	0	0.0%	0.141	21	16.5%	2	3.5%	0.013
	At least once a week	147	86.5%	14	100.0%		106	83.5%	55	96.5%	
Grain cereals	Less than once a week	73	42.9%	0	0.0%	0.005	69	54.3%	8	14.0%	0.038
	At least once a week	97	57.1%	14	100.0%		58	45.7%	49	86.0%	
Sugar	Less than once a day	70	41.2%	5	35.7%	0.689	52	40.9%	23	40.4%	0.940
	At least once a day	100	58.8%	9	64.3%		75	59.1%	34	59.6%	
Sweets	Less than once a week	84	49.4%	7	50.0%	0.966	66	52.0%	25	43.9%	0.309
	At least once a week	86	50.6%	7	50.0%		61	48.0%	32	56.1%	
Confectionary (cakes, cream, ice-cream)	Rarely	59	34.7%	5	35.7%	0.939	55	43.3%	9	15.8%	<0.001
	At least monthly	111	65.3%	9	64.3%		72	56.7%	48	84.2%	
Stewed fruit	Less than once a week	81	47.6%	6	42.9%	0.730	72	56.7%	15	26.3%	<0.001
	At least once a week	89	52.4%	8	57.1%		55	43.3%	42	73.7%	
Alcoholic beverages (beer, wine, distilled drinks)	Less than once a week	103	60.6%	3	21.4%	0.004	81	63.8%	25	43.9%	0.011
	At least once a week	67	39.4%	11	78.6%		46	36.2%	32	56.1%	
Carbonated sweetened drinks	Rarely	77	45.3%	4	28.6%	0.226	67	52.8%	14	24.6%	<0.001
	At least monthly	93	54.7%	10	71.4%		60	47.2%	43	75.4%	
Coffee	Less than once a day	36	21.2%	0	0.0%	0.055	31	24.4%	5	8.8%	0.013
	At least once a day	134	78.8%	14	100.0%		96	75.6%	52	91.2%	
Herb teas	Less than once a day	71	41.8%	6	42.9%	0.937	61	48.0%	16	28.1%	0.011
	At least once a day	99	58.2%	8	57.1%		66	52.0%	41	71.9%	
Fast-food (hamburger, hot-dog, chips, pretzels)	Never/rarely	91	53.5%	7	50.0%	0.799	80	63.0%	18	31.6%	<0.001
	At least monthly	79	46.5%	7	50.0%		47	37.0%	39	68.4%	

*p-value from chi-square test
***Corn flour was excluded from the category of "cereals" because it is a staple food in the study area

Dyspeptic patients consumed canned food significantly more frequently; all of them consumed canned food (fish, meat, or vegetables) at least monthly. Grain cereals (p=0.05) and alcoholic beverages were consumed at least weekly (OR=5.58, 95% 0=1.58-25.74, p=0.004).

Also, GERD patients consumed canned food (13.6, 4.46-57.5, p<0.001), grain cereals (p<0.05), and alcoholic beverages (2.24, 1.18-4.27, p=0.011) significantly more frequently. They consumed the following foods more frequently: fresh fish (17.65, 6.47-60.45, p=0.022), processed meat (2.57, 1.31-5.22, p=0.005), milk (9.57, 2.56-61.32, p<0.001), cheese (3.34, 1.0314.69, p=0.047), animal fat (butter, lard) (4.89, 2.26-11.37, p<0.001), vegetables with a low content of carbohydrates (3.76, 1.62-9.66, p=0.002), pulses (12.4, 5.38-31.8, p=0.001), confectionary (4.04, 1.87-9.41, p<0.001), stewed fruit (3.64, 1.85-7.4, p<0.001), carbonated sweetened beverages (3.4, 1.71-7.02, p<0.001), coffee (3.34, 1.28-10.2, p=0.013), herb teas (2.35, 1.21-4.72, p=0.011), and fast food (3.66, 1.89-7.62, p<0.001).

Regarding the intake of the following foods, there were no statistically significant differences: red meat, poultry, eggs, vegetable oil, potatoes and other vegetables high in carbohydrates, white bread, fruits, sugar, and sweets.

Predictors of functional dyspepsia and GERD

In order to exclude confounding variables, a multivariate regression analysis was used to determine the predictors for dyspepsia (22.44, 3.36-150.1, p=0.001), consumption of canned food (2.38, p<0.05), and alcoholic drinks at least weekly (5.4, 1.23-23.61, p=0.025).

Advanced age was one of the GERD predictors (1.086, 1.0521.122, p<0.001) and the use of canned food (13.94, 3.61-53.98, p<0.001) or fast food (4.646, 1.773-12.177, p=0.002).

The predictors of overlap between GERD and functional dyspepsia were advanced age (1.057, 1.012-1.105, p=0.013) and the consumption of canned food (2.82, p<0.05).

In our investigation, 7.6% of patients had functional dyspepsia. In other studies, the prevalence of dyspepsia varied according to country and the definition used, i.e., from 1.8% to 57.0%, and was higher in women (OR 1.24; 95% CI 1.13-1.36) and smokers (Ford et al., 2015). The overall pooled prevalence of uninvestigated dyspepsia in a very recent meta-analysis of 100 separate study populations was 20.8%. The greatest prevalence was found when a broad definition for dyspepsia (29.5%) or upper abdominal or epigastric pain or discomfort (20.4%) were used [135]. The prevalence for functional dyspepsia may vary from 20% to 40%. In USA, functional dyspepsia was 29.2% and 15% if subjects with GERD were excluded. Approximately 20%-30% of the general population presents every year with uninvestigated dyspepsia [136]

In Romania, the prevalence is thought to be between 20 and 30 percent, with half of that percentage being functioning. However, there are no conclusive epidemiological studies, and it is clearly under-reported. At the Ministry of Health, records can be found with a prevalence of 60-70/100,000 inhabitants in 2002-2003 [137].

The prevalence of GERD varies worldwide for unknown reasons, but genetic differences, difference in the *Helicobacter pylori* prevalence, and lifestyle factors such as obesity might be an influence. The highest population-based prevalence is reported from Europe (23.7%) and USA (28.8%) [138]. In our sample, using Montreal criteria, the prevalence of GERD was higher. The older age and the high percentage of overweight participants may explain this high GERD prevalence.

In our sample, the overlap of functional dyspepsia and GERD was 22.4%. In a recent review, the prevalence of dyspepsia was 27%, and this overlapped partially with GERD (from 10% to 66%, depending on the diagnostic criteria used for each) [139,140]. More studies suggest common pathogenic mechanisms with other functional digestive disorders [140–142].

The difficulty of general practitioners to distinguish between the two conditions may also contribute to the overlap between functional dyspepsia and GERD. The term “dyspepsia” has been confusing in the past. Patients do not use the term and physicians have variable

interpretations, minimizing its usefulness [132,136]. The difficulty in differentiating between dyspepsia and GERD symptoms was also reported; a recent paper revealed an over-diagnosis of GERD and under-diagnosis of functional dyspepsia in a US community. Only 62.9% of subjects reporting GERD symptoms were correctly diagnosed with GERD, and only 12.5% of subjects reporting dyspepsia were correctly diagnosed [143]. In our unpublished data, looking for recent symptoms, we found that heartburn (epigastric pain) was frequently present in both diseases, and this suggests the same idea and explains the overlap.

Numerous studies reveal that dyspepsia is very common among women. In our study, the higher prevalence of functional dyspepsia in men than in women could be explained by the large overlap with GERD. Also, in our region, the presence of *H. pylori* is more common, particularly in men [144]. As in other studies, the prevalence of GERD increased with age, obesity, physical inactivity, a low education level, and with stress [145–147], but we did not observe an association with smoking.

The possible contribution of food and dietary habits as a cause or exacerbating factor of dyspeptic symptoms represents a new area for evidence-based research. Despite frequent reports by patients that their symptoms are often related to food ingestion, this association has not been formally assessed.

Dietary assessments have frequently implicated fatty foods in symptom induction, and these findings are supported by laboratory-based studies, particularly the demonstration that patients with functional dyspepsia more often experience symptoms after intra-duodenal infusions of fat than glucose. Some studies suggest that food intolerance has no remarkable influence on food pattern and nutritional status in most functional dyspepsia patients. Further studies on the potential role of dietary factors as a cause of dyspeptic symptoms are required to establish whether dietary therapies have any place in the management of functional dyspepsia [148]. Although GERD can have anatomical explanations, there may be a relationship between the presence of symptoms and food because of food allergies [149,150].

Consuming certain meals on a regular basis was linked to functional dyspepsia and GERD. Bhatia et al. [145] reported that the consumption of non-vegetarian and fried foods, aerated drinks, tea, and coffee were associated with GERD, and using multivariate analysis, the consumption of non-vegetarian food was independently associated with GERD symptoms. In a Chinese study, routine usage of greasy food was considered a significant independent risk factor for non-erosive reflux disease [151].

Canned foods appear as a predictor in both illnesses. Although the consumption of canned food was not so popular in the studied group (median frequency was monthly), it was significantly correlated with the presence of disease. Some components of cans (food additives, pH, and tin) may determine digestive symptoms by certain mechanisms (intolerance, interference with medication, etc.). Tin is present in low concentrations in most canned foods and beverages; the highest levels are found in products when plain uncoated internal surfaces are used. A limited number of case reports of acute gastrointestinal disorders after consumption of food containing high concentrations (700 ppm or above) of tin have been reported, but there is little evidence for an association between the consumption of food containing tin at concentrations up to 200 ppm and significant acute adverse gastrointestinal effects [152,153].

The study's approach, which involved inviting the chosen participants to a medical center, may have had an impact on the findings because it focused on presenting patients to doctors who had symptoms recently.

A cross-sectional study cannot establish causality but only a relationship between the studied elements. A correlation can have several possible explanations. Frequent consumption of a particular food may positively or negatively influence the presence of disease; for example, canned food, alcoholic drinks, or processed meat for upper gastrointestinal disorders. Also, the disease can lead to a certain lifestyle or diet, sometimes in compliance with dietary

recommendations or due to the subjects preconception about the protective role of food in diseases (they may frequently use herb teas or grain cereals). Both factors may be dependent on a third factor; for example, age or educational level, which influenced both the eating or lifestyle and the presence of disease. To reduce confounding factors, we used a multivariate regression analysis.

II.1.1.4. Conclusions

The prevalence of functional dyspepsia, as defined by Rome III criteria, was found to be 7.6% in an urban population of Romania, while the prevalence of GERD, as established by Montreal criteria, was 31.0%.

The overlap between the two disorders was found to be 22.4 %.

Both illnesses showed a higher incidence in older and less educated respondents, and they were linked to the use of canned food, grain cereals, and alcoholic drinks.

The mechanisms by which diet influences gastrointestinal disorders are not fully elucidated, but the findings suggest the need for extensive research and specific strategies tailored to each specific population to promote healthy eating and lifestyle habits.

II.1.2. Irritable Bowel Syndrome

II.1.2.1. Aim of the Study

Our study aims to provide a general overview of the effects of a low-FODMAP diet (LFD) on gastrointestinal symptoms, nutrient intake, and lifestyle quality, making it a useful resource for clinicians and researchers working in this field. Furthermore, the study suggests that a LFD may be a feasible first-line therapeutic strategy to reduce stomach discomfort, pain, bloating, and improve QoL for patients with irritable bowel syndrome (IBS).

The novelty of this paper is stated in the comprehensive review of current studies that have evaluated the efficacy of a LFD against other diets in both adults and children with IBS.

II.1.2.2. Materials and Methods

The research team conducted a thorough investigation of the effectiveness of the LFD in IBS treatment using 7 searchable databases: Cochrane Central Register of Controlled Trials (CENTRAL), Cochrane Database of Systematic Reviews (CDSR), Excerpta Medica Database (EMBASE), Medline, PubMed, Scopus, and Web of Science up to March 2023.

The search terms used were: “irritable bowel syndrome”, “irritable colon”, “fructose oligosaccharide”, “FODMAP or FODMAPs”, “diet restriction”, “carbohydrate diet”, “clinical trials”, “double-blind”, “blind”, “randomized controlled trials”, “meta-analysis”, etc. The search process was not limited to English.

The following criteria were stated as the study protocol: (1) randomized controlled trials (including cross-over trials), (2) patients older than 4 years, (3) Rome I, II, III, or IV diagnostic criteria, (4) effectiveness of LFD, (5) comparing LFD with a placebo/regular diet, (6) results such as reduction in IBS symptoms, improvement in QoL, and stool regularity/frequency. Exclusion criteria for the trials selected were those that included patients with IBD, dementia, diabetes, renal, cardiovascular, and hepatic disease, patients with previous GI surgery, patients using antibiotics, prebiotics, probiotics or narcotics, and patients with food allergies.

II.1.2.3. Results and Discussions

IBS is a widespread functional GI disorder that determines symptoms such as chronic abdominal pain, flatulence, bloating, and altered bowel habits [154,155]. Depending on diagnostic standards and the regional area, this pathology has a prevalence between 5% and

20% in adults [156–158]. IBS can occur among patients of any age, even among children, more precisely 13.5% worldwide [159], and adolescents, rarely manifesting in older patients. IBS has a slightly higher prevalence among women than males and between 18 and 39 years of age [156,157].

Until 2006, diagnosing it seemed difficult for doctors because symptoms could change over time, but with the formulation of diagnostic criteria, the work of physicians became easier. Based on Rome IV diagnostic criteria and their most recent revision in 2016, IBS represents recurrent abdominal pain, which occurred weekly three months prior, coupled with a minimum of two of the subsequent criteria: influenced by bowel movements, associated with changes in the frequency and/or appearance. Following that classification, patients are grouped into three categories according to the pattern of the most frequent bowel movements: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), IBS with mixed bowel habits (IBS-M), or IBS unclassified (IBS-U) [160].

The pathophysiology of IBS is complex and still incompletely understood, as it involves altered enteric neurotransmitters, intestinal microbiota imbalances, neuroendocrine disorders, visceral hypersensitivity, changes in intestinal barrier function, and changes in motility and the response to maladaptive stress response [161,162]. It has been found to be an alteration of bidirectional communication through the brain-intestinal axis caused by an intricate association of biological, psychological, and social variables that underlie the condition.

Bacterial overgrowth in the small intestine in the majority of patients provides evidence that gut microbiota is at the forefront of the pathophysiology of IBS [161]. Bloating, constipation, diarrhea, and flatulence are the main symptoms of intestinal bacterial overgrowth. In approximately 25% of patients, the onset of IBS precedes an enteric infection [163].

Food is an additional element that contributes to the pathophysiology of IBS [164,165]. Short-chain carbohydrate fermentation reveals the process through which enteric bacteria and the presence of food allergies, nonimmune food sensitivities, changes in gut hormones, and changes in the gut microbiome produce symptoms of IBS. The use of non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, infections, and stress are known triggers for IBS symptoms. However, [166] ingesting foods high in FODMAPs and foods high in biogenic amines, which produce histamine [164,167], has been associated with the onset of gastrointestinal symptoms in IBS [166,168,169].

However, it has been found that early life experiences (such as dysfunctional family factors and trauma from psychological and physical abuse) are linked to IBS susceptibility. Anxiety and depression influence pain sensitivity, gut motility, immune function, and QoL [169–172].

The diagnosis of IBS requires the presence of characteristic symptoms within the last 3 months and the appearance 6 months ago. The Bristol stool form scale can help with the problematic subtyping of IBS because it is based on stool form [123,173,174]. The diagnosis of IBS is made after complete anamnesis based on the characteristic symptoms and results of various preliminary laboratory analyses, including complete blood count, determination of C-reactive protein (CRP), rapid erythrocyte sedimentation rate, and serological tests for CD [175–178].

Fecal lactoferrin (FL) and fecal calprotectin (fCal) are two biomarkers of intestinal inflammation that are useful for diagnosis. Their analysis is superior to serological tests (e.g., rapid erythrocyte sedimentation rate and CRP) for differentiating inflammatory bowel disease (IBD) from IBS [179,180]. To rule out other symptoms, a digital abdominal and rectal examination is required. This could confirm stool consistency, including rectal impaction, and it can detect dyssynergic defecation (paradoxical contraction on rectal examination during exertion) or low rectal masses [176].

Endoscopy is the 'golden investigation' for diagnosis of diseases of the GI. It allows direct visualization and offers the possibility of performing biopsies and establishing a histological diagnosis. However, despite those benefits, it is unpleasant to patients and may cause complications [179].

For IBS, it is important to perform colon cancer screening with the help of colonoscopy. Colonoscopy is a frequent test performed to determine whether a disease, such as IBD, microscopic colitis, or colon cancer, is not the cause of a patient's digestive symptoms. Polyps, hemorrhoids, and diverticula are just some of the lesions identified in patients with IBS during colonoscopy [175,178].

Management of IBS includes three directions (Figure 16): pharmacological therapy (antidepressants, antispasmodics, and laxatives), interventions on hygienic-dietary revitalization [181–183], and psychotherapy (cognitive behavioral psychotherapy, dynamic psychotherapy, hypnotherapy, and biofeedback-assisted stress management intervention) [167,175,184].

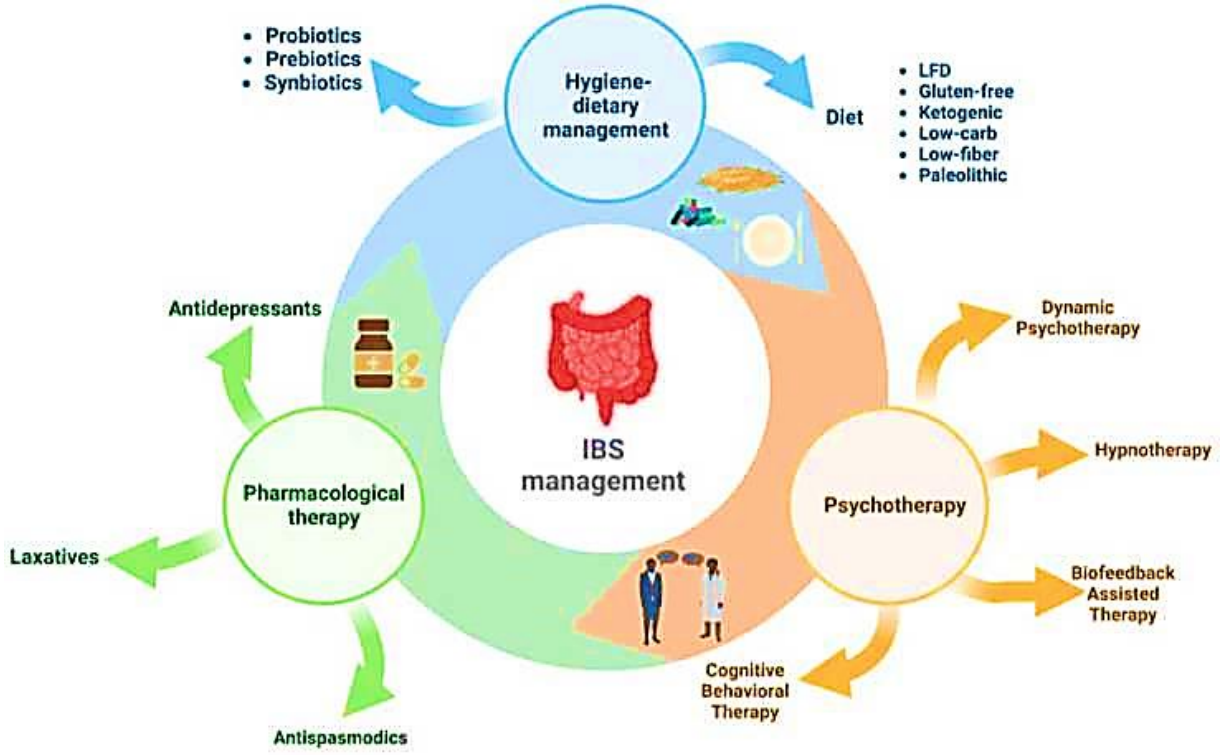

Figure 16. Therapeutic and nutritional management of irritable bowel syndrome

During the previous ten years, there has been an increase in interest in changing the lifestyle and the hygienic dietary regimen, with patients opting for one of the following diets: LFD, gluten-free diet (GFD), low-fiber diet, low-carb dies, ketogenic diet, and palaeolithic diet [160,185,186].

LFD is one of the most common nonpharmacological treatments for IBS. The acronym FODMAP stands for all foods that contain fermentable oligosaccharides, disaccharides, monosaccharides, and polyphenols [187]. These include fruits, vegetables, dairy, and cereals that contain short-chain carbohydrates that are harder to digest [188,189]. They can produce gas through intestinal microbial fermentation, particularly in the colon, and increased water retention via osmosis in the small intestine and colon due to insufficient absorption in the small intestine.

Short-chain carbohydrates ferment quickly, producing hydrogen, carbon dioxide, and methane. The other important fermentation products are short-chain fatty acids (SCFAs), which

enhance motility by enabling sodium and water absorption. Thus, luminal distention occurs through increased gas output and luminal water retention [190]. In susceptible individuals, these mechanisms produce luminal distension and characteristic GI symptoms, particularly gas production [190].

High intake of FODMAPs is also linked to visceral hypersensitivity, inflammation, intestinal barrier dysfunction, dysbiosis, and other conditions related to the pathogenesis and worsening of IBS [169].

Any food that exceeds any of the following amounts is considered high in FODMAPs: more than 4 g of lactose; more than 0.3 g of 0.3 g of mannitol; more than 0.3 g sorbitol; more than 0.3 g of galacto-oligosaccharides; more than 0.3 g of fructans if grain-based, otherwise, more than 0.2 g of fructans for grain-free products or more than 0.2 g of fructose [169].

Clinical research has shown the usefulness of the LFD, revealing that a restriction of FODMAP improved IBS symptoms in 70% of subjects [191]. In addition to the advantages obtained from following that diet, some disadvantages have also been reported: the complexity of diet monitoring among patients, the limitation of a certain food, the high costs, and the need for monitoring by a nutritionist to ensure an optimal nutrition intake.

Depending on the FODMAP content, the products are split into two categories: high-FODMAP foods versus low-FODMAP foods, as shown in Table XXXII.

Table XXXII. Products with high and low-FODMAP content.

Food Products	High-FODMAP Content	Low-FODMAP Content
vegetables fruits dairy and alternatives bread and cereals nuts and seeds	asparagus, garlic, onions, broccoli, green peas, sugar snap peas, mushrooms, cabbage, apples, pears, mangos, watermelon, nectarines, peaches, plums, dried fruits milk (cow, goat, sheep), condensed milk, yoghurt, cream, ice cream, cheese (fresh), soy milk, rye, wheat-containing bread, wheat-based cereals with dried fruit, wheat pasta, breakfast cereals pistachios and cashews	capsicum, carrot, corn, cucumber, eggplant, green beans, lettuce, pumpkin, tomato, zucchini, orange, mandarin, grapes, blueberries, lemon, kiwi, banana, strawberries lactose-free milk, almond/rice milk, lactose-free yogurts, ripened cheese, peanut butter, hard cheese, camembert/brie cheese, rice, quinoa, gluten-free bread, gluten-free pasta, sourdough, spelt bread, peanuts, walnuts, pumpkin seeds

In the scientific literature, we have identified 15 randomized control trials (RCTs) in adults. In Table XXXIII we summarize all the characteristics of those studies.

FODMAP occurs naturally in various foods that contain oligosaccharides and disaccharides (e.g., dairy products), such as fructans (e.g., garlic and onion), galacto-oligosaccharides (e.g., vegetables), and monosaccharides (e.g., honey), but also polyols used as sweeteners (e.g., sorbitol, mannitol, and xylitol). The amount of FODMAP is dependent on the species and the maturity of the product [192–195].

Implementing the LFD requires the guidance of specialists (nutritionists and gastroenterologists) because it requires careful guidance during each of its three phases. The first is the elimination phase, which involves eliminating FODMAP-rich products from the diet for 3-6 weeks. The results are already seen after 1-2 weeks from the start of the diet. The second phase is represented by the gradual reintroduction of foods containing high amounts of FODMAP. In the last stage, the diet is customized for each patient for the long term [191,196,197].

Table XXXIII. Characteristics of randomized control trial (RCT) studies in adults

Author	Type	Study Size	Study Characteristics	Conclusions
Ankersen et al. [198]	RCT	n = 29	Adults diagnosed with IBS according to Rome IV criteria. Comparing LFD with a moderate FODMAP diet. Exclusion criteria: patients with previous GI surgery, cardiovascular, liver, psychiatric, and neurological diseases, and other GI disease; patients with allergies or intolerance to food; and patients who used antibiotics within a month before the start of the trial.	LFD decreased the intensity of GI symptoms, including less frequent and firmer stool, when compared with moderate portions of the FODMAP diet. LFD seemed more helpful for IBS patients (IBS-D/IBS-M) with frequent loose stools than those with IBS-C.
Bodini et al. [199]	RCT	n = 127	RCT with adults diagnosed with IBS, according to Rome IV criteria, compares LFD with standard diet. Exclusion criteria: patients with moderate to severe disease, patients with previous GI surgery, and patients with coeliac disease, diabetes, and lactose intolerance.	The study highlighted the impact of LFD on the treatment of IBS and other intestinal diseases by evaluating some intestinal inflammatory markers (fCal and CRP dose in the beginning and after 6 weeks of the nutrition plan). A decrease in fecal biomarkers was observed, which was also associated with improvements in QoL.
Bohn et al. [200]	RCT	n = 67	RCT with adults diagnosed with IBS according to Rome III criteria compared LFD with NICE. Exclusion criteria: patients with cardiac, neurological, liver, psychiatric, or IBD.	The study showed that offering food guidance to patients with IBS in a medical environment helped improve GI symptoms; however, there were no obvious distinctions between LFD and NICE, as both reduced IBS symptoms.
Eswaran et al. [201]	RCT	n = 84	RCT with adults diagnosed with IBS-D according to Rome III criteria, compared LFD with mNICE. Exclusion criteria: patients with IBS-C, GI diseases, IBD, patients with previous GI surgery, pregnant patients, and patients using antibiotics or narcotics within a month before the beginning of the trial.	During a 4-week nutritional intervention, LFD diet significantly exceeded the mNICE diet to improve disease-specific QoL across all dimensions of the IBS-QoL questionnaire, except eliminating food. Following the introduction of LFD, a decrease in the average daily consumption of some micronutrients was observed, although there were no changes in the amount of energy consumed. Therefore, LFD was not immediately associated with significant nutritional deficits.
Grubel et al. [202]	RCT	n = 39	RCT with adults diagnosed with IBS, according to Rome IV criteria, which compared LFD with a low-lactose diet. Exclusion criteria: patients with coeliac disease, patients with food allergies, and patients using laxatives, antidiarrheal agents, and antibiotics.	LFD was associated with significantly fewer IBS symptoms than a low-lactose diet, highlighting the susceptibility of short-chain carbohydrates to poor digestion. That improvement was also due to the advice of the dietitian. Pain severity/frequency, bloating, and stool habits had better subscores when following an LFD.
Guerroiro et al. [203]	RCT	n = 70	A clinical trial with adult patients with IBS according to Rome IV criteria. Comparing LFD with standard diet. Exclusion criteria: patients with previous GI diseases and surgery, patients using antibiotics, prebiotics, and probiotics within a month before the start of the trial.	The global symptom frequency scores of both groups decreased significantly compared with baseline. However, the LFD group had a greater decrease in magnitude. LFD has been suggested to be more efficient than standard diet in reducing pain and diarrhea. Although standard diet decreased the frequency of constipation, there were no statistically

Author	Type	Study Size	Study Characteristics	Conclusions
				significant differences between the diets. Furthermore, the overall score for QoL increased significantly in both groups compared with baseline, with no statistically significant differences between the groups.
Hustoft et al. [204]	RCT	n = 20	A clinical trial with adult patients with IBS-D or IBD-M according to Rome III criteria, comparing LFD with FOS. Exclusion criteria: patients with IBS-C, pregnant women, and patients using probiotics or antibiotics.	In patients diagnosed with IBS-D or IBS-M, LFD was best at decreasing functional GI symptoms, and significantly more participants had symptom relief in response to a placebo (80%) than FOS (30%).
McIntosh et al. [205]	RCT	n = 37	According to Rome III criteria, a clinical trial of adult patients with IBS compares LFD with a high-FODMAP diet. Exclusion criteria: patients with previous GI surgery, patients using antibiotics, stool bulking agents, narcotics, or lactulose.	After 3 weeks, comparing patients diagnosed with IBS who received LFD with those who received a high-FODMAP diet, an overall decrease in GI symptoms was observed.
Menees et al. [206]	RCT	n = 43	According to Rome III criteria, adults diagnosed with IBS compare the effectiveness of an LFD vs. psyllium. Exclusion criteria: patients with dementia, diabetes, scleroderma, IBD, renal and hepatic disease, patients with previous GI surgery, and patients using antibiotics, prebiotics, probiotics, or narcotics.	The proportion of patients who reported a decrease of 50% in global symptoms was comparable for both groups. The psyllium group revealed a greater improvement in overall symptoms, but the LFD group reported a better QoL and stool consistency.
Naseri et al. [207]	RCT	n = 42	According to Rome IV criteria, adults diagnosed with IBS associated LFD with GFD. Exclusion criteria: patients with coeliac disease, IBD, liver disease, patients with precedent GI surgery, cancer, and patients using NSAIDs and drinking alcohol.	IBS patients who ingested LFD with GFD saw a substantial decrease in IBS symptoms and an adjustment of their gut microbiome. Intestinal inflammation can be reduced by association, which decreases IBS-SSS.
Patcharatrakul et al. [208]	RCT	n = 62	Adults diagnosed with IBS according to Rome III criteria, with moderate to severe GI symptoms, comparing LFD with BRD. Exclusion criteria: patients with previous GI surgery; coeliac disease; GI cancers; severe cardiovascular, liver, lung, neurological or mental diseases; and patients who used antibiotics, prebiotics, probiotics, or symbiotics within a month before the start of the study.	Compared with the BRD diet, the LFD proved its efficiency in decreasing visual analogue scale values. Following the LFD intervention, abdominal discomfort and bloating decreased considerably from their baseline values compared with those who received BRD. After both approaches, there were no significant improvements in belching or stool urgency.
Pedersen et al.[209]	RCT	n = 123	A clinical trial of adult patients with IBS according to Rome III criteria, comparing LFD with ND. Exclusion criteria: pregnant women, patients with GI surgery.	After 6 weeks of dietary intervention, patients who followed LFD compared with ND had a significant reduction in the IBS-SSS average.
Tuck et al. [210]	RCT	n = 80	A questionnaire was used to gather information about how LFD impacts patients with IBS.	Half of the patients reported an improvement in GI symptoms, but many did not reach the therapeutic level of FODMAP intake level, especially in the absence of the diet physician's guidance.
Wong et al. [211]	RCT	n = 16	Adults diagnosed with IBS according to Rome III criteria analyses the impact of LFD in Asian patients. Exclusion criteria: patients with frequent	11 of 16 patients (68.8%) reported an improvement in their general symptoms, which were classified in the following order: abdominal pain (60%), bloating /

Author	Type	Study Size	Study Characteristics	Conclusions
			organic diseases (cancer and IBD).	distension (70%), and flatulence (87.5%).
Zahedi et al. [212]	RCT	n = 101	According to Rome III criteria, the study involved the clinical response in patients with IBS-D after LFD vs. GDA. Exclusion criteria: patients with coeliac disease; IBD; cardiovascular, liver, kidney, and neurological diseases; diabetes; and thyroid disorders.	After six weeks, patients with IBS-D had a satisfactory reduction in GI symptoms with both LFD and GDA. However, LFD had greater benefits in improving IBS, such as a reduction in the severity, frequency, and status of abdominal pain and abdominal distention. However, in contrast with the GDA group, LFD did not affect QoL.

Effects on Global Symptoms, Abdominal Pain, and Bloating in Adults

The LFD had favorable effects on IBS symptoms, particularly in relieving abdominal pain, bloating, and diarrhea [213]. Furthermore, there was an improvement in intestinal movements and stool characteristics for those who followed a LFD [214,215].

After LFD intervention, Wong et al. [211], reported that 68% of patients with IBS had improved GI symptoms, noticeable even after the first week of the diet. Among the most common symptoms of IBS, abdominal pain decreased by 60%, bloating by 70%, and flatulence by 87.5%. Regarding stool formation, those with IBS-D had a more significant improvement.

The analysis performed by Bohn et al. [200] highlighted an improvement in global symptoms among patients who followed the LFD compared with the traditional IBS diet. Since the 29th day, a significant improvement compared with the baseline value for the frequency and intensity of abdominal pain was observed in the group who followed the LFD. Unlike the baseline value, there was a statistically significant reduction in the number of bowel movements in the LFD group ($p < 0.0001$), whereas there was none in the traditional IBS diet group. Furthermore, according to Zahedi et al. [212], compared with general dietary advice (GDA), the LFD demonstrated a substantial decrease in GI symptoms (abdominal pain, bowel movement, and bloating). Following six weeks of LFD compared with GDA, the status of bowel habits, the consistency, and the frequency had statistically substantial improvement. However, the results of these data were more pronounced in patients with IBS-D [212]. Additionally, Patcharatrakul et al. [208] observed an improvement in GI symptoms in 60% of patients who responded after the LFD compared with 28% of patients after brief advice on a commonly recommended diet (BRD) ($p = 0.001$). Following LFD, opposite to BRD, there was a substantial decrease in GI symptoms such as abdominal pain, severity of discomfort, and bloating compared with baseline ($p > 0.05$).

Ankersen et al. [198] revealed that the LFD decreases the intensity of GI symptoms and also has an effect on bowel habits due to the decrease in stool frequency and an increase in consistency, a factor that was not observed after a diet with a moderate FODMAP diet. Therefore, LFD may be more effective for IBS patients (IBS-D/IBS-M) with frequent soft stools compared with those [216] with less frequent and firm stools (IBS-C).

At the same time, GI symptoms were reduced in patients who received a LFD as in those who followed a moderate-FODMAP diet. Although it was carried out in only 40 patients, a global reduction in symptoms was also noticed in those who received the LFD compared with those who received a diet rich in FODMAP (RR = 0.44; 95% CI: 0.23 up to 0.83) in the study of McIntosh et al. study [205].

In various studies conducted by Hustoft et al. [204], LFD was compared with a high fructose-oligosaccharide diet (FOS), and all GI symptoms improved significantly after 3 weeks of LFD. The most statistically significant improvement included reduced burping (39.4; $p < 0.001$), regurgitation (24.3; $p < 0.001$), and exhaustion (21.2; $p = 0.001$). When those in the LFD group were compared with those in FOS or to those in the placebo group, the placebo

group reported a better symptom decrease (80%) compared with that FOS (30%, $p = 0.13$).

Following LFD dietary intervention compared with the normal diet (ND), Pedersen et al. [209] showed a significantly greater reduction in abdominal pain (OR: 2.97, 95% CI: 1.12-7.89, p = 0.03), stool consistency, and frequency (OR: 2.43,95% CI: 0.97-6.12, p = 0.06).

However, patients with IBS were observed for 4 weeks after nutritional intervention in the Guerreiro et al. [203] study that compared LFD to the standard diet. The results demonstrated that the total score for the frequency considerably decreased in both groups compared with the baseline value (LFD: $p < 0.001$; standard diet: $p < 0.05$), although the LFD group noticed a greater amplitude of the decrease ($p = 0.041$). In terms of treating individual symptoms, it was discovered that an LFD was superior to a standard diet in relieving abdominal pain and diarrhea. Although the standard diet decreased the frequency of constipation, there were no statistically significant differences between these two diets. Furthermore, a questionnaire reported that the LFD group had a 56.4% success rate in improving symptoms overall compared with that of the standard diet group at 22.2% ($p = 0.016$).

Regarding the effectiveness of the LFD compared with other treatment methods, in the study developed by Menees et al. [206], they evaluated the impact of an LFD versus psyllium treatment. The results revealed that after 4 weeks of LFD intervention, the mean FISI scores for stool consistency improved considerably compared with baseline (39.2 vs. 32.6, p = 0.02), but not after psyllium therapy (35.2 vs. 32.5, p = 0.22).

Effects on Quality of Life in Adults

LFD substantially improves QoL for patients with IBS compared with those who follow standard dietary recommendations and a high-FODMAP diet.

The therapeutic effect of an LFD can be measured using the standardized complex score (IBS-SSS). Through it, the frequency and severity of abdominal pain, bloating, frustration with bowel habits, and QoL are measured on a visual analogue scale are measured. Thus, using that score, the positive effect of the LFD was demonstrated by Bohn et al. [203,217], as well as its superiority over a traditional diet. A relevant reduction in total IBS-SSS following the LFD, compared with a low-lactose diet, was also confirmed three years later by Grubel et al. [202,218] in a randomized control trial underlining the importance of patient counselling and supervision by a dietitian. Furthermore, in Hustoft et al. [204], they research reported a mean decrease in IBS-SSS of 163.8 (95% CI: 135.7-500), which was reported [204] after 3 weeks of LFD. Each patient experienced an overall decrease of at least 50 (range: 57-275) [204]

The analysis performed by Guerreiro et al. [203] observed an improvement in QoL after an LFD intervention compared with an standard diet, as evidenced by an increase in the overall score for QoL. Compared with baseline, it considerably increased in both groups (LFD: $p < 0.001$; standard diet: $p < 0.05$), although there was no statistically significant difference between the groups (p = 0.2727). However, LFD significantly reduced the negative effects of IBS on dysphoria, interference with daily activities, body image, sexual life, and interpersonal connections with others.

In a study by Naseri et al. [207], the association of LFD with GFD in terms of QoL was also evaluated. In 73% of the patients, a clinically relevant improvement in IBS-SSS compared with the baseline value was observed after 6 weeks of dietary intervention (p = 0.001). In total, 53% of the patients presented a reduction in IBS-SSS of 30 to 60 points after completing the diet, while only 3.3% obtained a decrease of more than 60 points.

Furthermore, three studies by van Lanen et al. [215], Wang et al. [214], and Black et al. [213] evaluated the IBS-SSS score in a meta-analysis carried out on a large sample, more precisely, on 4537 patients from 14 RCTs, respectively, 1164 patients from ten RCTs, and 944 patients from 13 RCTs, observing a mean reduction of 45 points in patients who followed an LFD compared with a control diet; thus, this is consistent with previous studies.

The reviewed studies provide evidence that the LFD can substantially improve the

QoL of patients with IBS compared with those following standard dietary recommendations or a high-FODMAP diet. The therapeutic effect of LFD can be measured using the standardized IBS-SSS score, which has been used in multiple studies and consistently demonstrated the positive effect of the LFD on reducing abdominal pain, bloating, frustration with bowel habits, and improving QoL. Studies also emphasize the importance of patient counselling and supervision by a dietician during dietary intervention. Furthermore, LFD was found to significantly reduce the negative effects of IBS on dysphoria, interference with daily activities, body image, sex life, and interpersonal relationships with others. The studies also showed that combining LFD with a GFD can lead to clinically relevant improvements in IBS-SSS score. In general, these studies suggest that LFD can be an effective dietary intervention for patients with IBS to improve their QoL.

Effects on Bowel Water Content in Adults

The effects of foods with a higher FODMAP content demonstrated an increase in bowel water content because of the osmotic effect and the increase in gas synthesis by the microbiota in the colon. They exacerbated the symptoms of IBS and functional GI disorders primarily by causing distention and having an osmotic laxative effect.

Studies have shown that fermentable carbohydrates were osmotically active, showing that a diet rich in polyols, sucrose, and fully fermentable carbohydrates caused a doubling of the total wet weight of the effluent due to water retention. With the help of magnetic resonance imaging, it was observed that healthy individuals who drank approximately 18 g of mannitol solution exhibited a 10-fold increase in intestinal water compared with those that drank the same amount of glucose solution. Comparable results were also found after 40 g of fructose, with an increase in bowel water compared with the ingestion of 40 g of glucose [219].

Humans have incomplete absorption of fructose and mannitol in the small intestine, leading, through fermentation, to increased gas production in the colon. Increased volume of water in the intestines can worsen abdominal pain and cause diarrhea [219,220]

The decrease in fructose causes a reduction in the water content in the small intestine, causing a change in the osmotic load in those following an LFD [221].

According to Guerreiro et al. [203], an LFD is beneficial for patients with IBS-D because it decreases osmolarity and, thus, it decreases the water content in the intestinal lumen, which is advantageous in the management of IBS-D. In addition to alleviating symptoms such as abdominal pain and distention that were typically present in all subtypes of IBS, LFD might also help reduce intraluminal fermentation.

On the other hand, Bohn et al. [200] suggested that LFD is the most efficient recommendation to treat IBS, reducing symptoms, healthcare, and social costs [128,175,222,223].

In summary of the five studies considered, we underline and discuss the effects of fermentable carbohydrates on bowel water content and gas synthesis, which exacerbate symptoms of IBS and functional GI disorders. Incomplete absorption of fructose and mannitol in the small intestine leads to increased gas production in the colon, which worsens abdominal pain and causes diarrhea. Several studies have suggested that LFD is the most effective recommendation for treating IBS to reduce its symptoms and related healthcare and social costs. LFD benefits patients with IBS-D because it decreases the osmolarity and water content in the intestinal lumen, alleviating symptoms such as abdominal pain and distention. In addition, LFD may help reduce intraluminal fermentation.

Effects on Biochemical Markers of Disease Activity in Adults

fCal is an antimicrobial protein secreted primarily by neutrophils, is used for the diagnosis and management of IBS, and is currently preferred due to its specificity over typical inflammatory biomarkers (e.g., CRP). This biochemical marker allows the differentiation of IBS from other organic GI disorders [216].

Following LFD implementation, a decrease in fCal was noticed in the study by Bodini et al. [199] after following a nutritional plan for 6 weeks (T0: 88.4 mg/kg; IQR, 50,220.4 mg/kg vs. T1: 50 mg/kg; IQR, 50.681 mg/kg; p = 0.004) compared with that after following a standard diet (T0: 88.4 mg/kg; IQR, 50,220.4 mg/kg vs. T1: 87 mg/kg; IQR, 50,235.6 mg/kg; p = 0.175). Therefore, there was a decrease of 34.7% in fCal for patients following LFD compared with 4.4% after a standard diet. This suggests that a LFD may be beneficial in managing IBS symptoms and reducing inflammation in the gut. However, more studies are needed to confirm these findings and determine the long-term effects of a LFD on gut health.

Effects on Nutrient Intake in Adults

Exclusion diets, such as gluten- or dairy-free diets, can cause nutritional deficiencies. The same question was asked about LFDs, regarding the intake of micronutrients.

It was hypothesized that patients following an LFD risked a reduced fiber and micronutrient intake (e.g., calcium, zinc, iron, vitamin D, folic acid, natural antioxidants).

During a 4-week dietary intervention comparing LFD and modified National Institute of Health and Clinical Excellence dietary intervention (mNICE) by Eswaran et al. [201], a decrease in the average daily intake of thiamine (p = 0.01), riboflavin (p = 0.05), calcium (p = 0.01), and sodium (p = 0.001) was observed; however, that reduction was not sustained after adjusting for energy intake. The causes of the decrease could have been due to the decrease in consumption of grains that typically contained those specific micronutrients.

Additionally, calcium intake was low, probably as a result of the limited dairy intake in LFDs. Therefore, substantial micronutrient deficits were not immediately linked to LFDs.

Moreover, Staudacher et al. [217] revealed no significant variations in energy and macronutrients in those who followed the LFD compared to those who followed the control diet. There were indications that the LFD improved overall dietary intake, given the higher vitamin B12 compared with those who followed a standard control diet.

According to a questionnaire, the LFD was evaluated in the study by Tuck et al. [210] in patients with IBS, whether they followed the diet prescribed by a gastroenterologist or dietician. In total, 30% of the patients followed the dietitian's recommendations, and a specialist did not guide 70%. As a result, it was observed that patients who followed the diet as advised by the dietitian ingested around 12 g of FODMAP (p = 0.02), compared with those who were not consulted by a specialist and had lower levels (p = 0.04). Furthermore, when each subgroup's intake was evaluated separately, patients who followed dietician recommendations had significantly fewer polyols than those who did not (p = 0.04), which resulted in a decrease in the tendency to consume excess fructose (p = 0.08). In terms of micronutrient intake, the group of patients who followed the LFD on the advice of a dietitian had greater values of folate (322 mg vs. 295 mg), iron (13 mg vs. 11 mg), niacin (22 mg vs. 11 mg), and zinc (13 mg vs. 11 mg) compared with those who were not examined by a specialist. The authors concluded that to maintain an optimal intake of micronutrients and macronutrients, patients must be monitored by a dietician throughout the diet.

This section refers to three studies exploring the potential micronutrient deficiencies associated with the LFD for the management of IBS. Studies suggest that LFD may lead to a reduction in the intake of some micronutrients such as thiamine, riboflavin, and calcium, but these reductions were not sustained after adjusting for energy intake. However, the LFD did not cause significant micronutrient deficits, and there were indications that it improved overall dietary intake. Patients who followed the LFD under the guidance of a dietitian had a greater intake of folate, iron, niacin, and zinc compared with those who did not receive specialist advice. Therefore, to maintain an optimal intake of micronutrients and macronutrients, patients should be monitored by a dietitian while following the LFD.

Effects on Global Symptoms, Abdominal Pain, and Bloating in Children

In the scientific literature, we have identified four RCTs for children. The main characteristics of the investigations are summarized in Table XXXIV.

Table XXXIV. Characteristics of randomized control trial (RCT) studies in children

Author	Type	Study Size	Study Characteristics	Conclusions
Boradyn et al. [218]	RCT	n = 29	RCT with a parenteral opinion about LFD on children (age: 5-12 years) diagnosed with FAP, according to Rome III criteria. Exclusion criteria: patients with organic GI disorders, patients with food allergies, patients with acute infection, and patients with antibiotics, within two months of starting the study.	The effectiveness of LFD was evaluated after 4 weeks of dietary intervention based on parents' opinions on the intensity of their children's abdominal pain. LFD and BDA/NICE diets required the supervision of a pediatric dietician to obtain an effective result in children, thus avoiding nutritional deficiencies.
El Gendy et al. [227]	RCT	n = 50	RCT evaluated the effects of LFD in children (age: 3-18 years) diagnosed with FAP, according to Rome IV. Exclusion criteria: patients with a family history of IBD, coeliac disease, peptic ulcer disease, dysphagia, vomiting, blood loss, odynophagia, diarrhea, arthritis, and weight loss.	After 2 months of LFD intervention, a decrease in pain intensity was observed in 74% of the patients, as well as an increase in QoL, without detrimental effects on body weight.
Joishy et al. [228]	RCT	n = 74	The RCT evaluates fCal and FL in children (age 4-17 years) with IBD.	fCal and FL were evaluated as highly precise and non-invasive indicators for the preliminary identification of IBD, Crohn's disease, and ulcerative colitis in children. They could help distinguish between IBD and other non-IBDs such as IBS.
Nogay et al. [226]	RCT	n = 15	RCT evaluating the effect of LFD in children (age: 6-17 years) with ASD together with IBS according to Rome IV. Exclusion criteria: patients with previous GI surgery, patients with IBD, cystic fibrosis, liver and cardiovascular disease, and patients using antibiotics.	After 2 weeks, the LFD intervention had benefits in children diagnosed with autism with abdominal pain and/or constipation, as it was effective in reducing constipation and other GI problems without affecting the intake of nutrients.

In recent years, the effectiveness of LFDs has also been evaluated among children (age 4-18 years). Unfortunately, we have identified a few RCTs that have shown the effectiveness of that diet, in a small number of patients.

Functional abdominal pain is a common pediatric GI disorder characterized by chronic or recurrent abdominal pain that is not associated with any structural, inflammatory, or metabolic causes [224,225]

One of the studies on children, published by Boradyn et al. [218] in 2020, divided the subjects into two categories: the first group was represented by those who followed an LFD, and the second group followed the diet recommended by NICE. The results of the randomized control trial did not show a significant reduction in symptoms after LFD compared to the diet recommended by NICE. The study was carried out on 171 parents of children diagnosed with functional abdominal pain (FAP) to assess their opinion about the LFD. The results showed that while 70% of parents had never heard of the diet before, after being informed about it, most were willing to try it as a dietary intervention for their children's FAP. However, parents also expressed concerns about the complexity and feasibility of the diet, as well as the potential risk of nutrient deficiencies. In general, the study suggested that parental opinion and support play an important role in the success of the LFD as a dietary intervention for children with FAP. The

findings also highlighted the need for healthcare professionals to provide clear information and support to parents considering the LFD as a dietary intervention for their children's FAP [224].

Nogay et al. [226] evaluated, for the first time, the effectiveness of LFD on GI and behavioral issues in children with autism spectrum disorders (ASD) (e.g., self-harm, repetitive behavior, screaming, anxiety), considering the strong impact that behavioral problems play in the etiopathogenesis of GI problems.

Rhys-Jones et al. [159] evaluated through meta-analysis the use of the LFD in pediatrics and its impact on macronutrient intake by analyzing five RCTs. The research results identified a valid decrease in the frequency and consistency of stool in children diagnosed with IBS. Those improvements due to the decrease in carbohydrate intake were visible in the first few weeks of the diet. However, implementing the LFD among children raised some concerns about the intake of nutrients since the LFD is a restrictive food diet. There were no discernible differences between the groups that followed the LFD and the control diet in terms of nutrient intake, except for vitamins B12 and K. Vitamin B12 had lower levels for those who followed the LFD compared with the group that followed the control diet, most likely as a result of the decrease in dairy product intake. Some reported a lower calcium intake due to reduced consumption of certain dairy products or a decrease in vitamin B2 and increased levels of vitamin B3 and vitamin B6 in the group that followed the LFD compared with a control diet for 4 weeks. In some cases, the intake of vitamin C, vitamin B6, and vitamin E was improved by supplementing the portions of vegetables and proteins in the pediatric dietitian.

In addition to all those highlighted aspects, regarding the intake of macronutrients for children, additional studies are necessary to assert whether the temporary restriction of FODMAPs impacts the child's harmonious growth and development. According to the text, there is limited evidence from RTCs to support the effectiveness of the LFD in the treatment of FAP in children. Additional studies are necessary to determine the impact of temporary FODMAP restrictions on children's balanced growth and development.

Effects on Quality of Life in Children

Most studies performed on adults use IBS-SSS to evaluate QoL, but it does not apply to pediatrics.

El Gendy et al. [227] evaluated the QoL based on the KIDSCREEN-10 index to assert the subjective health and well-being of children and adolescents. It was created as a self-reporting tool, which is easily applicable to healthy and chronically ill children and adolescents. The LFD food intervention in the research of El Gendy et al. [227] showed a decrease in the pain score for 84% of the patients, where the median score at the beginning of the study was 8 (IQR: 6-10) in the range of 4-10. After two months of the diet, it had a value of 4 (IQR: 4-6) and the range was between 0-10 ($p = 0.0000$). That pain reduction in children was later associated with an improved QoL. Therefore, the LFD demonstrated a reduction in intestinal pain and QoL in young patients with FAP and even showed a positive increase in weight among children and adolescents because the diet was carefully monitored by a pediatric dietitian specialized in gastroenterology to ensure optimal intake of calories, vitamins, and minerals appropriate for their age and constitution [227].

Boradyn et al. [218]showed that parents perceived FAP to have a significant impact on their children's QoL, and many reported that their children had missed school or social activities due to their symptoms, so by alleviating those symptoms they perceived the diet to have a positive impact on their children's QoL.

Nogay et al. [226] revealed that preschoolers with ASD had a higher prevalence of GI symptoms, such as abdominal pain and bloating, compared with typically developing children. That study also found a significant relationship between GI symptoms and behavioral problems in preschoolers with ASD, such as irritability and hyperactivity. Furthermore, the severity of GI symptoms was found to be related to the severity of ASD symptoms, suggesting that there

may be a complex interplay between GI symptoms and ASD symptoms. Overall, the study highlighted the importance of addressing GI symptoms in preschoolers with ASD, as they could significantly impact the child's behavior and QoL. It also suggested that there might be a need for more comprehensive medical evaluations and interventions to address GI symptoms in children with ASD. In this text, three studies are considered. In summary, these studies highlight the importance of addressing GI symptoms in children and adolescents, as they could significantly impact their behavior and QoL. It is worth mentioning that only three studies were taken into account in this text.

Effects on Bowel Water Content in Children

Children with IBS may experience alterations in water content, which can contribute to their symptoms [229,230]. Research studies have found that children with IBS had a lower stool water content compared with healthy children, indicating that their fecal material was drier and harder to pass. Additionally, low water intake had been associated with a higher severity in children with IBS. The altered bowel water content in children with IBS could have been related to underlying factors such as abnormal intestinal permeability, changes in the intestinal microbiota, and increased levels of nitric oxide in the intestine. Understanding these factors might help develop targeted treatment strategies to alleviate symptoms in children with IBS [159,231]. A study used endoscopy to obtain rectal biopsies of children with IBD and healthy children. The biopsies were then analyzed for the expression of nitric oxide synthases enzymes and the presence of nitric oxide. They found that children with IBD had increased expressions of nitric oxide synthase enzymes and higher levels of nitric oxide in their rectal mucosa compared with healthy children. Higher levels of nitric oxide were associated with an increase in water content in children with IBD. The authors concluded that nitric oxide played an important role in the pathogenesis of IBD in children and may contribute to intestinal dysfunction by altering water content [232].

Effects on Biochemical Markers of Disease Activity in Children

The potential use of these markers in diagnosing, assessing disease activity, and predicting outcomes in patients with IBD is of high interest.

A study conducted by Fodor et al. [233] discussed the limitations and challenges associated with these markers, including the lack of specificity and sensitivity of some markers and the need for standardized assays and interpretations. Furthermore, emerging markers, including fecal biomarkers, genetic markers, and microbiome-related markers, have been discussed that have shown promise in recent studies for the diagnosis and monitoring of IBD. Overall, the article concluded that the use of biochemical markers in the management of IBD is still evolving, and further research is needed to identify specific and reliable and specific markers that can be used in clinical practice [233].

The research carried out by Joishy et al. [228] discusses the use of fCal and FL as non-invasive markers of IBD, including Crohn's disease and ulcerative colitis, in children. The study found that fCal and FL were reliable markers for the detection of IBD, with high sensitivity and specificity, and that they could differentiate between IBD and other non-inflammatory bowel conditions such as IBS.

Effects on Nutrient Intake in Children

The study of Boradyn et al. [218] revealed that, while parents were generally willing to try the diet as a dietary intervention for their children's FAP, they also expressed concerns about the possible risk of nutrient deficiencies. The article suggests that while LFD may effectively reduce symptoms of FAP in children, it is important to ensure that the diet is nutritionally adequate and does not cause nutrient deficiencies. It is recommended that the LFD be implemented under the guidance of a healthcare professional, which can ensure that the diet is nutritionally adequate, and that the child's nutritional status is monitored. Overall, the research highlights the importance of ensuring that any dietary intervention implemented in

children with FAP effectively reduces symptoms and does not lead to nutritional deficiencies [218]. Nogay et al. [226] suggested that nutritional deficiencies can play a role in the development and severity of symptoms of ASD and that addressing nutritional deficiencies can positively impact the behavior and development of children with ASD. However, the article does not provide a detailed analysis of nutritional intake or specific nutrient deficiencies in preschoolers with ASD. It is highlighted that adequate nutrition is needed while implementing nutritional interventions for children with FAP and ASD.

II.1.2.4. Conclusions

To conclude, adopting a diet that restricts the intake of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols presents a promising strategy for managing irritable bowel syndrome by ameliorating abdominal symptoms such as discomfort, pain, flatulence, and bloating, consequently enhancing the quality of life for both adults and children.

Nonetheless, the implementation of a low-FODMAP diet poses challenges due to potential alterations in intestinal microbial flora and nutrient deficiencies when not guided by a registered dietitian.

To solidify the efficacy of this dietary approach relative to others, there is a necessity for randomized controlled trials with larger participant cohorts and extended follow-up periods. Furthermore, a dearth of research pertaining to the effects of the low-FODMAP diet on children necessitates additional investigations in that demographic.

Our comprehensive review aimed to furnish detailed insights into the outcomes of following a low-FODMAP diet. We have delineated distinct sections addressing the primary symptoms of IBS, intending to facilitate a more robust endorsement of this dietary strategy as a viable option for managing the diverse manifestations of the condition.

II.1.3. Celiac Disease - Lupus Erythematosus

II.1.3.1. Aim of the Study

The present narrative review aims to present a summary of data from the specialized literature regarding the relationship between CD and SLE by analyzing the most recent studies published on PubMed.

CD-SLE correlation represents a crossroads in the study of autoimmune diseases, both in terms of their onset as well as their development, overlap, and treatment.

II.1.3.2. Materials and Methods

The research team conducted a thorough investigation of the correlation between CD-SLE using the following searchable databases: Cochrane Central Register of Controlled Trials (CENTRAL), Excerpta Medica Database (EMBASE), Medline, PubMed, Scopus, and Web of Science.

II.1.3.3. Results and Discussions

CD is associated with several autoimmune diseases, of which thyroid disease and type 1 diabetes (T1D) are defined as “associated conditions” or conditions with increased prevalence but not directly related to gluten ingestion. Loci in the HLA region common to those identified in SLE have been observed, SLE being among the top three autoimmune diseases developed by first-degree relatives of patients with CD. In addition, there is evidence of non-celiac autoimmune diseases in the spouses of patients with CD, which contradicts the claim that the involvement of genetics is the only predisposing cause, since the partners do not share genetic characteristics with each other, but only environmental factors and possibly the microbiome, with an impact on the risk of developing autoimmunity [234,235]. About 30% of all patients

with CD have one or more autoimmune conditions, while in the general population, there is a prevalence of 3% to 9.4%. Currently, the available evidence suggests that the common genetic background is the main factor that determines the high prevalence of the association, but it is not clarified whether extrinsic factors related to gluten, such as age at first introduction, concomitant breastfeeding, duration of exposure to gluten and GFD, influence the link between CD and autoimmune diseases [234].

The importance of effective screening among patients with SLE in order to detect CD was debated in a study carried out in the Middle East, the results showing the increase in prevalence among patients with autoimmune diseases, with a higher correlation in the groups of patients with SLE versus controls. Although the frequency of CD markers is considered to be high in SLE patients, only anti-endomysial (EMA) and TGA showed significant differences compared to controls (data present in the literature). The present study still found that 9.6% of the subjects tested positive for anti-gliadin antibodies (either AGAG or AGAA), 3.5% for TGA and 2.6% for EMA. The negative impact of the lack of screening resides in the possibility of incorrect diagnosis and management, which can lead to unnecessary exposure to immunosuppressive drugs associated with side effects [236].

In support of the previously stated findings, another study carried out at Colentina Hospital and the Institute for Mother and Child Care, Bucharest, Romania, demonstrated the increase in the prevalence of gluten-induced autoimmunity among patients with SLE compared to the general population, identified for TGA-IgA, but not for EMA [237].

SLE and CD are two complex diseases, encountered in all age groups (including in pediatric practice), with diverse clinical manifestations involving multiple organ systems and an evolution marked by relapses and remissions, dependent on both environmental factors and individual response to therapy.

The pathogenesis of CD can be attributed to a combination of inflammation, nutrient deficiency caused by malabsorption, and enzyme-mediated autoimmune response. The clinical picture provides an evolutionary description over the years, starting from presentation in the form of diarrhea with malabsorption syndrome and reaching that is nowadays a systemic disease with a serious clinical and histological picture.

The most frequent clinical manifestations described in the literature are represented by constitutional symptoms (fever, fatigue, weight loss), skin damage (malar rash, oral ulcers, vasculitic eruptions, photosensitivity, alopecia, discoid lesions, Raynaud's phenomenon), muscle-skeletal (often symmetrical, non-erosive polyarthritis of large and small joints, myalgia, rarely myositis), hematological (autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura, leukopenia, granulocytopenia, positive Combs test), cardiac (pericarditis, myocarditis, valvular damage, coronary damage due to arteritis or arteriosclerosis), neuropsychological (from headaches, memory loss to global cerebral dysfunction manifested by paralysis or convulsions), pulmonary (pleurisy, pneumonia, pneumothorax, diffuse interstitial damage, hypertension, and pulmonary hemorrhage) and renal (lupus nephritis, classified in six stages, starting from the mild form and culminating in end-stage renal disease) [238]

SLE and CD therefore share common systemic manifestations such as the production of autoantibodies, multiple inflammation, and the deposition of immune complexes. Worthy of emphasis are also the involvement of immune complexes and inflammation in the pathogenesis of the two diseases.

Dermatitis herpetiformis is another autoimmune manifestation encountered in the form of pruritic vesicles (appearing especially on the elbows, forearms, buttocks, knees, and scalp) associated with gluten-sensitive enteropathy. From an immunological point of view, this is described as the coexistence of aggregates of granular or, to a lesser extent, linear deposits of IgA type (predominantly IgA1, rarely IgA2) at the level of the dermo-epidermal junction and circulating immune complexes containing IgA, in the absence circulating IgA antibodies

directed against dermal structures. It was also highlighted with the help of immunoadsorption that the complexes found in dermatitis herpetiformis and CD formed on the basis of the two immune fractions (IgA1 and IgA2) that circulate separately, at the same time identifying different properties and the absence of pathogenic involvement, unlike SLE [239–242].

Inflammation is also a common characteristic of the two conditions and is found in particular in the composition of each in the form of a pro-inflammatory intestinal environment, which leads to an expansion of gliadin-specific T cells (in people with genetic sensitivity) and the perpetuation of a pro-inflammatory phenotype in the case of CD, or, on the contrary, of a vascular inflammation mediated by IL-6 found in SLE, which has as a consequence the appearance of perivascular and vascular leukocyte infiltration and vascular dysfunction with the implicit increase in cardiovascular mortality [243,244].

In humans, the intestinal microbiota tends to stabilize and reach a greater diversity around the age of three years, influencing a multitude of physiological or pathological processes of the host either directly (at the digestive level) or remotely by creating links with vital organs such as the heart, working together to maintain homeostasis. In evolution, until adulthood, Gram-positive bacteria such as *Clostridium, Bifidobacterium, Lactobacillus, Ruminococcus, Streptococcus,* and Gram-negative bacteria such as *Bacteroides* and *Escherichia* appear in the intestine. Intestinal dysbiosis observed in autoimmune diseases is associated with a decrease in both bacterial function and diversity, damage to the intestinal barrier function, increased inflammation, and a decrease in regulatory T cells in the intestine, as well as, possibly, with molecular mimicry and T-cell activation, favoring a pro-inflammatory or posttranslational modification of luminal proteins. Worthy of discussion is the variable course of autoimmune diseases in each individual, including monozygotic twins, an observation that reiterates the contribution of environmental factors to the pathogenesis of the disease [245–248]. More and more studies are focused on the correlation between changes in the intestinal microbiota and the occurrence of autoimmune diseases, the main causal evidence regarding CD and SLE predominantly involving the bacterial genera *Bifidobacterium* and *Ruminococcus.* Thus, we identified a lower risk of developing SLE, correlated with a higher risk of CD, in people with a high level of *Bifidobacterium,* together with a higher risk of SLE, but a negative association of CD with respect to *Ruminococcus*, and a decrease in the *Firmicutes/Bacteroidetes* ratio in SLE. It therefore remains open to debate the possible beneficial influence or not of probiotic treatment, with *Bifidobacterium* among children with CD, depending on the type of strain used [249].

It has been shown that immune cells, including dendritic cells, macrophages, and T and B cells, express the vitamin D receptor and 1a-hydroxylase, substances with a role in both calcium homeostasis and the mineralization of the collagen matrix, as well as immunomodulators in innate and adaptive immunity (through control of immune cell growth and differentiation), being anti-inflammatory, antioxidant, and anti-fibrotic. Another known effect of vitamin D is maintaining the integrity of the intestinal barrier (essential in preventing dysbiosis) by regulating the colonic mucus, influencing the composition and functions of the intestinal microbiota, and modulating the release of zonulin. Regarding the intake of vitamin D administered daily (in case of minimal exposure to the sun), the consensus was reached that it represents 400 IU/day for ages older than 1 year, 600 IU/day for ages between 1 and 70 years, and 800 IU/day for 71 years and older, while the upper tolerable level varies between 1000 and 4000 IU/day, comprising 1000 IU for infants 0-6 months, 1500 IU infants 6-12 months, 2500 IU for children 1-3 years, 3000 IU for children 4-8 years, and 4000 IU for children 9 years and older. The prevalence of insufficiency (21-29 ng/mL or 52-72 nmol/L) and vitamin D deficiency (below 20 ng/mL or 50 nmol/L) were also studied among children, recording values of 61% and 9%, respectively, in a study group made up of 6275 participants, with data proving that vitamin D supplementation for five years, with or without omega 3 fatty acids, can reduce

the risk of autoimmune diseases by 22%. The association between vitamin D and autoimmune disease has also been observed to be subject to seasonal variation (higher prevalence in spring-born children) as well as latitude (higher prevalence in northern countries with less UVB radiation) [250–253].

An important element that contributes to the appearance of fatigue is direct or indirect damage to the central nervous system [254]. Psychological stress, including social stress, also seems to be a risk factor in the alteration of the intestinal barrier (consisting of a layer of mucus, intestinal epithelial cells, tight junctions, immune cells, and intestinal microbiota), outlining a vicious circle together with the psychological impact determined by the disease, which can either trigger or aggravate autoimmune manifestations in children [255–257]. In addition, a longitudinal cohort study, which included 2.192.490 children together with their parents, concluded that autoimmune disease was more frequent among children and adolescents with a parental history of autoimmunity (respectively, 7.1% compared to 4.3%) and in girls (54.3% versus 45.7%), with little evidence of an association between autoimmune diseases and most parental mental disorders (e.g., reduced risk for CD identified among children with maternal non-psychotic affective disorders). This finding is delimited by the data in the literature regarding the increased risk of CD among first-degree relatives (parents/siblings) of people with schizophrenia [258]. An important role in the health of these patients is played by the practice of physical activity, which has known effects in terms of both reducing stress and improving cognitive status, but has recently shown a positive impact on the homeostasis of the immune system by modulating the number and function of immune cells, inhibiting the body's systemic inflammatory response, and delaying the onset and development of autoimmune diseases [259,260].

With birth, a complex process is started, a process that affects the mother as well as the fetus (depending on the time and the circumstances in which it was born). The latter goes through an intense period of adaptation to environmental factors during childhood and adolescence, an adaptation that leaves its mark on their health and the balance of later adult life [261]. All of these marks can potentiate the occurrence of autoimmune diseases, a group of diseases which, although considered heterogeneous, have many common aspects from a pathogenic and evolutionary point of view, aspects emphasized with the help of the CD-SLE association model. Studies regarding the involvement of diet in the modulation of the immune system are in continuous development. The first argument for this theory is represented by the observation of an increase in the incidence of these types of diseases, especially in Western countries, possibly in association with the great diversity of diets and unhealthy eating habits based on high contents of fat, sugars, and total calories added, in contrast to a low fiber content (which is accentuated by its inclusion in a GFD for children with CD and an imbalance in the use of fatty acids. These mistakes can promote the alteration of the intestinal barrier function, together with the decrease in the diversity of the microbiome, directly proportional to the diversity of the diet [262–265].

The mechanism by which dietary fibers influence the intestinal barrier and the immune system involves their fermentation by intestinal bacteria, with the subsequent production of SCFAs. These represent both an energy substrate for intestinal cells and a pawn in the development and differentiation of regulatory T cells, thus strengthening beliefs about the diet-microbiota-immunity triad [266]. Other food factors whose balance influences the pathological process (excluding vitamin D, already discussed) are vitamin A, vitamin E, selenium, calcium, iron, magnesium, zinc, omega-3 fatty acids, phytoestrogens, and flavanols, compounds that seem to influence at the same time regulatory T cells and cytokine production and also the development of extra-intestinal manifestations from the neurological, psychiatric or locomotor system spheres. Thus, their supplementation is beneficial for increasing life expectancy and its quality [265,267–271].

At the same time, numerous studies present in the literature highlighted the impact played by epigenetic changes such as DNA methylation (hypomethylation) or histone acetylation in the onset and evolution of SLE. The substances with a beneficial role in influencing this process proved to be folic acid, methionine, choline, and some vitamins from the B complex (methyl donor nutrients) [272].

Reviewing the most important aspects of each food constituent involved in regulating the compensation-decompensation balance of autoimmunity, we observed the following:

Fats consumption is misleading, especially among CD patients, where it seems that lipids are the main constituents of gluten-free products (bread and pasta). Another unfortunate aspect regarding the consumption of lipids is represented by the incorrect dosage of the ratio of saturated fatty acids versus unsaturated fatty acids in the diet [265].

Fiber required 25-31 g/day. There are components that seem to be in deficit both in children under a gluten exclusion regime and in children with a ND (possibly also because of the tendency toward a Western diet, excluding foods rich in fiber in favor of processed ones), but being more accentuated in the first case. Results have led to increased interest in alternating GFD by including compounds such as quinoa, buckwheat, and amaranth [262,265].

Omega-3 fatty acids (eicosapentaenoic and docosahexaenoic) represent substrates for the synthesis of signaling molecules, also exhibiting immunomodulating action on lymphocytes, cytotoxic T cells, natural killer cells, macrophages, monocytes, and neutrophils. It is well known that diet is the main source of essential fatty acids (linoleic and alpha-linoleic) [262,267].

Vitamin A having as its origin pro-VitA, retinol, or retinyl ester, vitamin A in insufficient concentrations deregulates the function of regulatory T cells, causing an excess of T helper 1 in favor of T helper 2, an observation that sparked interest in the study of all-trans retinoic acid, the main metabolite of vitamin A [270].

Vitamin B Complex represents one of the lines of micronutrients affected in autoimmune diseases, and it is therefore essential to know the inverse correlation between the level of total homocysteine in the plasma and that of the vitamins in the B complex (B1, B2, B6, B9), a marker that also proved its effectiveness in a study of the QoL among adult patients with CD [265].

One in ten patients with CD suffer from mineral deficiencies, the gender ratio being equal in terms of calcium and magnesium, while zinc and iron (influenced by factors such as the severity of villous atrophy and the degree of fortification of wheat flour) are deficient in men and women, respectively. Aspects worthy of the clinician's attention regarding the evolution of subjects with CD, especially children, are the increased frequency of the association between iron deficiency anemia and CD; the role played by zinc in protein synthesis, thus imprinting the body's growth, the inflammatory response (Il-2 and Il-2 receptor alpha), and cellular changes; but also the life-threatening consequences of copper deficiency, affecting numerous processes such as the synthesis of hemoglobin and neurotransmitters, iron oxidation, cellular respiration, the formation of pigments and connective tissue [265,267].

Flavanols required 4.2 mg-3 g/day. They interact with the pro-inflammatory cytokines of type Il-1 beta and Il-2, with TGF-beta1 and TNF-alpha, the beneficial effects being best demonstrated by the study of spirulina, known for its content rich in flavanols and sulfalipids [267]

As two autoimmune diseases that share many overlaps in terms of pathogenesis and clinical practice, SLE and CD also follow similar general lines in therapeutic management that include the avoidance of triggering factors, the use of steroid agents in the treatment of the acute phase, and the induction of symptomatology remission and pharmaceutical preparations directed against the various stages of the pathogenic cascade, as presented in Table XXXV.

Recent studies are contradictory regarding the identification of the impact obtained by

the introduction of a GFD among patients with autoimmune comorbidities such as thyroiditis and T1D, without being able to eliminate the risk of bias. The possible positive causal link between the establishment of a GFD in the context of CD and the evolutionary course of other associated autoimmunities is therefore emphasized; in order to define it more accurately, further studies are needed [278–280].

Table XXXV. General directions in the therapeutic management of SLE and CD (adapted from Al-Toma A et al., Fanouriakis A et al., Rubio-Tapia A et al., Pan L et al., and Fanouriakis A et al.) [273–277]

	Celiac disease	Systemic lupus erythematosus
Acute hives	• Hospitalization and parenteral nutrition-in celiac crisis (risk of hemodynamic instability, orthostatic hypotension, neurological and renal dysfunction, metabolic acidosis, hypoalbuminemia, and electrolyte disorders); • Steroids;	• Hospitalization and rebalancing of renal, hematological, neuropsychological, and other affected organ systems; • Pulsations with intravenous methylprednisolone (250-1000 mg/day for 3 days), doses adapted according to severity and body weight, administered after excluding infections;
Background treatment	• Avoiding the factors that precipitate the disease: GFD (safety limit between 10-100 mg/day), with the elimination of cereals and food products derived from wheat, barley, or rye; • Supplying nutritional deficiencies: iron, calcium, copper, zinc, folate, vitamin B12, B6, D; • Fiber-based diet (corn, potatoes, vegetables); • For refractory CD: - Steroids-Oral Budesonide/Azathioprine (2-2.5 mg/kg/day) + Prednisone; - Infliximab; - Mesalamine; - Purine analogue inhibitors (Cladribine or Fludarabine): 0.15 mg/kg/day for 5 days; - Jak3 inhibitor (Tofacitinib) or anti-Il-15; - Transplantation of autologous hematopoietic stem cells.	• Avoiding exposure to ultraviolet radiation, smoking and drugs with the potential to induce SLE; • Hydroxychloroquine (<5 mg/kg)-presents a risk of retinopathy; • Glucocorticoids (<7.5 mg/day); • Cyclophosphamide-may increase the risk of malignancies; • Immunomodulatory agents (Methotrexate, Azathioprine, Mycophenolate); • Belimumab; • Rituximab (anti-CD20)-with response on refractory SLE; • Tocilizumab (anti-Il-6); • Etanercept (anti-TNF).

Therefore, new paradigms are introduced regarding the control of autoimmune diseases with the help of balanced, carefully chosen diets. Starting from the premise "we are what we eat", we demonstrated in the previous sections that, although there are multiple links at the level of the etiological factors determining autoimmune diseases and the clinical aspects (which can sometimes go as far as overlapping pathologies), one of the most consistent roles in the control of the condition is played by dietary constituents. This idea must be considered by the clinician and exploited in the patient's therapeutic course, it being an easy treatment measure with a low cost and psychological impact, but with numerous benefits in increasing the patient's QoL.

Continuing the CD-SLE model, we discuss a list of foods in the composition of which high concentrations of the most important nutrients in autoimmune diseases are found:

- Folic acid: green vegetables, bell peppers, beans, lentils;
- Methionine: eggs, yogurt, cheese, red meat;
- Choline: beef liver, egg, soy, potatoes, quinoa, peanuts, carrots, apples, broccoli;
- B complex vitamins: rice, quinoa, apple, strawberries, bananas, watermelon, walnuts, spinach, onions, tomatoes, chickpeas, beans, potatoes, salmon, tuna, beef liver, milk, yogurt, cheese;

- Flavanols: cocoa, red grapes, tea, berries, apples;
- Vitamin A: liver, carrots, sweet potatoes;
- Vitamin E: quinoa, amaranth;
- Omega-3 fatty acids: chia/flax seeds, sardines, milk, beans, salmon, soy [265,267,270,272,281].

II.1.3.4. Conclusions

The current work presented the interactions between the two pathologies from the perspective of their common aspects, starting from similar diagnostic models (presence of autoantibodies, circulating immune complexes, inflammation and multisystem damage, with histological impact on the target organs), the influence at the genomic level, the impact of viral infections and IgA deficiency, the endogenous and exogenous factors that modulate the clinical expression, and also the common therapeutic lines in acute and long-term management. It was thus emphasized that the two pathologies are strongly correlated on multiple levels; there are also presented in the literature cases of the overlap of these, with or without other autoimmune diseases, which draws attention to the way in which they influence each other. A good knowledge of the many interactions that occur at the level of the autoimmune system is important in future medical practice, as the evolution of treatment means must go in tandem with the evolution of prophylaxis means in order to maintain an optimal QoL.

II.1.4. Dysbiosis - Heart Failure

II.1.4.1. Aim of the Study

The aim of this review is to analyze the current evidence available in the literature regarding gut-heart interactions and the insights of the "gut hypothesis" of heart failure (HF), highlighting the importance of the gut microbiota and their derived metabolites as a new frontier in HF research and a potential treatment target.

II.1.4.2. Materials and Methods

The research team conducted an investigation to highlighting the correlation between gut microbiota and HF using the following searchable databases: Cochrane Central Register of Controlled Trials (CENTRAL), Excerpta Medica Database (EMBASE), Medline, PubMed, Scopus, and Web of Science.

II.1.4.3. Results and Discussions

The human gut microbiome is recognized as an organ unto itself with significant interactions inside the human system, getting involved in a variety of immunological, neurological, metabolic, and endocrine responses [247]. The highest concentration and diversity of microorganisms from the human body lies in the GI, consisting of more than 500 distinct species of bacteria, viruses, fungi and protozoa [282,283]. The GI microbiota are represented by five primary bacterial phyla: the *Firmicutes* (synonym *Bacillota)* and *Bacteroides* (synonym *Bacteroidota)* phyla predominate the microbiome and represent more than 90% of total bacterial communities, while the *Proteobacteria* (synonym *Pseudomonadota), Actinobacteria* (synonym *Actinomycetota),* and *Verrucomicrobia* phyla are represented in smaller proportions [247,284]. Although the *Bacillota* phylum consists of more than 200 different genera such as *Bacillus*, *Lactobacillus*, *Enterococcus*, *Ruminococcus* and *Clostridium,* and the *Clostridium* genus represents 95% of the phylum. The *Bacteroidota* phylum is predominated by the *Prevotella* and *Bacteroides* genera. The *Actinomycetota* phylum is significantly less abundant than *Bacteroidota* phylum and the *Bifidobacterium* genus is its

main representative [285].

The microbiome is not inherited, but acquired, and its composition is changing through different stages of each individual's life, with a unique composition and microbial diversity [286,287]. Its development starts early, in prenatal life, and continues during birth and through senescence [288,289]. The following interfere with microbiome composition, leading the way to health or disease: sex; genetics; the mother's influence during pregnancy and birth; feeding practices in early childhood; dietary habits; antibiotics; tobacco and alcohol use; a sedentary lifestyle associated with the socioeconomic conditions; household pets; pollution; and geographical distribution [283,288–291].

The activities of the human gut microbiota are substantially similar between people, despite the fact that each person's gut microbiota is defined by a unique collection of bacterial species due to various inter- and intra-individual changes throughout human life [247]. In addition to one's genetic susceptibility, the diversity of the microbiome's composition plays a key role in each individual's personalized response to different environmental exposures such as diet, xenobiotics and medical treatments [292].

Inflammatory response can be directly controlled by the microbiota in the gut, inducing either innate or adaptive immune responses, or it can alter the immune cells' function using active metabolites, including SCFAs, trimethylamine N-oxide (TMAO) and indoleacetic acid (IAA) [293–295]. It appears that dysbiosis of the gut bacterial communities produces alterations to the microecological environment of the GI, becoming a pathogenic factor in a wide spectrum of diseases, including gastrointestinal disorders, inflammatory, respiratory, metabolic, and neurologic diseases [247,292].

Impaired Gut Barrier Function and inflammation in Heart Failure

The "gut hypothesis" in HF suggests that the gut microbiota, its metabolites, and HF pathophysiology are highly correlated, as shown in Figure 17 [292,296]. Although this bidirectional communication is not fully understood, evidence indicates that this bacterial translocation appears in HF resulting from various mechanisms leading to structural and functional alterations of the GI tract, from splanchnic congestion to the host's immunological defense system [297].

There is evidence that HF is related with impaired intestinal epithelial function: an alteration that seems to be the result of a reduced intestinal perfusion and ischemia [287,298]. A decreased cardiac output leads to an adaptive re-distribution of the systemic circulation to several end-organs [292]. Consequently, there appears to be an increase in intestinal wall edema, with the bowel wall thickening being positively related to increased markers of intestinal permeability, blood leukocytes and circulating levels of CRP [299]

A reduction in absorptive capacity and an increase in the gut's epithelial permeability are two additional characteristics of HF in addition to intestinal wall edema, facilitating the passage of several intestinal bacterial and/or endotoxins, such as lipopolysaccharides (LPS), from the gut to the systemic bloodstream [299,300]. LPS is a biologically active constituent of the Gram-negative bacterial wall with potential immunostimulatory activity by using the Toll-like receptor 4 pattern recognition receptor [301]. In HF patients, high LPS concentrations found in the hepatic veins sustain the hypothesis of an intestinal translocation process of gut microbes [302]. Moreover, it has been postulated that LPS itself can contribute to mucosal barrier functional deterioration, leading to HF progression [303]

Intestinal endotoxin absorption leads to the increase in inflammatory cytokine levels throughout the body [287]. According to current data, HF appears to be correlated with a chronic state of inflammation that can be induced or accelerated by this microbial translocation, indirectly affecting cardiomyocytes' normal function [304].

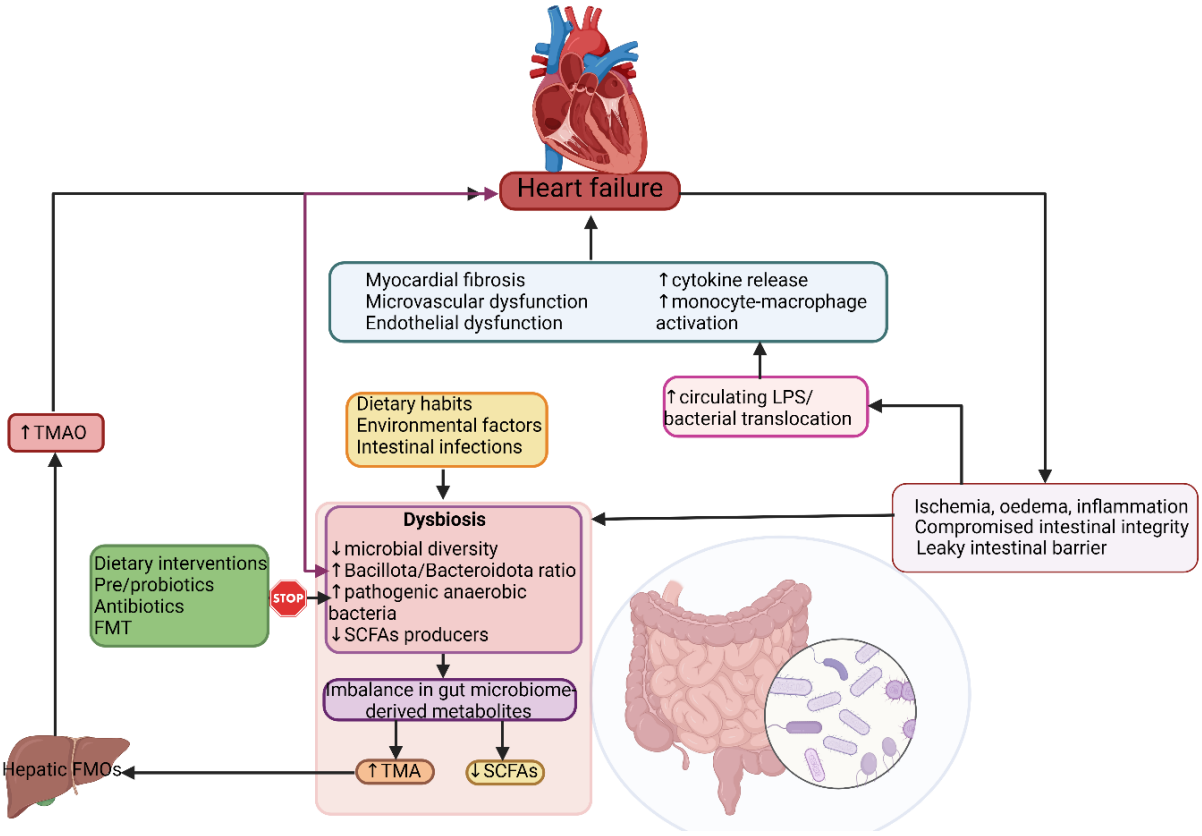

Figure 17. Concept of the gut-heart axis adapted to HF

It seems that increased levels of circulating cytokines correspond to more severe clinical symptoms and to a worse prognosis in HF patients' survival [305,306]. Serum levels of TNF-alpha, IL-1 and IL-6 of HF patients are directly influenced by the amount of existing LPSs, currently thought to be leading elements of a hyperinflammatory condition [307]. While in decompensated HF patients, LPS levels appear to be directly associated with systemic inflammation markers, and they decrease following HF recompensation. Treatment administration is not always followed by a decrease in plasma cytokine levels, suggesting a sustained effect as the disease progresses [287,308]. According to two large, randomized placebo-controlled trials, neither of the TNF-alpha antagonists' administration decreased the risk of hospital admission or death in HF patients [309,310].

Dysbiosis in Heart Failure

It has been demonstrated that the gut microbiota, which are the most significant active components, have a massive effect on HF. Besides the correlation with inflammation and increased intestinal permeability, an analysis using fluorescence in situ hybridization described the presence of bacterial overgrowth as mucosal biofilm and an increased bacterial adhesion in the sigmoid colon mucus of HF patients. The increased intestinal juxta mucosal bacterial biofilm has been correlated with an amplified immunoglobulin A-anti-LPS response [287,298].

In stable chronic HF with reduced ejection fraction (HFrEF) patients, an increased level of pathogenic bacteria such as *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia* species, as well as yeasts including *Candida* species, have been reported as assessed by microbial culture methods; their levels being correlated with HF severity [311]. Consistent with these results, there is evidence that the *Escherichia/Shigella* genus is increased in the same patient known with HF, during its decompensated compared to the compensated phase of disease [312]. Indeed, pathogen overgrowth increases the risk of developing invasive gastrointestinal infections in HF patients. A U.S. nationwide study of hospitalized patients, treated with

antibiotics, revealed that HF is more likely to develop an additional *Clostridium difficile (C. difficile)* infection and have substantially worse in-hospital prognosis, in comparison to non HF controls [313]

Using 16S rRNA gene sequencing Sun and colleagues [314] analyzed fecal samples of patients with severe forms of chronic HF and compared the results with the one obtained from healthy controls. They reported reduced alpha diversity in chronic HF patients and important differences in beta diversity between the two groups. *Bacillota* phylum was found to be dominating the chronic HF patient's fecal microbiota, but in smaller levels than the controls. *Pseudomonadota* and *Actinomycetota*, however, were reported to be more abundant than in the control samples. Moreover, *Pseudomonadota* phylum was the second most abundant phylum found in severe chronic HF patients instead of *Bacteroidota* phylum. *Pseudomonadota* phylum is composed of Gram-negative bacteria, mainly pathogens, and is thought to be a microbial signature of dysbiosis in gut bacterial communities [314]. Zhang et al. [315] reported similar results, with reduced amounts of the *Bacillota* phylum and an augmentation in the *Bacteroidota* phylum [315]. At the genus level, the microbiota of chronic HF patients was found to be less abundant with *Faecalibacterium* and more abundant with *Escherichia, Shigella, Enterococcus* and *Klebsiella* spp. than that in the healthy controls [314]. The increased abundance of Gram-negative bacteria is responsible for the amount of LPS translocated into the bloodstream, accelerating HF progression [316].

Yuzefpolskaya and colleagues [317] have evaluated the microbiome of HF patients with different degrees of severity and reported that alpha diversity was reduced as disease severity levels increased and remained low despite receiving interventional treatment such as left ventricular assist device or heart transplantation, probably due to persistent inflammation. Alpha diversity seemed to be negatively correlated with levels of inflammation and endotoxinemia (LPS and sCD14). Therefore, as HF evolves into advanced stages, levels of endotoxinemia and systemic inflammation increase and the gut diversity of bacterial communities decreases [317]. Several studies of the intestinal bacterial profile in patients with acute decompensated or stable HFrEF have reported that HF patients have a significantly reduced alpha and beta diversity compared to healthy individuals, providing evidence for the HF-induced community composition shifting of the gut microbiota [247,318–323]. Similarly, another small study on HF patients reported a different microbiota composition between individuals with congestive heart failure and healthy volunteers, although between ischemic and dilated cardiomyopathy no noticeable differences could be identified [324].

In a much more comprehensive study, Jia et al. [325]reported elevated levels of several Streptococcus species and genera of the *Enterobacteriaceae* family, and a decreased abundance of *Faecalibacterium prausnitzii* and *Roseburia intestinalis*, known producers of the SCFA butyrate [326]. Zhu and colleagues [327] reported an elevated abundance of *Enterococcus, Escherichia* and *Shigella* spp. and a decreased abundance of *Roseburia, Faecalibacterium* and *Eubacterium rectale*, known as butyrate producers [327].

Kamo and colleagues [328] reported reduced diversity of microbial communities in the GI tract of HF patients, but also stated that HF-associated gut dysbiosis varied according to the patient's age. Compared to younger patients known with HF, older patients seemed to display decreased levels of *Bacteroidota* and elevated amounts of *Pseudomonadota. Dorea longicatena and Eubacterium rectale*, members of the *Lachnospiraceae* family, were decreased in all patients known with HF while *Clostridium clostridioforme* and *Faecalibacterium prausnitzii*, members of the *Ruminoccaceae* family, were found in smaller amounts in older HF patients compared to younger patients [328].

Studies on the gut microbiota in HF patients are summarized in Table XXXVI.

Table XXXVI. Gut microbiota composition studies in patients with heart failure (HF)

Study	Patients	Patients Age	Sample size	Method	Gut Microbiota Profile
Kamo et al. [328]	Acute HF or exacerbation of chronic HF	47.4±2.8 years 73.8±2.8 years	n = 12 HF < 60 years n = 10 HF > 60 years n = 12 controls	16S rRNA	↓*Eubacterium rectale, Dorea longicatena* Depletion of *Faecalibacterium* in older patients
Sandek et al. [299]	Chronic HF	67±2 years	n = 22 Chronic HF n = 22 control	Fluorescence in situ hybridization	↑*Eubacterium rectale Faecalibacterium*
Pasini et al. [311]	Chronic HF	65±1.2 years	n = 60 HF n = 20 control	Traditional culture techniques	↑Campylobacter Shigella Salmonella Yersinia enterolytica Candida
Sun et al. [314]	Chronic HF	60.69 years	n = 29 HF n = 30 controls	16S rRNA	↓*Ruminococcaceae Lachnospiraceae Dialister* ↑*Enterococcus Enterococcaceae*
Zhang et al. [315]	Chronic HF	65-86 years	n = 29 NYHAIII HF n = 29 NYHA IV HF n = 22 controls	16S rRNA	↑*Escherichia* and *Bifidobacterium* (NYHA III) ↑*Klebsiella* and *Lactobacillus* (NYHA IV)
Luedde et al. [323]	Chronic HF: 70% exacerbation, 30% stable	65±3.2 years	n = 20 HF n = 20 controls	16S rRNA	↓*Coriobacteriaceae, Erysipelotrichaceae, Ruminococcaceae* (family level) ↓*Blautia* (genus level)
Kummen et al. [318]	Chronic HF	NA	n = 40 discovery n = 44 validation n = 266 control	16S rRNA	↓*Lachnospiraceae* family:
Cui et al. [324]	Stable chronic HF: Ischemic or dilated cardiomyopathy	58.1±13.3 years	n = 53 HF n = 41 controls	16S rRNA	↑*Ruminococcus gnavus* ↓*Faecalibacterium prausnitzii*
Beale et al. [334]	HFpEF	40-70 years	n= 26 HFpEF n = 67 control	16S rRNA	↓*Ruminococcocus* spp.
Wang et al. [335]	Chronic HF	65±3.17 years	n = 26 HF n = 26 controls	16S rRNA	↑*Escherichia Shigella Ruminococcaceae, Lactobacillus Atopobium Romboutsia Streptococcus Haemophilus Klebsiella*
Katsimichas et al. [336]	Non-ischemic HFrEF	18-70	n = 28 HFrEF n = 19 controls	16S rRNA	↑*Streptococcus* spp. *Veillonella* spp. ↓SMB53
Hayashi et al. [312]	De novo acute decompensated HF/acute worsening of chronic HF	72±18 years	n = 22 HF n = 11 controls	16S rRNA	↑*Actinomycetota* phylum *Bifidobacterium* genus ↓*Megamonas* genus

D. longicatena is a bacterium that produces acetic acid, an SCFA, as a fermentation product. However, acetate can be further used as a substrate in order to generate butyrate [329]. *Eubacterium rectale,* another butyrate producer bacterium, was identified at increased levels in gut mucosal biofilms of HF patients by Sandek and colleagues [298]. In contrast, another study

by Kamo et al. [328] reported decreased levels of the bacteria as characterizing HF [328]. *Faecalibacterium prausnitzii*, another butyrate-producing commensal bacteria with anti-inflammatory properties, was found to be decreased in abundance in HF patients, negatively affecting the intestinal permeability [324,330,331]. Butyrate-producing bacteria are essential for the state of well-being of each individual, as butyrate is used as an energy source for intestinal epithelial cells, and it regulates the integrity of the epithelial barrier and suppresses the intestinal and extra-intestinal inflammation [332,333]. The decreased levels of *F. prausnitzii* and increase in *Ruminococcus gnavus* were found to be important characteristics of gut microbiota in chronic HF patients [326].

Risk Factors for HF and Gut Microbiota

HF patients are known to have a variety of critical factors, but the majority of them have hypertension, obesity, dyslipidemia, diabetes, a genetic predisposition to HF, smoking, a sedentary lifestyle, or unhealthy food habits [337–340]. New evidence suggests that gut microbiota and its metabolites could have an impact on HF risk factors as well.

Dietary Choices

The western diet is known for its high intake of sugar and processed carbohydrates, which have a high glycemic index; content that inhibits nitric oxide synthase, resulting in myocardial oxidative dysfunction, cardiac hypertrophy and cardiomyocyte remodeling, all known to be predisposing factors for HF [341]. This diet rich in fast-food aliments and glucose leads to dysbiosis state characterized by elevated *Pseudomonadota* and *Bacillota* levels, which increases the levels of TMAO and ceramides, promotes cholesterol accumulation in macrophages and promotes atherosclerosis development [342]. The western diet also leads to lipid accumulation in the myocardium, chronic inflammation and obesity [343]. Increased levels of salt and dietary additives used in fast-food alimentary processing, including nitrites and phosphates, have been associated to an increased risk of HF. They alter the *Bacillota* to *Bacteroidota* ratio [344]. Moreover, this diet alters gut barrier permeability, characterized by the decreasing levels of *Bacteroidetes* spp., *Bifidobacterium* spp., *Clostridiales* spp., *Lactobacillus* spp. and *Akkermansia muciniphila,* as well as all gut barrier-promoting bacteria. Furthermore, the intestinal wall integrity seems to be disrupted by an increase in *Desulfovibrio* spp. and *Oscillibacter* spp. [344].

Obesity

Savji and colleagues [345] in their study reported that obesity and its associated dysmetabolism, including hyperlipidemia, hyperglycemia and insulin resistance, are strongly correlated with HF [345]. A pro-inflammatory environment characterized by elevated levels of pro-inflammatory cytokines is promoted by obesity and its associated cardiometabolic factors (insulin resistance, dyslipidemia and abdominal adiposity) [346]. The endothelial dysfunction and the nitric oxide unavailability might lead to left ventricular hypertrophy and systolic and diastolic dysfunction in HFpEF [346,347]. Furthermore, obesity can cause modifications in vasculature and blood volume which, associated to the increased consumption of oxygen, conducts to ventricular hypertrophy, increased mean pulmonary arterial pressure and elevated left ventricular diastolic pressure [348].

In both animal and human studies, obesity seems to be associated to a modified ratio between *Bacillota* and *Bacteroidota* phylum in most research, with a decrease in *Bacteroidota* and an increase in *Bacillota* [349]. The amount of *Bacteroidetes* found the intestinal microbiota has been reported to be relevant in obesity. Obese people that follow a calorie-restricted diet and lose weight seem to have an elevated ratio of *Bacteroidetes* species in their gut microbiota [350]. Specifically, *Clostridium bartlettii, Akkermansia muciniphila* and *Bifidobacteria,* all SCFA producers, have been negatively associated with obesity induced by a high fat diet and its metabolic complications [351,352].

Type II Diabetes Mellitus

T2DM is a major risk factor for cardiovascular failure and other CVDs. Patients known to have T2DM present a decreased level of bacterial genera such as *Faecalibacterium, Bifidobacterium, Akkermansia, Bacteroides* and *Roseburia. Roseburia, Bacteroides* and *Akkermansia* have anti-inflammatory effects. *Bacteroides* and *Akkermansia* in decreased levels lead to an under expression of tight junctions' genes, elevated "leaky gut", and, in consequence, endotoxemia [353]. Furthermore, the reduced abundance of the butyrate-producing *Faecalibacterium prausnitzii* and *Roseburia intestinalis* dysregulates the metabolism of fatty acids, leading to oxidative stress and its associated cardiometabolic adverse manifestations [354,355]. On the other hand, T2DM is positively associated with bacteria from *Fusobacterium* and *Ruminococcus* genera, and the phylum *Bacillota*, all with pro-inflammatory activity [356].

Hypertension

Constantly high blood pressure patients present a higher (up to five-fold) *Bacillota-to-Bacteroidota* ratio in comparison to normotensive controls [357]. Moreover, the intestinal microbiota is dominated by lactate-producing genera (e.g., *Turicibacter* and *Streptococcus),* while SCFA-producing ones appear to be reduced (such as *Clostridiaceae, Bacteroides and Akkermansia)* when hypertension is present [358,359]. Some of these associated perturbations in gut microbiota homeostasis are partially related to HF pathogenesis and increase the risk of HF progression.

Gut-Derived Metabolites as Possible Biomarkers Related to Intestinal Dysbiosis in HF

Gut microbial-derived metabolites can also play a significant role in the pathogenesis of HF. It appears that the gut microbiome acts similarly to an endocrine organ. By generating active biometabolites including SCFAs, trimethylamine (TMA)/ TMAO, and bile acids, the gut microbiome influences the host physiology. Several studies described the association of the gut's microbiome metabolites and different pathologies including hypertension, atherosclerosis, HF, obesity, chronic kidney disease, and T2DM [292,297,360–363]. These metabolites can be considered as biomarkers of intestinal dysbiosis and can predict inflammation in patients known with HF [363]. These patients with elevated plasma levels of phenylalanine display increased levels of inflammatory cytokines (IL-8, IL-10), CRP and associate higher mortality [364], whereas glycine manifest anti-inflammatory effects and seem to offer protection to the cells and heart [365]. Furthermore, in an analysis of data gathered from the FINRISK and PROSPER cohorts, phenylalanine was reported to be an independent predictor of HF [366]. A recent study conducted by Hayashi and colleagues [367], used whole genome shotgun sequencing for analyzing fecal samples and mass spectrometry-based profiling of amino acids and identified a possible correlation between amino acid metabolic disturbances and gut dysbiosis in patients diagnosed with HF [367].

Alterations of gut microbiota composition, especially elevated TMAO levels are correlated with the risk of developing HF [368]. TMAO is a metabolite produced by gut bacteria including *Bacillota* and *Pseudomonadota,* obtained from choline, phosphatidylcholine, and L-carnitine fermentation [292]. Chen and colleagues [368] reported that an elevated level of TMAO resulted from a diet high in saturated fat and sugar can lead to fibrosis, myocardial inflammation and to impaired diastolic function. Individuals with an increased abundance of *Ruminococcus*, *Prevotella* and *Clostridium* genera and the *Lachnospiraceae* family, and decreased levels of *Bacteroidota,* revealed higher levels of TMAO in their plasma [369,370]. HF-associated dysbiosis is characterized by high levels of circulating TMAO, that are able to stimulate cardiac remodeling through promoting myocardial fibrosis and pro-inflammatory effects [292,341,371,372]. Available evidence reports that the overexpression of cytokines with pro-inflammatory action, including Il-1p, and TNF-a and the attenuation of IL-10 and other cytokines with anti-inflammatory properties are both stimulated by increased levels of TMAO

[342,373].

Acetate, propionate, and butyrate are examples of SCFAs that are produced by gut bacteria including *Bacteroide*s, *Bifidobacterium* and *Faecalibacterium* spp. [319]. They are the most important metabolites produced through colon bacteria fermentation of resistant starch and dietary fibers [363]. Most evidence sustains the fact that SCFAs have a protective role against HF and play a significant role in maintaining the integrity of the intestinal barrier: in mucus production and they are active in anti-inflammation protection [374]. However, increased SCFA levels in fecal samples are considered to be a marker of hypertension, central obesity and cardiometabolic disease subclinical measures [375]. There is evidence that SCFAs are closely associated to atherosclerosis [376]. In a rodent model, butyric acid supplementation through the diet inhibited the atherosclerotic lesions of apolipoprotein E by reducing the macrophage migration rate and increasing the collagen deposition and plaque stability [377].

In chronic HF patients, there was an increase in microbial genes responsible for LPS biosynthesis, lipid metabolism, tryptophan, and particularly TMAO production [324]. On the other hand, microbial genes for butyrate-acetoacetate CoA transferase, a vital enzyme for butyrate synthesis as well as SCFA-producing bacteria, were importantly reduced in chronic HF patients [378]. Levels of ricinoleic acid, a gut microbiota metabolite with anti-inflammatory proprieties, were found to be highly decreased in these patients' plasma [378]. Moreover, ricinoleic acid levels were reported to be negatively associated with the bacterial communities found to be enriched in chronic HF patients' guts and positively correlated to those dominating the microbiota of controls [324]. Elevated levels of cardiovascular-harmful metabolites including sphingosine 1-phosphate and a diminished value of beneficial cardiovascular metabolites such as orotic acid was also reported [324]. This functional alteration sustains the link between chronic HF and an imbalance of gut microbial communities and their metabolites.

Another study team focused their attention on a limited number of elderly people with persistent heart failure. When evaluating the relationship between gut microbiota representatives and its metabolites, Wang et al. [335] reported that *Escherichia* and *Shigella* spp. were negatively associated with riboflavin and biocytin. *Haemophilus* spp. was negatively associated with cellobiose, alpha-lactose, lactose, isomaltose, sucrose, melibiose, turanose and trehalose. *Klebsiella* spp. was positively associated with ethylsalicylate and bilirubin, and negatively related to hexanoylcarnitine, citramalate, isovalerylcarnitine, inosine, methylmalonate and riboflavin. The authors concluded that the gut microbiota alteration in chronic HF is associated with various modifications of the serum metabolic map [335].

Luo et al. [379], in a two-sample mendelian randomized study, demonstrated that *Candida*, *Campylobacter* and *Shigella* spp. were not correlated with an increased incidence of HF. However, when analyzing the genetic prediction, it was suggested that for every 1 unit increase in *Shigella* concentration, there is an increase of 38.1% in the relative risk for myocarditis and an increase of 13.3% for hypertrophic cardiomyopathy. Moreover, for every 1 unit increase in *Candida* concentration, there is an increase of 7.1% in the relative risk of chronic kidney disease. As for intestinal metabolites, the genetic prediction report suggested that the relative risk of myocardial infarction and HF increases by 1.4% and 1.7% separately, for every 1 unit increase in betaine [379].

Interactions between the Gut Microbiome and Cardiovascular Drugs

Individual responses to medication therapy can be influenced by age, sex, nutritional condition, illness states, genetics, and environmental exposures [380]. The human microbiome is known for its involvement in drug metabolism and pharmacological efficacy, but among them there is bidirectional communication, as drugs can also influence microbiota composition.

Drug absorption is a complex process that depends on a number of variables, including the medications' interaction with host and microbial enzymes, their solubility and stability in GI fluids, pH, GI transit time, and permeability through epithelial membranes [381]. The human

gut microbiota is genetically capable of producing enzymes involved in oral drugs' metabolism, facilitating their absorption across the gut and through the bloodstream [380]. Dysbiosis of the gut's bacterial communities can further alter drug pharmacokinetics; the activation of prodrugs can contribute to the production of unwanted toxic metabolites and the inactivation of drugs [382]. Variation in drug response can also be present in a "healthy" gut, due to inter-individual differences in intestinal bacterial species [247].

Related to the cardiovascular medication used in HF patients, metagenomic sequencing of stool samples from HF patients revealed that the use of several pharmaceutical agents such as statins, beta-blockers, angiotensin-converting enzyme inhibitors and platelet aggregation inhibitors has an important influence on gut microbial composition [383]. Despite the fact that specific underlying mechanisms are unknown, partial results of this study were reproduced by another British group of researchers [384]. Examples of microbial biotransformations are listed in Table XXXVII.

Table XXXVII. Known and proposed mechanisms by which the gut microbiota may influence cardiovascular drug outcomes, adapted from Tuteja et al. [380]

Drug	Bacteria	Mechanisms	Outcome
Known drug-microbiota interaction			
Digoxin [385]	*Eggerthella lenta*	Inactivation by reduction	Bacterial reductase activity reduces the quantity of active drug reaching target tissues
Proposed drug-microbiota interaction			
Simvastatin [390]	unknown	Microbial derived bile acids competing for host uptake transporters Disruption in bacterial communities with bile salt hydrolase (bsh) activity	Reduced amount of drug reaching target tissues FXR receptor signaling variability
Rosuvastatin [391]	unknown	Disruption in host gene expression of bile acid metabolism pathways Disruption in bacterial communities with bsh activity	FXR receptor signaling variability
Atorvastatin [392]	unknown	Reduced quantity of secondary bile acids	FXR receptor signaling variability
Amlodipine [393]	unknown	Pre-systemic metabolism by dehydrogenation	Reduced quantity of active drug reaching target tissues
Captopril [380]	unknown	unknown	Improved villi length and reduced intestinal permeability
Aspirin [394]		unknown	Bacterial communities alteration
Warfarin [395]		Antibiotics eliminate vitamin K producing bacteria	Increased bleeding events

Cardiac Glycosides

Digoxin, a drug frequently recommended in HF is a good example of microbiota influencing drug bioavailability. Some strains of *Eggerthella lenta* are responsible for converting digoxin into an inactive microbial metabolite, limiting the quantity of active drug absorbed into the systemic bloodstream in an important 10 percent of patients [385,386]. Recent studies offered proof that coadministration of digoxin together with antibiotics or an arginine rich diet both resulted in elevated systemic digoxin levels and clinically relevant fluctuations in drug levels [385,387].

Blood Thinners and Gut Microbiota

Aspirin is a NSAIDs commonly used to decrease the risk of cerebrovascular and

cardiovascular disorders [386]. Existing evidence demonstrates its ability to disrupt the gut's microbiota composition. Patients using aspirin present variations of *Ruminococcaceae, Prevotella, Barnesiella* and *Bacteroides* bacterial levels in comparison to individuals not using or using other types of NSAIDs. Furthermore, the gut's bacterial communities' composition seems to exert influence on aspirin metabolism. While oral antibiotic administration can decrease the gut microbiota's metabolic activity by slowing its degradation, increasing its bioavailability and prolonging its anti-thrombotic action, probiotics containing *Bifidobacterium breve* Bif195 bacteria can protect against an aspirin intake adverse reaction, such as intestinal wall damage and aspirin-induced gastric ulcers [388,389].

Warfarin, a frequently used anticoagulant expresses its effect by inhibiting vitamin K-dependent activation of clotting factors II, VII, IX and X. Bleeding episodes associated with warfarin use increased when given together with antibiotics [396]. Two mechanisms have been cited. Antibiotics can interfere with warfarin use through inhibition or induction of CYP enzymes and can also alter the intestinal bacterial composition, eliminating vitamin K-producing bacteria, such as the *Bacteroides* genus [395,397].

The Effects of Beta-Blockers, ACEi, and ARBs on Gut Microbiota

Studies on both animals and people have been done to investigate the effects of antihypertensive drugs. Despite expectations, the association between the use of beta-blockers, angiotensin receptor blockers (ARBs) and angiotensin converting enzyme inhibitors (ACE inhibitors) can modify the composition of gut microbiota. A positive association was reported from a large metagenomics study, between calcium channel blockers, ACE inhibitors and bacterial composition of the gut [398]. Moreover, ACE inhibitors, including captopril, have been shown to have beneficial effects on hypertensive rats by diminishing gut dysbiosis, ameliorating the intestinal wall's permeability and increasing villi length [380,394].

Statins and Gut Microbiota

The capability of statins to lower cholesterol and low-density lipoprotein-C (LDL-C) levels makes them attractive medications. Inter-individual variations in the response to statin treatment are well-known and are not related to a specific statin agent or dose [383]. Studies have proven their action on modulating gut bacterial communities' composition [380,391]. Individuals treated with atorvastatin presented an increased level of anti-inflammatory gut bacteria such as *Faecalibacterium prausnitzii* and *Akermansia muciniphila,* whereas untreated patients known with hypercholesterolemia displayed an increased level of bacterial species with pro-inflammatory effects, such as *Collinsella* and *Streptococcus* [399]. LDL-C levels seem to be negatively correlated to the phyla *Bacillota* and *Fusobacteria,* while *Lentisphaerae* and *Cyanobacteria* spp. were positively associated with LDL-C [391]. Existing evidence suggests that the LDL-C response to statin treatment can be influenced by bacteria containing bsh. Administration of *Lactobacillus reuteri,* one of the gut's bacteria with elevated bsh activity, resulted in an important reduction of LDL-C levels [400]. The same study reported that individual variations in LDL-C levels were inversely correlated with circulating bile acids. The *Bacillota* phylum, previous negatively associated with LDL-C levels, has also been associated with bsh activity [391,401]. Several animal models sustain the beneficial effect of statin therapy on microbial communities of the gut [392,399,402].

Modulation of Dysbiosis as a Potential Target in Heart Failure

The ability to identify each patient's gut microbiota and his disease-related dysbiosis permits the development of a customized, focused treatment strategy. Although there are various ways to manage and modulate the dysbiotic intestinal microbiota, such as dietary interventions (which also include the use of prebiotics, prebiotics and postbiotics) and fecal transplantation, several reports from the available literature place diet modification and probiotic use as the main interventions for microbiota modulation.

Diet has always been considered as a significant factor in determining the composition

and function of the microbiota that is associated with the gut. A 5-day adjusted diet has been shown to produce beneficial changes in the number and species of the gut microbiota [403]. Often cited in the medical literature, the Mediterranean diet (MD) consists of elevated levels of polyunsaturated fatty acids, dietary fiber, polyphenols, and a small quantity of red meat [341]. Among its recognized benefits on human health, an MD provides an increased abundance of probiotics, greater biodiversity, elevated SCFAs, and reduced TMAO [404,405]. Adherence to an MD was associated with a decreasing HF incidence up to 74% [406]. Moreover, it seems that the high compliance to MD is negatively associated with HF and improved the long-term prognosis for HFpEF patients, as it results from a 10-year follow-up. The MD might have an anti-inflammatory effect, as the beneficial action correlates with CRP levels [407,408]. The Dietary Approaches to Stop Hypertension (DASH) eating plan represents a diet that is rich in whole grain aliments, vegetables, fruit and low-fat dairy foods, and offers a significant potential in decreasing the HF incidence [409,410].

A high-fiber diet has recently been demonstrated to improve gut dysbiosis (described by the *Bacilliota* and *Bacteroidota* ratio), reduced blood pressure, improved cardiac function and normalized cardiac hypertrophy in a hypertension-induced HF experimental model [411]. Additionally, fermentation of fiber results in augmented SCFA production, with their beneficial actions on human health [408].According to World Health Organization, probiotics are living microorganisms that benefit the host when administered in the proper amounts [412]. Among their beneficial effects, we recall their capacity of regulating the altered intestinal microbiota, the protection of the integrity of the epithelial barrier, their capacity to inhibit the adhesion of pathogenic microbiota through competition, their encouragement of the production of B-cell-secreting IgA, mucin, as well as SCFAs with immune modeling and anti-inflammatory effects [412–415]. The most used probiotics are different strains of bifido bacteria, yeasts, and lactic acid bacteria [308,416].

In a rat model, oral administration of *Lactobacillus plantarum 299v* and *Lactobacillus rhamnosus GR-1* induced beneficial cardiac effects [417–419]. Lactobacillus supplementation seems to promote SCFA-producing bacteria such as *Eubacterium*, *Roseburia* and *Ruminococcus* in order to facilitate the dietary fiber-fermented byproduct SCFA, with critical roles in maintaining a healthy cardiovascular activity [420,421]. Although most studies on probiotic administration efficacy in HF are in animal models, there have also been a few reports describing clinical improvement by gut microbiota-mediated therapy in patients with HF [422]. In a small double-blind, placebo-controlled pilot study on HF patients (NYHA class II or III, with left ventricle ejection fraction < 50%), were randomized to probiotic treatment receiving *Saccharomyces boulardii* (1000 mg per day for 3 months) or placebo. HF patients that followed probiotic treatment showed a reduction in total cholesterol levels and in uric acid levels, reporting an improvement in cardiac systolic function when compared with the placebo group [423]. Another three months' treatment with rifaximin or *S. boulardii* reported no clinically significant effect on left ventricle ejection fraction, circulating levels of TMAO, microbiota diversity and function or systemic inflammation in HF with reduced ejection fraction [424].

Results of antibiotic usage in HF patients' gut microbiome modulation are controversial. In animal models, oral vancomycin use induced smaller left ventricular infarct size, and improved recovery cardiac function following ischemia/reperfusion experiments in treated, compared to untreated, rats [425]. Rifamixin, besides its bactericidal and bacteriostatic effect, also has the capacity to reduce translocation of bacteria and toxicity, has an anti-inflammatory effect and can positively regulate the composition of the intestinal microbiota, promoting the growth of *lactobacillus* and bifidobacterial [426,427]. As for human clinical trials, the results are contradictory. The use of a cocktail of tobramicyn and polymixin B, in HF patients, normalized the level of intestinal Gram-negative bacilli, significantly decreased pro-inflammatory cytokines and improved flow-mediated dilation: evidence of endothelial

dysfunction [428]. However, the results were limited to the treatment administration period. Furthermore, when prescribing an antibiotic therapy, side effects such as polymyxin B toxicity and macrolides' increased risk of myocardial infarction must be considered [429]

Prebiotics are "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits) upon host health" [430]. Prebiotics use could increase the amount of *Bifidobacterium* and promotes a higher body weight loss, which decreased systolic and diastolic blood pressure [431]. A recent study reported that prebiotic oligofructose reduces infiltration of inflammatory cells in rats [432]. Prebiotic administration can promote the development of beneficial bacteria, including *Bifidobacterium* and *Lactobacillus* spp, reducing body weight and inflammation and an improving glucose and insulin tolerance [433], all associated to better HF outcomes [339].

Regarding the regulation of the harmful metabolite production by the gut microbiota, preclinical studies reported beneficial effects of DMB administration as well as both dietary TMAO removal and administration of choline TMA lyase inhibitor, iodomethylcholine, in decreasing serum TMAO levels, ameliorating cardiac remodeling and reducing the expression of pro-inflammatory cytokines [434,435]. Resveratrol has also been shown to stimulate the growth of beneficial bacteria in the intestinal tract through the reconstitution of intestinal microflora, thus decreasing the production of TMAO [430]

Fecal microbiota transplantation (FMT) has been proven to be an effective method of reconstructing normal intestinal function and treating microecological imbalance in several disorders by introducing bacteria or metabolites from donor feces into diseased receptors [323,436–439]. A recent study reported that FMT and tributyrin treatment improved early cardiac dysfunction and increased the catabolism of branched chain amino acids in a diet induced pre-HFpEF rodent model [283]. On human subjects, FMT normalized insulin sensitivity of obese individuals with metabolic syndrome, but the effects were short-term [440]. Currently, there are no clinical studies available to evaluate FMT outcome in HF patients, but FMT has great therapeutic potential and represents a promising direction for future research [363,441]. According to ClinicalTrials.gov there are four clinical trials focused on the efficacy and safety of different strategies regarding gut microbiota modulation in HF patients. Another 21 studies share the same objective, but they include patients with different CVD: two of them evaluating the effect of gut microbiome restoration via FMT.

Limitations in the Study of Gut Microbiota and Their Implications in HF

There are still difficulties that need to be solved in the research of the gut microbiome, despite the vast characterization of the microbiome that has been done recently. Variation in study design as well as confounding variables in different studies frequently result in discordant results. The available experimental and bioinformatics methods leave space for bias and unreliable results [442]. There is also a lack of compatibility between existing databases, mainly because there is not a correct scale to be used when comparing the taxonomy and the functions associated with the human microbiome [443]. Currently, there are no quantitative definitions regarding microbial dysbiosis available, as this concept seems to be host-specific and disease-specific. There is a huge amount of data resulting from human microbiome studies and artificial intelligence techniques, although not available on a large scale, which would be useful after synthesizing them [444].

As for the research of the gut microbiome influence on HF, most studies are focused on bacterial communities while other members in gut microbiota such as virus, fungal, or archaea are not widely studied and thus their roles in human disease remain underappreciated. The exact microbial composition of HF patients in current studies is not the same and a common microbiome associated with HF has not yet been established. The great heterogeneity of HF populations has an important influence on common factors influencing microbiota composition. Another negative aspect would be that most of the existing research includes small size groups,

and the outcome is not adjusted for various factors and medications that may affect the growth of the gut bacteria, thus offering results with low statistical relevance. Moreover, HF risk factors vary depending on age, and the composition of gut microbiota and metabolites may also change with age [445]. Therefore, the critical role of age may affect the stability of the results.

II.1.4.4. Conclusions

The objective of this research was to compile the information that is now available on the influence of gut microbiota and the metabolites they produce on HF and the risk factors that go along with it. Gut dysbiosis, low bacterial diversity, intestine overgrowth of potentially pathogenic bacteria, and a decline in SCFA-producing bacteria have all been associated with HF. HF development is associated with increased intestinal permeability that permits microbial translocation and the transport of compounds generated by bacteria into the circulation.

Dysbiosis is a key factor in HF pathogenesis and disease evolution, and targeting the disrupted gut microbiota could be considered an effective therapeutic objective. There are many methods available in order to modulate the dysbiotic intestinal microbiota, such as dietary interventions (which include prebiotics, probiotics, and postbiotics) and fecal transplantation. Treatment results vary, however, as they highly depend on the baseline characteristics of each individual, including genetic background, gut barrier function and microbiome diversity. Therefore, the creation of customized microbiome therapies is essential for the therapeutic intervention of HF.

II.1.5. Dysbiosis - Celiac Disease

II.1.5.1. Aim of the Study

In order to get a better understanding of the potential processes behind the gut microbiota's role in CD pathogenesis, this review summarizes the most recent microbiome research on CD. We have also explored the potential relevance of microbiota alteration in customized medical treatment, both as a therapeutic and preventive approach.

II.1.5.2. Materials and Methods

The research team conducted an investigation on the possible mechanism that stands behind the gut microbiota's involvement in CD pathogenesis using the following searchable databases: Cochrane Central Register of Controlled Trials (CENTRAL), Excerpta Medica Database (EMBASE), Medline, PubMed, Scopus, and Web of Science.

II.1.5.3. Results and Discussions

Genetic and Environmental Determinants of Celiac Disease

Genetics play a significant role in CD since a gluten-rich diet causes diverse regional differences in CD prevalence. HLA testing in CD has a low positive predictive value, while its negative value has a high predictive validity [446]. HLA-DQ2/8, which is characteristic to CD, can also be found in 40% of the general population, suggesting that genetics are not enough for CD progression [447].

An additional factor involved in CD development is gluten. As a protein mixture, gluten's main components are gliadin and glutein. Gliadin is the principal antigen that triggers CD thorough its components glutamine and proline. When they arrive in the small intestine, these proline-rich peptides have a longer period of degradation, increasing the chance of activating an immune response. Once both the innate and the adaptive responses are triggered, this will further lead to crypt hyperplasia, villus atrophy, and to the infiltration of the intestinal epithelial inflammatory cells [448]. In addition, these pathological modifications will be

followed by an increase in intestinal permeability and to intestinal epithelial cell destruction, clinically translated as diarrhea, emaciation, abdominal pain, abdominal meteorism, and dermatitis herpetiformis, as well as features of CD pathogenesis [446].

Due to their capacity to alter microbiota composition, several variables, such as the type of birth, baby feeding practices, intestinal infections, and medication exposure, appear to have a role in CD etiology [449], as shown in Figure 18**.**

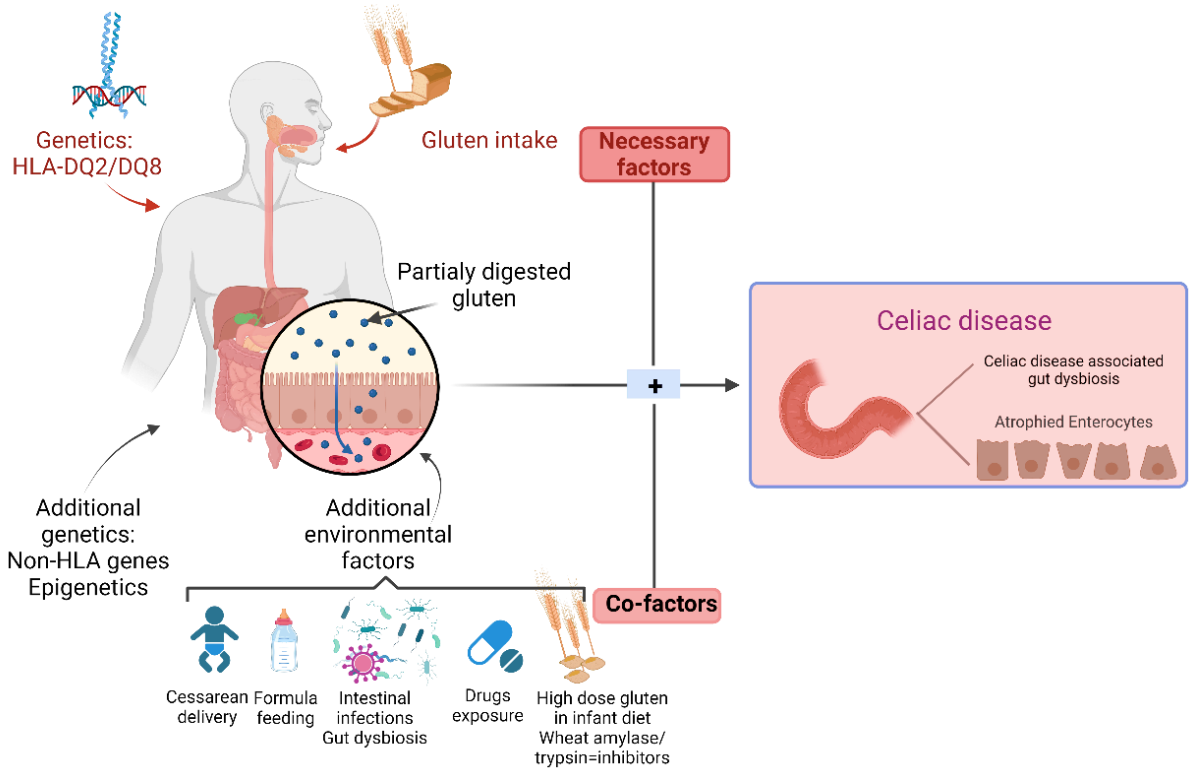

Figure 18. Genetic and environmental factors in CD

Dysbiosis of the gut bacterial community has the potential to change the gastrointestinal microecological environment and function as a pathogenic factor in a variety of ailments, including gastrointestinal, cardiac, respiratory, neurological, and metabolic diseases [247,248,450]. The recent advances in human microbiome study have offered evidence that diet is a major determinant of the intestinal microbiota's composition and function, and gluten can have an important influence on the gut microbiota's stability.

Gut Microbiota Profile in CD Progressors

Evaluating the gut microbiota composition of children at risk of developing CD before the onset of the disease might be useful in identifying possible CD progression markers. Pozo-Rubio and colleagues [451] analyzed the fecal microbial composition of children at risk of CD and reported some association between some pre-selected microbial taxa and the delivery mode, feeding practices of the infant, the administration of the rotavirus vaccine, and antibiotic exposure [451].

Similarly, Leonard et al [452] focused their efforts on analyzing the influence of genetic and environmental risk factors on the composition of the intestinal microbiota, before solid food and gluten introduction, in infants known to be at risk for CD. In their report, infants who were genetically predisposed to CD displayed a decreased amount of several *Coprococcus*, *Streptococcus*, *Parabacteroides*, and *Veillonella* species and *Clostridium perfringens* at four and six months of age [452], which was similar to the results of Hov and colleagues [453], who also identified lower levels of *Coprococcus* in individuals genetically at risk of different

autoimmune disorders [453]. Infants with a genetic risk of CD presented increased amounts of *Bacteroides* and *Enterococcus* species, as proven in several different studies [452,454,455]. As for the type of delivery, the intestinal microbiota of children delivered via cesarean section was characterized by an elevated abundance of *Enterococcus faecalis* at 3 months after birth, while several species of *Parabacteroides* and *Bacteroides* were reported in lower amounts compared to infants born through vaginal delivery, at all-time points [452]. The association of cesarean section with decreased levels of *Bacteroides vulgatus* and *Bacteroides dorei* strengthens the influence of the birth method on CD risk. These beneficial species, in increased amounts, are reported to lower the production of microbial lipopolysaccharide, leading to an improvement in the host's immune response [456]. Furthermore, infants born via cesarean section displayed decreased folate synthesis and riboflavin metabolism at the ages of four and six months, changes that might be associated with an altered immune response to viral aggression and a reduced natural killer cell activity [452,456]. Some authors correlate the last finding with the increased probability of developing T1D, a disorder that is often associated with CD [457].

Table XXXVIII provides a summary of the main findings relating to the microbiome profile of children at risk of CD previous to the development of the disease.

Table XXXVIII. Microbiota alterations before CD onset in children with CD genetic risk

Study	Subjects	Sample and Techniques	Microbiota Alterations
Pozo-Rubio et al. [451]	55 infants	Blood-sample-flow cytometry analysis Fecal sample quantitative PCR analysis	-Infants born through cesarean delivery: ↓*B. catenulatum* ↑*B. angulatum* -Antibiotic use during pregnancy ↓B. *angulatum* -Formula feeding ↓*B. angulatum* -Antibiotic use during first the 4 months of life ↑*Bacteroides fragilis* ↑*B. angulatum* ↓*Bifidobacterium* spp. *B. longum* -Rotavirus vaccine ↓*Bacteroides fragilis* -Allergy and dermatitis ↓*B. angulatum*
Leonard et al. [452]	21 genetically predisposed infants 5 genetically nonpredisposed infants	Fecal sample metagenomic analysis	-Standard and high risk of CD: ↓*Streptococcus* spp. ↓*Coprococcus* spp. ↓*Veillonella* spp. ↓*Parabacteroides* spp. ↓*Clostridium perfringens* ↑*Bacteroides* spp. ↑*Enterococcus* spp. -Infants born through cesarean delivery: ↓*Bacteroides* spp. ↓*Parabacteroides* spp. t ↑*Enterococcus faecalis* -Formula feeding ↓*Bifidobacterium breve* ↓*Staphylococcus epidermis* ↑*Bifidobacterium adolescentis* ↑*Ruminococcus gnavus* ↑*Lachnospiraceae bacterium* -Infant antibiotic exposure ↑*Bacteroides thetaiotaomicron*

Study	Subjects	Sample and Techniques	Microbiota Alterations
			↑*Propionibacterium* spp. ↑*Subdoligranulum* spp. ↓*Bifidobacterium merycicum* ↓*Streptococcus lutetiensis*
de Palma et al. [454]	20 infants	Fecal sample fluorescence in situ hybridization analysis	-High-risk infants ↑Gram-negative bacteria ↑*Bacteroides-Prevotella* group ↑E. coli ↑Streptococcus-Lactococcus spp. ↑E. rectale-C. coccoides -Sulfate-reducing bacteria ↑C. lituseburense ↑C. histolyticum
Olivares et al. [455]	22 infants	Fecal sample 16S rRNA gene pyrosequencing and real-time quantitative PCR analysis	-High-risk infants ↑Bacillota phylum ↑Pseudomonadota phylum ↑*Corynebacterium* genus ↑*Gemella* genus ↑*Clostridium* sensu stricto ↑*Escherichia/Shigella* ↑Actinomycetota phylum ↑*Bifidobacterium* spp
Leonard et al. [458]	20 infants	Fecal sample analysis using shotgun sequencing and metabolomic profiling	↓*Bacteroides vulgatus* str_3775_S_1080 Branch ↓*Bacteroides uniformis_* -American Type Culture Collection (ATCC)_8492 ↓*Streptococcus thermophiles* ↓*Faecalibacterium prausnitzii* ↓*Clostridium clostridioforme* ↓*Veillonella parvula* ↑*Dialister invisus* strain DSM 15470r ↑*Parabacteroides* species and strains ↑*Lachnospiraceae bacterium* ↑*Bifidobacterium longum* ↑*Bifidobacterium breve* ↑*Escherichia coli* ↑*Clostridium hathewayi* ↑*Eubacterium eligens*
Girdhar et al. [459]	33 children	Fecal sample 16S rRNA sequencing and flow cytometry analysis Blood plasma sample metabolomic analysis	↑IgA-coated bacteria and unique targets of IgA in their gut microbiota
Rintala et al. [461]	27 infants	Fecal sample 16S rRNA sequencing	No statistically significant differences in early microbiota composition between children that later developed CD and healthy controls were found
Olivares et al. [462]	127 infants	Fecal sample 16S rRNA sequencing	-High risk of CD, both formula and breastfeeding ↑ETEC -Formula feeding ↑C. *perfringens* ↑C. *difficile*
ETEC-enterotoxigenic *E. coli*; ↑increased levels; ↓decreased levels.			

In a recent longitudinal and cross-sectional metagenomic investigation of the fecal microbial populations of infants at risk for CD, Leonard and colleagues [458] evaluated the gut microbiota composition and its associated metabolites in infants who progressed to CD, in comparison to matching non-affected controls. The surveillance started 18 months before the disease onset. The cross-sectional analysis that was performed at the moment of the onset reported differences in the abundance of several bacterial strains and derived metabolites in the

infants who developed CD compared to the controls, while the pathway abundance and microbial species evaluation revealed no modification. *Bacteroides vulgatus* and *B. uniformis* were found in decreased levels, which was further associated with a reduced efficacy of the immune defense mechanisms. The longitudinal study before the disease onset, however, described elevated levels of different microbial species, strains, and metabolites. Some microbial species were also reported to be associated with some other autoimmune disorders, suggesting that they could be used as possible biomarkers in autoimmune disease identification. *Dialister invisus* was found in elevated levels at all of the time points when compared to its levels at CD onset [458], while other studies reported an increased abundance in children with pre-type-1 diabetes and individuals who further developed CD [459,460].

Gut Microbiota Profile at CD Onset

Enterococci, lactobacilli, and bifidobacteria appear to be less prevalent in the fecal microbiota of children with CD [463]. In their study, El Mouzan and colleagues [464] reported that CD patients display lower levels of the *phylum Actinomycetota*, mainly representatives of the genus *Bifidobacterium*, which are bacteria with immunomodulatory effects, often used as probiotics. *Roseburia* and *Lachnospiraceae* species, also known as beneficial bacteria, were found in decreased abundance in newly diagnosed CD children [452,464,465].

Depending on the clinical characteristics of the patient, intestinal microbial diversity in CD patients may differ [466]. The microbiota of patients known to have CD associated with gastrointestinal symptomatology is mainly represented by the Pseudomonadota phylum and decreased microbial diversity, whereas the Bacillota phylum dominates the microbiota of patients with dyspepsia or dermatitis herpetiformis as their main manifestation [467]. These findings offer evidence that intestinal dysbiosis can have an essential role in CD pathogenesis and clinical courses.

Di Biase and colleagues [468], in their pilot study, tried to identify a pattern between the gut microbiota composition and the associated clinical elements present in children at CD onset. After analyzing the stool and duodenal mucosa samples of CD patients in comparison with healthy controls, it was reported that the duodenal microbiota of patients with CD is mainly represented by the *Enterobacteriaceae* family, followed by the Bacteroidota phylum and *Streptococcus* species as major representatives. The stool samples of these patients, on the other hand, are characterized by decreased levels of genus such as *Akkermansia*, *Bacteroides*, and *Prevotella*, and a reduction in levels of the *Staphylococcaceae* family [468]. Furthermore, the authors have pointed out a possible correlation between the presence of abdominal pain in some CD patients and elevated levels of pro-inflammatory microbiota such as *Enterobacteriaceae* and *Bacillaceae* family representatives. In addition to these findings, the levels of bacterial-derived metabolites such as SCFAs and *Bacteroides fragilis*-derived polysaccharide A also appeared to be modified in CD patients [468].

Fungi are widely known as having interactions with both bacteria and the human immune system. The current evidence sustains their role in gastrointestinal disorders, including IBS and IBD [469,470]. A study of fungal dysbiosis recently reported in children suggests that fungi might also be an important element in CD pathogenesis. In a metagenomic analysis of mucosal and fecal samples of children known to have CD, taxa such as *Saccharomyces cerevisiae* and *Saccharomycetaceae* were reported to be more abundant in the fecal samples, whereas *Pichiaceae* and *Pichia kudriavzevii* were found in decreased abundance. The mucosal samples were more abundant in taxa including *Sacchharomycetes* and *Candida*, while *Pneumocystis* and *Pneumocystis jirovecii* levels were decreased compared to controls [471].

Saccharomyces cerevisiae might represent an important factor in CD-associated dysbiosis. Found in increased levels in patients with CD, several studies have noted its reduction or complete disappearance in patients under a GFD, suggesting its potential role in CD pathogenesis [472,473].

Candida albicans is one of the yeasts whose immunologic involvement in CD has been the subject of research. It seems that *C. albicans* hyphal wall protein 1 can be covalently associated with endomysium components and to tissue transglutaminase, due to its amino acid sequences, which are similar to CD-related gamma-gliadin and alpha-gliadin T-cell epitopes [474]. Several studies have confirmed the correlation between CD and *C. albicans*, indicating a possible connection with CD initiation and progression [471,475,476].

Table XXXIX provides a summary of the major findings on the microbiota profile of children at the beginning of CD.

Table XXXIX. Gut microbiota alterations in children at CD onset

Study	Subjects	Sample and Techniques	Microbiota Alterations	Other Findings
El Mouzan et al. [464]	20 CD children 20 fecal controls 19 mucosal controls		Duodenal samples of CD ↑Pseudomonadota phylum ↑*Lactobacillus acidophilus, Neisseria* spp ↑*Coprococcus* spp. Fecal samples of CD ↑*Verucomicrobia* spp. ↑*Clostridium* spp. ↑*Escherichia* spp. ↑*Lachnospiraceae bacterium oral* ↓*Bifidobacterium* genus ↓*Bacteroides* spp.	Fecal samples were more diverse and richer in bacteria compared with mucosal samples *Bacillota* and *Bacteroidota* were the most abundant phyla in both fecal and mucosal samples
Zafeiropoulou et al. [465]	20 CD children 45 CD under GFD 57 healthy controls 19 children at risk with CD	Fecal sample 16S rRNA frequency	Untreated CD ↓*Clostridium sensu stricto 1* genus ↓*Ruminococcus* genus	Microbial dysbiosis was not reported in CD compared to healthy controls *Alistipes* was correlated with the presence of symptoms of CD
Di Biase et al. [468]	21 CD children 16 healthy controls	Fecal sample Duodenal sample 16S rRNA sequencing	Duodenal samples of CD ↑*Enterobacteriaceae* family ↑*Bacteroidetes/Streptococcus* spp. Fecal samples of CD ↓*Bacteroides-Prevotella* ↓*Akkermansia* spp. ↓*Staphylococcaceae* family	Patients with abdominal pain ↑*Bacillaceae* family ↑*Enterobaeriaceae* family Patients with diarrhea ↓*Clostridium* cluster XIVa ↓*Akkermansia* ↑*Bacillaceae* ↑*Fusobacterium*
Schippa et al. [477]	20 CD children before and after GFD 10 healthy controls	Duodenal sample 16S,ribosomal DNA analysis compared with TTGE	In CD patients vs. controls ↑*Bacteroides vulgatus* ↑*Escherichia coli* Active CD vs. Inactive CD prevalence *B. vulgatus* (80% vs. 90%) *Clostridium coccoides* group (50% vs. 90%) *Bifidobacterium* spp (20% vs. 40%)	Mean interindividual similarity index: 54.9%±14.9% Active CD 55.6%±15.7% remission state 21.8%±30.16% controls Similarity index between CD children before and after GFD: 63.9%±15.8%
Sample et al. [478]	22 CD children before and after GFD 17 healthy controls	Fecal ,sample16S ribosomal RNA sequencing	Active CD vs. Controls ↑*Haemophilus* genera ↑*Alistipes* genera ↑*Bacteroides* genera	

Study	Subjects	Sample and Techniques	Microbiota Alterations	Other Findings
El Mouzan et al. [479]	40 CD children 39 controls	Fecal samples Duodenal sample metagenomic analysis of microbial DNA	Fecal samples of CD ↓*Bacteroides intestinalis* ↓*Burkholderiales bacterium 1-1-47* Mucosal samples of CD ↓*Human endogenous retrovirus K*)	
El Mouzan et al. [480]	40 CD children 39 controls	Fecal samples Duodenal sample metagenomic analysis of microbial DNA	Fecal samples ↓*Human polyomavirus 2, Enterobacteria phage mEpX1, Enterobacteria phage mEpX2*	Mucosal samples- no association with CD
El Mouzan et al. [471]	40 CD children 39 controls	Fecal samples Duodenal sample metagenomic analysis of microbial DNA	Fecal samples of CD ↓*Pichiaceae* family ↓*Pichia kudriavzevii* ↑*Saccharomycetes* family ↑*Saccharomyces cerevisiae* ↑*Tricholomataceae* family f ↑Mucosal samples of CD ↑*Saccharomycetaceae* family ↑*Candida* spp. ↓*Pneumocystis* spp. ↓*Pneumocystis jirovecii*	Fecal fungal communities were more abundant than those observed in mucosal samples
Sanchez et al. [481]	32 active CD on GFD 17 8 healthy controls	Duodenal mucosa sample 16S ribosomal RNA sequencing	Active CD ↑Pseudomonadota phylum ↑*Enterobacteriaceae* family ↑*Klebsiella oxytoca* ↑*Staphylococcus epidermidis* ↑*Staphylococcus pasteuri* ↓Bacillota phylum ↓*Streptococcaceae* family	Non-active CD *t Streptococcus mitis* group
TTGE-temporal temperature gradient gel electrophoresis; ↑increased levels; ↓decreased levels.				

CD Prevention Strategies

In order to prevent CD, several gluten interventions have been recommended during recent years. Breastfeeding was thought to have a protective effect against CD, and an early introduction of gluten during a breastfeeding period in infants from four to six months of age was expected to have beneficial outcomes on disease onset [482].

As for dietary intervention in children with a known genetic risk of CD, several clinical trials have evaluated the outcomes of a delayed gluten introduction at 6 and 12 months of age. The BABYDIET clinical trial observed its subjects for 3 and 8 years, concluding that, in genetically at-risk children, a delayed gluten introduction at the age of 12 months does not offer a reduction in the risk of celiac disease autoimmunity (CDA) or an impact on the persistence of tTGA positivity [483,484]. However, there is evidence that a delayed exposure to gluten might lead to a delayed onset of the disease [485,486], while an early introduction of a small, fixed amount of gluten in infants with a known genetic risk reported no effect on disease onset [487].

Data from children with disease onset by the age of six years have revealed that these children had a rich gluten-containing diet after two years of age [488], whereas high gluten consumption during the second year of life increased the risk of CD [489,490]. Two ongoing clinical trials are evaluating the outcome of a late introduction of gluten/a restricted gluten intake up to an age of three and five years old, respectively. The researchers behind these studies

aim to obtain CD prevention, or at least to delay the disease onset to an older age, in children at genetic risk [491]. Furthermore, the Prevention of Celiac Disease in Skåne (PreCiSe) study evaluated whether the intake of a daily probiotic during the first 3 years of life would prevent CD onset up to the age of seven, in comparison with keeping a GFD, or with no dietary intervention. Two strains of Lactobacillus *(Lactobacillus plantarum* HEAL9 and *Lactobacillus paracasei* 8700:2) were chosen, due to their positive actions on the intestinal environment, to modify the underlying mechanisms of CD pathogenesis. The use of *Lactobacillus plantarum* HEAL9 aims to restore the normal permeability of the intestinal mucosa, while *Lactobacillus paracasei* 8700:2 exerts its immunomodulatory effects by regulatory T cells stimulation [492,493].

It has been suggested on several occasions that viral infection episodes at the time of establishing gluten tolerance may significantly increase the probability of developing CD.[491]. An increased exposure to enterovirus B during a child's first 2 years of life might elevate the risk of CDA, while a diet rich in gluten, in association with an enterovirus B infection history, would offer a cumulative effect regarding the risk of CD [494]. While children who have experienced rotavirus infections have an increased risk of CDA, those who received a rotavirus vaccination seem to be protected from CDA, as long as gluten is introduced in their alimentation before their 6th month of life, suggesting that limiting an infant's viral exposure might have a beneficial influence on CD risk [495,496].

Modulation of Gut Microbiota as a Potential Target in Celiac Disease

Dysbiosis was proved to be an important factor in CD pathogenesis and progression. Dietary exclusion of gluten, the key therapeutic option for CD, is not able to fully restore the disrupted intestinal microbiota of CD patients, even after long periods of adherence to a GFD [463,478]. Table XL provides a summary of the data available for gut dysbiosis in individuals undergoing a GFD.

Table XL. Gut microbiota alterations in CD patients under a GFD

Study	Subjects	Sample and Techniques	Microbiota Alterations	Other Findings
Di [463]	19 CD children under GFD (T-CD) 15 non-celiac controls	Fecal samples Duodenal samples, both on PCR and DGGE analysis	Duodenal biopsy ↑Eubacteria in T-CD ↑*Bacteroides* spp. ↑*Staphylococcus* spp. ↑*Salmonella* spp. ↑*Shigella* spp. ↑*Klebsiella* spp.	
Sampl [478]	22 CD children before and after GFD 17 healthy controls	Fecal sample 16S ribosomal RNA sequencing	CD after GFD vs. controls ↑*Haemophilus* genera ↑*Alistipes* genera ↑*Bacteroides* genera ↑*Holdemania* genera ↑*Blautia* genera	*Faecalibacterium* and *Roseburia* were enriched in patients whose aTTG levels did not normalize after GFD
DGGE - denaturing gradient gel electrophoresis; ↑increased levels; ↓decreased levels.				

As indicated in Figure 19, dietary treatments (prebiotics, probiotics, and postbiotics are also included) and fecal transplantation are two of the numerous currently available techniques for modifying the CD-associated dysbiotic intestinal microbiota in adult patients. However, in pediatric patients, several reports from the available literature have placed diet modification (GFD) and probiotic and probiotic use as the main options for the modulation of the gut microbiota.

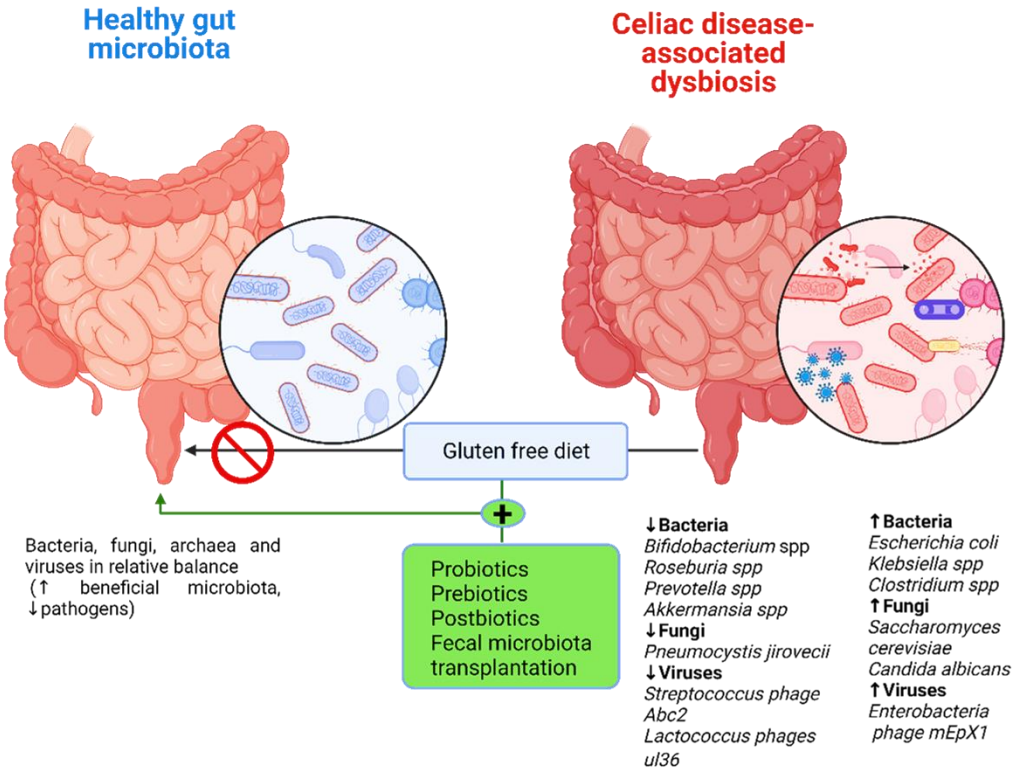

Figure 19. Microbiota modulation therapeutic options in CD

Bifidobacterium strains have shown their potential as probiotics in restoring the normal *Bacillota/Bacteroidota* ratio in children with CD by increasing *Bacillota* phylum abundance. Furthermore, *Bifidobacterium breve* BR03 and B632 administration in CD patients under a GFD restored *Lactobacillaceae* family levels close to those reported in healthy individuals [497]. The re-establishment of the *Bacillota/Bacteroidota* ratio was negatively associated with TNF-alpha serum levels after probiotic intake [498–500].

Prebiotic usage has demonstrated its ability to help CD patients regain intestinal balance. A randomized placebo-controlled trial evaluated the effect of oligofructose-enriched inulin (Synergy 1) supplementation in CD children under a GFD compared to a placebo. After 3 months, the patients receiving the prebiotic presented a significant increase in *Bifidobacterium* count. Furthermore, prebiotic administration increased acetate and butyrate fecal levels, evidence of the stimulation of bacterial metabolite production in CD patients [501].

Given the dearth of clinical studies in CD children, these favorable findings encourage more investigation. There is a need to generate new evidence regarding the efficacy and safety of these methods of microbiota modulation, since a GFD alone is not enough to re-establish gut microbiota eubiosis.

Limitations in the Study of Gut Microbiota and CD

Due to developments in the study of the structure and function of the microbiome, progress has been made in comprehending the intricate relationship between gut microbiota and CD. Our understanding of the role played by the gut microbiota in the etiology of pediatric celiac disease, however, still has a lot of gaps.

Most frequently, this is due to the reduced amount of existing prospective and cross-sectional studies, their small number of participants, and their different applied methodologies (16s ribosomal RNA sequencing, fluorescence in situ hybridization-PCR assay, and denaturing gradient gel electrophoresis). This lack of uniformity leads to different results, which interferes with the aim of identifying a distinctive microbial signature in children with CD. Recognizing early changes in the composition of the intestinal microbiota of children who are genetically

susceptible to CD and monitoring the influence of several environmental factors on the gut microbiota's profile over time is another challenge in CD study that would benefit from further research, as these results could be used to develop a future prevention strategy.

Additional therapies are required to alleviate CD patients' symptoms and QoL as a GFD is the only approved therapy. A major obstacle for researchers interested in CD is the adoption of numerous treatment methods based on microbiota manipulation into pediatric therapy, which have previously demonstrated their efficacy and safety in adult services.

II.1.5.3. Conclusions

Recent advancements in the study of the human microbiome have provided compelling evidence suggesting the involvement of gut-associated microbiota in the pathogenesis of CD. Among children afflicted with CD, there is a noticeable overabundance of various Gram-negative bacterial genera such as Bacteroides, Escherichia, and Prevotella. In contrast, beneficial bacteria like lactobacilli and bifidobacteria are reduced in number. This disruption is also accompanied by imbalances in fungal and viral populations, contributing to an overall dysbiosis in the gut environment.

Notably, deviations from the usual composition of the microbiota can be observed even before the disease manifests, particularly in children who are at a heightened risk of developing CD. This underscores the substantial impact of environmental factors on the gut microbiota, predisposing individuals to CD. Despite the efficacy of GFD in mitigating clinical symptoms and improving duodenal histopathology, it falls short of fully restoring the original equilibrium of gut microbiota.

The findings stemming from the exploration of gut microbiota in CD patients lead to a compelling hypothesis: the utilization of specific strains of Lactobacillus and Bifidobacterium, in conjunction with a GFD, could potentially rectify the perturbed intestinal microbiota. Encouragingly, therapies focused on modulating the microbiota show promise in the treatment of children with CD. However, it is crucial to emphasize that further extensive research is indispensable to fully understand the potential of microbiota modulation therapy in this context.

II.2. OBESITY

In recent times, human life is characterized by chronic imbalances characteristic of modern man's diet, including caloric excesses, mainly lipids and carbohydrates, from concentrated and refined sources. Thus, obesity is very widespread both in super developed countries and in those with little financial potential.

Moreover 50% of people in the majority of European nations are overweight, and 20–30% are obese. In Romania, obesity is still a growing public health concern, making it difficult to design modern anthropogenic food with a balanced nutritional profile, tailored to specific individual metabolic demands, and sensitive to recent changes in eating patterns.

World Health Organization reported for Romania 51.0% overweight (49.1% females and 53,1% males) and 19.1% obesity (21.2% females and 16.9 % males).

One of the biggest public health concerns of our century is obesity. Methods to prevent and treat obesity include nutritional education, physical activity, diets, medication, psychological therapies, or bariatric interventions. Industrialization, urbanization, ultra-technology, sedentarism create the conditions for the expansion of these metabolic pathologies such as obesity, diabetes, cardiovascular diseases, cancer, etc. Healthy eating requires a balance between food intake and the body's energy consumption. The QoL is dependent on the quality and quantity of food, and a diet poor in nutrients will cause negative effects on the health of the

human body.

Foods intended to be used in energy restricted diets for weight reduction are: total diet replacement products and meal replacement products for weight control. The compositional criteria include requirements on energy, protein quantity and quality, fat quantity and type, minimum and maximum levels for dietary fiber and minimum levels for certain vitamins and minerals [502]. Nutritional substances that may be used in the manufacture of these products are laid down in European Union Commission Regulation No 953/2009. Foods for weight reduction are a category of calorie-controlled diets which include nutritional fortified shakes snack bars, and low-calorie frozen meals [503]. Low-energy liquid diets of around 3MJ (750 kcal) daily have been popularized, often as part of an overall behavior modification program, or in the form of sachets intended to be used as foods for weight reduction. Both approaches have been shown to have potential for success in short-term studies lasting up to 1 year.

II.2.1. Use of Food for Weight Reduction in Obesity Management

II.2.1.1. Aim of the Study

The purpose of our study was to assess the effectiveness of MR (mendelian randomization) diets in body weight management and the impact in body composition for working age people, when the participants purchased the products and follow the weight loss program outside the clinical trial.

II.2.1.2. Materials and Methods

Our research was conducted in the Department of Environmental Health of the Institute of Public Health for weight and body composition monitoring in a framework of a weight loss program.

Participants in this retro-prospective study has been overweight or obese adults who followed personalized low-calorie diets for more than two months. The selected subjects were invited to participate as volunteers in this study and the rules of bioethics and research ethics were followed.

Informed written consent was obtained from all the participants.

Subjects were monitored at least once a month and we recorded the evolution of parameters. In this study we present measurements of the first investigation, at the end of weight loss (final weight) and at the end of monitoring in the maintenance of weight gained.

The study consisted of 79 subjects, 56 women and 23 men. Mean age of patients at study entry was 39.98±13.9 years, ranging from 18 to 69 years. The basic epidemiological characteristics - age, marital status, number of children and level of education - were similar by gender.

During the weight loss period we recommended daily calorie intake close to basic energy expenditure (BEE) of the person, which means 1100-1500 calories/day for women and 1300-1700 calories/day for men. BEE (kcal/day) was calculated using the following formula: 370+(21.6×lean mass in kg) [504].

We monitored the following anthropometric indicators: height (measured in the morning), weight (measured on an electronic scale with a deviation of ±100g, morning fasting and after using the toilet), BMI, body perimeters measured using metric ribbon (waist - abdomen, hip - buttocks), percentage body fat. Self-measurement of these indices and their reporting can have sufficient accuracy for trained subjects [505], but we prefer to be performed by the same assessor.

Optimal weight - with minimal health risk - was calculated using the Metropolitan Life Insurance formula that takes account of height in cm (h), age in years (a) and gender:

Theoretical optimal weight (kg) = 50 + 0.75 (h-150) + 0.25 (a-20) for men; in women, the result is multiplied by 0.9 [504]

For statistical analysis, data were loaded and processed by use of Microsoft Excel and EPIINFO 7.2 CDC. For descriptive statistics we use mean and SD. We used x2 test to compare frequencies and t-Student test for calculation of significant difference between the two media. Significance was agreed for p value <0.05. All analyses were performed separately for males and females.

II.2.1.3. Results and Discussions

The initial anthropometric measurements of the subjects are shown in Table XLI. Average height and weight were significantly higher in men than in women (p<0.001). BMI did not differ significantly between sexes (p>0.05), while average body fat percentage (FAT%) was significantly higher in women compared with men (p<0.001) as it can be seen in Table XLII.

In an averaged time of 3.98±2.29 months, the entire group lost an average of -10.19±6.12kg i.e., 11.34% of body weight, -11.47±9.15cm of waist circumference, -5.29±5.46% of body fat (Table XLIII). All the changes were statistically significant (p<0.001). The significance is retained (p<0.05) also for a hypothesized mean difference of body weight of 7% for men and 10% for women.

Table XLI. Initial anthropometric characteristics (mean±SD) by gender

	Women (n=56)	Men (n=23) |	t-test	p value
Height (cm)	163.8±6.02	175.8±8.21	6.32	<0.001
Weight (kg)	85.9±15.07	99.17±20.7	2.76	<0.01
Optimal weight (kg)	59.8±4.47	74.5±8.56	7.78	<0.001
BMI (kg/cm^2)	32.1±5.80	31.8±5.04	0.21	>0.05
FAT%	41.8±5.91	32.7±6.21	6.06	<0.001

Table XLII. Evolution of anthropometric characteristics during the weight loss program (t-test: paired two samples for means, two-tail)

Total (n=79)	Initial					Final (end of weight-loss period)					p value
	Mean	SD	Median	Min.	Max.	Mean	SD	Median	Min.	Max.	Initial vs. final
Height (cm)	167.30	8.6	167	150	192						
Weight loss period (months)						3.98	2.3	3	1.5	13	
Weight (kg)	89.80	17.9	86.5	58.0	170.0	79.6	17.2	77	52.7	162.0	<0.001
BMI (kg/m^2)	32.03	5.6	31	22.4	47.1	28.3	5.5	27.7	20.8	44.9	<0.001
Fat (%)	39.19	7.3	39.9	21.1	53	33.9	7.0	34.6	18.0	47.7	<0.001
Waist (cm)	109.9	14.4	109	80	164.0	99.0	13.9	97	71.0	159.0	<0.001
Hip (cm)	113.4	11.8	112	86	144.0	105.3	**11.7**	104.0	83.0	133.0	<0.001
Women (n=56)											
Height (cm)	163.8	6.0	163	150	181						
Weight loss period (months)						4.21	2.4	3	1.5	13	
Weight (kg)	85.9	15.1	83.5	58	120	75.1	13.2	73	52.7	114	<0.001
BMI (kg/m^2)	32.1	5.8	30.95	22.4	45.6	28.1	5.7	27.5	20.8	41.2	<0.001
Fat (%)	41.8	5.9	42.1	24.6	53.0	36.3	5.7	37.1	23	47.7	<0.001
Waist (cm)	108.9	14.2	107.7	80	136.0	96.1	13.0	96.5	71.0	126.0	<0.001
Hip (cm)	114.7	12.5	114	86	144.0	105.3	12.7	104.5	83.0	133.0	<0.001
Men (n=23)											
Height (cm)	175.8	8.2	176	159	192						
Weight loss period (months)						3.43	1.9	3	1.5	10	
Weight (kg)	99.2	20.8	96	66	170	90.5	20.5	86	61	162.0	<0.001

Table XLIII. Evolution during the weight loss program

Total (n=79)	Initial					Final (end of weight-loss period)					p value
	Mean	SD	Median	Min.	Max.	Mean	SD	Median	Min.	Max.	Initial vs. final
BMI (kg/m^2)	31.8	5.0	31	26.1	47.1					29.1	
Fat (%)	32.7	6.2	33.1	21.1	46.5	28.07	6.5	28.3	18.0	43.2	<0.001
Waist (cm)	112.4	15 0	112	93	164	104.4	14.6	103.5	89.5	159.0	<0.001
Hip (cm)	110.1	9.6	109	94	136	104.4	9.2	102	92	125.0	<0.001

Most of participant lost 5-10% of body weight, but 14% of them lost more than 20% of their initial body weight (Figure 20).

Weight loss was made on account of fat mass, which was significantly reduced by 24% compared to initial (average 20.8% in men and 25.2% in women) and insignificant on account of fat-free mass (3%) (Figure 21).

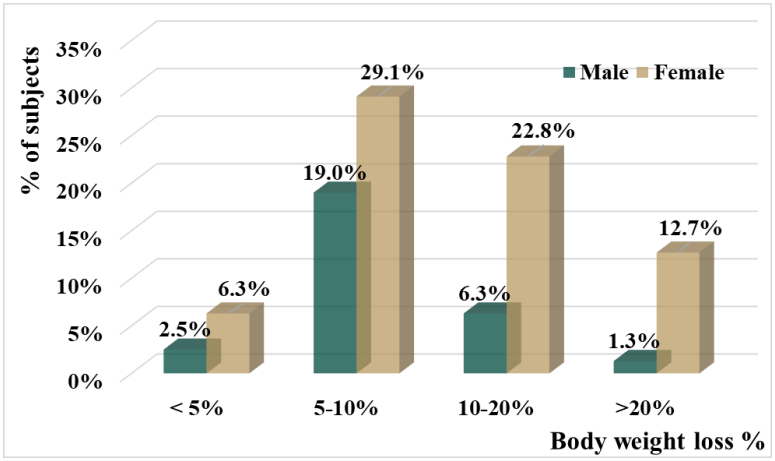

Figure 20. Distribution of percentage of weight loss (C2=4.72; df=3; p=0.193)

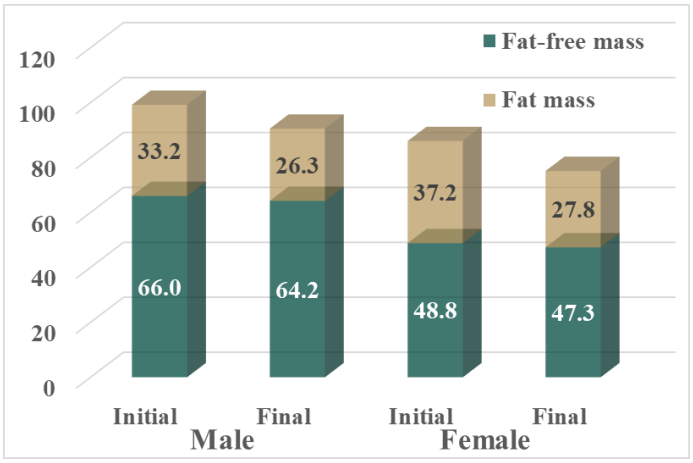

Figure 21. Evolution of average values of fat and fat-free mass (kg) during weight loss

WHR dramatically decreased for both men and women, going from 1.02 for males to 0.99 for women. Waist circumference as an indicator of central fat distribution recorded significant changes, but the final results were not within the accepted limits. Adherence to the proposed weight loss schedule was higher in women (who have followed the rules for 4.21±2.4 months) than in men (3.43±1.9 months).

The average weight loss was -2.71 kg/month, more in men (average -2.91 kg/month, up to -6.8 kg/month) than in women (average -2.6 kg/month, up to -5.5 kg/month). Most common causes of participants' left the weight loss program before reaching the target were:

financial problems (37%) - monthly cost of imported products was close to minimum wage, lack of motivation for loss weight (33%), monotony (13%), ineffectiveness of program compared to initial expectations (9.2%).

Subsequently, in the period of maintenance of weight gained, subjects were followed for an average of 11.28±8.1 months and the entire group of participants reached an average weight gain of +2.86±3.02 kg compared to the minimum weight (31.5% of weight lost) (Figure 22).

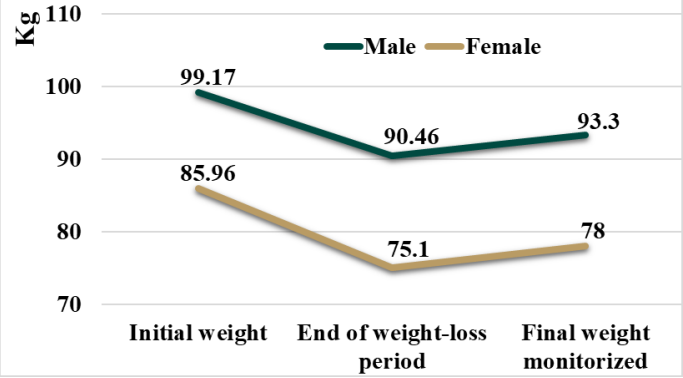

Figure 22. Evolution of body weight during monitoring

We compared the group that in the maintenance of weight lost replaced one meal a day with MR at least 3 times a week (n=30) with those who rarely or never did it (n=49); these groups were significantly different in the percent of weight regain from totally weight loss (t-test, $p<0.001$, 12.3% vs. 43.3%).

Our research showed that the weight reduction in a population not included in a clinical trial was equal to or greater than the findings of those studies. A randomized controlled study investigated the effect of 12 weeks-long energy-restricted modified diet with or without MRs for weight control on weight loss and body composition in 87 overweight women. Dietary intervention resulted in a significant weight loss in both groups (-5.98±2.82 kg, $p<0.001$ and -4.84±3.54 kg, $p<0.001$, respectively).

However, the rate of responder (weight loss >5%) was higher in MR (77%) versus non-MR group (50%) ($p=0.010$). A significant reduction was observed in waist circumference and body fat mass in both groups [506]. Other study evaluated the impact of a 30-day MR diet (1200 kcal/day, with unlimited fresh vegetables and fruits) in 32 adult primary care patients; mean weight loss was -2.7±2.6 kg [507].

Several studies have suggested that proteins are the most important mediators of macronutrient sense of satiety, and their increased consumption leads to weight loss with retention of lean mass. An increase in dietary protein content has been proposed to streamline regulation of body weight through effects on satiety, thermogenesis and maintenance or accretion of fat-free mass [508,509].

Evaluation of the efficacy of two low-calorie diets with partial meal replacement plans (a high-protein plan (HP) and a nutritionally balanced conventional plan (C-plan) in a 12-week randomized double-blind study with 75 participants) showed that the overall mean weight loss was 5kg in the HP-plan group and 4.9kg the C-plan group ($p=0.72$). Body fat mass decreased 2.5kg in the HP-plan group ($p<0.05$) and was more effective in reducing body fat among subjects with ≥70% dietary compliance [510].

Over a period of 4 months, our participants lost 8.29±5.26 kg of body fat mass. These better results can be explained by a better adherence to the program and a stronger motivation, possibly because of the price paid for products [511].

II.2.1.4. Conclusions

An increase in protein consumption on MR energy-restricted diets led to a small reduction in body fat while maintaining muscle mass. Loss of fat was distributed to the entire body by reducing all perimeters and percentage of body fat. The cost of the products and the monotony of the diet were the main causes of dismissing the MR diet. Subsequently, keeping the weight obtained was better in the subjects that replacing one meal a day with MR. Further study is necessary since maintaining weight loss over the long term is still challenging.

Due to their effectiveness, simplicity, and simplicity of use, MR diets may be a significant approach in the fight against obesity. They simplify the composition of energy-restricted diets (defined contents of nutrients and calories), reduce confusion, and increase compliance. Such diets are expensive for a large part of the population and become monotonous for long term use. However, most dieticians still maintain that a meal replacement diet is not an appropriate substitute for a healthy lifestyle.

II.2.2. The Impact of Food Supplements and Diet on Body Weight

II.2.2.1. Aim of the Study

The aim of the study was to assess the impact of using meal replacement diets in body weight and composition in adults.

II.2.2.2. Materials and Methods

Participants in this retro-prospective study have been overweight or obese adults who followed personalized low-calorie diets for more than two months. The selected subjects were invited to participate as volunteers in this study and the rules of bioethics and research ethics were followed. Informed written consent was obtained from all the participants.

The study consisted of 97 subjects, 68 women and 29 men. Mean age of patients at study entry was 43.1±14.07 years, ranging from 18 to 74 years. The basic epidemiological characteristics - age, marital status, number of children and level of education - were similar (j2 test, $p>0.05$) by gender.

The evolution of the parameters was monitored while subjects were observed at least once every month. In this study we present measurements of the first investigation, and at the end of weight loss (final weight).

The recommended daily calorie intake has been close to BEE of the person, which means 1100-1500 calories /day for women and 1300-1700 calories / day for men. BEE (kcal/day) was calculated using the following formula: 370 + (21.6×lean mass in kg) [504]

Protein ingestion was substantially increased (1-1.6 g/kg, 20-35 % of energy intake) to preserve muscle mass. Main source of protein in foods for weight reduction was soy and milk protein (whey and casein). Fat intake was low (15-25% of energy intake), over of the fat was a source of essential or unsaturated fatty acids. Carbohydrates covered 35-60% of the necessary and were low or medium glycemic index. Combinations of different nutritional supplements were used to provide a daily intake of nutrients. For example, the main formula provides 39 g of protein, 19.8 g of carbohydrates, 12.7 g of lipids per 100 g of powder, and prepared with 250 ml of skimmed milk (1.5% fat) provides 18.9 g of protein, 18.1 g of carbohydrates and 16.3 g of lipids per portion. For an increased intake of protein (esp. for men) another formula provided 83 g of protein per 100 g. For the weight loss period two meals per day were replaced with meal replacement and one additional meal was from conventional food, respecting the recommendations of appropriate nutrition.

The following anthropometric indicators were monitored: height (measured in the morning), weight (measured on an electronic scale with a deviation of ±100g, morning fasting

and after using the toilet), BMI and the percentage of body fat. So, we assessed anthropometric parameters using also waist and hip circumferences and waist-hip ratio [504,512], because excess of abdominal fat is an independent risk factor for associated diseases [513].

Body fat percentage is relevant for metabolic implications and was measured by Bioelectrical Impedance Analysis (BIA) using an OMRON BF306 bimanual Body-Fat Monitor, respecting the conditions specified by the manufacturer. Using BIA is relatively simple, rapid, non-invasive, the results are immediate and reproducible with an error of 1-4% for repeated measurements [504]. Validity of bimanual method (Omron BF 306BIA) was tested on participants from Asia and results in the assessment of FAT% showed acceptable levels of bias (SEE = 4.5%), due in particular age and length of arms [514,515]. A normal percent varies from one author to another, but it would correspond to 13-20% for men and 1730% for women. Fat content exceeding 25% in men and, respectively, 35% of women identify people with obesity [516,517]. Optimal weight - with minimal health risk - was calculated using the Metropolitan Life Insurance formula that takes account of height in cm (h), age in years (a) and gender: Theoretical optimal weight (kg) = 50 + 0.75 (h-150) + 0.25 (a-20) for men; in women, the result is multiplied by 0.9 [504].

For statistical analysis, data were loaded and processed by use of Microsoft Excel and EPIINFO 7.2 (CDC, 2013). For descriptive statistics we use mean and SD. We used j2 test to compare frequencies and t-Student test for calculation of significant difference between the two media. Significance was agreed for p value < 0.05. All analyses were performed separately for males and females.

II.2.2.3. Results and Discussions

Characteristics of participants

The initial anthropometric characteristics of the individuals are shown in Table XLIV. Average height and weight were significantly higher in men than in women ($p<0.001$). BMI did not differ significantly between sexes ($p>0.05$), while average FAT% was significantly higher in women compared with men ($p<0.001$).

Table XLIV. Initial anthropometric characteristics by gender

Characteristic (mean±SD)	Women (n=68)	Men (n=29)	t-test	p value
Height (cm)	164.2±6.1	176.5±7.0	8.62	<0.001
Weight (kg)	92.4±13.9	106.1±21.3	3.69	<0.01
Optimal weight (kg)	61.3±4.74	76.5±7.8	11.7	<0.001
BMI (kg/cm^2)	34.4±5.7	33.8±5.0	0.48	>0.05
FAT%	43.9±5.6	34.1± 5.7	7.77	<0.001

Excess weight over optimal weight was 31.1±13.0 kg for women (51.1%) and 29.5±17.2 kg (38.3%) for men ($p>0.05$). The fat mass was 30.5±9.56 kg for women and 29.2 ±13.7 for men ($p>0.05$). Calculated BEE was significantly lower in women (1366±142 kcal /day) than in men (1929±280 kcal /day) and determined the type of weight loss program and the recommended daily intake (average 1275 kcal/day and 1550 kcal/day, respectively).

Evolution of the participants during the weight loss program

In a time of 4.1±2.34 months, the entire group lost an average of -12.14±6.79 kg, i.e., 12.8±7.2% of body weight (Table XLV). All the changes were statistically significant ($p<0.001$). Adherence to the proposed weight loss schedule was higher in women (who have followed the rules for 4.55±2.6 months) than in men (3.05±0.93 months). The average rhythm of weight loss was -3.2±1.3 kg/month, up to -6.7 kg/month. Weight loss was made on account of fat mass, which was significantly reduced by 9.8±6.16 kg (24±13.7%) compared to initial (average 21.8±15.3% in men and 25.8±12.8% in women) and insignificant on account of fat-free mass (4.3±4.1%) (Figure 23).

Table XLV. Evolution of anthropometric characteristics during the weight loss program (t-test: paired two samples for means, two-tail)

Initial				Final			p value initial vs. final
Parameters	Mean	SD	Median	Mean	SD	Median	
Total (n=97)							
Height (cm)	167.80	8.5	167				
Weight loss period (months)				4.1	2.3	3	
Weight (kg)	96.5	17.6	92.5	84.4	18.6	80.5	<0.001
BMI (kg/m^2)	34.2	5.4	32.6	29.8	5.3	29.2	<0.001
Fat (%)	40.9	7.2	40.7	35.1	7.1	36.2	<0.001
Women (n=68)							
Height (cm)	164.1	6.1	163				
Weight loss period (months)				4.55	2.6	4	
Weight (kg)	92.4	13.9	90.5	79.3	14.2	79	<0.001
BMI (kg/m^2)	34.4	5.7	33.9	29.4	5.24	28.6	<0.001
Fat (%)	43.9	5.6	43.5	37.7	5.6	38.1	<0.001
Men (n=29)							
Height (cm)	176.5	7.0	176				
Weight loss period (months)				3.05	0.9	3	
Weight (kg)	106.1	21.3	96	96.3	22	87	<0.001
BMI (kg/m^2)	33.8	4.9	32	30.7	5.2	29.2	<0.001
Fat (%)	34.1	5.7	33.1	29.1	6.6	29.2	<0.001

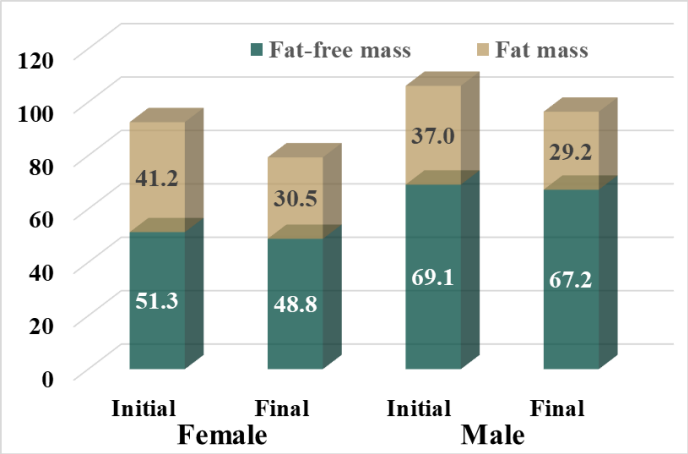

Figure 23. Evolution of fat and fat-free mass (as average values in kg)

These results were comparable to those from clinical studies that were published [518–520]. A randomized, controlled 2-arm trial was carried out on 101 overweight/obese (BMI 29.2+/-2.4 kg/m^2) females aged 18 to 44 years and, at the end of two weeks, the mean reductions in body weight and waist circumference were significantly greater in the intervention group, -0.53 kg for body weight which had a significantly higher increase in dietary intakes of certain vitamins, fiber and sugar, and significantly higher reductions in total and polyunsaturated fats and sodium intakes, as compared to the control group ($p<=0.05$) [519]. A randomized controlled study investigated the effect of 12 weeks-long energy-restricted modified diet with or without foods for weight reduction on weight loss and body composition in 87 overweight women. Dietary intervention resulted in a significant weight loss in both groups (-5.98±2.82 kg, $p<0.001$ and -4.84±3.54 kg, $p<0.001$, respectively). However, the rate of responder (weight loss >5%) was higher in meal replacement (77%) versus non-meal replacement group (50%) ($p=0.010$). A significant reduction was observed in body fat mass in both groups [506].

Although processed foods or preserved formulas may favor some gastrointestinal symptoms [181,521], the digestive tolerance of weight loss products was good; but a major

disadvantage was monotony, as has been observed in other studies [522].

According to several studies, proteins are the most significant macronutrient satiety controllers, and eating more of them helps people lose weight while maintaining lean body mass. An increase in dietary protein intake has been shown to improve weight loss maintenance and the source of the dietary proteins influenced changes in body weight, body composition, and cardiometabolic risk factors to streamline regulation of body weight [523]. In a randomized controlled trial soy and casein meal replacement shakes were compared with energy-restricted diets for obese women and concluded that both study groups with a highly structured behavioral 16 week long program incorporating four foods for weight reduction and vegetables and fruits lost significant amounts of weight and that differences in weight loss and body composition changes between casein and soy treatments were not significant [524]. Evaluation of the efficacy of two low-calorie diets with partial meal replacement plans (HP and a C-plan in a 12-week randomized double-blind study with 75 participants) showed that the overall mean weight loss was 5 kg in the HP-plan group and 4.9 kg in the C-plan group ($p=0.72$). Body fat mass decreased 2.5 kg in the HP-plan group ($p<0.05$) and 2.3 kg in the C-plan group ($p<0.05$) and the HP-plan was more effective in reducing body fat among subjects with >=70% dietary compliance [510]. Our participants, during 4 months period, lost 8.29±5.26 kg of body fat mass - these better results can be explained by a better adherence to the program and a stronger motivation.

Because of its effectiveness, simplicity, and ease of usage, the use of nutritional supplements and foods for weight loss may be a significant approach in the drive against obesity [509]. These formulas may increase compliance of patients, reducing confusion and simplifying the composition of energy-restricted diets (defined contents of nutrients and calories).

II.2.2.4. Conclusions

Our study revealed that using weight loss foods and nutritional supplements in a well-run weight loss program caused substantial changes in body composition, particularly on the reduction of body's fat mass.

The prevalence of excess body weight remains a significant public health concern on both a global and national scale. Efforts to find effective methods for weight loss continue to be pursued at both individual and population levels. A contemporary challenge is to devise diets, foods, and supplements that align with the evolving patterns of lifestyle and dietary preferences. These solutions should also cater to a well-balanced nutritional profile tailored to individual metabolic requirements.

Achieving a reduction in fat mass can be accomplished through personalized diets that take into consideration an individual's fundamental energy expenditure and caloric demands. Research has demonstrated that a promising approach for achieving weight loss involves the replacement of two daily meals with specially designed foods aimed at reducing body weight.

Diets that are energy-restricted and incorporate nutritional supplements or weight-reduction foods have been shown to be effective, particularly when they facilitate a slightly elevated intake of protein. This approach not only leads to the loss of fat mass but also ensures the preservation of muscle mass.

In essence, the pursuit of weight loss solutions necessitates a nuanced understanding of individualized dietary needs and the integration of innovative dietary strategies. This approach acknowledges the complex interplay between dietary patterns, metabolic demands, and the goal of achieving sustainable and healthy weight loss.

SECTION II. ACADEMIC AND PROFESSIONAL FUTURE DEVELOPMENT

The research activity cannot be separated from the didactic one, as there is an interdependent relationship, which is why the studies carried out allowed me to always be up to date with all the news in the field of environmental and food chemistry, food toxicology, diet therapy, as well as in the field of adjacent areas (medicine, biology, biochemistry). All that allowed me to be able to optimize educational programs, to implement new technologies in laboratory activity, to disseminate the results of scientific research, to increase the interest of students and residents towards scientific research.

Education has been and remains a priority for any society that is planning its future, and therefore teachers are essential to guide every young person in their intellectual and spiritual training. The teaching career is a noble one, based on dynamism, receptivity, flexibility in thinking, dedication and devotion. This implies awareness of the continuous need for training, increased interest in the use of current information and communication methods and technologies, creativity in approaching new didactic methods, promoting a high-quality educational style.

Motivated university teaching staff means motivated and determined students for a sustained and persistent effort in achieving the proposed educational goal, as well as the training of competitive specialists adapted to the labor market.

Therefore, a focus on the achievement of immediate and future goals will allow me to identify opportunities for personal development, as well as for professional, teaching and research development, with recognition and satisfaction guaranteed.

Based on the principle of continuity and expansion, I want to expand my professional profile in the coming years, being aware that this will require involvement, perseverance, conscientiousness, transparency, dynamism, support, friendship and permanent feedback.

Perspectives in academic activity

As didactic activity coordinator of the Discipline of Environmental and Food Chemistry, I consider it of major importance to implement a strategy for organizing and coordinating the students' activity to ensure that the intellectual work is carried out in the best possible conditions.

In this sense, I will contribute to the permanent updating of the content of the courses and the presentation of well-documented lectures, of high scientific level, with a solid knowledge base. I will also appropriately adjust the amount of detailed knowledge and the complexity of the information to the different categories of students and residents in order to make the right connections with the other subjects covered.

I will be able to achieve this priority objective by using and integrating in the educational process the newest and most effective pedagogical methods in order for the students to assimilate the fundamental knowledge, with operational value, from the main fields of

activity.

The teaching activity requires a permanent development of skills, knowledge and competences, thus responding to the challenges and, in addition, to the demands of our students. Therefore, I propose innovative and interactive approaches for the continuous expansion of the general culture horizon, which facilitate the formation of professional culture and creativity of students.

Permanent availability for dialogue with the student, proven by accepting and encouraging their questions, alternative points of view, constructive criticism and personal solutions, will provide continuous support in the learning process.

The permanent revision and enrichment with new bibliographic and web sources of all courses, as well as the development of course and practical works materials, will ensure the efficiency of the educational act.

To generate transparency and trust in my teaching activity, I will pay maximum attention to the evaluation and self-evaluation of the efficiency and quality of the educational activity. I will periodically inform the students about the criteria and ways of evaluating their work and professional results.

I believe that through these concepts, the learning process will become student-centered and will allow the dynamic growth of teacher-student interactivity, as well as the rapid evaluation of acquired knowledge.

The development of students' research activity will involve encouraging and stimulating them for their participation with valuable papers at various scientific events (congresses, conferences, symposia), as well as the development of well-structured and documented bachelor theses for final year students.

Perspectives in scientific activity

To develop and expand the research activity, I wish to participate in as many national and international scientific events as possible, as they are real opportunities for the dissemination of research results, but also for the exchange of experience and information with researchers in other institutions in the field. These opportunities are particularly beneficial for my development as a scientific researcher, but it will contribute substantially to increasing the prestige of the discipline, the department, the faculty and the university to which I am affiliated.

The main objectives that I will focus on in the future research activity are:

1. Identification and promotion of new research directions, with possible applications in the field of environmental and food chemistry, nutrition and diet therapy;
2. Implementation of research methods and results in the didactic process (courses, seminars, laboratories), thus increasing its value;
3. Development of research activity within projects financed from international, national, central, regional or local sources, won through competitions, launched by funding forums.
4. Capitalizing on research results by developing and publishing articles in internationally, nationally or regionally recognized journals.
5. Capitalizing on research results by communicating them at international and national conferences and publishing them in conference volumes.
6. Interdisciplinary collaboration with research structures from other universities and research centers.
7. Carrying out experience/research exchanges or internships with similar educational/research units in our country and abroad.

In the next period I will continue the current research directions and I propose to participate in grant competitions to secure the funding and material support necessary to achieve the proposed scientific objectives. I will also remain and intensify my presence in the space

reserved for pharmaceutical sciences, especially in the field of environmental and food chemistry, nutrition and dietetics, in order to make the results of my research, carried out with my colleagues, of high scientific value and as visible as possible on a national and international level.

To achieve this objective, I propose, on the one hand, to use the means that I have at hand and that have been developed and diversified by me up to now, and on the other hand, to be attentive, receptive and combative in identifying opportunities that might arise to support my research activity.

I propose to create a collaboration with research/training centers in the country and abroad and to attract the necessary funds for research. Establishing collaborations at national and international level, to realize projects of common interest. Establishing and cultivating professional collaborative relationships with specialists from related fields of interest, with a view to an integrative multidisciplinary approach to the researched field.

I include in this area of reflection also citations, as well as point observations of a scientific nature. In this sense, I hope to increase the number of citations and increase the international visibility of the scientific results obtained.

Final remarks

The framework through which I propose to build my academic career is based on a set of extremely important values: feedback, objectivity, communication, transparency, openness to new things, flexibility, teamwork, and a set of communities with mutual support.

I want to create lasting relationships with all colleagues in the academic community to build open, engaged, and friendly teams, groups, and communities.

Developing a community and effective professional relationships is based on the transmission and incorporation of feedback. I will support and use feedback in the activities in which I am involved, of a didactic nature (feedback from students or within teams of collaborators), of a scientific and professional development nature.

In such a dynamic field as the medical and pharmaceutical sciences, openness to the new is mandatory for any professional. New technologies must be discovered and evaluated from the moment they appear.

In the future, I will work intensively to achieve the teaching and research objectives that are my responsibility as a researcher and teacher.

This skill thesis will give me the opportunity to train PhD students, being an excellent motivation to continue my professional, teaching and research development.

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